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Research Report

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.

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

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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

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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 (Fig. 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 (Figs. 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 ms × 5 with 950-ms 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 ms × 10 with 90-ms intervals) efficiently introduced plasmids into the spinal cord without any apparent physical damage.

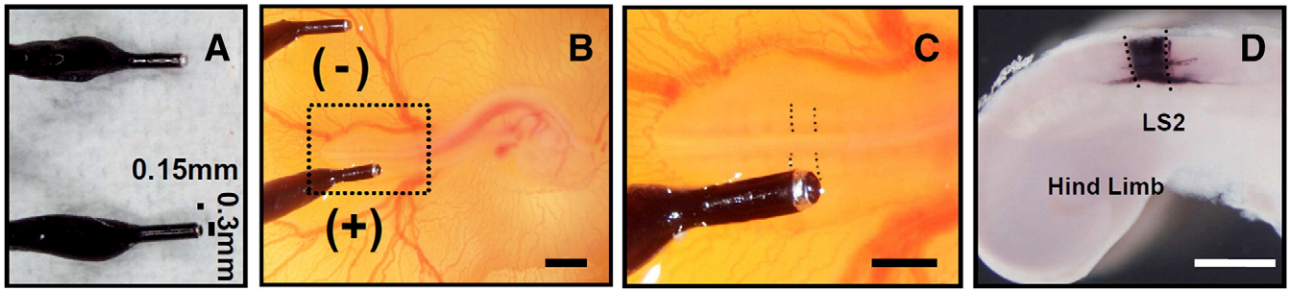

Fig. 1 – Introduction of expression vectors by *in ovo* electroporation into the chick embryonic spinal cord limited to around one somite length. A. Electrode prepared for the experiments. The diameter and the length of the electrode are indicated. B and C. The HH18 chick embryo setup for electroporation. 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: 22 V 10 ms × 10 times with 90-ms 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).