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Axons arrest the migration of Schwann cell precursors

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SUMMARY

The neural crest gives rise to a variety of cell types including Schwann cells of the peripheral nervous system. Schwann cell precursors begin to differentiate early and migrate along specific pathways in the embryo before associating with nerve trunks. To determine whether motor axons direct the migration of Schwann cell precursors along specific pathways, we tested the effect of ablating the ventral half of the neural tube, which contains motor neuron cell bodies. The ventral neural tube was removed unilaterally from lumbar regions of chicken embryos at stage 17, when neural crest cells are just beginning to migrate and before motor axons have extended out of the neural tube. At several stages after ventral tube ablation, sections of the lumbar region of these embryos were stained with anti-acetylated tubulin to label developing axons, HNK-1 to label migrating neural crest cells and 1E8 to label Schwann cell precursors. In many embryos the ablation of motor neurons was incomplete. The staining patterns in these embryos support the idea that some Schwann cells are derived from the neural tube. In embryos with complete motor neuron ablation, at stage 18, HNK-1-positive neural crest cells had migrated to normal locations in both control and ablated sides of the embryo, suggesting that motor axons or the ventral neural tube are not required for proper migration of neural crest cells. However, by stage 19, cells that were positive for HNK-1 or 1E8 were no longer seen in the region of the ventral root, nor ventral to the ventral root region. Because Schwann cell precursors require neural-derived factors for their survival in vitro, we tested whether neural crest cells that migrate to the region of the ventral root in ventral neural tube-ablated embryos then die. Nile Blue staining for dead and dying cells in ventral neural tube-ablated embryos provided no evidence for cell death at stage 18. These results suggest that motor axons arrest the migration of Schwann cell precursors during neural crest migration.

Key words: neural crest, migration, peripheral nerve, Schwann cell, chicken

INTRODUCTION

Neural crest cells give rise to a variety of cell types, including Schwann cells of the peripheral nervous system. Neural crest cells migrate away from the dorsal aspect of the neural tube along particular pathways to different locations in the embryo (Weston, 1963; LeDouarin, 1982; Rickmann et al., 1985; Bronner-Fraser, 1986a; Loring and Erickson, 1987). Crest cells differentiate into specific cell types largely in response to environmental cues. It is not known, however, how particular neural crest cells become committed to specific cell lineages.

The observation that the abundant myelin glycoprotein P_0 is expressed on a subset of migrating neural crest cells (Bhattacharyya et al., 1991) suggests that Schwann cell precursors begin to differentiate early, long before association with axons. These Schwann cell precursors migrate along the ventral and ventrolateral pathways, but not along the subectodermal pathway (Bhattacharyya et al., 1991). The cues that direct Schwann cell precursors along pathways that are appropriate for their ultimate fate as Schwann cells are unknown. Because Schwann cells and their precursors require contact with axons for survival, for proliferation and for terminal differentiation (Wood and Bunge, 1975; McCarthy and Partlow, 1976; Salzer and Bunge, 1980; Salzer et al., 1980a,b; DeVries et al., 1982; Pleasure et al., 1985; Ratner et al., 1987; Jessen and Mirsky, 1991), it is reasonable that motor neurons and/or their axons may provide local cues to guide Schwann cell precursors along specific pathways and to stimulate or stabilize their differentiation.

To test whether motor neurons or their axons direct the migration of neural crest cells and subsequent differentiation into Schwann cells, we ablated the ventral neural tube from embryos at stage 17, just before motor axon outgrowth and neural crest migration, and then compared migration of neural crest cells and differentiation of Schwann cells in the presence and absence of motor axons. The results suggest that neural crest cells migrate normally to the region of the ventral root even in absence of motor axons, but that the motor axons are necessary to arrest the migration and promote the differentiation of Schwann cell precursors.

MATERIALS AND METHODS

Ventral neural tube ablations

Ventral neural tube ablations were performed as described in Landmesser and Honig (1986). Briefly, stage 17-18 (Hamburger and Hamilton, 1951) chick embryos were opened along the dorsal midline using a tungsten needle, exposing the inner surface of the neural tube. The ventral third to half of the neural tube was removed unilaterally from 5-6 somites in the lumbar region of each embryo using a micropipette and applied suction. After surgery, the embryos were allowed to develop to various stages and stained with Nile Blue or fixed in 4% paraformaldehyde for immunocytochemistry.

Antibodies and reagents

The degree of motor neuron removal was evaluated along the rostrocaudal extent of the ablated area by staining serial transverse sections of ventral neural tube-ablated embryos with a monoclonal antibody (6-11-B1) to acetylated α-tubulin (Piperno et al., 1987). This antibody labels axons early in development (Chitnis and Kuwada, 1990). Conditioned medium from hybridoma cells was a kind gift of Dr Gianni Piperno, Mount Sinai School of Medicine. To identify migrating neural crest cells, we used the monoclonal antibody HNK-1, which recognizes a carbohydrate epitope common to migrating neural crest cells and their derivatives (Tucker et al., 1984; Vincent et al., 1984). HNK-1 hybridoma cells were obtained from American Type Culture Collection. The 1E8 monoclonal antibody is specific for Schwann cells and their precursors in the chick and reacts with P_0 (Bhattacharyya et al., 1991). 1E8 IgG was partially purified from ascites fluid, and was used as described (Bhattacharyya et al., 1991). FP1 is a monoclonal antibody specific for the notochord and floorplate (Yamada et al., 1991). FP1 ascites fluid was a generous gift of Drs Tom Jessell and Jane Dodd, Columbia University.

Immunohistochemistry

Embryos were staged according to Hamburger and Hamilton (1951) and fixed in 4% paraformaldehyde. The embryos were then infiltrated with 20% sucrose and the lumbosacral regions were embedded in Tissue-Tek OCT embedding medium and quick frozen in liquid nitrogen-cooled isopentane. Serial transverse sections (10 μm) were cut on a Micron cryostat at –20°C and the sections mounted on gelatin-coated slides. Non-specific staining was reduced by incubation in 10% normal goat serum (NGS) and 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 1 hour. For double-label immunofluorescence, the sections were then incubated with anti-acetylated α-tubulin (1:30 of 6-11-B1 conditioned media in PBS + 10% NGS + 0.1% Triton X-100) overnight at 4°C followed by 1 hour in rhodamine-conjugated goat anti-mouse IgG (Cappel, Charlotte NC; 1:200 in PBS + 10% NGS + 0.1% Triton X-100). The sections were then incubated in conditioned medium from HNK-1 hybridoma cells (1:30 in PBS + 10% NGS) overnight at 4°C followed by 1 hour in FITC-conjugated goat anti-mouse IgM (Kirkegaard Perry Labs, Gaithersburg MD; 1:200 in PBS + 10% NGS). All steps were done at room temperature unless specified and three 5-minute washes in PBS were included between each step. The sections were mounted in glycerol-PBS (Citifluor, London) and viewed with a Zeiss fluorescence microscope. For single-label immunofluorescence, alternate serial sections were processed as above and incubated with either the FP1 monoclonal antibody (1:1000) or 6-11-B1 followed by rhodamine-conjugated goat anti-mouse IgG.

Sections to be immunostained with the 1E8 monoclonal antibody were incubated in 1E8 ascites fluid (1:5000) in PBS + 0.1% Triton X-100 overnight at 4°C and processed for immunoperoxidase staining using an avidin-biotin-horseradish peroxidase (HRP) procedure (Vectastain, Vector Laboratories, Burlingame CA) followed by diaminobenzidine-HCl (DAB). These sections were mounted in aqueous mounting medium (Shandon, Pittsburgh PA) and viewed on a Leitz microscope.

Nile Blue sulfate staining

Nile blue sulfate has been used to visualize patterns of cell death during embryogenesis (Saunders et al., 1962; Jeffs and Osmond, 1992; Jeffs et al., 1992; see Hinchcliffe, 1981 and Bowen, 1981 for review). As positive controls for Nile blue staining, we repeated the experiments of Saunders et al. (1962), obtaining the same patterns of Nile Blue staining in the developing limbs (Fig. 5A). After motor neuron ablation, embryos were stained with Nile Blue sulfate (Jeffs et al., 1992). Briefly, embryos were removed from the egg and placed in 10 ml Hank's balanced salt solution (HBSS) or L-15 medium (Gibco, Grand Island NY) with 30 μl of Nile Blue sulfate (1 mg/ml in water; Sigma, St. Louis MO; lot number 42H4372) for 30-40 minutes at room temperature with gentle shaking. Embryos were then washed for 1 hour in HBSS or L-15 under the same conditions. Embryos were fixed in 4% paraformaldehyde for 1 hour, infiltrated with 20% sucrose for 1 hour, and sectioned on a Micron cryostat at –20°C. Serial 25 μm sections through the ablated region were mounted in DPX (BDH Lab Supplies, Poole, U.K.) and the number of Nile Blue-positive cells on control and ablated sides were counted.

RESULTS

To test whether neural crest cells that will become Schwann cells require motor axons or signals from the neural tube for their migration or differentiation, we removed the ventral neural tube from chick embryos and evaluated the subsequent pathways taken by neural crest cells. Additionally, we analyzed the embryos for the presence of Schwann cell precursors. The ventral neural tube was removed in lumbar segments from embryos at the time when motor neurons are just starting to extend axons out of the neural tube (Hollyday, 1983; Tosney and Landmesser, 1985). At this time, neural crest cells in the lumbar region are beginning their migration away from the dorsal aspect of the neural tube (Rickman et al., 1985; Thiery et al., 1982; Bronner-Fraser, 1986a; Loring and Erickson, 1987).

Evaluation of ventral neural tube ablations

A segment of the ventral neural tube, approximately 5 somites in length, was ablated in each experimental embryo. After incubating the embryos for 6-18 hours, the extent of motor neuron removal was evaluated by staining alternate serial sections of the ablated segments with a monoclonal antibody to anti-acetylated α-tubulin (Piperno et al., 1987). This antibody has been used to label axons early in development and is useful for detecting single axons (Chitnis and Kuwada, 1990). Anti-acetylated α-tubulin staining revealed that motor neuron removal was usually complete in at least the region of the middle 3 somites of the ablated area. Fig. 1A,C shows representative sections from a stage 18 (A) and a stage 19 (C) embryo stained with anti-acetylated α-tubulin followed by a rhodamine-labelled secondary antibody. Over 100 operations were performed, but the success of motor neuron removal varied between embryos and within segments of the same embryo. All embryos, including embryos in which the surgery resulted in incomplete removal of motor neurons were examined for HNK-1 immunoreactivity. Only those segments in which motor neuron ablation was complete were used in neural crest migration analysis at stage 18 and 19.

Additionally, in some embryos, the entire rostral to caudal extent of the ablated area of ventral neural tube-ablated embryos was examined for the presence of the floorplate by

staining with the FP1 monoclonal antibody, which specifically labels the notochord and floorplate in developing embryos (Yamada et al., 1991). Fig. 1B and 1D show sections of a stage 18 (B) and a late stage 19 (D) embryo respectively, stained with FP1. Analysis of alternate serial sections of several embryos revealed that in the absence of motor axons, the floorplate is still present, confirming that the effects of the ablation (see below) do not result from removal of the floorplate. Interestingly, in older ventral neural tube-ablated embryos (stage 20 and older), the position of the floorplate was often shifted away from the midline, toward the ablated side of the neural tube. This distortion may be due to the growth of the embryo and shifting of the neural tube toward the space created by ventral neural tube removal.

Neural crest cells migrate along normal pathways in the absence of motor axons

To test whether neural crest cells require motor axons or the ventral neural tube to migrate properly to the ventral root and associate with motor axons, serial sections from embryos in which the ventral neural tube had been ablated were double-labelled with anti-acetylated α-tubulin, to locate motor axons, and with HNK-1, to label migrating neural crest cells. Examination of embryos (n=4) sectioned and stained at stage 18 (6-9 hours after ventral neural tube ablation) revealed HNK-1-positive neural crest cells in the sclerotome between the neural tube and the dermamyotome in both the control and ablated sides of the embryo, in agreement with previous studies of normal embryos (Thiery et al., 1982; Bronner-Fraser, 1986; Loring and Erickson, 1987). Some differences in crest migration patterns relative to unoperated embryos were seen, however, whereas some migrating neural crest cells are normally closely apposed to the neural tube, at the level of the somite, HNK-1-positive cells in operated embryos were seldom seen directly alongside the neural tube on either the control or ablated sides of the embryo (Fig. 2). Instead, there was a gap between the neural tube and the cells in the ventral migratory pathway. The apparent shift of the ventral migratory pathway alongside the neural tube appears to be due to a distortion caused by the bending open of the neural tube when it is opened along the dorsal midline during surgical manipulation. Other critical features of crest migration were unchanged in operated embryos. For example, HNK-1-positive neural crest cells and anti-acetylated α-tubulin-positive axons were only present in the anterior half of the somites (not shown), confirming that the segmental pattern of crest migration and axon outgrowth was not disrupted by the ventral neural tube ablation (not shown).

In caudal sections (early stage 18) of these embryos double-labelled with HNK-1 and anti-acetylated α-tubulin, motor axons have not yet emerged from the neural tube and HNK-1-positive migrating neural crest cells can be observed beneath the dermamyotome (Fig. 2A,B). Fig. 2 also shows more rostral sections (late stage 18) double-labelled with anti-acetylated α-tubulin (C) and HNK-1 (D). In these sections, no motor axons are evident on the ablated side, while motor axons on the control side have extended out of the neural tube. HNK-1-positive cells can be seen in the sclerotome between the neural tube and the dermamyotome on both sides. On the control side of ventral neural tube-ablated embryos, even very short ventral root fibers, as visualized with the anti-acetylated α-tubulin antibody, always had HNK-1-positive cells associated with them. This close association of neural crest cells with ventral root fibers has been reported previously in studies of neural crest migration (Loring and Erickson, 1987) and motor axon outgrowth (Rickmann et al., 1985). Finally, no HNK-1-

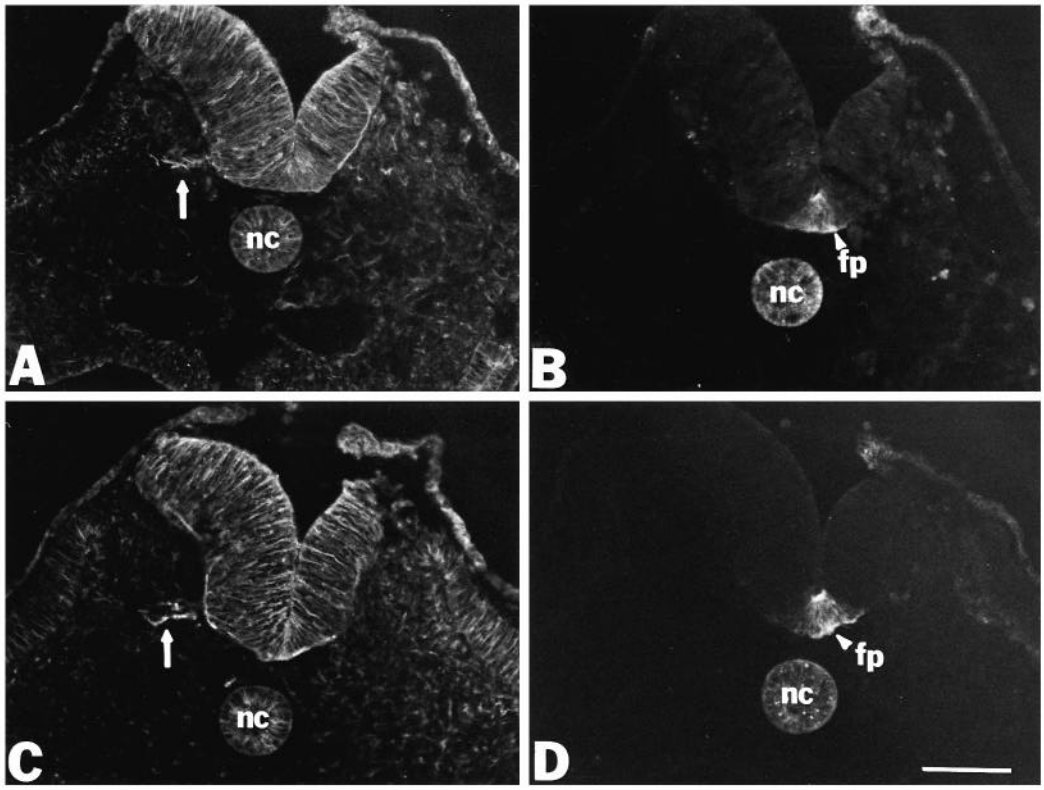

Fig. 1. Evaluation of the effects of ablation on the development of the ventral neural tube. Alternate sections of a stage 18 (A,B) and a stage 19 (C,D) embryo in which the ventral neural tube was ablated, were stained with anti-acetylated α-tubulin (A,C) or FP1 (B,D) followed by a rhodamine-conjugated secondary antibody. In each section, the control side is on the left. Axons are labelled by anti-acetylated α-tubulin on the control side, but none are present on the ablated sides (A,C; arrows). The FP1 antibody labels the cells of the floorplate and notochord and can be seen in sections of ventral neural tube-ablated embryos (B,D; arrowheads). nc, notochord; fp, floorplate. Bar, 100 μm.

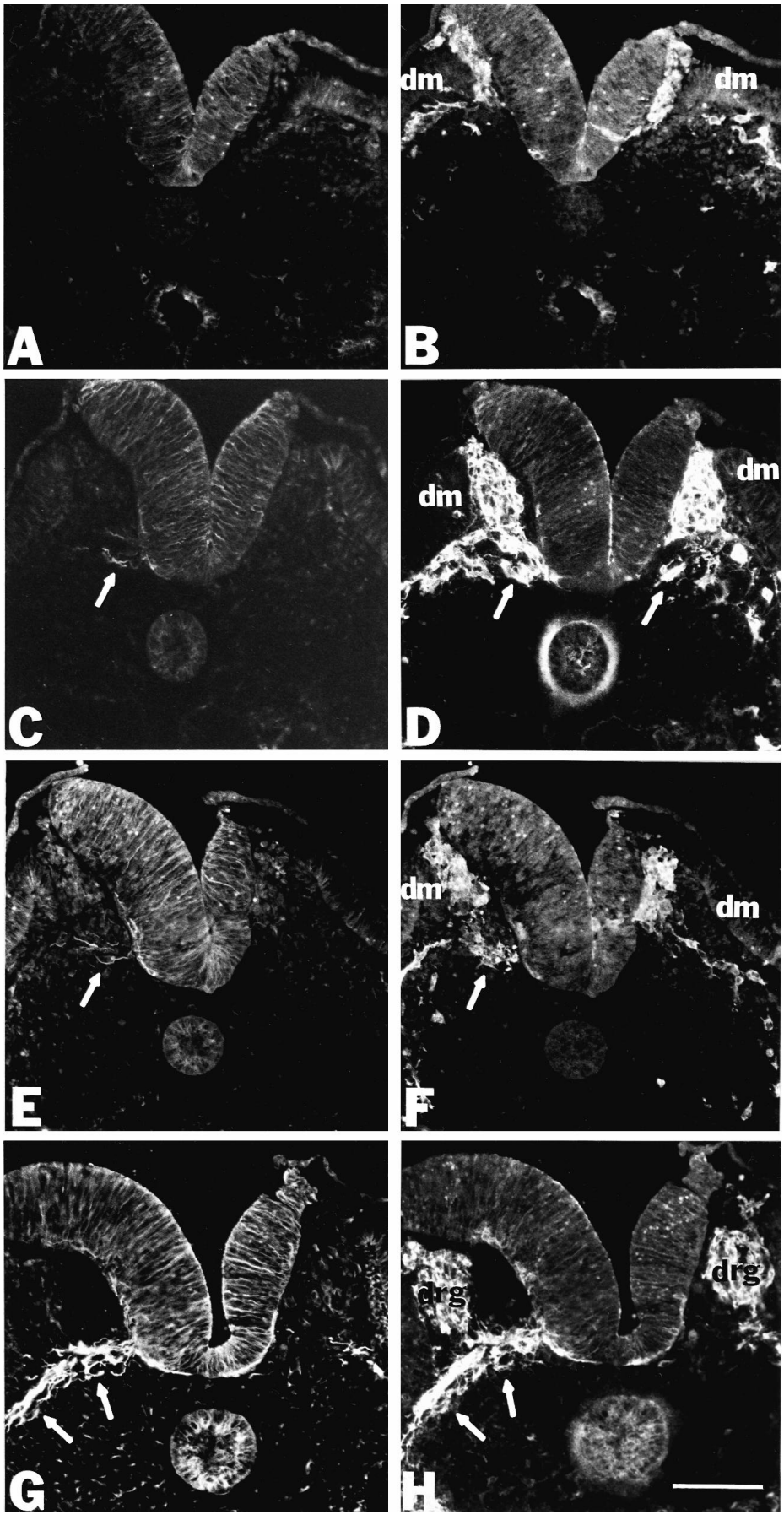

Fig. 2. Neural crest cells do not remain in the region of the ventral root in the absence of motor axons. Sections of ventral neural tube-ablated embryos were double-labelled at various stages of peripheral nerve development with anti-acetylated α-tubulin followed by a rhodamine-conjugated secondary antibody (A,C,E,G), and HNK-1 followed by a fluorescein-conjugated secondary antibody (B,D,F,H). The control side of the embryo is on the left. A and B show a section of an early stage 18 embryo, taken from the caudal region of the ablated segments, in which no axons are present on either the control or ablated side (A) and HNK-1-positive neural crest cells are present alongside the neural tube and beneath the dermamyotome (B). C shows a section of a stage 18 embryo in which motor axons, labelled by anti-acetylated α-tubulin, are present on the control side (arrow), but not on the ablated side. In the same section stained with HNK-1 (D), neural crest cells are located between the neural tube and dermamyotome and in the region of the ventral root on both sides (arrows). E and F show a section of a stage 19 embryo. Motor axons can be seen on the control side in E (arrow) and neural crest cells can be seen migrating between the neural tube and dermamyotome on both sides (F), but neural crest cells are present in the region of the ventral root only on the control side (arrow). Motor axons, labelled by anti-acetylated α-tubulin can be seen extending from the neural tube on the control side (arrows) in a section of a stage 21 embryo (G). In the same section, HNK-1-positive neural crest cells are beginning to form dorsal root ganglia (DRG) on both sides, but neural crest cells are associated with the ventral root only on the control side (arrows). dm, dermamyotome; drg, dorsal root ganglia. Bar, 100 μm.

Table 1. Analysis of HNK-1-positive cells in the ventral root region following ventral neural tube ablation

Stage	Number of embryos	Control	Ablated	*P*
18	3	35.0±6.1	22.5±1.2	0.0124*
18+	3	24.1±0.8	16.1±2.3	0.0022†
19	6	72.1±28.0	1.4±1.2	<0.0001‡

HNK-1-positive cells were counted on the control and ablated side of serial sections through the ablated segments of ventral neural tube-ablated embryos. Cells were counted in the region from the ventral root to the dorsal aorta (illustrated in Fig. 3). The cell counts are expressed as the number of cells per 200 μm (the approximate length of a somite). At all stages, there were significant differences in the number of HNK-1-positive cells in the ablated side, compared to the control side. The number of HNK-1-positive cells decreased over developmental time on the ablated side. *P* values were obtained using an unpaired *t*-test.

*Significant, †very significant, ‡extremely significant.

positive cells were observed ventral to the ventral root fibers (visualized by anti-acetylated α-tubulin) on the control side or ventral to the neural tube on the ablated side.

In the absence of motor axons, neural crest cells are not present at the ventral root region at later stages

Analysis of stage 19 embryos (*n*=5; 9-12 hours after ablation) revealed that HNK-1-positive cells in the region of the ventral root were virtually absent from the sclerotome on the ablated side relative to the control side. Fig. 2E,F shows sections of a stage 19 embryo double-labelled with anti-acetylated tubulin (E) and HNK-1 (F). Staining with anti-acetylated tubulin shows no motor axons on the ablated side, while motor axons on the control side have extended out of the neural tube and into the sclerotome. Migrating neural crest cells, labelled by HNK-1, can be seen between the neural tube and dermamyotome in both the control and ablated sides. However, although HNK-1-positive cells are associated with motor axons on the control side, no HNK-1-positive cells are observed within 50-100 μm of the neural tube in the ventral root region on the ablated side. The lack of HNK-1-positive cells in the ventral root region is maintained as peripheral nerve development progressed. G and H show a section of a stage 21 embryo in which neural crest cells are associated with extending axons on the control side, but are absent from the ablated side.

The number of HNK-1-positive cells in the region of the ventral root on the control and ablated sides of ventral neural tube-ablated embryos at stage 18 is summarized in Table 1. HNK-1-positive cells were counted in the region of the ventral root from the midline laterally to the edge of the ventrolateral migration pathway (defined as a stream of HNK-1-positive cells) and from a line perpendicular to the central canal of the neural tube at the ventral edge of the canal ventrally to the dorsal aorta. HNK-1-positive cells are found on both the control side and ablated side. Although the distibution of HNK-1-positive cells appears normal on the ablated side of embryos at stage 18, there are actually significantly fewer cells on the ablated side (Table 1). The greater number of HNK-1-positive cells on the control side (approximately 10 cells per somite) may be due to proliferation of HNK-1-positive cells that associate with the motor axons in the control side and/or a possible contribution of HNK-1-positive cells from the ventral neural tube.

Whereas approximately 100 HNK-1-positive cells were observed over the area of the ablation (approximately 3 somites) in the control side in each embryo, virtually no HNK-1-positive cells were found on the ablated side by stage 19. It should be noted that the few HNK-1-positive cells counted in the ablated side at stage 19 were located at the lateral boundary of the analyzed region, suggesting they might be crest cells migrating in the ventrolateral pathway.

Schwann cells are not present at the ventral root region in the absence of motor axons

To verify that not only HNK-1-positive cells but also presumptive Schwann cell precursors are absent from the ventral root region, sections of ventral neural tube-ablated embryos were stained with anti-acetylated α-tubulin, HNK-1, and 1E8, a marker for Schwann cells and Schwann cell precursors (Bhattacharyya et al., 1991). We have previously shown that 1E8-positive cells are a subset of HNK-1-positive cells (Bhattacharyya et al., 1991). Fig. 3 shows sections of a stage 18 embryo in which alternate sections were double-labelled with anti-acetylated tubulin (A,D) and HNK-1 (B,E) or stained with the 1E8 monoclonal antibody (C,F). 1E8-positive cells are found in the same locations as HNK-1-positive cells. On the control side, 1E8-positive cells are associated with ventral root axons, labelled by anti-acetylated α-tubulin, but 1E8-positive cells are absent from the ventral root region on the ablated side.

In all embryos analyzed, 1E8-positive Schwann cell precursors were never observed in the subectodermal pathway taken by presumptive melanocytes (not shown) on either the control or ablated side (not shown). This observation is consistent with the idea that, in the absence of motor axons, Schwann cell precursors do not migrate along an inappropriate pathway.

Cell death analysis

One hypothesis to explain why HNK-1-positive cells in ventral neural tube-ablated embryos are found in the region of the ventral root at stage 18, but are not present at later stages, is that HNK-1-positive neural crest cells migrate to the ventral root region, but subsequently die, perhaps due to lack of a survival factor normally provided by the neural tube. We tested this hypothesis by using Nile Blue sulfate to label dead or dying cells in the ventral root region after ventral neural tube ablation. Nile Blue sulfate has been used to visualize patterns of cell death during embryogenesis (Saunders et al., 1962; Jeffs and Osmond, 1992; Jeffs et al., 1992). Embryos were stained with Nile Blue sulfate 4, 6, 8, and 12 hours after ventral neural tube ablation and the number of Nile Blue-positive cells was counted in the area of the ventral root on the control and ablated sides of the embryo in 25 μm serial sections. Fig. 4 shows a control section from a region of the hindlimb of a normal stage 23 embryo known to contain dying cells, stained with Nile Blue sulfate (A) corroborating that the technique can be used to visualize dead or dying cells. In contrast, a section of an embryo stained with Nile Blue sulfate 8 hours after ventral neural tube ablation (B) contains hundreds of cells in the region of the ventral root, but contains no Nile Blue-positive cells. The Nile Blue-positive cell counts are shown in Table 2. Virtually no Nile Blue-positive cells were observed on either the control or ablated sides of the embryos. Similar results were obtained after propidium iodide staining of sections; dead cells were restricted to the site of surgical

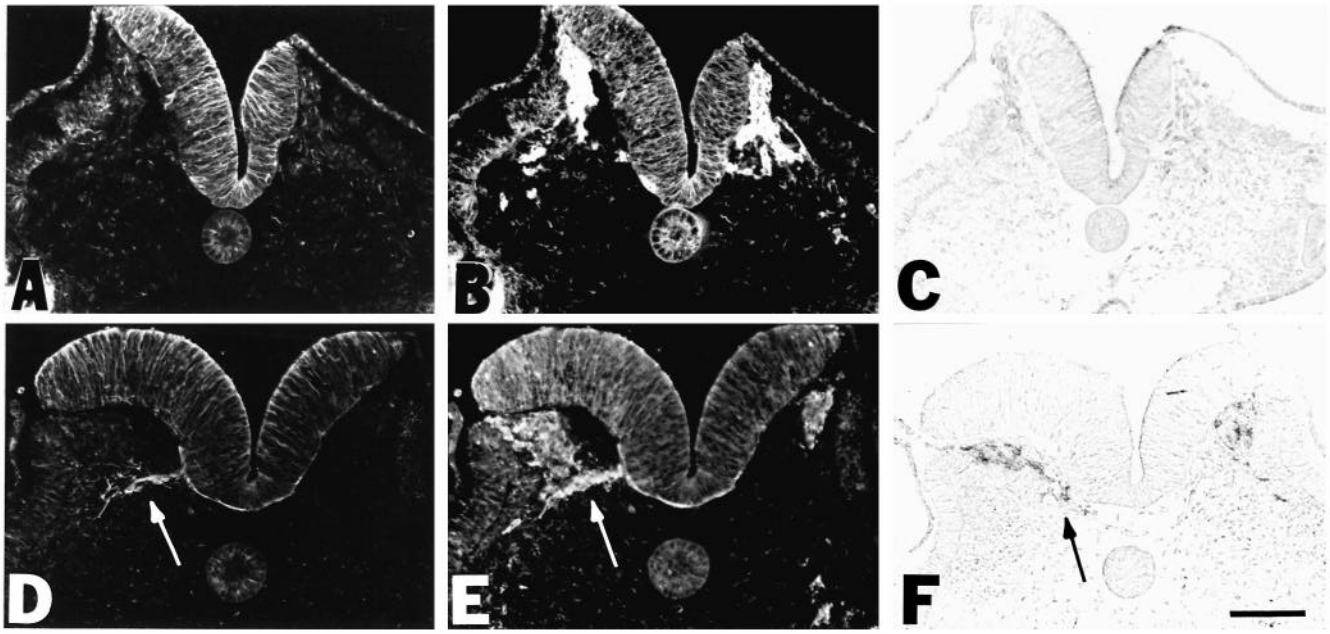

Fig. 3. Schwann cell precursors do not remain in the region of the ventral root in the absence of motor axons. Sections of a stage 18 (A,B,C), and a stage 19 (D,E,F) ventral neural tube-ablated embryo were processed for immunocytochemistry to visualize axons, neural crest cells and Schwann cell precursors. Alternate sections were double-labelled with anti-acetylated α-tubulin followed by a rhodamine-conjugated secondary antibody to visualize developing axons (A,D) and HNK-1 followed by a fluorescein-conjugated secondary antibody to visualize neural crest cells (B,E), or labelled with 1E8 followed by avidin-biotin-HRP and DAB to visualize Schwann cell precursors (C,F). The control side is on the left in all sections. At stage 18 (A-C), motor axons are not yet present on the control side (A). In B, neural crest cells can be seen in similar patterns, located between the neural tube and dermamyotome and in the region of the ventral root, on both the control and ablated sides. Similarly, in the adjacent section stained with 1E8 to label Schwann cell precursors (C), 1E8-positive cells are located between the neural tube and dermamyotome and in the region of the ventral root on both sides. In stage 19 sections (D,E,F), motor axons are present on the control side only (D, arrow) and neural crest cells are located between the neural tube and dermamyotome on both sides (E), but in the region of the ventral root only on the control side (E, arrow). 1E8-positive cells in the adjacent section (F) are also present between the neural tube and dermamyotome on both sides, but in the region of the ventral root only on the control side (arrow). The distal part of the ventral root is out of the plane of section in F. Bar, 100 μm.

incision and were absent from the ventral root region (not shown). These results strongly suggest that, in ventral neural tube-ablated embryos, the absence of neural crest cells in the ventral root region at stage 19 does not result from the death of crest cells that were present in this region at stage 18.

Incomplete ablations

Often, ablation of the ventral neural tube resulted in an incomplete removal of motor neurons, as assessed by anti-acetylated α-tubulin staining. These embryos proved to be useful in addressing the association of neural crest cells with axons. Fig. 5 shows sections of stage 18 (A,B), stage 19 (C,D), and stage 21 (E, F) embryos in which motor neurons, visualized by anti-acetylated α-tubulin (A,C,E) were incompletely ablated. Double-labelling of these sections invariably showed HNK-1-positive cells (B,D,F) associated with ventral root fibers on both the control and partially ablated sides of the embryo. Additionally, ventral neural tube surgeries occasionally resulted in the displacement rather than removal of motor neurons, leading to ectopic motor axon extensions from the neural tube. C and D show a section through a stage 19 embryo in which a single axon is extending out of the neural tube dorsal to the ventral root (C). HNK-1-positive cells are colocalized with this axon (D). In many of these cases, analysis of serial sections of the area of incompletely ablated embryos revealed that the HNK-1-positive cells associated with the axon on the ablated side were not continuous with the bulk of neural crest cells that were migrating between the neural tube and the dermamyotome.

Table 2. No dead or dying cells are observed in ventral root region following ventral neural tube ablation

	Mean number of Nile Blue-positive cells in ventral root region		
Hours after ablation	Number of embryos	Control	Ablated
4	6	0	0
6	4	0	0
8	9	3	2
12	6	0	2

After surgery, embryos were allowed to develop for 4, 6, 8, or 12 hours before Nile Blue sulfate staining and fixation. Serial sections from ablated segments of the embryos were mounted and the mean number of Nile Blue-positive cells was determined. The region between the neural tube and the dermamyotome on the control and ablated sides was analyzed for Nile-Blue-positive cells. Virtually no Nile Blue-positive cells were observed on either the control or ablated sides of the embryos.

DISCUSSION

Previous studies indicate that Schwann cells associated with

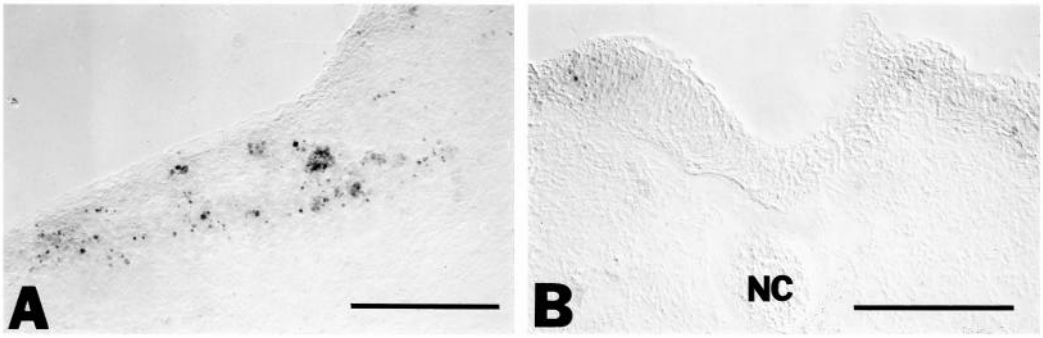

Fig. 4. No dead cells are present in the ventral root region in ventral neural tube-ablated embryos. Embryos were stained with Nile Blue sulfate (see Materials and Methods), sectioned and viewed. A shows a 25 μm section of the hindlimb of a stage 23 embryo stained with Nile Blue sulfate. The dense, granular staining indicates dead and dying cells. B shows a 25 μm section of an embryo stained with Nile Blue sulfate 8 hours after ventral neural tube ablation. NC, notochord. Bar, 100 μm.

peripheral nerves are derived largely from the neural crest (Harrison, 1924; Weston, 1963; LeDouarin, 1982; Carpenter and Hollyday, 1992) although some Schwann cells at the proximal portion of the ventral root appear to be derived from the ventral neural tube (Kuntz, 1922; Raven, 1937; Weston, 1963; Rickmann et al., 1985; Loring and Erickson, 1987; Lunn et al., 1987; Carpenter and Hollyday, 1992). Schwann cell precursors that are derived from the neural crest begin to differentiate early and then migrate along specific pathways before encountering and associating with axons (Bhattacharyya et al.,

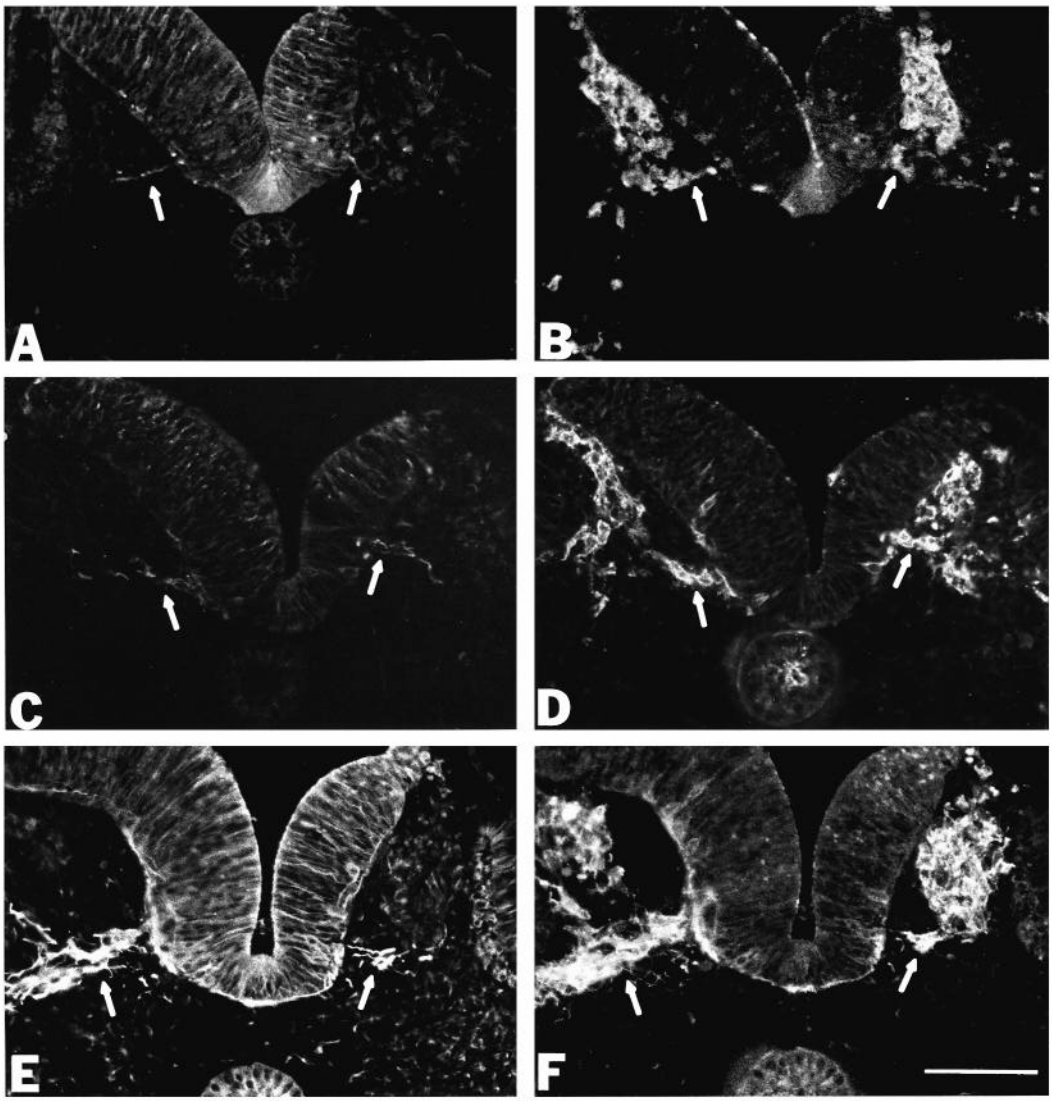

Fig. 5. Axons in incomplete ablations always have neural crest cells associated with them. Sections of ventral neural tube-ablated embryos were double-labelled at various stages of peripheral nerve development with anti-acetylated α-tubulin followed by a rhodamine-conjugated secondary antibody (A,C,E) and HNK-1 followed by a fluorescein-conjugated secondary antibody (B,D,F). The control side is on the left in all sections. A and B show a section of a stage 18 embryo in which motor axons are present on both the control and ablated side (A, arrows) and neural crest cells are associated with the axons (B, arrows). C and D show a section of a stage 19 embryo in which the surgery resulted in the displacement of motor neurons and ectopic motor axon extension on the ablated side (C, arrows). Neural crest cells are associated with the control axons as well as the ectopic axons on the ablated side (D, arrows). E and F show a section of a stage 21 embryo. Motor axons have extended out of the neural tube on the control side and some axons are present on the ablated side (E, arrows). Neural crest cells are associated with axons on both the control and ablated sides (F, arrows). Bar, 100 μm.

1991). The experiments described here were aimed at determining whether developing axons play a role in specifying the pathways taken by these migrating Schwann cell precursors. As discussed below, the major finding of this study was that removal of motor neurons before neural crest migration did not affect the initial migration of neural crest cells and Schwann cell precursors. Schwann cell precursors migrate from the neural crest to the region of the ventral root in both the presence and absence of motor axons. However, in the absence of motor axons, Schwann cell precursors are not retained in the region of the ventral root, suggesting that motor axons are necessary to arrest the migration of these Schwann cell precursors.

Several considerations suggest that the surgeries employed here did not cause non-specific disruptions of development. First, the dermamyotome and sclerotome formed normally and initial outgrowth of sensory neuron processes appeared to occur normally in the absence of motor axons. In addition, we saw no major defects in initial neural crest cell migration in either the presence or absence of motor neurons. Aberrant sensory axon projection patterns resulting from removal of motor axons (Yntema, 1943; Landmesser and Honig, 1986) occur at later stages than analyzed in our experiments. Thus, the changes in the migration of Schwann cell precursors that we observed at stage 18-19 in ventral neural tube-ablated embryos can most likely be ascribed specifically to the absence of motor axons.

Motor axons do not direct the migration of neural crest Schwann cell precursors

After ventral neural tube ablation, the pattern of neural crest migration on the control and ablated side of the embryos appeared similar at early stages. This observation provided the basis for a major conclusion of this study: that the presence of motor axons, motor neuron cell bodies or other ventral root constituents is not necessary for directing the early migration of neural crest-derived Schwann cell precursors. In contrast, the floorplate region of the ventral neural tube is known to provide chemotactic signals that direct the growth of axons from commissural neuron cell bodies in the dorsal neural tube (reviewed by Placzec et al., 1991).

Quantitation of neural crest cells at early stages did, however, reveal a significant difference, of approximately 10 cells per somite, between the number of cells on the control versus the ablated side. This difference in crest cell number at early stages could reflect the contribution of Schwann cells from the ventral neural tube on the control side. Previous studies indicate that some of the Schwann cells that populate the proximal ventral root are derived from the ventral neural tube (Kuntz, 1922; Raven, 1937; Weston, 1963; Rickmann et al., 1985; Loring and Erickson, 1987; Lunn et al., 1987) although a significant number of these Schwann cells are derived from the neural crest (Carpenter and Hollyday, 1992). Because Schwann cells are known to proliferate upon axonal contact (Wood and Bunge, 1975; McCarthy and Partlow, 1976; Salzer and Bunge, 1980; Salzer et al., 1980a,b; DeVries et al., 1982; Pleasure et al., 1985; Ratner et al., 1987), another possible explanation for the difference in number of Schwann cell precursors could be differences in proliferation of neural crest cells on the control and ablated sides of the embryo. In our studies, however, embryos were analyzed only 6-12 hours after ablation, which is barely enough time for these cells to reach the ventral root and then replicate, as previous studies have found that neural crest cells undergo 4-5 cell divisions in 48 hours in vivo (Fraser and Bronner-Fraser, 1991). Taking these explanations into consideration, we conclude that the initial migration of neural crest cells in the sclerotome between the neural tube and the dermamyotome is similar on the control and ablated sides of ventral neural tube-ablated embryos and the most probable explanation for the greater number of neural crest cells on the control side is the contribution of Schwann cells from the ventral neural tube.

Analysis of incomplete ablations in our study showed that even the few motor axons extending from the neural tube in incompletely ablated embryos invariably had HNK-1-positive cells associated with them as soon as they emerged from the neural tube. These cells were often disconnected from the bulk of neural crest cells, making it unlikely that these cells could have migrated from the neural crest. This interpretation is consistent with the idea that these Schwann cells were derived from the ventral neural tube. Despite the separations seen, it is also possible that the Schwann cells associated with these ectopic axons may have been recruited, via local cues, from the bulk of migrating Schwann cells. These possibilities could not be decisively distinguished by the limited data available.

Motor axons arrest migration of Schwann cell precursors

At later stages, the distribution of neural crest cells and Schwann cells precursors on the control versus ablated sides of ventral neural tube-ablated embryos was strikingly different. The possibility that this profound difference reflected the ablation of the ventral neural tube itself and, thus, the Schwann cells known to derive from the ventral nerual tube was considered. The recent study by Carpenter and Hollyday (1992) showed that in chick-quail chimeras both quail-derived neural crest cells and chick-derived cells, probably from the ventral neural tube, populate proximal regions of the ventral root. Thus, at least some of the cells in the ventral root are derived from the neural crest; while lack of ventral neural tube derived cells may contribute to the absence of cells in the region of the ventral root after ventral nerual tube ablation, our results cannot be explained simply by loss of ventral neural tube Schwann cells. Several other possible explanations for the reduction in the number of neural crest cells in the absence of motor axons were considered, including cell death, ectopic migration, or migration along other pathways. The first of these possibilities was excluded by Nile Blue staining in which no dead or dying cells were detected on the control or ablated side of ventral neural tube-ablated embryos. Second, no neural crest cells were observed in ectopic locations such as under the ectoderm or ventral to the region of the ventral root. Therefore, the most likely explanation is that Schwann cell precursors that normally associate with the emerging motor axons at the ventral root migrate with the bulk of neural crest cells in the ventrolateral pathway.

Several possible destinies exist for 1E8-positive Schwann cell precursors in the absence of the ventral root. The expression of the 1E8 antigen on cells during neural crest migration suggests that they have begun to differentiate and, therefore, may respond to cues, such as axonal cues, specific for their ultimate fate. 1E8-positive Schwann cell precursors

may encounter other axons, such as sensory axons, and differentiate into Schwann cells, retaining their 1E8-immunoreactivity. Alternatively, these Schwann cell precursors may be developmentally plastic and subsequently differentiate into other cell types in response to environmental cues and cease expressing the 1E8 antigen. We know that Schwann cells stop expressing the 1E8 antigen if removed from axons (Bhattacharyya et al., 1993), and that even relatively differentiated Schwann cells can transdifferentiate into melanocytes with proper cues (Ciment et al., 1986; Hess et al., 1988; Stocker et al., 1991). Therefore, Schwann cell precursors migrating from the neural crest may also stop expressing the 1E8 antigen if not associated with axons.

Mechanisms of Schwann cell precursor arrest

There are at least two possible mechanisms by which axons may stop the migration of Schwann cell precursors from the neural crest: by contact or via a diffusible cue. Complex permissive and inhibitory cues from the local environment are thought to direct the migration of neural crest cells. Neural crest cells migrate only in the anterior half of each somite (Rickmann et al., 1985; Bronner-Fraser, 1986a; Teillet et al., 1987; Loring and Erickson, 1987), suggesting that inhibitory cues in the posterior somite or permissive cues in the anterior somite direct neural crest migration. Neural crest cells are not seen in a region around the notochord, suggesting that inhibitory cues exist around the notochord (reviewed by Bronner-Fraser, 1993). Neural crest cells encounter numerous extracellular matrix and adhesion molecules during their migration (Newgreen and Thiery, 1980; Krotoski et al., 1986; Duband and Thiery, 1987; Perris et al., 1991) that have been implicated in the promotion of neural crest cell migration (Boucaut et al., 1984; Bronner-Fraser, 1986b; Poole and Thiery, 1986; Bronner-Fraser and Lallier, 1988). Schwann cell precursors migrating from the neural crest may simply find motor axons a more attractive substrate than the surrounding matrix. This mechanism is supported by the observation that motor axons always have neural crest cells associated with them (Rickmann et al., 1985; Loring and Erickson, 1987). Specific adhesion molecules, such as N-CAM, Ng-CAM (LI), and N-cadherin, may mediate and encourage contact of Schwann cells with axons (Hatta et al., 1987; Grumet and Edelman, 1984; Martini and Schachner, 1986; Letourneau et al., 1991). Subsequent signals from the axons may trigger further differentiation of the Schwann cells; the contact of Schwann cells with axons is known to stimulate the differentiation of Schwann cells later in development, including the expression of Schwann cell-specific antigens (Holton and Weston, 1982a,b; Jessen and Mirsky, 1991).

Alternatively, the ventral neural tube, motor neurons and/or motor axons may release short-range diffusible cues that attract or guide Schwann cell precursors to axons. Evidence for such a signal has been reported in cultures of rat neural crest cells, in which some cells express early Schwann cell markers (Smith-Thomas and Fawcett, 1989), but more cells express markers of mature Schwann cells in the vicinity of co-cultured axons, suggesting a diffusible factor from the axons stimulates Schwann cell differentiation (Smith-Thomas et al., 1990). Such a diffusible factor released by axons may lure Schwann cell precursors to axons, during neural crest migration, for further differentiation. In the absence of axons to attract Schwann cell precursors to the ventral root, therefore, Schwann cell precursors may continue to migrate with the bulk of neural crest cells.

Our results support the idea that the early differentiation of neural crest-derived Schwann cell precursors and their migration along specific pathways occurs without the influence of axons. Once the Schwann cell precursors reach the region of the ventral root, however, axons appear to be necessary to arrest the migration of these precursors and promote their further differentiation into Schwann cells.

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