Title: Paths, elongation, and projections of ascending chick embryonic spinal commissural neurons after crossing the floor plate.

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Abstract

The paths of embryonic chick spinal commissural neurons originating from the lumbosacral (LS) 2 spinal segment and born around Hamburger-Hamilton stage (HH) 18 were observed by labeling the axons with an *in ovo* electroporation method designed to limit the electroporated area to ~ one somite length. After crossing the floor plate, these axons followed two major paths, one ventral and one dorsal, and a minor path running between the major ones. These axons reached the brachial region by HH28, passed through the cervical region at HH29, and entered the medullary area by HH30. The dorsal axons entered the developing cerebellum by HH33, crossed the midline again, and spread into the rostral-ipsilateral area of the developing cerebellum so that most of them were confined to lobules II-III by HH39. A small population of ventrally running axons turned to enter the cerebellum, and the rest entered the superior medullary velum between the cerebellum and the midbrain. The LS2-originating axons that ascended ipsilaterally into the cerebellum followed a single path, and their extension was delayed compared with that of the commissural axons. Some of the ipsilateral axons innervated the cerebellum; the rest entered the superior medullary velum. These direct observations of the formation of part of the spinocerebellar projections in chick will be a useful reference for future analyses of the underlying mechanisms.

Section: Nervous System Development, Regeneration and Aging.

Keywords: spinal commissural neuron, spinocerebellar tract, chick, electroporation

Abbreviations: HH: Hamburger-Hamilton stage, LS: lumbosacral spinal segment, FP: floor plate, VSCT: ventral spinocerebellar tract, PLAP: human placental alkaline phosphatase, r: rhombomere, C: cervical spinal segment.

1. Introduction

Commissural neurons of the avian and mammalian spinal cord send their axons ventrally toward the floor plate (FP), where they cross the midline and then turn toward their targets. The molecular mechanisms governing the behavior of these neurons in reaching and crossing the midline, and in making the turn, have been extensively characterized (Reviewed by Colamarino and Tessier-Lavigne, 1995; Lyuksyutova et al, 2003; Bourikas et al, 2005). However, relatively little is known about the paths they follow after making the turn to reach their final destinations.

One of the destinations of these spinal commissural neurons is the cerebellum, in which case the axons function as part of the spinocerebellar tracts, which relay proprioceptive and exteroceptive information to the cerebellum (Jansen et al, 1967; Kim et al, 1986). The origins and destinations of these tracts have been extensively studied in the cat (Matsushita et al, 1979; Grant et al, 1982; Yaginuma and Matsushita, 1989; Xu and Grant, 1994), the rat (Yamada et al, 1991; Matsushita and Gao, 1997; Matsushita, 1999), and the chick (Lakke, et al, 1986; Okado et al, 1987) using anterograde and retrograde labeling techniques. In all mammals, the ventral spinocerebellar tract (VSCT) sends its axons across the midline to ascend contralaterally into the cerebellum (reviewed in Xu and Grant, 1994). In the cat, the somata of the contralaterally ascending axons of the spinocerebellar tract are located in lamina VIII and the ventromedial part of lamina VII (reviewed in Matsushita and Gao, 1997). These previous studies precisely demonstrated the connections between the somata and their axonal targets, and although they provided some information about the axonal paths (Okado et al, 1987; Xu and Grant, 1994), generally little information is available on the pathways taken by the axons to reach their destinations during development.

As a first step toward examining the molecular processes by which commissural axons find their targets in the cerebellum after crossing the floor plate, we sought a method for monitoring their behavior in the embryonic chick spinal cord. Gene expression in chick embryos can be easily modulated by *in ovo* electroporation to over-express or knock down molecules of interest (reviewed by Nakamura et al, 2004; Stern, 2005). In this method, the anode and cathode are placed on either side of the neural tube, into which the DNA solution is injected. The application of square electric pulses efficiently introduces DNA or RNA molecules into the progenitor cells that line the inside of the

neural tube proximal to the anode (Muramatsu et al, 1997). The axonal paths of the neurons generated by the progenitor cells can readily be observed by using this method to introduce vectors expressing appropriate markers. Furthermore, vectors designed to express or knock-down candidate molecules can be introduced by this method, and their effects, if any, should be readily visible. However, the currently available electroporation protocols introduce vectors into the spinal cord over a distance spanning several somites, which prevents both the observation of axons derived from a specific anteroposterior level of the spinal cord and a precise estimation of the elongation rate of these axons.

Here we report a method for introducing plasmids into a restricted area of the developing embryonic chick spinal cord (~1 somite in length) by *in ovo* electroporation. By using this technique to introduce a plasmid carrying the gene for GPI-anchored human placental alkaline phosphatase, we were able to follow the elongation of the ascending commissural axons born in the lumbosacral (LS) 2 spinal segment at Hamburger and Hamilton Stage (HH) 18, and the elongation time course and the path of the ipsilaterally ascending neurons, into the cerebellum.

2. Results

2.1. Conditions for the electroporation of plasmids into chick embryonic spinal cord limited to a specific anteroposterior position of ~1 somite in length.

To limit the area of the spinal cord to be electroporated, we first evaluated the effect of the shape of the electrode. We found that the length of the electrode could be reduced to 0.15 mm (Figure 1A) and the diameter thinned to 0.3 mm. Further minimization was technically difficult, because of difficulties placing the electrodes correctly on the embryo and the potential for the failure of the insulating enamel to maintain the exposed area of the electrode along an even shorter length. The length of the electrode, 0.15 mm, was comparable to the length of one somite of the HH18 chick embryo (Figure 1B, C).

We next optimized the conditions of the applied voltage and pulses. Under the conditions commonly used for the embryonic chick spinal cord (i.e. 20-25 V; 50 msec x 5 with 950-msec intervals), the treated embryos showed considerable damage at the electroporation site (data not shown), probably because the electric current was focused on a relatively small area. We found that multiple small pulses with shorter intervals (22 V; 10 msec x 10 with 90-msec intervals) efficiently introduced plasmids into the spinal cord without any apparent physical damage.

Next, using these electrode and pulse conditions, we tested the ability of the system to introduce plasmids into embryonic chick spinal cord at the LS2 segment. We performed the electroporation at HH18, when spinal cord is closed and spinal neurons begin to be actively produced. Around this stage, the lumbosacral spinal cord is relatively accessible, and the somites are readily recognizable. Of the segments in the LS region, LS2 was chosen because it is relatively easy to identify and access, and it is where the main motor pools innervating the major hind limb muscles are located (Landmesser, 1978). To visualize the axons of the electroporated cells, we introduced a plasmid expressing GPI-anchored human placental alkaline phosphatase (PLAP). Neither EGFP nor LacZ elicited strong enough signals to allow reliable tracking of the axons to their final destinations under these conditions, in which the number of electroporated cells was limited (data not shown). Of our first 157 attempts, we were successful in limiting the expression of the introduced gene to the area just around LS2 in 33 embryos (21%; Figure 1D). The success rate was readily improved, to ~80%, after several trials (see

Table 1). Thus, with practice we were able to obtain reproducibly a high percentage of embryos that expressed the electroporated genes in the intended limited position of the spinal cord.

2.2. Axonal paths of chick embryonic commissural neurons born in the LS2 segment at HH18.

We next used our optimized electroporation method to observe the axonal paths of chick embryonic commissural neurons born in the LS2 segment at HH18. The activity of the introduced alkaline phosphatase was high enough to label the entire axon tract, until HH40. In many cases, the tips of stained axons that were well isolated, and therefore readily visible, ended in a broader triangle-like shape (Figure 2A), which is consistent with the shape of a growth cone. This finding showed that single axons were readily visible under these conditions.

In 24 hours after the electroporation (HH22-23), the labeled axons reached the floor plate, started crossing the midline by HH24 (Supplemental Figure 1). The time needed to cross the midline was comparable to that observed previously for commissural neurons born around HH14 (Yaginuma, et al. 1990), suggesting that the PLAP protein had little effect on the axonal behavior. Two major pathways were recognizable at HH26 and clearly visible by HH27 (Figure 2B, C). One pathway ran dorsally and one ventrally, as previously reported (Imondi and Kaprielian, 2001). At HH27, a small minor pathway became visible between the two major paths (Figure 2C, D). All of these axons entered the brachial region by HH28 (Figure 2D). The positions reached by the majority of axons at the indicated stages are represented in Figure 2E-H. No significant difference in the speed of axonal elongation was observed between the two major paths.

We next followed the axonal elongations of the ascending LS2 commissural neurons even farther. These neurons continued to extend their axons beyond the brachial region at HH29 (Figure 3A), reaching the medulla oblongata around HH30 (Figure 3B). After passing the brachial region, the two major paths ran closer together, and the axons were less tightly constrained to a narrow trajectory, especially the axons of the ventral path. Mostly, the axons in the two paths rarely appeared to intermingle. However, a small population of axons running the ventral path changed their lane to the dorsal path around cervical (C) 10 spinal segment after passing the brachial area (Figure 3A, B).

The minor path running between the major ones approached and finally joined the dorsal path (Figure 3A). After entering the medulla oblongata, the paths again clearly bifurcated dorsally and ventrally around the region of rhombomere (r) 5-6 (Figure 4C, E).

The axons following the dorsal path started entering the developing cerebellum by HH33 (Figure 4A, C, E), in agreement with previous observations obtained by the anterograde labeling of the lumbar spinal neurons with wheat germ agglutinin-conjugated horseradish peroxidase (Okado et al, 1987), which added to the indications that the introduced PLAP had little effect on axonal growth. Upon entering the developing cerebellum, the axons following the dorsal path crossed the midline again (Figure 5A) and started spreading in the rostral-ipsilateral area (Figure 5B, C). The area where the axons spread became more confined as the cerebellum developed, to lobules I – III for the majority, by HH39 (Figure 5D-F). The time course of the axonal elongation and paths are schematically summarized in Figure 6.

In the ventral pathway, a small population of axons turned to enter the cerebellum around HH35 (Figure 4E). The rest of the ventral axons entered the superior medullary velum between the cerebellum and the midbrain. These ventral axons remained in this area until HH40, when the activity of the exogenous PLAP became faint.

2.3. Axonal paths of ipsilaterally ascending neurons born in the LS2 segment.

We also observed the ipsilaterally ascending axons that were labeled simultaneously with the commissural neurons by the electroporation. In contrast to the commissural neurons, the ipsilaterally ascending axons rarely strayed from their path. By HH28, the rate of their elongation was slower than that of the contralaterally ascending commissural neurons (Figure 2E-I, Supplemental figure 2). During their continued ascent through the cervical region, their elongation rate was comparable to that of the commissural neurons. The ipsilateral ascending axons reached the medulla oblongata around HH30 and approached the cerebellum by HH35 (Figure 4B, D, F, H). They followed a path in the medulla oblongata roughly identical to those observed on the contralateral side. Their path became broader as they passed the medullary region, and some dorsal axons then entered the cerebellum while the rest, running in a relatively

ventral pathway, entered the superior medullary velum between the cerebellum and the midbrain.

3. Discussion

Here we report the paths and elongation time course of the ascending spinal commissural axons, after they have crossed the floor plate. We followed these axons and those of the ipsilateral ascending neurons from the site of their birth, around spinal segment LS2 at HH18, to the cerebellum, one of the axonal targets. The axons were labeled histochemically by the activity of exogenous PLAP, which was introduced into the neurons by *in ovo* electroporation. The transparency of the developing chick central nervous system examined here enables us to observe the whole processes of the labeled axonal projections in whole mount preparations without preparing transverse sections. This system will be a useful tool for analyzing the effects of potential axon guidance molecules on their axonal behavior, by co-introducing genes of interest with the reporter gene. The observations described here can be used as a reference in evaluating such effects.

The PLAP activity in the axons was strong enough to permit the histochemical staining of the entire axonal track until HH40, showing that PLAP is a useful indicator for observing axon path-finding in the chick embryo. In many cases, individual axons were clearly visible. PLAP compares favorably with other indicators that are currently widely used, such as fluorescent proteins and β-galactosidase, especially for whole-mount detection. The stained cell bodies and axons were also visible in transverse sections (Supplemental figure 3). In addition to the spinal neurons described here, we successfully labeled motor axons innervating the muscles and dorsal root ganglia neurons innervating the spinal cord (data not shown). However, PLAP does not seem to be as useful in the mouse as in the chick, due to the higher level of endogenous AP activity (TY, unpublished observation).

In electroporation *in ovo*, expression plasmids are transferred randomly into progenitor cells facing the central canal of the developing neural tube, which raises the question of whether some populations of neurons fail to be labeled and are therefore overlooked. This is theoretically possible; however, we think the major populations of the ascending spinal commissural neurons born around the time of electroporation were detected in our experiments. We reproducibly observed essentially the same distribution of labeled axons over five or more trials in each case (see Table 1), and the labeled progenitors were evenly distributed after electroporation (data not shown). It is therefore unlikely

that a specific population of neurons generated at the time of electroporation consistently escaped labeling. Furthermore, the PLAP activity introduced into the neurons was strong enough to enable the visualization of individual axons, suggesting that even a few neurons belonging to a minor population can not be overlooked if they behave differently from others. These experimental conditions collectively indicate that the resultant images represent the major axons originating from neurons generated at the LS2 segment, during and soon after electroporation.

The connections of the commissural neurons innervating the cerebellum observed here matched those of the VSCT observed in mammals (reviewed in Xu and Grant, 1994; Matsushita, 1999). The VSCT is a crossed ascending tract, located in the ventrolateral spinal cord. The commissural neurons observed here re-crossed the midline in the cerebellum, consistent with previous observations in the cat showing that the VSCT neurons project predominantly to the ipsilateral side of the cerebellum (Yaginuma and Matsushita, 1986). These results collectively suggest that the dorsal commissural neurons projecting to the cerebellum observed here, at least in part, represented the chick anatomical counterparts of the mammalian VSCT neurons.

Most of the commissural neurons originating from LS2 and born around HH18 followed one of two paths, one ventral and one dorsal, confirming the previous observations (Imondi and Kaprielian, 2001). The axons running the dorsal path primarily reached the cerebellum. On the contrary, of the axons initially running the ventral path, some changed their path to the dorsal one at a certain point; around C10 after passing the brachial region, and around r5-6 in the medulla oblongata. In addition to these major paths, we found a minor but reproducible path running between the major pathways. The axons following the minor path elongated at a comparable rate to those in the major paths, and later joined the dorsal path. These observations raise a question: why they initially followed the different paths? One of the possibilities is that they just failed to follow the dorsal path after passing the floor plate by somehow and just returned to their original course. One of the other possibilities would be that they might need to project collaterals to nuclei that they cannot reach from the dorsal path. It is also conceivable that their final destination is not the cerebellum (i.e. reticular formation), however no such indication is observed at HH33 medulla oblongata (Figure5A; Supplemental figure 3D). Further analyses are needed to characterize these paths.

The commissural axons that took the dorsal path toward the cerebellum clearly

separated from the ventrally running axons again at the rostral medullary region, with clear bifurcation point at r5-6. Interestingly, a minor but substantial population of the ventrally running axons turned toward the cerebellum after passing this point as discussed above. This observation also supports the idea that signals may exist at r5-6 that attract the axons to the cerebellum. The commissural neurons that enter the 'developing' cerebellum project to a confined area, suggesting that they may utilize the same cues that participate in the cerebellar compartmentation, such as the Eph-ephrin system (Karam et al, 2000; Blanco et al, 2002), in defining their paths. In fact, EphA4 is expressed in the ventral cerebellar commissure, where the mossy axons enter and cross the cerebellum (Karam et al, 2000), suggesting that Eph ligands may indeed contribute to axonal pathway instruction in the cerebellum.

The targets and the fates of the ventrally running commissural axons are still uncertain. These axons reached the superior medullary velum and stayed there until HH40, when the exogenous PLAP activity faded. Further investigation is necessary to distinguish whether these axons pioneer the pathway and project nowhere, leaving the "follower" axons to make functional projections, or if their elongation is stalled by a barrier that disappears after HH40.

Ascending axonal tracts from the spinal cord projecting to the upper brain regions carry important information from the body's inner and outer environments. However, the mechanisms of their path finding and projection are still relatively poorly understood. Here we demonstrated the formation of some of these tracts in the chick. Our approach provides a useful basis for additional studies aimed at understanding such mechanisms.

4. Experimental Procedures

4.1. Construction of expression vectors.

Expression plasmids for PLAP were prepared by inserting a PCR-amplified fragment of chick MDGA1 cDNA encoding its GPI-anchoring signal sequence (amino acids 916-949) (Fujimura et al. 2006) into the *Xho*I site of AP-tag5 (Flanagan et al., 2000). The *NheI-PmeI* fragment (blunt ended by Klenow fragment) of the resultant plasmid was inserted to the *Xho*I site (blunt ended by Klenow fragment) of pCAGGS (Sunaga et al., 1997).

4.2. *In ovo* electroporation.

In ovo electroporation was performed essentially as described (Nakamura et al, 2000) with the modifications described in the text. The electrode developed here will be available upon inquiry.

4.3. Whole-mount alkaline phosphatase activity staining.

The developmental stages of chick embryos were determined according to the table of Hamburger and Hamilton (Hamburger and Hamilton, 1951). The staged embryos were fixed with 4% paraformaldehyde-PBS for 2 hr at 4ºC, and washed twice with PBS for 30 min at 4ºC. The fixed embryos were incubated at 65ºC in PBS for 8 to 16 hr to inactivate the endogenous AP activity. The treated embryos were washed with 100 mM Tris-Cl (pH 9.5) containing 100 mM NaCl and 50 mM MgCl2, and the residual PLAP activity was visualized by incubating the embryos with NBT/BCIP (Roche) in the same buffer at 4ºC for 24 hours. After extensively washing the embryos with PBS-5mM EDTA, the spinal cord, medulla oblongata, and cerebellum were exposed for observation. The positions where the majority of axon tips reached in spinal cord were determined by referring the somites, dorsal root ganglion, and ribs remaining in the other side of the body left intact for the purpose. The bifurcating point of the commissural axons in medulla oblongata was estimated by referring the places where cranial nerves exit (Barlow, 2002; Chandrasekhar, 2004).

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Figure legends

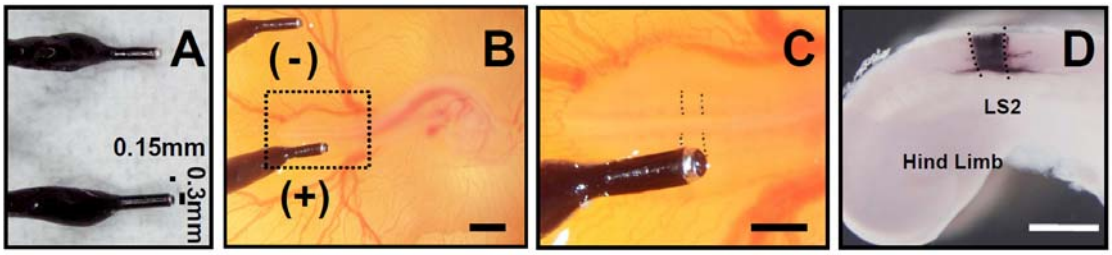

Figure 1. Introduction of expression vectors by *in ovo* electroporation into the chick embryonic spinal cord limited to around one somite length.

A. Electrode prepared for the experiments. The diameter and the length of the electrode are indicated.

B and C. The HH18 chick embryo set up for electroporation. The boxed area in B is enlarged in C. The length of the electrode was comparable to the length of one somite of the embryo (indicated by the dotted lines) at this stage.

D. The electroporated area at HH25 (ipsilateral side view) following electroporation at HH18 to introduce an expression vector for GPI-anchored human placental alkaline phosphatase. The pulse conditions were: 22V 10 msec x 10 times with 90-msec. intervals. The expressed PLAP activity was limited to the area around the LS2 segment (indicated by the dotted lines). Scale Bars = 0.5 mm (B, C), 1 mm (D).

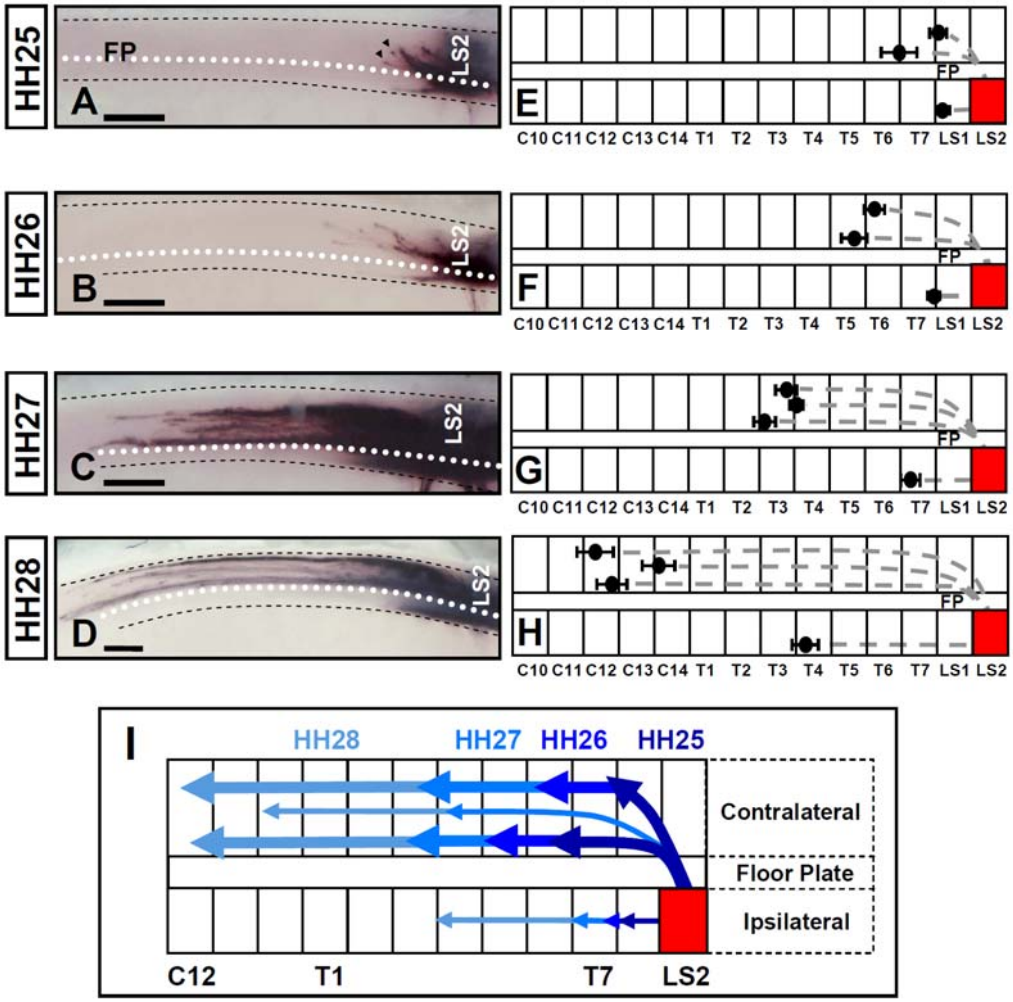

Figure 2. Approach of the commissural axons originating from the LS2 segment at around HH18 to the brachial region.

The labeled ascending commissural neurons were observed at HH25 (A), HH26 (B), HH27 (C), and HH28 (D). Two major ascending paths were readily observed with a small population of axons running between them. The axons frequently ended in a triangle-like shape (some examples are indicated by the black arrowheads in A). The positions of the observed axon tips are shown in E-H (error bars indicate standard errors) and summarized in I. The positions of the ipsilaterally ascending axon tips that originated from LS2 are also shown in E-H. The position of the floor plate is indicated by the white dotted line. The spinal cord is outlined by the black dashed lines. FP = floor plate; HH25 n = 11; HH26 n = 22; HH27 n = 17; HH28 n = 28; Scale Bar = 0.5mm.

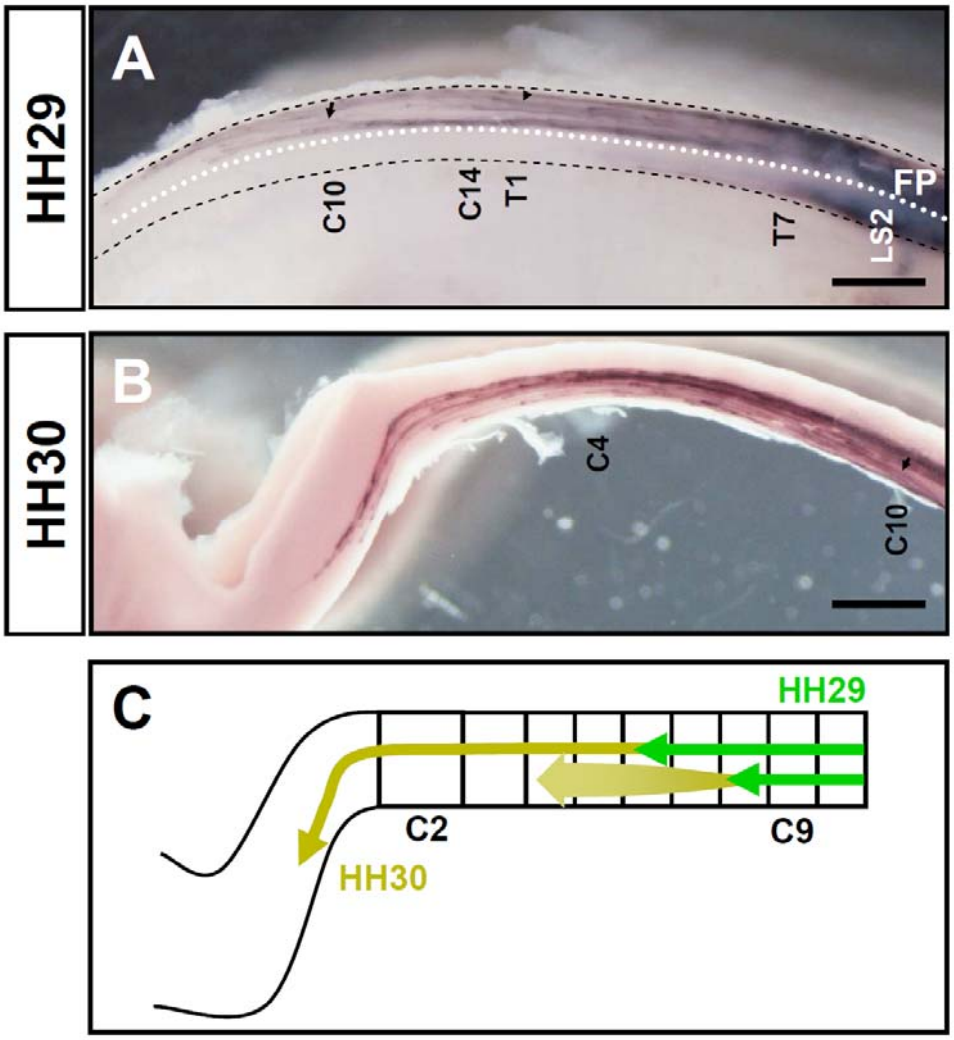

Figure 3. Approach of the commissural axons originating from the LS2 segment at around HH18 to the medulla oblongata.

The labeled ascending commissural neurons were visualized at HH29 (A) and HH30 (B). The position of the floor plate is indicated by the white dotted line, and the spinal cord is outlined by the black dashed lines. The axons changing their path from the ventral to the dorsal one are indicated by the arrows. The minor path running between the major ones and joining the dorsal path is indicated by the arrowhead. The paths and positions of axon tips are schematically summarized in C. The ventral path became broader and closer to the dorsal path as the axons ascended into the cervical region. HH29 n = 26; HH30 n = 26; Scale Bar = 1 mm.

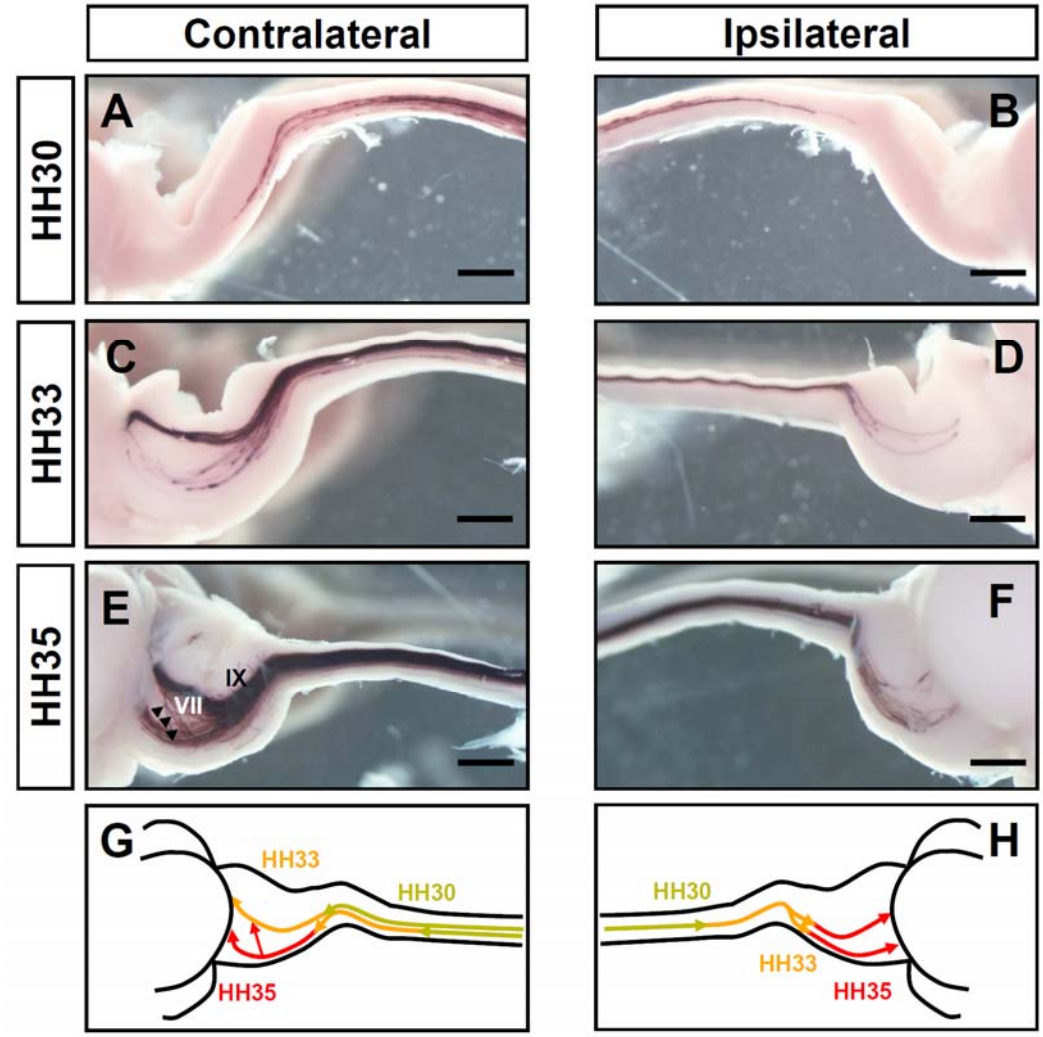

Figure 4. The commissural and ipsilateral ascending axons originating from the LS2 segment in medulla oblongata.

The labeled commissural (A, C, E) and ipsilateral (B, D, F) ascending axons were visualized at HH30 (A, B), HH33 (C, D), and HH35 (E, F). The paths and positions of the axon tips are schematically summarized in G (commissural) and H (ipsilateral). The commissural axons entered the medulla oblongata at HH30 (A) and again divided into two major paths. The dorsal population of axons approached the developing cerebellum, and the ventral population began diverting ventrally at HH33 (C). Some ventrally running axons turning to dorsal path were observed at HH35 (E) and indicated by arrowheads. The ipsilaterally ascending axons behaved in a similar manner but with a delayed time course (B, D, and F). The positions where the cranial nerves VII and IX exit are indicated in E. HH30 n = 26; HH33 n = 13; HH35 n = 21; Scale Bar = 1 mm.

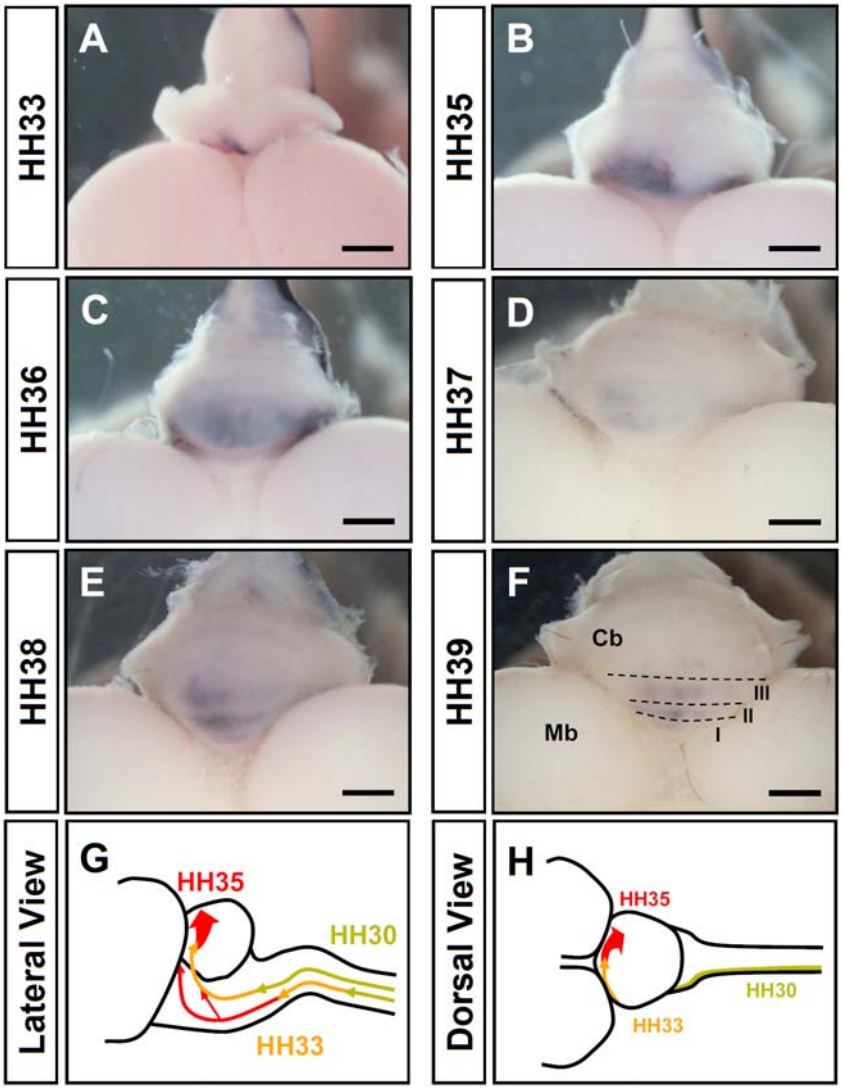

Figure 5. Ascending axons originating from the LS2 segment entering the developing cerebellum and their projections in the developmental cerebellum.

The labeled ascending axons were observed at HH33 (A), HH35 (B), HH36 (C), HH37 (D), HH38 (E) and HH39 (F); dorsal view is shown. Commissural axons running in the dorsal path entered the developing cerebellum by HH33 (A) and crossed the midline again. After re-crossing in the cerebellum, the dorsally running commissural axons projected to the ipsilateral side of the central lobules (B, C) mainly II-III, at around HH37-38 (D-F). The paths and projection patterns of the commissural ascending axons are schematically summarized in G (contralateral side view) and H (dorsal view). HH33 n = 13; HH35 n = 21; HH36 n = 9; HH37 n=9; HH38 n=10; HH39 n=10; Scale Bar = 1 mm.

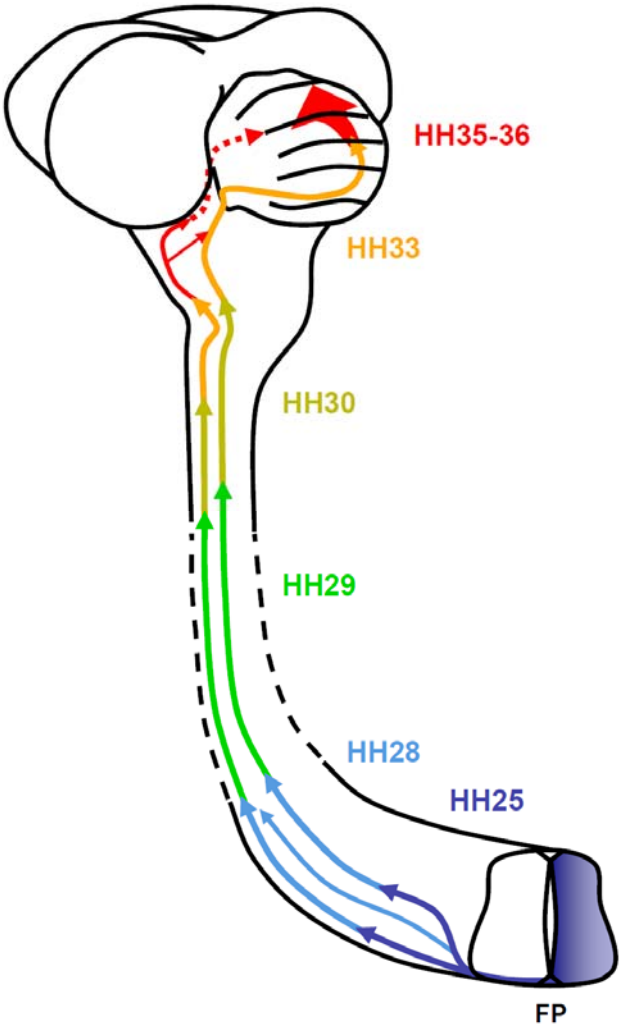

Figure 6. Paths and elongation time course of the commissural neurons originating from the LS2 segment at around HH18.

After crossing the floor plate, the commissural axons turned anteriorly, following two major paths and a minor path that ran between the major ones. These tracts ascended the spinal cord separately but approached each other more closely when passing through the cervical region. After reaching the medulla, they were clearly divided into two paths again. The dorsal population of axons entered the developing cerebellum and re-crossed the midline to project to the ipsilateral side of central lobules I-III. A small population of the ventrally running axons also turned to enter the cerebellum after passing the major bifurcation point. The majority of the ventrally running population entered the superior medullary velum and stayed there until HH40, when the exogenous PLAP activity faded.

Stage	HH22 (E3.5)	HH23 (E4)	HH24 (E4)	HH25 (E5)	HH26 (E5)	HH27 (E5)	HH28 (E6)	HH29 (E6)	HH30 (E7)	HH31 (E7)	HH33 (E8)	HH35 (E9)	HH36 (E10)	HH37 (E11)	HH38 (E12)	HH39 (E13)
Number of embryos	8	8	15	18	40	36	55	76	56	13	36	37	22	14	16	15
Ratio of successful expression (%)	62.5	75	80	94.4	77.5	83.3	80	80.3	82.1	84.6	86.1	78.4	63.6	71.4	62.5	66.7

Table 1. Number of electroporated embryos and the ratio (%) of successful gene expression limited to the area around the LS2 segment.