Development 108, 705-716 (1990)
Printed in Great Britain © The Company of Biologists Limited 1990

Identification of early developing axon projections from spinal interneurons in the chick embryo with a neuron specific β-tubulin antibody: evidence for a new 'pioneer' pathway in the spinal cord

HIROYUKI YAGINUMA[1], TAKASHI SHIGA[1], SHUNSAKU HOMMA[1], RIEKO ISHIHARA[2] and RONALD W. OPPENHEIM[1,*]

[1]*Department of Neurobiology and Anatomy, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC 27103, USA*
[2]*Department of Anatomy, Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, 305, Japan*

* Author for correspondence

Summary

The early development of interneurons in the chick embryo spinal cord was studied using a monoclonal antibody against a neuron-specific β-tubulin isoform. Early developing interneurons were divided into two cell groups on the basis of their location and the pattern of growth of their axons. One group is composed of cells that establish a primitive longitudinal pathway (PL-cells), whereas the other group contains cells constituting a circumferential pathway (C-cells). The onset of axonal development in both cell groups occurs at stage (st.) 15 (embryonic day, (E), 2) in the brachial segments, which is prior to axonogenesis of motoneurons. PL-cells develop in the region between the floor plate and the motoneuron nucleus. Their axons are the first neuronal processes ('pioneer axons') to arrive in the ventrolateral marginal zone and they project both rostrally and caudally to establish a primitive longitudinal association pathway at the ventrolateral surface of the neural tube. This pathway is formed before axons of C-cells arrive in the ventrolateral region. The first C-cells are initially located in the most dorsal portion of the neural tube, whereas later appearing C-cells are also located in both intermediate and ventral regions of the neural tube. The axons of C-cells project ventrally, without fasciculating, along the lateral border of the neural tube. Some of their axons enter the ipsilateral ventrolateral longitudinal pathway at st. 17. We often observed apparent contacts and interactions between preexisting axons of PL-cells and newly arriving axons of C-cells. The axons of commissural C-cells first enter the floor plate at st. 17 and cross the midline at st. 18. Axons of C cells begin to join the contralateral ventrolateral longitudinal pathway at st. 18+ to st. 19. In the floor plate region, contacts between growth cones and axons were often observed. However, axons in the floor plate at these stages were not fasciculated.

These observations establish the timing and pattern of growth of axons from two specific populations of early developing interneurons in the chick spinal cord. Additionally, we have identified an early and apparently previously undescribed 'pioneer' pathway that constitutes the first longitudinal pathway in the chick spinal cord.

Key words: ontogeny, immunocytochemistry, process outgrowth, pathway formation, axonal growth pattern, propriospinal, spinal cord, avian.

Introduction

Previous studies, including two reports from our laboratory, have shown that intersegmental projection neurons constitute one of the earliest developing pathways in the chick spinal cord (Ramón y Cajal, 1929; Windle and Orr, 1934; Hollyday and Hamburger, 1977; Holley, 1982; Okado and Oppenheim, 1985; Holley and Silver, 1987; Holley, 1987; Oppenheim *et al.* 1988). The cells of origin of these intersegmental neurons are located in the lateral margin of the neural tube and their axons project ventrally forming the circumferential pathway. In the ipsilateral ventrolateral region of the neural tube, some of these fibers change their direction of growth from circumferential to longitudinal, whereas others first cross the midline (commissural) and then also change the direction of growth from circumferential to longitudinal. The formation of this early longitudinal pathway occurs prior to that of the descending supraspinal system (Okado and Oppenheim, 1985; Oppenheim *et al.* 1988). Considering the easy accessibility of the chick spinal cord for experimental analysis, we believe

that this relatively simple interneuronal system provides an excellent model for the experimental analysis of axonal guidance mechanisms in the central nervous system of higher vertebrates.

Recently, Holley and Silver (1987), and Holly (1987) have examined the detailed projection pattern of axons of the C-cell system in the brachial segments of the chick embryo at stages 16, 17 (E 2.5) and 19 (E 3–3.5) using electron microscopy. They found that axons of the circumferential pathway grow ventrally in a non-fasciculative fashion along the lateral margin of the neural tube. They also reported the presence of unidentified longitudinal fibers in the vicinity of the ventral root at stage 17 (Holley and Silver, 1987). However, they neither examined stages prior to stage 16, nor did they study axonal projections in the ventral region of the neural tube. Accordingly, one of the major goals of the present study was to examine the origin of these early longitudinal fibers. A related goal was to determine the spatial–temporal relationship between this early pathway and axons arising from cells of the circumferential pathway. For this purpose, we utilized a monospecific antibody against a neuron-specific β-tubulin isoform, cβ4 (Frankfurter *et al.* 1986*a*,*b*; Sullivan *et al.* 1986; Lee *et al.* 1987) with whole mount preparations of the chick spinal cord. It is known that this antibody can label neuronal cells during or immediately after the final cell division (Moody *et al.* 1989).

Materials and methods

Fertilized eggs were obtained from Hubbard farm (Statesville, NC). The primary monoclonal antibody (TuJ1) was a generous gift from Dr A. Frankfurter at University of Virginia (Charlottesville, Virginia). Biotinylated secondary antibody and avidin–biotin–horseradish peroxidase complex were obtained from Vector.

The eggs were incubated in the laboratory (38°C, 60% humidity) until they reached the desired stage (stage 13–22). Embryos were removed from the shell, placed in a Petri dish containing phosphate-buffered saline (PBS) and carefullly staged through a dissecting microscope using the Hamburger–Hamilton (1951) morphological stage series. Following staging, whole embryos were placed in fixative (4% paraformaldehyde in 0.1 M phosphate buffer). In older embryos, the roof plate was opened to facilitate the penetration of antibodies. Two to five hours later, the neural tube was removed and placed in PBS. In some cases (stages 17–22), prior to fixation, the neural tube was removed, opened like a 'book' by cutting the roof plate and placed with fixative between two glass slides. Following thorough washing with PBS, the primary antibody (TuJ1) was applied at a dilution of 1:100 for 18 to 36 h in the refrigerator (4°C). After washing with PBS, the neural tube was immersed in biotinylated anti-mouse IgG antibody diluted at 1:200 for 3 h at room temperature. Solutions of primary and secondary antibodies contained 0.1% triton-X100. The avidin–biotin–horseradish peroxidase complex was applied for 1–2 h. The neural tube was processed for peroxidase histochemistry using diaminobenzidine (Sigma) as the chromogen. The 'opened' neural tube preparations were either dehydrated through a graded ethanol series and mounted with Entellan (Merck) or directly mounted with glycerol-gelatin (Sigma). In other whole mount neural tube preparations, the specimens were mounted with glycerin between two glass slides, observed and photographed. Following photography, these preparations were then dehydrated and embedded in epoxy resin. Twenty to 30 μm thick plastic sections were cut either horizontally, sagittally or transversally using a rotary microtome with a steel knife (Tosney and Landmesser, 1986). The sections were coversliped with Entellan. Because of the thickness of both sections and whole mount preparations, in many cases photographs were taken at several different focal planes and photomontages were made.

Results

Although observations were made from brachial, thoracic and lumber segments of the neural tube, for convenience of presentation we will focus primarily on the brachial cord. Other regions were identical to the brachial in the pattern of growth of both PL- and C-cells; only the timing was different. A rostral–caudal gradient of development was found in that the development of the brachial segments of neural tube, for example, occurred 6–8 h earlier than that of the lumbar segments. Even within the same region of neural tube (e.g. lumbar) a rostro-caudal gradient of development was observed.

The first β-tubulin immuno-positive cells were detected at stage (st.) 14 in brachial segments and at st. 16 in lumbar segments. Two different cell groups were identified based on their location in the neural tube and the direction of axonal growth at later stages. Cells of the first group (the primitive longitudinal pathway: PL-cells) were located adjacent to the floor plate. They were radially oriented, bipolar immature neurons that made contact with the ventricular lumen by their internal processes (Fig. 1A). Cells of the other group (the circumferential pathway: C-cells) were located in the most dorsal region of the neural tube. They were also radially oriented, bipolar immature neurons (Fig. 1B).

At the subsequent stages (st. 15 in the brachial and st. 17 in the lumbar segments), many primitive neurons at various stages of development were seen in the ventral region (Fig. 2). The cells were transformed from primitive bipolar neurons to monopolar neurons by losing their internal (medial) processes and by extension of axons from their distal (lateral) side. Most of the cell bodies of this group had migrated laterally to the ventrolateral region of the neural tube by the end of this stage. Their axons grew either rostrally or caudally in the ventrolateral region of the neural tube (Fig. 2B, D, E). Axons arising from cells that had not yet migrated to the ventrolateral region grew laterally at first then turned caudally or rostrally (Fig. 2A). Large, apparent lamellipodial type growth cones were observed that were flattened or extended mediolaterally (Fig. 2A,B, C). However, because immunocytochemical labelling of axons with β-tubulin may not visualize either filopodia or peripheral portions of growth cones (Forscher and Smith, 1988), the actual size of the growth cones may be even larger than they appear in these prep-

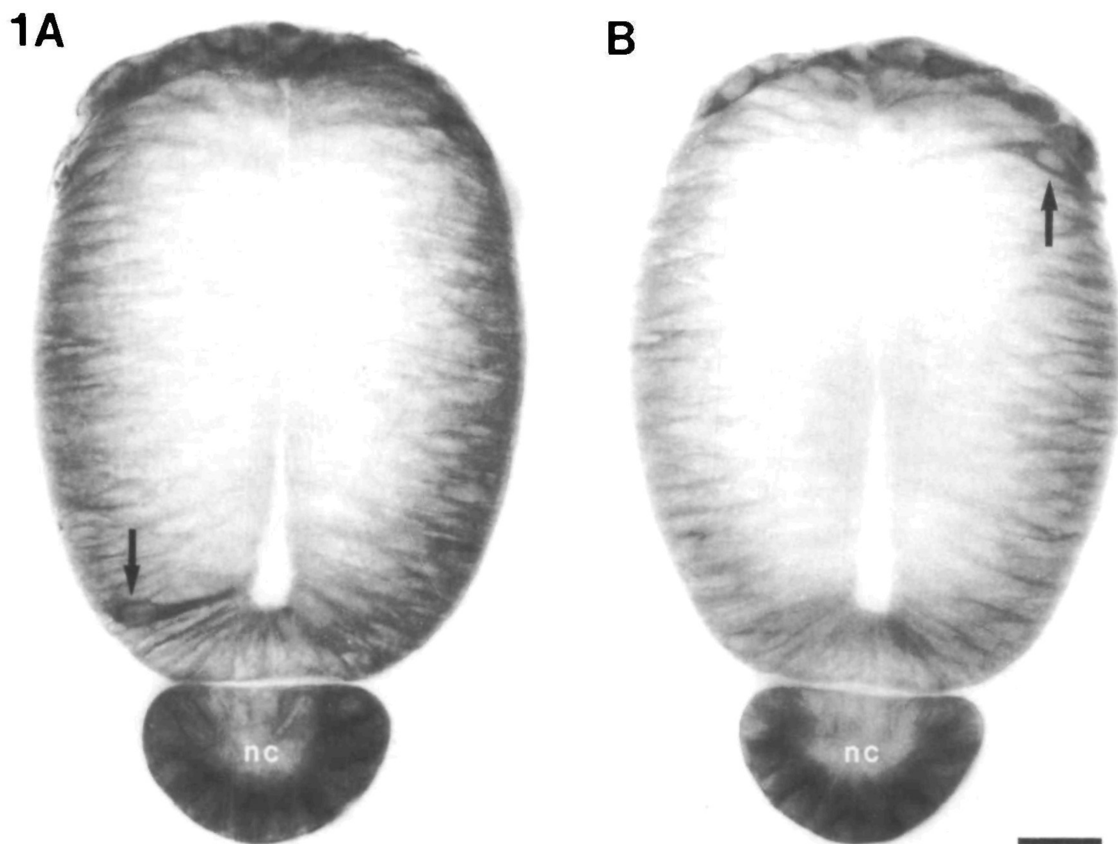

Fig. 1. Photographs of transverse sections of brachial neural tube of a st. 14 embryo. Note the presence of β-tubulin-positive cells (arrows) in the ventral (A) and dorsal regions (B). nc, notochord. Scale bar=20 μm for A and B.

arations. In the dorsal region, it was also found that the C-cells began to lose their internal process and to extend axons ventrally along the lateral surface of the neural tube (Fig. 2E).

Fig. 3 shows the pattern of axonal growth along the lateral surface of the brachial neural tube at st. 16. Axons of the two neuronal groups, C-cells and PL-cells, grew ventrally and longitudinally, respectively, and their lengths were almost identical. It was often observed that axons of PL-cells grew first in one direction for a short distance, but then made a U-turn and grew in a different direction (Fig. 3 inset). To determine whether or not there is a preferential direction (rostral *vs* caudal) for axonal growth of this cell group, the number of axons growing rostrally and caudally were counted in the brachial (st. 16, 4 cases) and lumbar (st. 18, 4 cases) segments. This analysis revealed that there was no significant directional preference in the brachial region (45:45) but that there was a small but significant preference for rostrally directed growth in the lumbar region (78:50, $P<0.05$ Chi-square). At this same stage, the axons of the β-tubulin-positive cells constituting the circumferential pathway first began to appear in the intermediate and ventral regions along the lateral surface of the neural tube. Their axons also grew ventrally and by the end of this stage some of them had arrived at the region where the axons of PL-cells were located. Presumptive motor neurons also began to express β-tubulin immuno-positivity at this stage. They appeared as radially oriented bipolar young neurons or spindle-shaped unipolar neurons and were located dorsally to the PL-cells. Later in this stage, some axons from the motoneurons began to project out of the neural tube (Fig. 3 arrows).

At st. 17 in the brachial segments and st. 19 in the lumbar segments, the number of cells contributing to the circumferential pathway increased, but the axons continued to be non-fasciculated (Fig. 4A). Some axons of this group had already made a right-angle turn to enter the ventrolateral longitudinal pathway (Fig. 4A–D), whereas others grew beyond this longitudinal pathway and entered the floor plate (Fig. 4A). However, only a few fibers crossed the midline at this time. We often observed axons of the circumferential pathway fasciculating or becoming closely associating with pre-existing longitudinal fibers (Fig. 4B,C). However, we have also observed that the axons of the circumferential pathway seem to be able to enter the longitudinal pathway without contacting axons of PL-cells (Fig. 4D). At this stage, the ventrolateral longitudinal pathway consists of axons arising from both PL- and C-cells. The axons in his pathway are partially fasciculated and are mainly located ventral to the outlet of motor neuron axons (Fig. 4A).

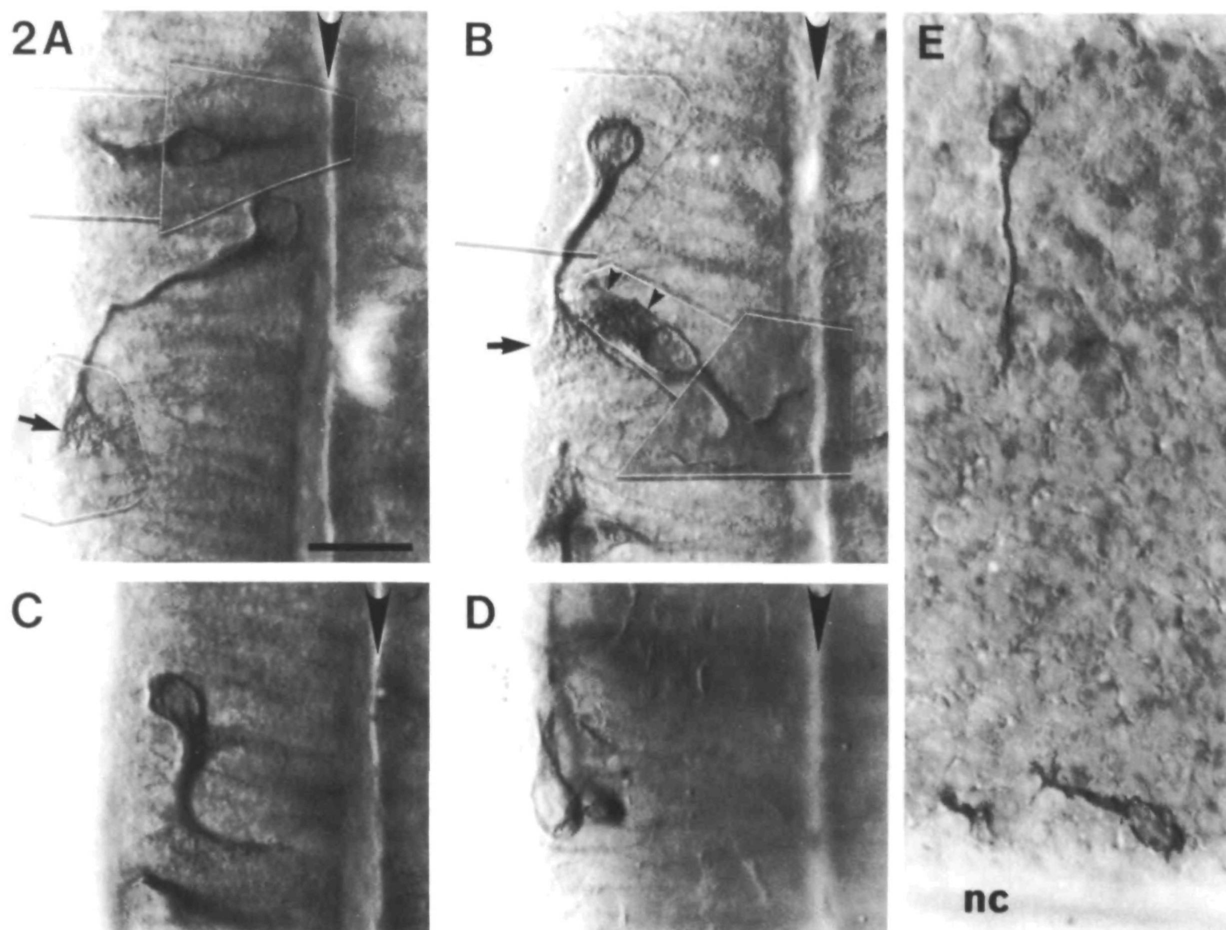

Fig. 2. Photographs and photomontages of young neurons observed in horizontal sections through the ventral portion of lumbar neural tube of st. 17 embryos (A–D) and in a whole mount preparation of brachial neural tube of a st. 15 embryo (E). The midline is indicated by large arrowheads (A–D). Rostral is towards the top in A–D and towards the left in E. Scale bar=20 μm in A–E. (A) Bipolar primitive neuron (upper) and monopolar neuron (lower) of PL-cells. Note that the cell body of the monopolar neuron is still located close to the midline. The axon of this cell grows laterally then turns caudally. A large growth cone is seen at the tip of the axon (arrow). (B) A monopolar neuron (upper) and a migrating primitive neuron (lower) of PL-cells. The cell body of the monopolar neuron has already migrated to the ventrolateral region of the neural tube. The axon with the large growth cone (arrow) is growing caudally. The migrating neuron has a large process on the distal side (arrowheads). A meshwork of microtubules can be seen in the process. (C) A presumptive monopolar PL-neuron. (D) A monopolar PL-neuron beginning to extend an axon rostrally. (E) A monopolar C-neuron (upper) extending an axon ventrally along the lateral surface of the neural tube and a monopolar PL-neuron (lower) extending an axon rostrally. Dorsal is to the top. nc, notochord.

Fig. 5 shows the projection pattern of axons of the commissural neurons in the ventral region of the brachial neural tube, including the floor plate region, at st. 18 to st. 21 (E3–3.5). The numbers of axons traveling in both this region and in the ventral funiculus increased rapidly during these stages. The axons first cross the midline at st. 18 and arrive at the contralateral ventrolateral longitudinal fascicules at late st. 18 to st. 19 (Fig. 5A,B). The majority of axons advanced across the floor plate by a relatively straight growth pattern with only slight sinuous growth and upon arriving at the contralateral ventrolateral longitudinal pathway, they projected either rostrally or caudally following right-angle or more gradual turns (Fig. 5C,D). However, we have also often observed some axons that advanced obliquely in the floor plate or that made turns before reaching the ventrolateral longitudinal pathway and then grew in parallel with it for a short distance (Fig. 5A, B). Most of the growth cones of the first axons entering the floor plate region grew without contacting other axons or growth cones (Fig. 5A). As the number of axons crossing the floor plate region increased, we often observed that later arriving growth cones made contact with other growth cones or with other axons that were growing either in the same direction or in the opposite direction (Fig. 5B). However, in spite of these contacts, the axons did not appear to fasciculate with each other over long distances. We have also sometimes observed regions in the ventral commissure containing greater numbers of axons separated by regions with fewer axons at st. 19 to st. 20 (Fig. 5B, C). At later stages these apparent groupings of axons were obscured

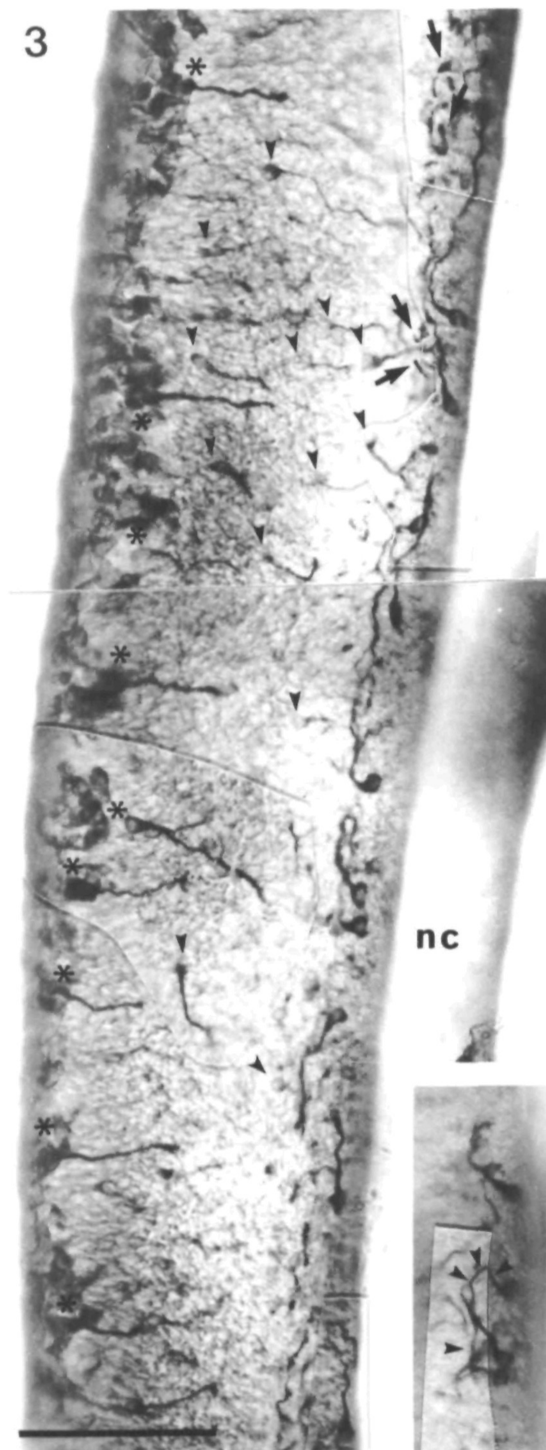

Fig. 3. Photomontage of a whole mount preparation of brachial neural tube of a stage 16 embryo showing the growth pattern of axons of both C-cells and PL-cells. Right side is ventral and top is rostral. Scale bar= 100 μm. Arrowheads indicate cells of the circumferential pathway that are located in the intermediate and ventral regions along the lateral margin of the neural tube. Arrows indicate the axons of motoneuron that project out of the neural tube *via* ventral roots. Asterisks indicate cells of the circumferential pathway located in dorsal regions of the neural tube. Inset shows an axon in a different embryo that makes a U-turn (arrowheads). nc, notochord.

by the greatly increased numbers of later growing fibers (Fig. 5D).

Longitudinal fibers were first observed in the dorsal funiculus at st. 20. Fibers in the lateral funiculus were not found until close to the end of the observation period (st. 21–22 in the brachial segments, not shown).

Discussion

In the present study, we have utilized immunocytochemical labelling of neurons and axons with a monoclonal antibody against a β-tubulin isoform, cβ4, which is neuron specific in the chick (Frankfurter *et al.* 1986*a*,*b*; Sullivan *et al.* 1986; Lee *et al.* 1987), in order to examine the early development of spinal cord interneurons in the chick embryo. It was shown previously that the β-tubulin antigen recognized by this antibody in both the central and peripheral nervous system appears either during or shortly after the terminal mitotic division (Moody *et al.* 1989). In the present study, we have been able to detect neuronal cells with this antibody beginning at very early stages (Figs 1, 2), thereby further demonstrating the usefulness of this probe for studying the early development of neuronal cells and their processes. Furthermore, by using a whole mount preparation of the neural tube, we were able to obtain much more comprehensive and detailed information about the temporal–spatial relationships between axons of specific groups of developing neurons. One possible technical limitation of this approach is that we cannot exclude the possibility that there are some neuronal cells that begin to differentiate as neurons but do not exhibit cβ4. However, we would argue that based on the following four lines of evidence this is rather unlikely. (1) cβ4 expression appears to be involved in early steps of neuronal differentiation that are shared by divergent neuronal systems. For instance, the early expression of cβ4 has been used as a marker for the initial development of trigeminal motor neurons and trigeminal ganglion neurons which are derived from different sources, placodes and neural crest (Moody *et al.* 1989). (2) The initiation of axonal growth observed by us with cβ4 is supported by results obtained from conventional electron microscopy using serial thin sectioning (Holley, 1987). (3) The location of cβ4-positive cells at st. 16 is consistent with the location of the earliest post-mitotic cells as revealed by tritiated thymidine autoradiography (Langman and Haden, 1970). (4) We have observed that double staining with cβ4 and another neuronal marker, Ng-CAM, indicates that Ng-CAM-positive neuronal cells are always cβ4 positive, whereas cβ4-positive cells are sometimes Ng-CAM negative at the earliest stages of differentiation (Shiga *et al.* in submission). Although none of these arguments taken separately are compelling, together they support our contention that cβ4 is a reliable marker for virtually all early differentiating central and peripheral neurons in the chick embryo. Another technical limitation is that this antibody probably does not visualize the most peripheral portions, including the

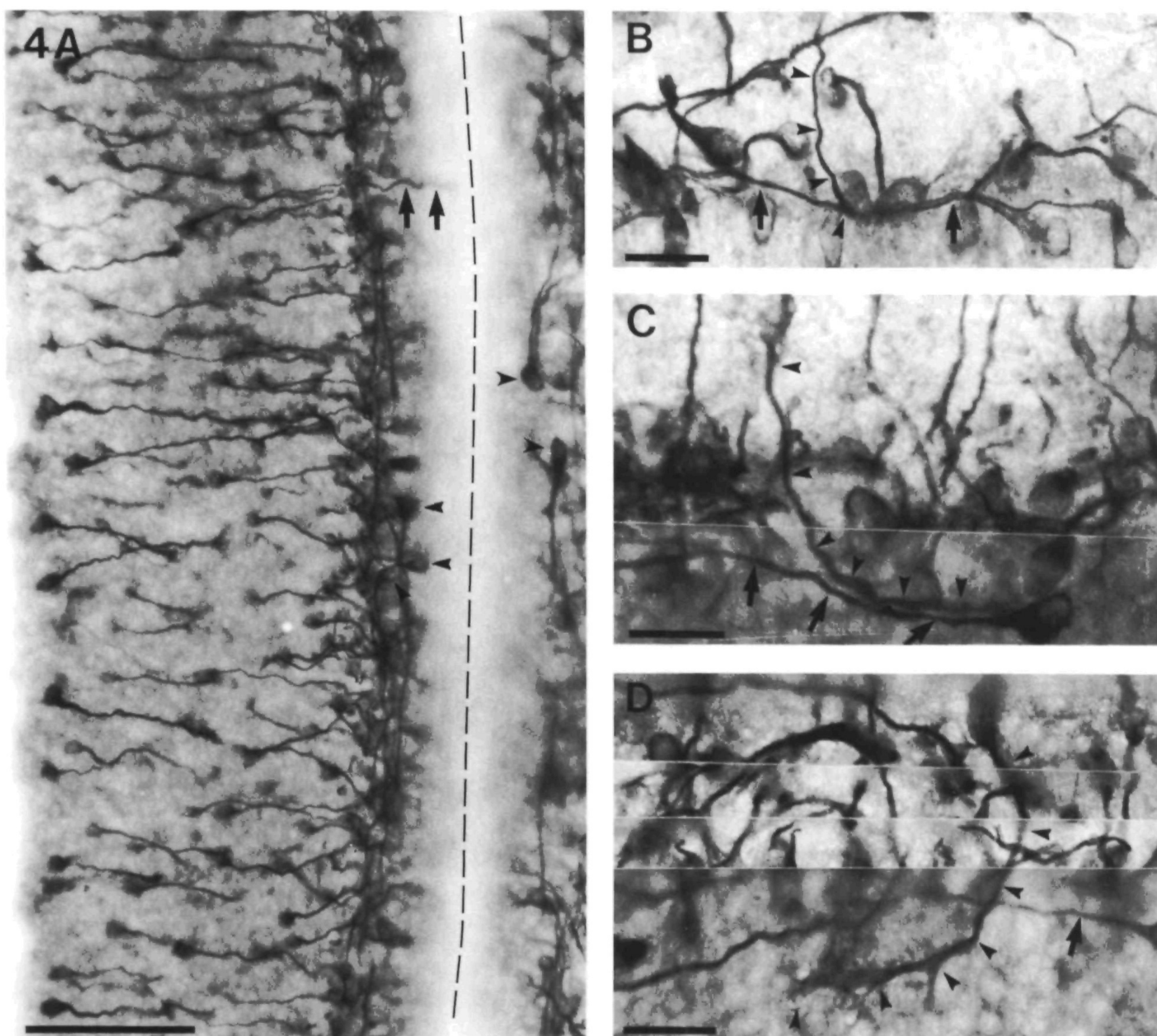

Fig. 4. Photographs and photomontages of whole mount preparations of brachial neural tubes of stage 17 embryos. Scale bars are 100 μm for A and 20 μm for B–D. (A) Lower magnification photograph of an 'opened' whole mount preparation showing the growth pattern of axons of both C-cells and PL-cells. The whole dorsoventral extent of the right side, the floor plate region and the ventral portion of the left side can be seen. The midline is indicated by a dashed line. Rostral is to the top. Arrows indicate an axon from a C-cell passing beyond the ventrolateral longitudinal pathway into the floor plate. Arrowheads indicate PL-cells. (B–D) Higher magnification photographs showing the relationship between axons of C-cells and axons of PL-cells. Arrowheads indicate the axons of C-cells that are turning and entering the ventrolateral longitudinal pathway. Arrows indicate the axons of PL-cells.

filopodia of growth cones, regions that are known to contain little if any tubulin molecules (Forscher and Smith, 1988). This limitation prevents us from determining more precisely the relationship between the growth cone and surrounding structures. However, ultrastructural studies currently in progress are providing more detailed information on growth cone interaction in this system.

Time schedule of early development of interneurons in the spinal cord

Fig. 6 provides a summary of the events of early development of spinal interneurons revealed by this study. Pathway formation in brachial segments precedes that of the lumbar segments by 6 to 8 h (also see Holley, 1982). This difference coincides with the reported differences in the birth dates of motoneurons in these regions as revealed by tritiated thymidine autoradiography (Hollyday and Hamburger, 1977). For convenience, the discussion below will focus only on events in the brachial segments.

We have demonstrated here that the earliest cβ4-positive cells are formed in the ventral and dorsal portions of the neural tube as early as st. 14 (Figs 1, 6A) and that these were primarily composed of radially oriented primitive neurons. At the next stage, these

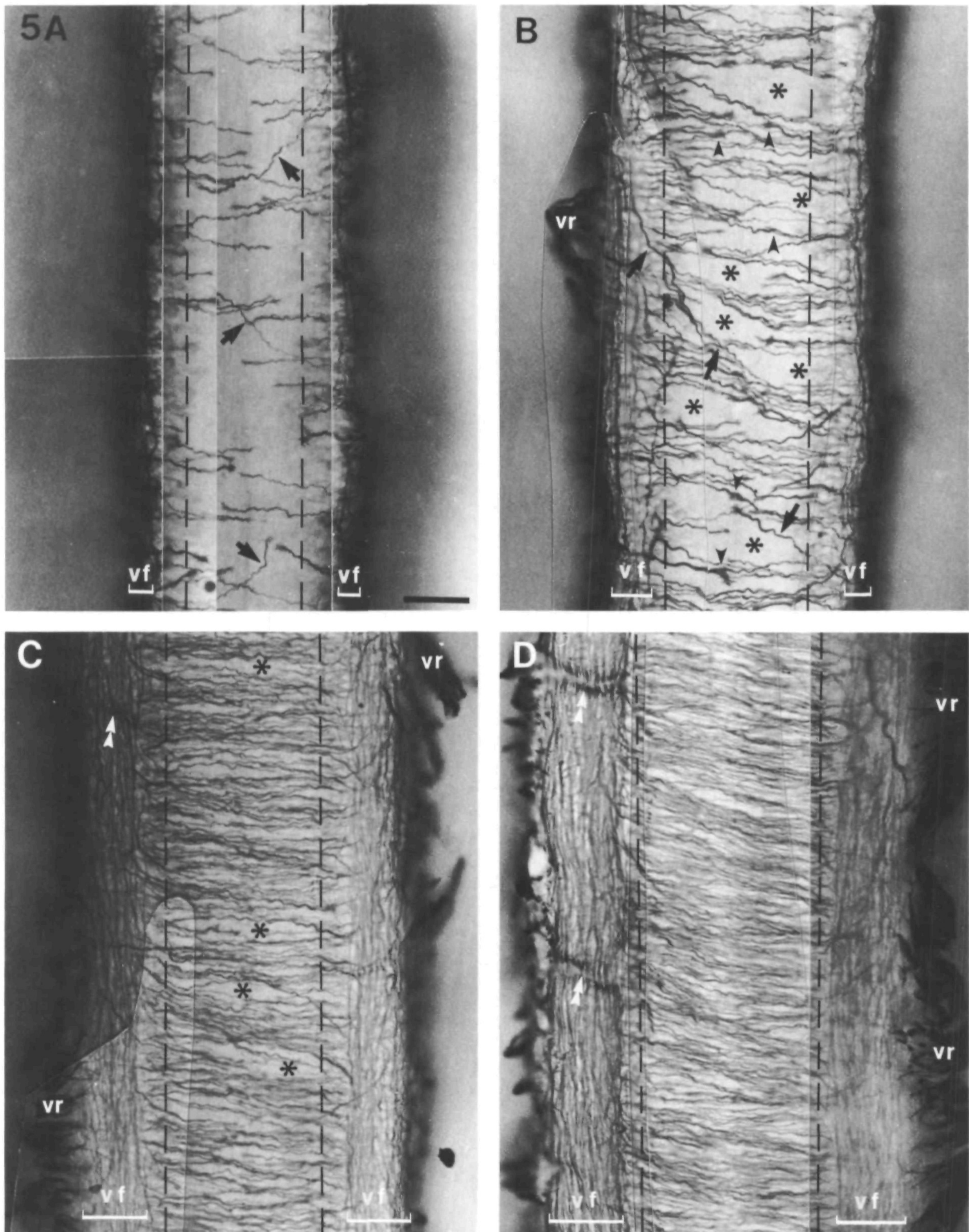

Fig. 5. Photomontages of ventral aspects of whole mount preparations of brachial neural tubes of stage 18 (A), stage 19- (B), stage 20- (C) and stage 21- (D) embryos. Lateral borders of the floor plate are indicated by dashed lines. vf, ventral funiculus. vr, ventral root. Scale bar= 50 μm for A–D. Arrows in A and B indicate axons which grow obliquely or turn longitudinally before they arrive at the contralateral ventrolateral marginal zone. Arrowheads in B indicate apparent contact between growth cones or growth cones and other axons. Note that there are loose groupings of axons at st. 19 and 20 (B and C) with regions containing greater numbers of axons separated by regions with fewer axons (asterisks). Double arrowheads in C and D indicate artifactual wrinkles in the preparations.

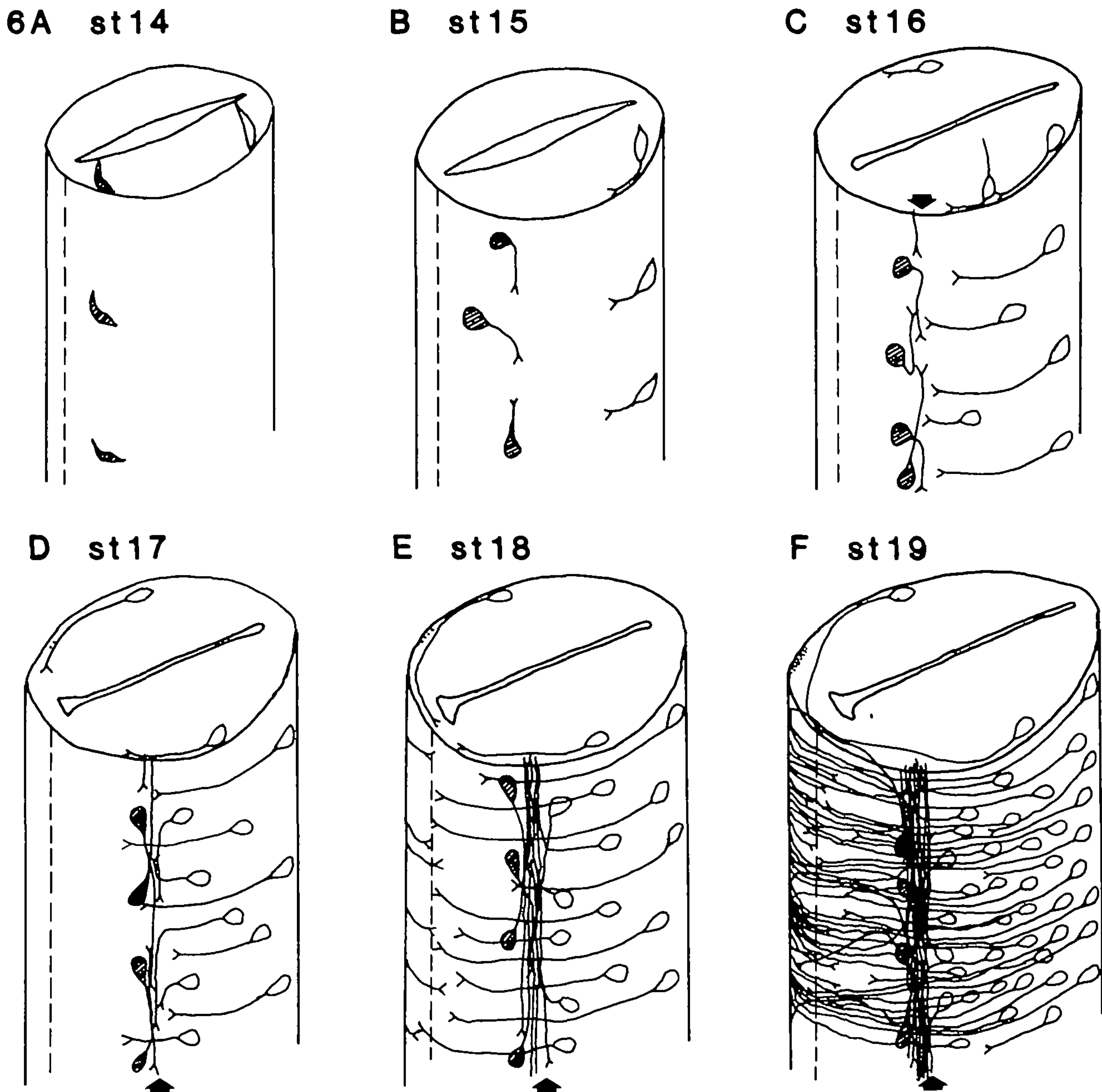

Fig. 6. Schematic diagrams summarizing the development of the circumferential and longitudinal pathways in the chick brachial neural tube. Development of motoneurons is omitted but the level of the ventral root is indicated by arrows. The midline is indicated by the broken lines. Ventral is to the left. Cells of the primitive longitudinal pathway are cross-hatched.

cells migrate laterally losing their contact with the lumen of the ventricle and begin to extend axons (Figs 2, 6B). The axons of the cells in the dorsal region (C-cells) grow ventrally along the lateral surface of the neural tube to form the circumferential pathway. By contrast, the axons of the cells in the ventral region (PL-cells) grow laterally then project rostrally or caudally, forming the primitive longitudinal pathway in the ventrolateral region of the neural tube. This pathway begins to form prior to the arrival of axons in the circumferential pathway (Figs 2, 6B). At the next stage (st. 16), additional cells of the circumferential pathway, located in the intermediate and ventral portion of the lateral margin of the neural tube, begin to extend axons (Figs 3, 6C). Motor neurons also begin to express a cβ4-positive reaction at this stage. Later in this stage, some axons of the C-cells (mainly axons originating from those cells in the intermediate or ventrolateral margin of the neural tube) arrive at the ventrolateral region of the neural tube where the primitive longitudinal pathway has formed. At the same time, axons of some motoneurons begin to grow out of the neural tube (Fig. 3). At stage 17, some axons of C-cells begin to turn to join the ipsilateral ventrolateral longitudinal pathway, whereas others first begin to enter the floor plate (Figs 4A, 6D). These latter fibers cross the

midline at st. 18 and begin to enter the contralateral ventrolateral longitudinal pathway at late st. 18 to st. 19 (Figs 5, 6E,F).

The circumferential pathway

The development of circumferential pathway neurons in the spinal cord has been described in several previous studies in the rat (Windle and Baxter, 1936), mouse (Holley, 1982; Wentworth, 1984), cat (Windle, 1931), human (Windle and Fitzgerald, 1937), sheep (Barron, 1944), and chick (Ramón y Cajal, 1929; Windle and Orr, 1934; Holley, 1982; Holley and Silver, 1987; Holley, 1987). According to the recent detailed studies by Holley and Silver (1987), and Holley (1987) on the chick, the cells of origin of this pathway are distributed along the lateral surface of both the dorsal and ventral portions of the neural tube. The axons of these cells were reported to grow ventrally in a non-fasciculated fashion. Our results are in general agreement with those of Holley and Silver (1987) and Holley (1987). However, by observing earlier stages and by using the β-tubulin antibody, we have been able to more clearly establish the time of onset and the pattern of projection in this pathway.

Using tritiated thymidine autoradiography, Hollyday and Hamburger (1977) demonstrated that there are some neurons that are born earlier than motoneurons and that are located in several different regions of the gray matter of the spinal cord at later stages. They concluded that the cells located at the base of the dorsal horn and at the lateral margin of the gray matter are the same neurons that were first described by Ramón y Cajal (1929) in the chick embryo and which are the first postmitotic neurons in the spinal cord. Our data provide additional support for the identity of these early developing interneurons.

The primitive longitudinal pathway

One of the most interesting observations of this study is our discovery of another early developing interneuron system, i.e. the primitive longitudinal pathway (PL-cells). As far as we know, this system has not been described previously either in the chick or in mammals. The reason for this oversight may be attributed to the location and small number of cells involved in this pathway. Because in the ventrolateral region of the neural tube the development of large numbers of motoneurons begins only slightly later than the cells of this primitive longitudinal pathway, the cells of this system may have been included as motoneurons in observations made from transverse sections of the neural tube. Indeed, we initially had some difficulty in distinguishing these cells from motoneurons in transverse sections. However, they can be easily distinguished from motoneurons by their orientation and by the direction of axonal growth when observed in whole mount preparations or in horizontal sections (Figs 2, 3).

Another reason why this system has not been described in the mammals may be owing to species differences between birds and mammals. It has been pointed out previously that there are, in fact, differences in the development of spinal pathways between mammals and birds (Windle and Orr, 1934; Windle and Baxter, 1936; Windle and Austin, 1936). Perhaps this pathway is not present in mammals.

Early developing longitudinal pathway systems have also been described in the spinal cord of frogs (Roberts and Clarke, 1982; Nordlander, 1984, 1989; Jacobson and Huang, 1985) and fish (Kuwada, 1986). Roberts and Clarke (1982) categorized spinal neurons of *Xenopus laevis* into nine groups based on their location, laterality and direction of axonal projections, and physiological function. They identified four groups that form longitudinal pathways: Rohon-Beard interneurons, descending interneurons, ascending interneurons and dorsolateral ascending neurons. Similar early pathways have been described in fish (Kuwada, 1986). The pathways described by us in the chick probably correspond to one or more of the early pathways described in fish and frogs.

As noted above, Holley and Silver (1987) reported the presence of longitudinal axons at the level of the ventral rootlet of the brachial spinal cord in st. 17 chick embryos, although they were unable to identify the cells of origin. According to our results, at st. 17 a few axons of the circumferential pathway have already joined the primitive ventrolateral longitudinal pathway. Therefore, the axons observed by Holley and Silver could belong to either the primitive longitudinal pathway or to the circumferential pathway.

Identification of the cells of origin of the primitive longitudinal pathway in later stages is not yet clear. In a previous study, we categorized intersegmental projection neurons into five groups according to their location, morphology and projections (Oppenheim *et al.* 1988). Judging from their location in relation to motoneurons, the cell groups previously designated as *ventral–medial* or *ventral horn* intersegmental neurons are the most likely candidates for the cells of origin of the primitive longitudinal pathway at later stages. This suggestion is supported by the observation that there are interneurons with early birthdates in these regions (Hollyday and Hamburger, 1977). Although these cells are known to have primarily commissural axons with some ipsilateral collateral branches (Hollyday and Hamburger, 1977; Oppenheim *et al.* 1988), the possibility that there are also some cells with only ipsilateral projecting axons in this region seems likely. Ramón y Cajal (1929) described similar large cells with axons, which enter the ipsilateral ventral marginal zones in the ventral horn in a E7 chick embryo. Therefore, we consider it likely that some of the cells in the *ventral–medial* or *ventral horn* intersegmental populations are the cells whose axons constitute the longitudinal pathway. Alternatively, the cells of this system may develop commissural collateral branches at later stages. It is also possible that cell death occurs in this system at later stages. Recently, transient projections of pioneer neurons have been reported in the vertebrate (McConnell *et al.* 1989), and invertebrate nervous system (Bate *et al.* 1981; Goodman *et al.* 1981; Loer *et al.* 1983; Stewart *et*

al. 1987; Klose and Bentley, 1989). These pioneer neurons have been observed to die or lose their initial projection after the adult patterns of axonal projections are established. We are currently examining the chick spinal cord at later stages (st. 20–25) in an attempt to identify dying cells in the ventrolateral region of the spinal cord that may be the cells of origin of the early longitudinal pathway. Although we have found some dying cells in this region at st. 20 (Homma and Oppenheim, unpublished observation), the identification of these as the cells of the primitive longitudinal pathway has not yet been firmly established.

Projection pattern of axons and mechanisms of axonal guidance

The two early developing interneuron systems described here appear to be 'pioneer neurons', in that their axons delineate the first pathways taken by other later developing axonal systems. Accordingly, it will be of considerable interest to examine the mechanisms that guide these axons along stereotyped pathways to their targets and to determine the role these axons may play in the guidance of axons of later developing systems.

Holley and Silver (1987) examined the projection pattern of axons of C-cells in the lateral surface region of the chick spinal cord, using serial parasagittal thin sections, and found that the axons grow ventrally in a non-fasciculative fashion. Based on their observations, they excluded the possible role of the basal lamina, mechanical guidance, pioneering axons, and guidepost cells as cues in the guidance of early C-cell axons. They, instead, favored a gradient mechanism, whereby chemotactic, differentially adhesive substrates or electromagnetic gradients in the dorso-ventral plane serve as cues for ventrally directed projections. In a companion paper, Holley (1987) proposed that the differential adhesivity of neuroepithelial cells and axons may play a role in guiding the axons ventrally. Katz and Lasek (1985), using computer simulation techniques, showed that by making certain assumptions, including differential adhesivity among axons and non-neuronal cells, initial polarity, internal constraints of axonal turning and axonal density, one can simulate the pattern of axonal growth of C-cells *in vivo*. Evidence in favor of chemotaxis or chemotropism by diffusible factors has been reported by Tessier-Lavigine *et al.* (1988). They provide evidence indicating that the ventral portion of the neural tube of the rat, including the floor plate, secretes a chemotropic factor(s) that guides the axons of commissural neurons toward the floor plate. Following various kinds of experimental manipulations of the chick embryo spinal cord *in vivo*, we have also observed that the floor plate region appears to exert a chemotropic-like effect on the projection of C-cell axons. For instance, following 180° rotation of three segments of thoracic neural tube around the dorso-ventral axis, it was observed that the axons of C-cells located in the vicinity of the junction between the graft and the unrotated segments grew in a U-shaped pathway turning across the junction and advancing towards the adjacent floor plate, which, however, was in an ectopic position for these cells. That is, the presence of an ectopic floor plate appeared to act as an attractant causing the nearby C-cells to alter their direction of growth (Yaginuma and Oppenheim, unpublished observation).

In the present study, we have examined the projection pattern of pioneer axons of the circumferential pathway in the ventral region of the neural tube, including the floor plate region. There are two fundamental questions regarding the cues that these axons follow as they navigate this pathway. (1) What mechanisms are resposible for determining that the axons of commissural neurons from the two sides, which are identical except for laterality, grow in opposite directions and cross the midline to reach the contralateral ventral longitudinal pathway? (2) Why do commissural axons ignore the ipsilateral ventral longitudinal pathway which probably provides cues identical to those of the contralateral side? One possible answer to these questions is provided by studies on the development of commissural axons in the rat spinal cord by Dodd *et al.* (1988). They found that the axonal cell surface glycoprotein, TAG-1, is expressed on axonal segments of commissural neurons in the spinal cord grey matter but is reduced or absent in longitudinal fiber tracts. By contrast, they found that the L1 glycoprotein is expressed predominantly in longitudinal fiber tracts. On the basis of these observations, Dodd *et al.* (1988) suggested that the spatio-temporal regulation of membrane glycoproteins may be involved in the projection pattern of the axons, especially in switching them from a non-fasciculative, circumferential pathway to a fasciculative, longitudinal one. They also suggested that the floor plate cells may play a role in this transition, based on the observation that the transformation of membrane glycoproteins occurs during the passage of axons across the floor plate (Dodd and Jessell, 1988; Dodd *et al.* 1988). Although this is a plausible scenario for the projection of C-cell axons in the rat embryo, it is unlikely that it applies to the chick embryo. We have observed that C-cells and their axons express the avian L1 homolog, Ng-CAM, all along their ipsilateral pathways toward the floor plate, as well as in the longitudinal pathway (Shiga *et al.*, in submission). Therefore, in the chick, L1/Ng-CAM is not likely to be responsible for altering the projections from circumferential to longitudinal.

We have observed that in the floor plate, axons continue to grow in a non-fasciculative fashion, similar to their earlier dorsal-to-ventral pattern of growth in the lateral margin of the neural tube (Fig. 5). However, by observing anterogradely labelled (HRP) C-cell axons in the floor plate with electron microscopy, it appears that the guidance mechanisms in this region are different from those in the lateral margins of the neural tube outside the floor plate. We have found that in the floor plate region, the growth cones exhibit a different morphology than in other regions. Furthermore, most of the growth cones in the floor plate contact the basement membrane through filopodia or by the main

body of the growth cone, and the axons are separated from each other by processes of the floor plate cells (Yaginuma *et al.* 1989 and unpublished observation). At a slightly later stage (st. 19), presumptive contacts between axons and growth cones were often seen in the floor plate (Fig. 5C) and axons tended to become loosely grouped together. Although the axons do not actually fasciculate with each other, we observed zones with more axons alternating with zones with fewer axons distributed along the rostrocaudal axis (Fig. 5B, C). This grouping of axons may indicate that pioneer axons are guiding the growth cones of later projecting axons by contact guidance.

The direction of axonal projections in the primitive longitudinal pathway is both rostral and caudal. The choice of direction does not seem to be determined by the polarity of original outgrowth from the cell but rather by cues provided by the environment surrounding the growing axons. We have often observed axons that extended in one direction but which then made a U-turn to grow in another direction. No preferential direction of growth was found in the brachial segments, but a slight rostral preference was found in the lumbar segments. The significance of this difference is unknown.

The axons of the C-cells first encounter the axons of the PL-cells at the ventrolateral region of the neural tube at st. 16+ to st. 17 and some fibers then turn at this point to join the longitudinal pathway. Although we have not yet been able to demonstrate a direct causal interaction between these two pathways, it seems likely that the pre-existing longitudinal axons may provide critical cues that initiate the directional choice of the axons of C-cells. Contacts between growth cones of C-cell axons and the pre-existing longitudinal fibers and fasciculation of circumferential axons into the longitudinal pathway have been reported for older embryos (st. 19) by Holley and Silver (1987). In related studies on insects, it has been shown that provisional contacts between growth cones and longitudinal axons *via* filopodia and selective fasciculation play an important role in the guidance of axons of G and C neurons when they switch their growth from commissural to longitudinal (Goodman *et al.* 1982; Raper *et al.* 1983*a*,*b*; Bastiani *et al.* 1984; Raper *et al.* 1984). Similar observations in fish embryos also supports the role of early developing longitudinal axons in the guidance of later appearing axons (Kuwada, 1986). We are presently examining axons from normal and experimentally manipulated chick embryos in the electron microscope in an attempt to substantiate the role of the primitive longitudinal pathway in altering the directional growth of the axons of C-cells from a circumferential to a longitudinal pathway.

We thank Dr A. Frankfurter for his gift of the TuJ1 antibody, Dr S. Moody for her help and advice, and Ralf Künzi for his conscientious technical assistance. This research was supported by an NSF grant (BNS 8707290) to R. W. Oppenheim.

References

Barron, D. H. (1944). The early development of the sensory and internuncial cells in the spinal cord of the sheep. *J. comp. Neurol.* **81**, 193–225.

Bastiani, M. J., Raper, J. A. and Goodman, C. S. (1984). Pathfinding by neuronal growth cones in grasshopper embryos III. Selective affinity of G growth cone for the P cells within the A/P fascicle. *J. Neurosci.* **4**, 2311–2328.

Bate, C. M., Goodman, C. S. and Spitzer, N. C. (1981). Embryonic development of identified neurons: Segmental differences in the H cell homologues. *J. Neurosci.* **1**, 103–106.

Dodd, J. and Jessell, T. M. (1988). Axon guidance and the patterning of neuronal projections in vertebrates. *Science* **242**, 692–699.

Dodd, J., Morton, S. B., Karagogeos, D., Yamamoto, M. and Jessell, T. M. (1988). Spatial regulation of axonal glycoprotein expression on subsets of embryonic spinal neurons. *Neuron* **1**, 105–116.

Forscher, P. and Smith, S. J. (1988). Actions of cytochalasins on the organization of actin filaments and microtubules in a neuronal growth cone. *J. Cell Biol.* **107**, 1505–1516.

Frankfurter, A., Binder, L. I. and Rebhun, L. R. (1986*a*). Identification of neuron-specific beta-tubulin. *Neurosci. Abstr.* **12**, 1123.

Frankfurter, A., Binder, L. I. and Rebhun, L. R. (1986*b*). Limited tissue distribution of a novel β-tubulin isoform. *J. Cell Biol.* **103**, 273a.

Goodman, C. S., Bate, C. M. and Spitzer, N. C. (1981). Embryonic development of identified neurons: origin and transformation of the H cell. *J. Neurosci.* **1**, 94–102.

Goodman, C. S., Raper, J. A., Ho, R. K. and Chang, S. (1982). Pathfinding by neuronal growth cones in grasshopper embryos. *Symp. Soc. Dev. Biol.* **40**, 275–316.

Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of chick embryo. *J. Morph.* **88**, 49–92.

Holley, J. A. (1982). Early development of the circumferential axonal pathway in mouse and chick spinal cord. *J. comp. Neurol.* **205**, 371–382.

Holley, J. A. (1987). Differential adhesivity of neuroepithelial cells and pioneering circumferential axons. *Devl Biol.* **123**, 389–400.

Holley, J. A. and Silver, J. (1987). Growth pattern of pioneering chick spinal cord axons. *Devl Biol.* **123**, 375–388.

Hollyday, M. and Hamburger, V. (1977). An autoradiographic study of the formation of the lateral motor column in the chick embryo. *Brain Res.* **132**, 197–208.

Jacobson, M. and Huang, S. (1985). Neurite outgrowth traced by means of horseradish peroxidase inherited from neuronal ancestral cells in frog embryos. *Devl Biol.* **110**, 102–113.

Katz, M. J. and Lasek, R. J. (1985). Early axon patterns of the spinal cord: Experiments with a computer. *Devl Biol.* **109**, 140–149.

Klose, M. and Bentley, D. (1989). Transient pioneer neurons are essential for formation of an embryonic peripheral nerve. *Science* **245**, 982–984.

Kuwada, J. Y. (1986). Cell recognition by neuronal growth cones in a simple vertebrate embryo. *Science* **233**, 740–746.

Langman, J. and Haden, C. C. (1970). Formation and migration of neuroblasts in the spinal cord of the chick embryo. *J. comp. Neurol.* **138**, 419–432.

Lee, M., Tuttle, J. B. and Frankfurter, A. (1987). Pattern of expression of a neuron-specific beta-tubulin in early developmental stages of chick embryo. *Neurosci. Abstr.* **13**, 701.

Loer, C. M., Steeves, J. D. and Goodman, C. S. (1983). Neuronal cell death in grasshopper embryos: variable patterns in different species, clutches and clones. *J. Embryol. exp. Morph.* **78**, 169–182.

McConnell, S. K., Ghosh, A. and Shatz, C. J. (1989). Subplate neurons pioneer the first axon pathway from the cerebral cortex. *Science* **245**, 978–982.

Moody, S. A., Quigg, M. S. and Frankfurter, A. (1989). Development of the peripheral trigeminal system in the chick

revealed by an isotype-specific anti-beta-tubulin monoclonal antibody. *J. comp. Neur.* **279**, 567–580.

NORDLANDER, R. H. (1984). Developing descending neurons of the early *Xenopus* tail spinal cord. *J comp. Neur.* **228**, 117–128.

NORDLANDER, R. H. (1989). HNK-1 marks earliest axonal outgrowth in *Xenopus*. *Dev. Brain Res.* **50**, 147–153.

OKADO, N. AND OPPENHEIM, R. W. (1985). The onset and development of descending pathways to the spinal cord of the chick embryo. *J. comp. Neur.* **232**, 143–161.

OPPENHEIM, R. W., SHNEIDERMAN, A., SHIMIZU, I. AND YAGINUMA, H. (1988). Onset and development of intersegmental projections in the chick embryo spinal cord. *J. comp. neur.* **275**, 159–180.

RAMÓN Y CAJAL, S. (1929). *Studies on Vertebrate Neurogenesis.* Revised and translated by L. Guth. Springfield, IL: Thomas.

RAPER, J. A., BASTIANI, M. J. AND GOODMAN, C. S. (1983*a*). Pathfinding by neuronal growth cones in grasshopper embryos. I. Divergent choices made by the growth cones of sibling neurons. *J. Neurosci.* **3**, 20–30.

RAPER, J. A., BASTIANI, M. J. AND GOODMAN, C. S. (1983*b*). Pathfinding by neuronal growth cones in grasshopper embryos. II. Selective fasciculation onto specific axonal pathways. *J. Neurosci.* **3**, 31–41.

RAPER, J. A., BASTIANI, M. J. AND GOODMAN, C. S. (1984). Pathfinding by neuronal growth cones in grasshopper embryos. IV. The effects of ablating the A and P axons upon the behavior of the G growth cone. *J. Neurosci* **4**, 2329–2345.

ROBERTS, A. AND CLARKE, J. D. W. (1982). The neuroanatomy of an amphibian embryo spinal cord. *Phil. Trans. R. Soc. Lond.* **B296**, 195–212.

SHIGA, T., OPPENHEIM, W. R. AND GRUMET, M. (1990). Neuron glia cell adhesion molecule (Ng-CAM) expression in the chick embryo spinal cord: Observations on the earliest developing intersegmental interneurons. (in submission).

STEWART, R. R., GAO, W.-Q., PEINADO, A., ZIPSER, B. AND MACAGNO, E. R. (1987). Cell death during gangliogenesis in the leech: Bipolar cells appear and then degenerate in all ganglia. *J. Neurosci.* **7**, 1919–1927.

SULLIVAN, K. F., HAVERCROFT, J. C., MACHLIN, P. S. AND CLEVELAND, D. W. (1986). Sequence and expression of the chicken $\beta 5$- and $\beta 4$-tubulin genes define a pair of divergent β-tubulins with complementary patterns of expression. *Mol. Cell. Biol.* **6**, 4409–4418.

TESSIER-LAVIGNE, M., PLACZEK, M., LUMSDEN, A. G. S., DODD, J. AND JESSELL, T. M. (1988). Chemotropic guidance of developing axons in the mammalian central nervous system. *Nature* **336**, 775–778.

TOSNEY, K. W. AND LANDMESSER, L. T. (1986). Neurites and growth cones in the chick embryo. Enhanced tissue preservation and visualization of HRP-labeled subpopulations in serial 25-μm plastic sections cut on a rotary microtome. *J. Histochem. Cytochem.* **34**, 953–957.

WENTWORTH, L. E. (1984). The development of the cervical spinal cord of the mouse embryo. II. A Golgi analysis of sensory, commissural and association cell differentiation. *J. comp. Neurol.* **222**, 96–115.

WINDLE, W. F. (1931). The neurofibrillar structure of the spinal cord of cat embryos correlated with the appearance of early somatic movements. *J. comp. Neurol.* **53**, 71–114.

WINDLE, W. F. AND AUSTIN, M. F. (1936). Neurofibrillar development in the central nervous system of chick embryos up to 5 days' incubation. *J. comp. Neurol.* **63**, 431–463.

WINDLE, W. F. AND BAXTER, R. E. (1936). Development of reflex mechanisms in the spinal cord of albino rat embryos. Correlations between structure and function, and comparisons with the cat and the chick. *J. comp. Neurol.* **63**, 189–209.

WINDLE, W. F. AND FITZGERALD, J. E. (1937). Development of the spinal reflex mechanism in human embryos. *J. comp. Neurol.* **67**, 493–509.

WINDLE, W. F. AND ORR, D. W. (1934). The development of behavior in chick embryos: Spinal cord structure correlated with early somatic motility. *J. comp. Neurol.* **60**, 287–307.

YAGINUMA, H., HOMMA, S., KÜNZI, R. AND OPPENHEIM, R. W. (1989). Growth cone morphology of commissural interneurons in the chick spinal cord. *Neurosci. Abstr.* **15**, 877.

(*Accepted 7 February 1990*)