Cell interactions that affect axonogenesis in the leech *Theromyzon rude*

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Summary

The leech nervous system comprises a relatively simple network of longitudinal (connective) and transverse (segmental) nerves. We have followed the normal pattern of axon development in the glossiphoniid leech *Theromyzon rude* by immunostaining embryonic preparations with antibody to acetylated α-tubulin. The dependence of the normal pattern of axon growth on cells in the mesodermal (M) and ectodermal (N, O, P and Q) lineages was examined by selectively ablating subsets of these lineages in developing embryos. We found that ablating mesoderm severely disrupted overall axonogenesis, while various ectodermal ablations induced a range of more specific phenotypes. In particular, formation of the posterior segmental nerve (PP) was abnormal in embryos deficient in primary neuroectoderm (N lineage). More specific ablations demonstrated that a subset of N-derived cells were required for establishing the PP nerve root. Previous studies have shown that the PP nerve root is normally pioneered by an O lineage-derived neuron (P_D). Our results suggest that the role of the N lineage-derived cells is to induce the migration of neuron P_D to its normal position in the posterior compartment of the hemiganglion.

Key words: *Theromyzon rude*, Leech, Nervous system, Cell lineage, *engrailed*

Introduction

The leech nervous system comprises a relatively simple network of longitudinal and segmentally iterated transverse nerves, yet provides adequate circuitry to support a broad behavioral repertoire. As a result, leeches have been used as a preparation to study the neural circuitry underlying behaviors ranging from shortening and local bending to forms of learning and memory (for reviews, see Kristan et al., 1988; Brunelli et al., 1997). Historically, hirudinid genera (*Macrobdella*, *Haemopis* and especially *Hirudo*) have been used for studying the adult nervous system and relatively late aspects of neural development (Retzius, 1891; Ramon y Cajal, 1904; Muller et al., 1987; Macagno et al., 1990; Stent et al., 1992; French and Kristan, 1994). By contrast, glossiphoniid genera (*Helobdella*, *Haementaria* and *Theromyzon*) are particularly useful for examining embryogenic processes because their embryos are large (~800 μm for *Theromyzon*) and develop normally outside of their cocoons; in addition, leech embryos undergo stereotyped cleavages (Whitman, 1878; Schleip, 1936; Sandig and Dohle, 1988) that give rise to identifiable cells, including neurons (Kramer and Weisblat, 1985). Moreover, these embryos are accessible to experimental manipulation throughout development.

Essential features of glossiphoniid leech development are shown schematically in Fig. 1. Segments arise sequentially from five bilateral pairs of stem cells called M, N, O, P and Q teloblasts. Each teloblast divides repeatedly, giving rise to segmental founder cells in a coherent column called a bandlet. During gastrulation, bandlets coalesce, first bilaterally and then longitudinally along the ventral midline to form the germinal plate. Cells in each lineage divide throughout gastrulation, leading eventually to the differentiation of definitive mesodermal and ectodermal progeny within the germinal plate. Both mesodermal (M) and ectodermal (N, O, P and Q) lineages contribute neurons to the 32 bilaterally symmetric, segmentally iterated ventral neuromeres of the central nervous system (CNS). In addition to a rostral, unsegmented, supraesophagael ganglion, the CNS comprises four fused neuromeres in the anterior subesophageal ganglion, seven fused neuromeres in a caudal ganglion associated with the posterior sucker and 21 neuromeres occurring as distinct midbody ganglia, separated from adjacent ganglia by interganglionic connective nerves (Stent et al., 1992). Each midbody ganglion contains ~300-400 neurons, depending on the species (Macagno, 1980). Cells arising from N, O, P and Q lineages differentiate into specific subsets of central and peripheral neurons and epidermal cells (Kramer and Weisblat, 1985; Braun and Stent, 1989a). More than two-thirds of the ganglionic neurons arise from the N teloblasts (Kramer and Weisblat, 1985); consequently, the N lineage has been used to follow morphological aspects of gangliogenesis (Fig. 1) (Shain et al., 1998; Shain et al., 2000).

Major features of axonal architecture within the 21 midbody segments are conserved between hirudinid and glossiphoniid leeches, but there are also significant differences. In adult *Hirudo,* bilaterally paired anterior and posterior segmental nerves exit the ganglion in each segment, then branch to yield

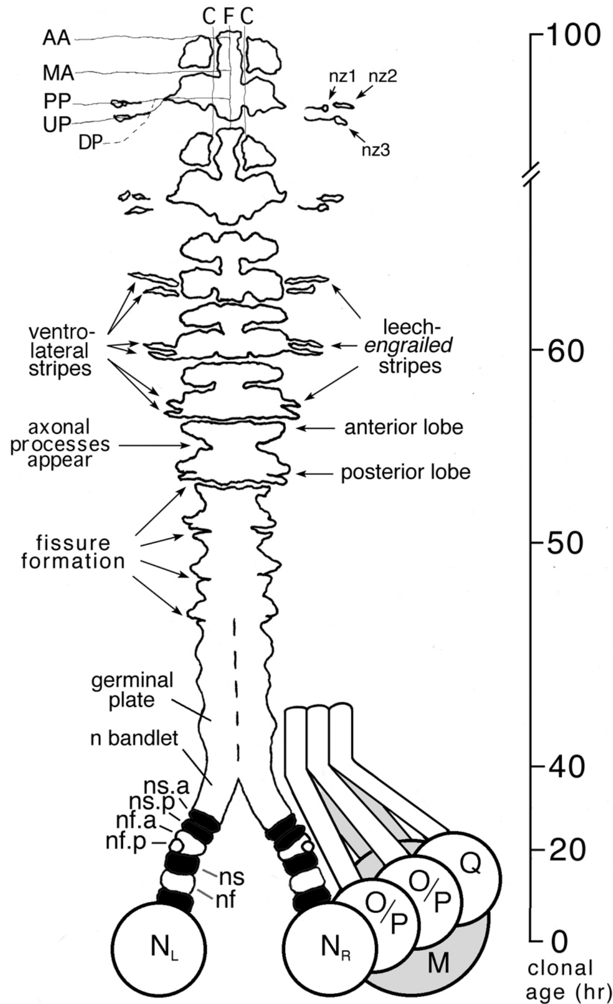

Fig. 1. Segmental development in *Theromyzon rude* illustrating the temporal progression of the main neurogenic lineage (N) and final positions of major segmental and longitudinal nerves. The relative positions of the mesodermal (M) and ectodermal (O/P, O/P and Q) teloblasts and their progeny are indicated on the right side only. Bilaterally paired N teloblasts (N_L and N_R) give rise to coherent columns of cells (n bandlets). Within the germinal plate, contralateral n blast cell clones lie in apposition across the ventral midline (broken line) and subsequently give rise to the bulk of the segmental ganglia of the ventral nerve cord, along with segmentally iterated peripheral neurons (nz1, nz2 and nz3) and a few epidermal cells (not shown). The O, P and Q teloblasts on each side give rise to distinct lineages that generate progressively more lateral and dorsal ectoderm (not shown here) (Weisblat et al., 1984). Ganglionic primordia result from the formation of transverse fissures that arise when the parent blast cell clones are ~50 hours old (Shain et al., 1998). Immediately following fissure formation (~50-55 hours), the first axonal processes appear between the anterior and posterior lobes of the N lineage, as evidenced by acetylated α-tubulin (ACT) antibody staining (see Fig. 2). The outgrowth of two ventrolateral stripes of cells from each posterior lobe (the anterior of which expresses the leech *engrailed*-class gene) occurs later at ~65 hours clonal age. By ~100 hours, the major nerve tracts have acquired their final positions in the juvenile leech (AA, anterior-anterior; MA, medial-anterior; PP, posterior-posterior; DP, dorsal-posterior; UP, ultraposterior; C, connective; F, Faivre's nerve). Approximate clonal ages for n blast cells and their derivatives are indicated at right. Anterior is upwards. Not drawn to scale.

anterior (AA), medioanterior (MA), posterior (PP) and dorsoposterior (DP) nerves. Developmentally, these arise as four discrete, roughly parallel tracts, which later condense to form the anterior and posterior nerves (Jellies et al., 1996). In glossiphoniid species, by contrast, three major nerve roots (AA, MA and PP), rather than two, project laterally from each hemiganglion (Kramer and Goldman, 1981) (Fig. 1). In addition, *Helobdella* and *Theromyzon* feature an additional, ultraposterior (UP) segmental nerve that branches from the PP nerve immediately outside the ganglionic margin (Braun and Stent, 1989a) and the DP nerve is less prominent in glossiphoniid species than in *Hirudo* (Kramer and Kuwada, 1983; Braun and Stent, 1989b).

In this study, we sought to identify modifications in development that account for the observed differences in the mature nervous systems of these leeches. In addition, because *Theromyzon* embryos are accessible to experimental manipulation during early development, we were able to ablate specific cell lineages, or subsets of lineages, to determine the cellular requirements for forming specific nerves. We were particularly interested in determining the significance of two transverse stripes of N lineage-derived cells that transiently connect the posterior margin of the ganglion to ventral body wall (Shain et al., 1998), and which contribute three N-derived, peripheral neurons to the nervous system (nz1-3) (Braun and Stent, 1989a). Previous studies indicated that these cells may play a role in establishing the normal projection of the PP segmental nerve (Shain et al., 1998). We show here that cells within the anterior, transverse stripe of N-derived cells appear to position an O lineage-derived neuron, P_D, that pioneers the PP segmental nerve tract.

Materials and methods

Embryos

Theromzyon rude embryos were obtained from specimens collected in the ponds of Golden Gate Park (San Francisco, CA), and were cultured as previously described (Torrence and Stuart, 1986), except that they were maintained at 12°C or 23°C.

Lineage tracer injections and cell ablations

Fluorescent lineage tracer [either fluorescein-dextran amine (FDA, Molecular Probes)] or tetramethylrhodamine-dextran amine (RDA, Molecular Probes) was injected into M, N, O, P or Q teloblasts as previously described (Weisblat et al., 1980). Cell ablations were achieved by irradiating FDA-labeled cells with the focused beam of a 488 nm argon laser (Hobbs or Lexel, Model 65) (Braun and Stent, 1989b) or by 'over-injecting' the cell of interest as described (Shain et al., 2000) with DNase (Blair, 1982) or ricin A chain (Nelson and Weisblat, 1992).

Immunohistochemistry

Fixed, dissected germinal plates were prepared and immunostained as previously described (Shain et al., 2000). Monoclonal antibodies specific to either leech muscle (Lan 3-14) (Zipser and McKay, 1981) or mouse acetylated α-tubulin (Sigma) were used at a 1:1000 dilution; Cy3- and Cy5-conjugated secondary antibodies (Jackson Lab) were diluted 1:400. TOTO-3 nuclear staining was performed according to the specifications of the manufacturer (Molecular Probes).

Microscopy

Germinal plates were viewed and photographed using either a Zeiss Axiophot microscope or a confocal microscope (BioRad model MRC-

1000/1024). Specimens were photographed using Ektachrome 400 film (Kodak) and scanned with a SprintScan 35 Plus (Polaroid) slide scanner. Adjustment of color levels and merging of images was performed with Adobe Photoshop 6.0. In generating digitally merged fluorescence images, the shades used to pseudocolor different signals were selected to optimize clarity and consistency, rather than to mimic the spectra of the fluorophores employed.

Results

Normal axonal development in *T. rude*

Axonal outgrowth during *T. rude* development was examined in germinal plates dissected from embryos fixed at progressively later stages and then immunostained with a crossreacting antibody directed against acetylated α-tubulin (ACT). Previous studies demonstrate that ACT antibody preferentially labels axonal processes in leech (Jellies et al., 1996). Because leech embryos generate homonymous segments sequentially from a posterior growth zone (see Fig. 1), it was possible to reconstruct axonal development chronologically by observing consecutive segments in several different embryos. Using this approach, a schematic representation of axonal development was assembled by tracing axon architecture at representative stages and fusing these images digitally (Fig. 2). For this purpose, we indicate the developmental state of individual ganglia in terms of the approximate 'clonal age' of their N lineage components, i.e. the time elapsed since the birth of the primary n blast cells whose progeny populate the ganglion (Shain et al., 1998). In presenting these results, we refer to the first detectable neurites in a particular pathway as 'pioneering' that pathway, without meaning to imply that they have any special role in its establishment.

By these criteria, the first axon processes appeared at ~50 hours clonal age on either side of the midline (Fig. 2). These bilateral processes projected medially and met at the ventral midline, where they took an anterior route. It is likely that these are the pioneering processes of the medial, unpaired Faivre's nerve, which spans from the subesophageal ganglion to the caudal ganglion in the adult leech CNS. Approximately 5 hours later (~55 hour clonal age), bilaterally paired, longitudinal fibers occupying positions of the future connective nerves were apparent on either side of the midline, and commissures formed a ladder-like structure along the ventral midline. The presumptive pioneering axons of the AA and MA segmental nerves appeared between 55-58 hour clonal age. The MA axon fiber(s) originated from a peripheral group of neurons corresponding in position to the P-derived pz8/cf3 cell cluster (Braun and Stent, 1989a). Pioneering of the MA nerve from the periphery is consistent with what has been observed in *Hirudo,* although these fibers are not ACT-positive in *Hirudo* (Jellies et al., 1996). The pioneering MA fibers in *T. rude*

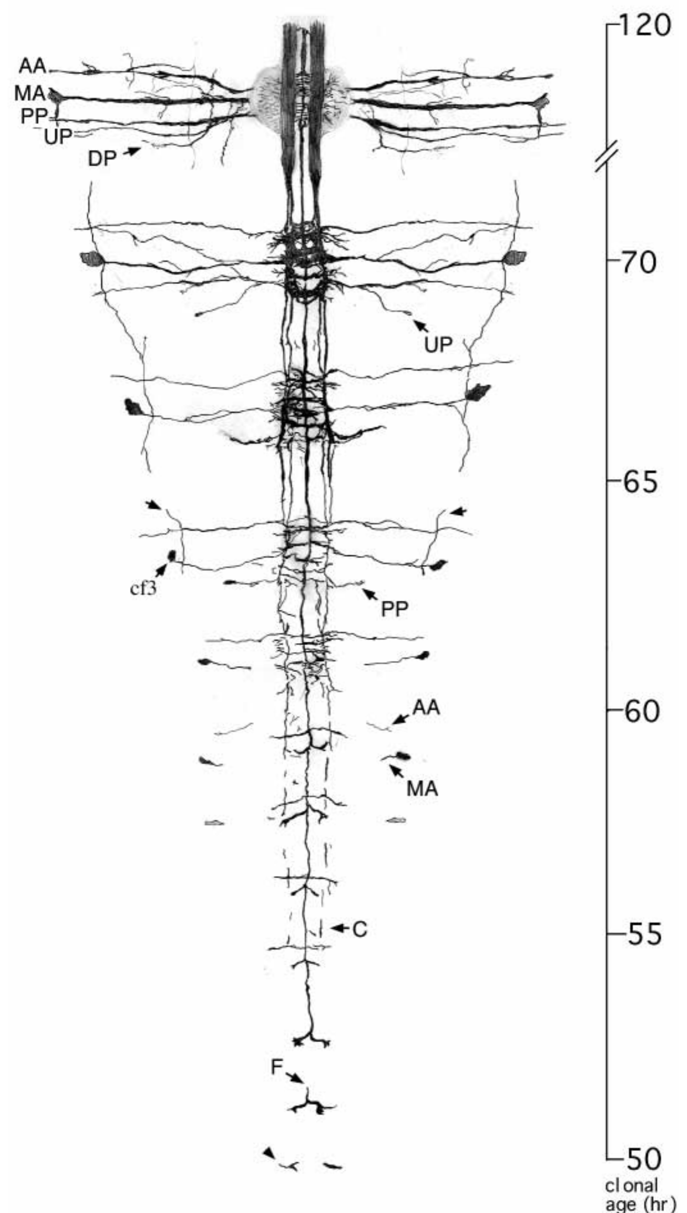

Fig. 2. Development of axonal processes in the leech nervous system. A monoclonal antibody to acetylated α-tubulin (ACT; Sigma) was used to stain axons in embryos of different stages. Tracings of nerves from representative segments were fused into a single image that depicts the major events during *T. rude* axogenesis. The time line indicates the approximate clonal age of axon fibers with respect to the corresponding birth of blast cells within the neuroectodermal (N) lineage. The first axon processes were observed at ~50 hours (arrowhead), appearing on either side of the prospective midline. These projected medially and met at the midline ~1 hour later, upon which they extended anteriorly to pioneer Faivre's nerve (arrow identified by F). Paired longitudinal axons occupying positions of the future connective nerves (arrow C) appeared at ~55 hours clonal age. The pioneering process of the MA and AA segmental nerves (arrows MA and AA, respectively) appeared almost simultaneously between ~55-58 hours clonal age; the MA axon fiber(s) originated from a peripheral location corresponding to pz8/cf3 (Braun and Stent, 1989a), while AA fibers appear to extend both medially and laterally from unidentified cells at the ganglionic margin. Processes of the PP segmental nerve (arrow PP) appeared at ~60 hours clonal age. A transient, lateral longitudinal nerve (arrows) was apparent at ~65 hours clonal age, positioned slightly medial to peripheral floret cf3 along the MA nerve. An axon marking the UP nerve (arrow UP) appeared at ~70 hours clonal age. At ~120 hours clonal age, the medial portion of the DP nerve was apparent (arrow DP), the segmental nerves were compressed longitudinally forming a cluster of three nerve roots and the ganglion was surrounded by glial sheaths. Anterior is upwards.

projected medially and connected with the central aspect of the ganglion by 60 hour clonal age. The AA axon fiber(s) originated near the anterior, lateral aspect of the ganglion and appeared to project processes both medially and laterally to form the AA segmental nerve.

At ~60 hours clonal age, axon fibers projected laterally from the posterior aspect of the ganglion to establish the PP nerve. The appearance of these processes coincided approximately in time and location with the formation of two transient, ventrolateral stripes of N lineage-derived cells near the posterior ganglionic boundary; cells in the anterior stripe express the leech *engrailed*-class protein (Wedeen et al., 1991; Lans et al., 1993; Shain et al., 1998).

Peripheral, longitudinal fibers originated from the pz8/cf3 cluster at ~65 hour clonal age. These bilaterally paired nerves projected across segmental boundaries and also moved laterally with the expanding germinal plate until ~100 hours clonal age, at which time they retracted. The first fiber(s) corresponding to the UP segmental nerve were observed at ~70 hours. Initially, these appeared to project from the N-derived nz3 neuron into the ipsilateral root of the PP nerve, and later sent processes lateral and parallel to the PP nerve. Processes of the DP nerve appeared at ~80 hours clonal age as a branch of the PP nerve, distal to the bifurcation of UP and PP. DP nerve projections of the Retzius neuron in glossiphoniid leeches have been described previously (Stuart et al., 1987; Elsas et al., 1995).

By clonal age ~120 hours, the neuroarchitecture of the *T. rude* nervous system was essentially as described for a mature glossiphoniid leech (Braun and Stent, 1989a). In contrast to *Hirudo* (Jellies et al., 1996), there was no condensation of the AA and MA nerves, thus the three segmental nerves remained distinct at the edge of the ganglion; also by this time, more than 10 well-defined commissures were evident across the midline. The longitudinal connectives and Faivre's nerve were considerably larger in diameter than at earlier stages, and ganglia had become encapsulated by connective tissue sheaths.

Normal axon contributions from each teloblast lineage

To establish the contributions of each teloblast lineage (M, N, O, P and Q) to the development of specific nerves, subsets of neurons were labeled by unilateral injections of FDA into each teloblast and the distribution of labeled axons was observed by epifluoresence in the nerves of midbody ganglia in which the N lineage cells were ~100 hours clonal age. A summary of data obtained from this analysis is presented in Table 1. The neuroectodermal (N) lineage displayed the broadest distribution of axon projections, contributing processes bilaterally to all 11 nerve tracts that were scored. The other ectodermal lineages (O, P and Q) contributed primarily to ipsilateral segmental nerves (AA, MA and PP) and longitudinal connectives, while the mesodermal (M) lineage, contributed processes only to the longitudinal connectives and Faivre's nerve.

Segmental nerve patterning in experimentally perturbed embryos

To distinguish autonomous from non-autonomous aspects of the nerve patterning process, we sought to identify changes in the normal patterns of nerve outgrowth induced by systematic, unilateral ablations of each lineage (M, N, O, P and Q). Typical results are illustrated in Fig. 3. Mesodermal ablations produced severe disruptions in the normal pattern of nerve fibers, resulting in a disorganized array on the ablated side (Fig. 3A). Any specific effects of M lineage ablations on nerve patterning were masked by the fact that this treatment also disrupts gangliogenesis (by disrupting the normal distribution of ectodermal progeny) (Blair, 1982; Torrence et al., 1989).

Table 1. Projections of midbody M-, N-, O-, P- and Q-derived neurons into longitudinal and segmental nerves

	M (10)	N (6)	O (12)	P (5)	Q (6)
Ipsilateral					
AA	0	6	7	3	6
MA	0	4	7	5	6
PP	0	6	12	3	2
UP	0	6	1	0	0
C	5	3	8	5	6
Contralateral					
AA	0	6	1	0	0
MA	0	6	0	0	0
PP	0	6	0	0	0
UP	0	6	0	0	0
C	10	6	6	3	4
Midline					
F	5	3	12	5	0

Teloblasts were injected unilaterally with FDA and the embryos were fixed ~100 hours later. Only well-labeled preparations with the normal distribution of cell bodies were scored. Numbers in parentheses represent the total number of midbody ganglia that were scored, some of which were in the same embryo. Projections into the DP nerve were not apparent in the preparations examined. Abbreviations are given in Fig. 2.

Ectodermal ablations gave more specific and interpretable aberrations in nerve patterning. In particular, unilateral ablations of the N lineage resulted in the apparent condensation of the MA and PP nerves within the ganglion. Only two, instead of three, segmental nerve roots formed on the ablated side, at the positions of the normal AA and MA nerves. Presumptive PP nerves arose as abnormal, posterior branches from the MA nerve tract immediately lateral to the ganglia (Fig. 3B). Unilateral N ablations also resulted in a medial shift of the ipsilateral connective, to a position near Faivre's nerve (Fig. 3B).

No dramatic modifications of the normal nerve pattern were observed upon unilateral O teloblast ablations. However, the PP nerve appeared to be retarded in its growth and deviated from its normal projection parallel to the AA and MA nerves (Fig. 3C). Unilateral P teloblast ablations resulted in deficiencies in the size and position of the MA nerve tract. Although an MA nerve often formed in the absence of ipsilateral, P-derived progeny, its diameter was reduced, suggesting a significant decrease in the number of axons occupying that nerve. In addition, the MA nerve often projected into the ganglion either anterior or posterior to its normally central position (Fig. 3D). [P lineage ablations are feasible in *T. rude* because the O-to-P fate change that invariably results from loss of the P lineage teloblast in *Helobdella* (Weisblat and Blair, 1984; Shankland and Weisblat, 1984; Huang and Weisblat, 1996) occurs in only some cases in *Theromyzon* (Keleher and Stent, 1990).] Unilateral Q teloblast ablations induced no gross changes in the normal pattern of axons (not shown).

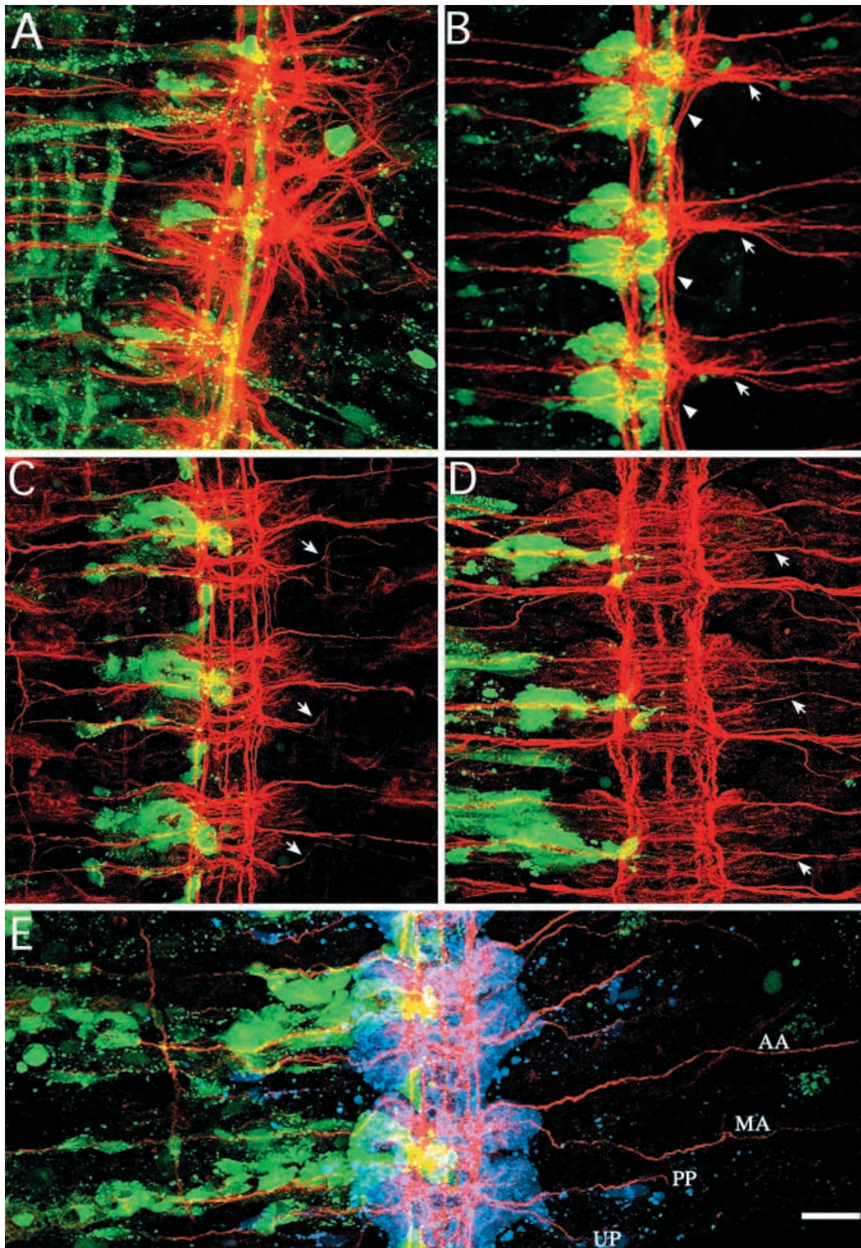

Fig. 3. Axonal architecture following lineage-specific ablations. Each panel shows the medial region of two (E) or three (A-D) midbody segments immunostained for ACT to reveal axon patterning (red). (A-D) Germinal plates from embryos in which one teloblast of a given pair was injected with lineage tracer (green; left side) and its contralateral homolog was ablated (right side). (A) Unilateral M lineage ablation resulted in a random array of axon fibers. (B) Unilateral N lineage ablation resulted in coalescence of the PP and MA segmental nerves at the ganglionic margin (arrows). The ipsilateral connective was often shifted towards the midline and appeared smaller in diameter (arrowheads). (C) Unilateral O lineage ablations resulted in a truncated and/or displaced PP segmental nerve (arrows) on the experimental side relative to the contralateral control at the same clonal age. (D) Unilateral P lineage ablations had little effect other than a decrease in diameter and moderate displacement of the medial end of the MA segmental nerve (arrows). The transient, lateral longitudinal nerve failed to appear (not shown). (E) A similar preparation in which O, P and Q lineages are labeled on the left side and missing from the right side, while both N lineages were labeled with a different lineage tracer (blue). Unilateral OPQ ablations resulted in abnormalities of all the segmental nerves ranging from displacement to reduction in size and/or length, but all nerves could be recognized. Anterior is upwards. Scale bar: 25 μm.

In a related study, Braun and Stent (Braun and Stent, 1989b) unilaterally photoablated several segments' worth of n, o, p and q progeny within the germinal plate of *Helobdella triserialis*, and monitored axon growth by labeling contralateral, N-derived motoneurons that crossed the midline. Our experiments were conducted in *Theromyzon* using teloblast ablations so that the lineage in question was never present within the germinal plate. Although the results of the two studies are generally consistent, several phenotypes observed here (e.g. MA/PP fusion, developmental delays, abnormal position of some nerves) were not observed in the earlier study. We attribute these differences to the timing and efficacy of the ablations, although species-specific effects cannot be ruled out.

These differences notwithstanding, preventing the normal contributions of the individual O, P and Q lineages had no large effects on nerve formation in either species (i.e. *H. triserialis* or *T. rude*), despite the fact that these lineages contribute processes to all ipsilateral nerve tracts except UP. To test for the possibility that these three ectodermal lineages have redundant effects on nerve patterning, we examined germinal plates from embryos in which the O, P and Q lineages had all been ablated on one side. In such embryos [and in embryos with bilateral OPQ ablations (not shown)], all of the major longitudinal and segmental nerves still formed, but their positions were abnormal (Fig. 3E). This suggests that axons from the remaining cells (primarily ipsilateral and contralateral N-derived neurons) have the capacity to establish all of the major nerve tracts in leech. Conversely, embryos in which both N teloblasts were ablated (resulting in the absence of ~2/3 of ganglionic neurons) also formed the major longitudinal and segmental nerves, although the MA and PP nerves coalesced as described above and there was a marked reduction in the

size of the AA nerve (not shown), suggesting that O-, P- and Q-derived neurons can also pioneer most nerve tracts in the absence of the N lineages.

Correlation between muscle fiber and segmental nerve placement

The severe disorganization of nerve tracts following unilateral mesodermal ablations (Fig. 3A) prompted us to examine the relationship between nerve and muscle fibers during development. For this purpose, we labeled the neuroectoderm by injecting an N teloblast with RDA, fixed and dissected the embryos after ~100 hours further development, and then differentially labeled axons (ACT) and muscle fibers (Lan 3-14) in the dissected germinal plates (Fig. 4). More than 30 bilateral pairs of longitudinal muscle fibers and between 20-25 circular muscle fibers per segment were apparent in our preparations. These were arranged in stereotypical patterns so that homologous muscle fibers could be recognized in each segment. By this criteria, the MA nerve, which enters each ganglion approximately at its center (see Fig. 2), always projected between the two circular muscle fibers that displayed the greatest inter-fiber separation (Fig. 4). The AA and PP nerves consistently ran between sets of circular muscle fibers that were about five fibers anterior and posterior of the MA nerve, respectively. The UP nerve appeared less constrained, running between muscle fibers that were approximately three to seven fibers posterior to PP. Aside from the short, oblique regions of segmental nerves that exited the ganglion, the segmental nerves showed a strong propensity to maintain their position between these specific pairs of muscle fibers. Similar results have been reported previously (Braun and Stent, 1989a), who found that a distance of at least 5 μm (in the smaller embryos of *Helobdella*) separates nerve tracts from their nearest circular muscle fiber. Similar rules may apply for longitudinal nerves that run between stereotypical sets of longitudinal muscles on either side of the midline (Fig. 4). In light of the severe disruptions on nerve patterning induced by mesoderm ablations (Fig. 3A), these results suggest that proper patterning of the segmental nerves requires cues provided by the muscle fibers, or that both segmental nerves and muscle fibers are patterned in parallel by some other set of mesodermally derived cues.

Development of the posterior (PP) segmental nerve

As noted above, the PP nerve appears at ~60 hour clonal age within the posterior lobe of the ganglionic primordion (see Fig. 2). At about the same time, two transverse stripes of N-derived cells (approximately four to six cells in each stripe) extend ventrolaterally from the dorsal, posterior aspect of the hemiganglion, transiently connecting the ganglion with the ventral body wall (Shain et al., 1998); the anterior stripe arises from the nf.a clone and the posterior stripe arises from the nf.p clone (see Fig. 1). By ~75 hours clonal age, the stripes break down; medial cells in the stripes disappear (presumably by cell death or migration into the ganglion), while peripheral cells in the anterior and posterior stripes differentiate into three peripheral neurons, nz1-2 and nz3, respectively (Weisblat et al., 1978; Weisblat et al., 1984; Torrence and Stuart, 1986). The apparent coincidence of the PP nerve and the anterior stripe of N-derived cells prompted us to examine their relationship during the period of ~65-75 hours clonal age in *T. rude*. We found that pioneering axons of the PP nerve were closely associated with N-derived cells as they moved peripherally, and that their association with the differentiating nz1 and nz2 neurons was maintained (Fig. 5A).

Previous observations have identified an O-derived, pressure-sensitive mechanosensory neuron, P_D, that lies within the ganglion near the base of the posterior segmental nerve root (Kramer and Goldman, 1981; Kuwada and Kramer, 1983; Kramer and Weisblat, 1985; Kuwada, 1985). P_D sends one process medially that bifurcates at the ipsilateral connective and another laterally out the posterior nerve root,

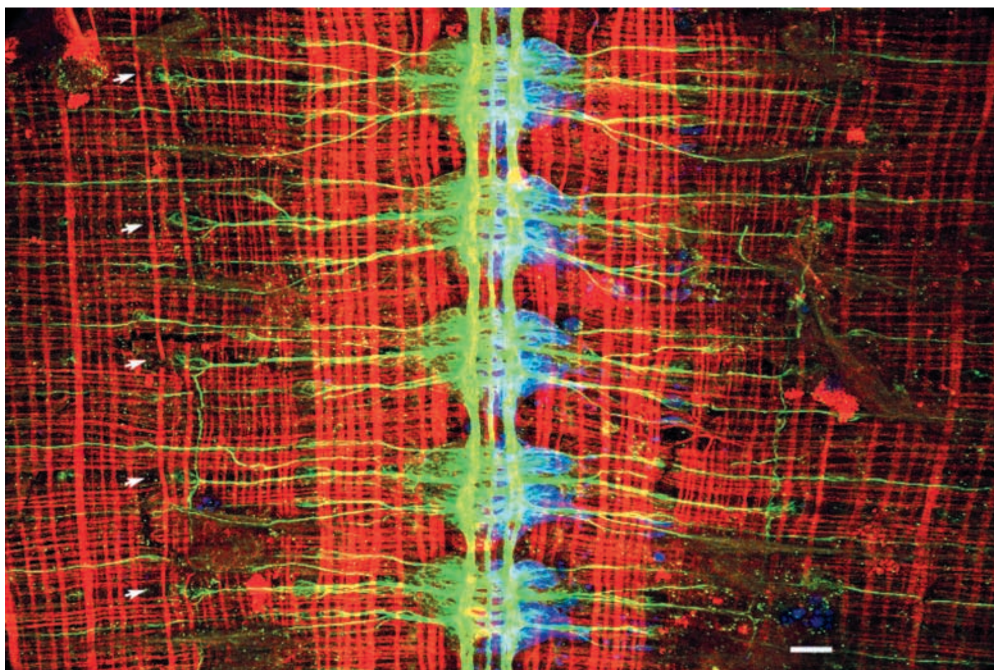

Fig. 4. Coincidence of nerve and muscle fibers in *T. rude.* A view of five segments (~95-105 hours clonal age) in a germinal plate immunostained with anti-ACT (green) and Lan3-14 (red) to identify axons and muscle fibers, respectively. The right N teloblast was labeled with RDA (blue). More than 30 bilaterally paired, longitudinal muscle fibers are apparent; between 20-25 circular muscle fibers can be identified per segment. Arrows indicate the largest gap between identifiable circular muscle fibers and correspond to the position of the iterated MA nerve tract. Other nerve tracts (AA, PP and UP) consistently align between characteristic sets of muscle fibers. Anterior is upwards. Scale bar: 25 μm.

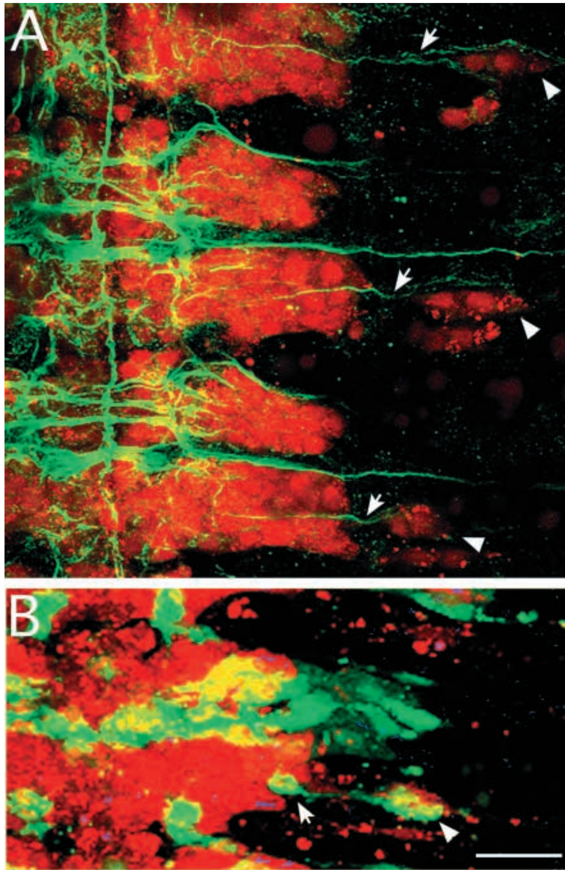

Fig. 5. Early development of the PP nerve. Each panel shows one or more hemiganglia on the right side of a germinal plate in which the N lineage was labeled with RDA. (A) A series of three hemiganglia (~65-70 hours clonal age), from a preparation that was also immunostained for ACT (green), reveals the close association of the developing PP nerve (arrows) with the laterally migrating nz1 and nz2 axons (arrowheads). (B) A single hemiganglion at the same age from a preparation in which the O lineage was labeled with FDA lineage tracer (green). There is a close association between processes originating from the P_D neuron (arrow) and the peripheral oz2 and/or oz3 neurons (lower arrowhead). O-neurons P_D, oz2 and oz3 are coincident with the PP nerve and the latter two (oz2 and oz3) are closely associated with N-derived neurons nz1 and nz2 (overlap appears yellow). Anterior is upwards. Scale bar: 25 µm.

eventually within the DP nerve (Kramer and Weisblat, 1985; Kuwada, 1985). To examine the association between P_D and the stripe of N-derived cells, we prepared embryos in which the N and O lineages were labeled with FDA and RDA lineages tracers, respectively (Fig. 5B). As previously described, we noted that several O-derived neurons migrate from a central cluster of ~20 cells to positions along the PP nerve tract (Torrence and Stuart, 1986; Braun and Stent, 1989a). Among these were the central neuron P_D, and peripheral neurons oz2 and oz3 (Torrence and Stuart, 1986). As oz2 and oz3 migrated laterally from the posterior lobe of the ganglionic primordium they projected medial neurites that remained closely associated with P_D (Fig. 5B). Similar observations describing the behavior of oz2 have been reported (Braun and Stent, 1989a). We also saw that oz2 and oz3 maintained close association with peripheral neurons nz1 and nz2 in the anterior, N-derived stripe (Fig. 5B). Thus, P_D, oz2 and oz3 appear to contact cells in the anterior ventrolateral stripe of N-derived cells as the PP nerve forms.

The close association of N- and O-derived cells near the nascent PP nerve suggests that these cells might be required for the normal formation of PP. Indeed, unilateral ablations of N or O teloblast lineages resulted in characteristic modifications of the PP nerve (see Fig. 3B,C). To identify cells in the N lineage involved in PP nerve patterning, individual primary n blast cells (nf or ns; see Fig. 1) were ablated and the resultant embryos were later immunostained with ACT. Ablation of either nf or ns prevents approximately one-half (~75) the normal complement of N-derived neurons from forming in the experimental hemiganglion; an ns ablation causes a deficiency in the anterior lobe of the hemiganglion, while an nf ablation causes a deficiency in the posterior lobe, including both ventrolateral stripes (Ramirez et al., 1995; Shain et al., 2000). In all segments from which the contributions of the ns primary blast cell were missing, the patterning of the PP nerve appeared normal (*n*=4; Fig. 6B). In segments showing the effects of nf primary blast cell ablations, however, the putative PP nerve arose as a posterior branch of the MA nerve near the ganglionic margin (*n*=9; Fig. 6C), a phenotype similar to that observed in unilateral N teloblast ablations described earlier (*cf.* Fig. 3B). In many injections, blast cells were not killed but rather went on to produce an abnormally low number of progeny (between 5-10); in these cases, no ventrolateral stripes were formed (e.g. Fig. 6C) and the PP nerve was condensed with the MA nerve.

In normal development, the O lineage-derived P_D neuron migrates posteriorly from a central cluster of ectodermal cells to a position at the base of the PP nerve (Fig. 7A,B), where it is thought to pioneer this nerve tract (Kuwada, 1985; Jellies et al., 1994). We therefore speculated that the failure of this migration could lead to the MA/PP nerve coalescence (Fig. 3B, Fig. 6C), i.e. that P_D may project its axon into the MA nerve instead of PP, resulting in the coalescence of the PP and MA nerves within the ganglion. To test this hypothesis, we caused the MA/PP coalescence phenotype by unilaterally ablating the N teloblast (as in Fig. 3B,) while concomitantly labeling the ipsilateral O teloblast with lineage tracer, thus allowing us to observe the behavior of P_D in the absence of N-derived progeny. We found that in these preparations, P_D invariably failed to migrate to its normal position in the posterior lobe of each respective hemiganglion; rather, it remained within the central aspect of the hemiganglion near the MA nerve root (Fig. 7A). By contrast, oz2 and oz3 migrated to their normal peripheral positions in the absence of N-derived progeny. Importantly, ACT immunostaining revealed neurites linking ectopically situated P_D neurons to oz2 and oz3 (Fig. 7C,D), as observed during normal PP nerve formation (see Fig. 5B). Thus, the position of P_D (i.e., posterolateral in normally developing ganglia versus central in the absence of N-derived progeny) determines whether the PP nerve root exits the hemiganglion at a posterior or medial location, respectively. Collectively, these observations provide strong evidence that cell-cell interactions between the O-derived, P_D neuron and N-derived cells are required for establishing the normal projection of the PP nerve root from the posterior lobe of each hemiganglion.

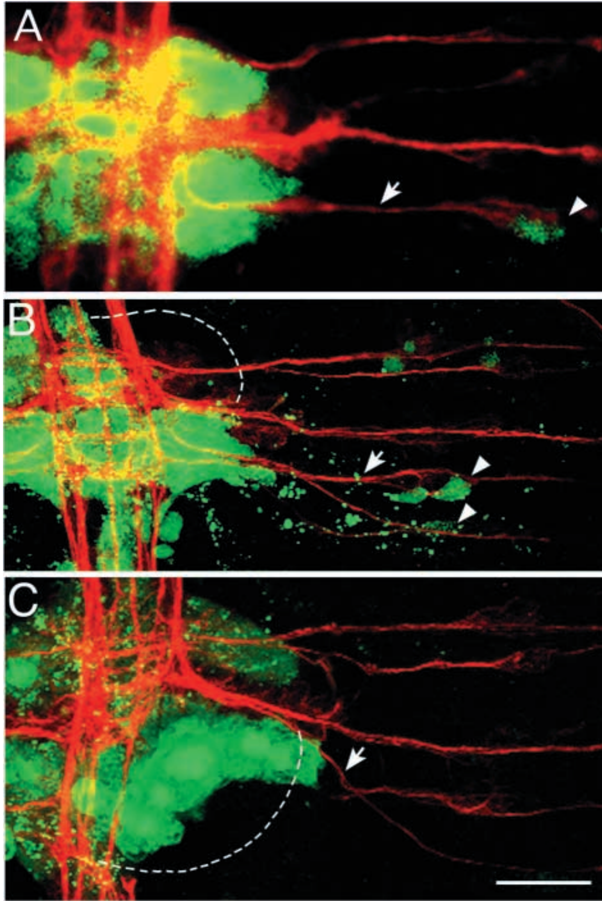

Fig. 6. Effects of specific N lineage ablations on PP nerve patterning. Each panel shows a right hemiganglion (~80 hours clonal age) from a preparation in which the N lineage was labeled with FDA (green) and axons were labeled by ACT immunostaining (red). The PP nerve is indicated by an arrow in each panel. (A) An unablated control preparation shows the normal, posterior origin of the PP nerve and its association with peripheral nz neurons (arrowhead). (B) Ablation of an ns primary blast cell resulted in a significant reduction in size of the anterior region of the ganglion (broken outline approximates the normal contour), but the PP nerve root maintained its normal position. (C) Ablation of an nf primary blast cell resulted in a significant reduction in size of the posterior region of the ganglion (broken line shows the normal contour). The putative PP nerve branched from the MA nerve at the margin of the ganglion. Anterior is upwards. Scale bar: 25 μm.

Discussion

We have followed the pattern of axon development in the glossiphoniid leech, *Theromyzon rude*, and identified a crosslineage neuronal migration required for establishing a major segmental nerve. Like nervous system development in other invertebrates (Ho and Goodman, 1982; Keshishian and Bentley, 1983a; Keshishian and Bentley 1983b; Hartenstein, 1988), nerve pathways in *T. rude* are established by contributions from both central (CNS) and peripheral (PNS) neurons. In addition, cell-dependant neuronal migrations within the CNS are required for normal axon patterning in a range of organisms, including vertebrates (Hatten, 2002). Our results identify several commonalities with nervous system development in the medicinal leech *Hirudo medicinalis* (Jellies et al., 1996): the initial axon fibers appearing along the ventral midline of each leech pioneer Faivre's nerve; and intraganglionic commissures and bilateral connectives appearing prior to segmental nerves. *Hirudo* and *Theromyzon* differ, however, in the number, order of appearance and secondary condensation of their segmental nerves. Ultimately, *Hirudo* projects four main segmental nerves from two segmental nerve roots in each hemiganglion, whereas *T. rude* and other glossiphoniid leeches project five main segmental nerves from three segmental nerve roots in each hemiganglion.

In *Hirudo,* the first neurites projecting from the ganglion correspond to the DP nerve, which forms the future posterior nerve root (Jellies et al., 1996). The MA nerve, which occupies the position of the future anterior nerve root, appears a few hours later and is followed by the PP and AA nerves, both of which form distinct projections from the ganglion (Jellies et al., 1996). Thus, four distinct nerve roots (AA, MA, PP and DP) appear initially in *Hirudo*. Later condensation of the AA nerve with the MA nerve, and the PP nerve with the DP nerve leaves just two segmental nerve roots in the adult.

The first nerves to appear in *T. rude* are MA and AA (see Fig. 2). MA is pioneered by a peripheral, P-derived cell(s) as in *Hirudo* (Jellies et al., 1996). The PP nerve root forms in *T. rude* after MA and AA are well established, which is in reverse order of that described in *Hirudo* (Jellies et al., 1996). And an additional prominent segmental nerve called the ultraposterior (UP) nerve (Kramer and Goldman, 1981; Braun and Stent, 1989a), which is absent in *Hirudo,* branches from the PP nerve outside of the ganglionic margin. The DP nerve also branches from PP outside of the ganglion and is distal to the PP/UP branch point (Braun and Stent, 1989a). Collectively, *T. rude* projects five segmental nerves from three segmental nerve roots in each hemiganglion, and no condensation is observed. [Although the AA and PP nerve roots shift towards the center of the ganglion as in *Hirudo,* they remain distinct (see Fig. 2).] We note that the initial condensation step in *Hirudo* joins the DP and PP nerves, while the AA and MA nerves remain independent. At this stage of *Hirudo* development, the axon architecture resembles that of *T. rude* and other glossiphoniid leeches (Braun and Stent, 1989a; Braun and Stent, 1989b), and may represent an ancestral condition.

The development of the DP nerve also differs between *Hirudo* and *Theromyzon.* In *Hirudo*, DP is the first segmental nerve to emerge from the ganglionic primordia and forms the most prominent projection (Jellies et al., 1996). By contrast, the DP nerve in *T. rude* is not well defined, appearing only late in the development of the segmental nerves as a relatively minor bifurcation of the PP nerve (see Fig. 2). The significance of this observation remains unclear, but there is a correlation between swimming behavior and the prominence of the DP nerve that transcends taxonomic groupings. The glossiphoniid leeches *Theromyzon* and *Helobdella*, which do not swim, project a rudimentary DP nerve late in development (Braun and Stent, 1989a) (Fig. 2), while *Hirudo* (Family Hirudiniformes) and also the glossiphoniid leech *Haementeria ghilianii,* both of which do swim, form a prominent DP nerve early in embryogenesis (Kuwada, 1985; Jellies et al., 1996).

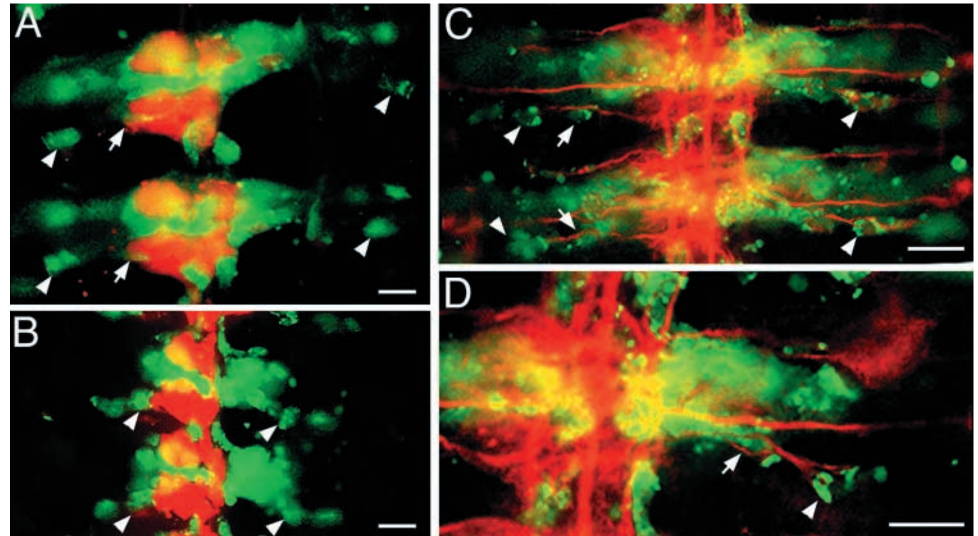

Fig. 7. N-derived progeny are required for the normal intraganglionic migration of the O-derived P_D neuron. Each panel shows one (D) or two (A-C) segmental ganglia from germinal plates in which both the left and right O lineages were labeled with lineage tracer (green). P_D (arrows) and oz2/oz3 (arrowheads) are indicated when they can be distinguished from other O-derived cells. In A and B, the N lineage was labeled with lineage tracer (red) on the left side and is absent on the right side, owing to teloblast ablation. (A) By 80-85 hours clonal age, P_D and oz2/oz3 were readily distinguished on the control (left) side. In the absence of the N lineage (right side), oz2/oz3 migrated to their normal peripheral positions, but P_D apparently remained within a central cluster of O-derived cells. (B) Earlier in development (50-55 hours clonal age), P_D, oz2 and oz3 assumed their normal positions, independent of the N lineage (compare positions of O lineage cells on left and right sides). P_D has not yet separated from the central cluster of O-derived cells (compare with A). (C,D) ACT immunostaining (red) of axon projections reveals that PP nerve fibers still connect P_D and oz2/oz3 in N-depleted hemiganglia (right side). As P_D has failed to migrate normally, the PP nerve now branches from a central position in N-depleted hemiganglia (compare right side to left side). Ganglia are between 80-85 hours clonal age. Anterior is upwards. Scale bars: 25 μm in all panels.

Cell ablations

In the leech nervous system, identified neurons arise by stereotyped patterns of cell division and migration from each of the five teloblast lineages (M, N, O, P and Q) (Zackson, 1984; Kramer and Weisblat, 1985; Torrence and Stuart, 1986; Bissen and Weisblat, 1987; Shankland, 1987; Bissen and Weisblat, 1989; Braun and Stent, 1989a; Shain et al., 1998). In other animals, specific cells have been shown to provide positional cues used by navigating growth cones (Bentley and Keshishian, 1982; Palka et al., 1992; Goodman and Shatz, 1993), and similar results have been obtained in leech (Braun and Stent, 1989b). In this study, we removed subsets of definitive progeny from one side of the embryo by ablating specific teloblast lineages or sublineages, so that developing axons were challenged to find their targets in the absence of normal positional cues. The effects of mesodermal deficiencies were most dramatic, including severely disorganized arrays of axons (Fig. 3A). These results are consistent with previous studies showing that mesodermal ablation leads to massive disorganization of ectodermal patterning in *Helobdella* (Blair, 1982; Torrence et al., 1989). The strong correlation between the position of muscle fibers and nerve tracts (see Fig. 4) further supports the notion that mesoderm is a crucial component of axon guidance in leech (Torrence et al., 1989).

Unilateral ablations of other teloblast lineages displayed less severe, but more specific effects on nerve patterning. In the absence of N lineage-derived progeny, the ipsilateral UP nerve failed to form and the ipsilateral PP nerve appeared to have coalesced with the MA nerve within the hemiganglion and arose instead as a posterior branch of the MA nerve at the margin of the ganglion. The absence of UP is consistent with previous studies demonstrating that the N-derived neuron nz3 is required for UP nerve formation (Braun and Stent, 1989b). Ablation of the O lineage also disrupted ipsilateral PP nerve formation although to a lesser extent. These observations suggest either a cooperative or epistatic interaction between N and O lineage derivatives is required to form the normal PP nerve, as discussed below.

Formation of the PP segmental nerve

Several lines of evidence suggest that the O lineage-derived mechanosensory neuron P_D plays a role in establishing the normal trajectory of the PP segmental nerve. Previous studies have shown that P_D homologs in other glossiphoniid species extend processes medially toward the ipsilateral connective and also peripherally via the PP nerve root (Kuwada and Kramer, 1983; Kramer and Weisblat, 1985; Kuwada, 1985; Kramer and Goldman, 1981; Braun and Stent, 1989a). In this study, ACT immunostaining confirmed those results for *T. rude* and also revealed an axon process growing laterally from the ipsilateral connective towards P_D (see Fig. 2). When P_D was missing (i.e. in unilateral O ablations), a PP nerve tract eventually formed and exited the ganglion but its peripheral trajectory was abnormal (see Fig. 3C). Together, these observations suggest that the process(es) emanating from the ipsilateral connective can pioneer the PP nerve in the absence of the P_D neuron, but that they normally grow out along a pre-existing pathway established by the P_D axon.

We have shown previously that a ventrolateral stripe of N lineage-derived cells forms a transient bridge that connects the ganglion with the ventral body wall (Shain et al., 1998). The close association of these cells with early fibers in the PP nerve tract (see Fig. 5) suggested that this interaction is required for establishing the PP nerve. Previous studies indicate that the two distalmost cells in the anterior ventrolateral stripe (i.e. the future nz1 and nz2 neurons) lie along the PP nerve pathway (Braun and Stent, 1989a). But ablating these cells did not disrupt formation of the PP nerve (Braun and Stent, 1989b), in apparent contradiction with results presented here (see Fig. 6C,D). This discrepancy appears to result from the difference

in timing and also in the number of cells that were ablated in each study. Braun and Stent (Braun and Stent, 1989b) did not ablate nz1 and nz2 until after they had separated from the hemiganglion. Our results indicate that the PP nerve has already formed by that time (see Fig. 5A). In our present experiments, ablations were performed prior to the lateral migration of cells within the stripe, well before the first fibers of the PP nerve appeared. This lesion not only prevented formation of nz1 and nz2, but also cells at the medial end of the N-derived ventrolateral stripe that are closely associated with the O-derived P_D neuron (see Fig. 5B, Fig. 7). Although Braun and Stent (Braun and Stent, 1989b) made unilateral ablations in the n bandlet, these appear not to have removed all N-derived progeny in the hemiganglion and the remaining cells were presumably sufficient to effect the migration of the P_D neuron. P_D arises as part of an O-derived cell cluster within the medial aspect of the ganglionic primordia, then migrates posteriorly before initiating axonogenesis (Braun and Stent, 1989a; Torrence and Stuart, 1986) (Fig. 7). In N teloblast ablation experiments, P_D fails to migrate posteriorly (Fig. 7) before differentiating and therefore projects its peripheral process from the central cluster of O-derived cells along the MA nerve. Apparently, the axons growing out from the ipsilateral connective detect the ectopic P_D axon and follow it to the periphery instead of pioneering a posterior tract (as they would if P_D were absent and the ipsilateral N lineage were present) (Fig. 3C,E); this results in coalescence of the MA and PP tracts within the ganglion. Interestingly, the displaced PP axons assume their normal posterior position shortly after exiting the ganglion, apparently in response to cues from the O-derived oz2, oz3 neurons, which may act as guidepost cells, similar to those found in the embryonic appendages of insects (Bate, 1976; Keshishian and Bentley, 1983a; Keshishian and Bentley, 1983b).

In summary, we propose that the posterior migration of P_D is required for normal formation of the PP nerve root, and this migration is dependent upon N-derived progeny. In the absence of the N lineage (specifically, one or more cells within the nf.a clone), the O lineage-derived P_D neuron fails to migrate posteriorly. Its peripheral process exits the ganglion abnormally via the ipsilateral MA nerve as a result and other neurons that would normally project out the PP nerve also adopt this route, leading to coalescence of the PP and MA nerves. In the absence of the O lineage-derived P_D neuron, other axons, possibly from N-derived progeny (Table 1), may pioneer the PP nerve independently, but only after a developmental delay (see Fig. 3C).

This model not only explains the dual requirement for N and O lineage derivatives in forming the normal PP nerve, but also leads us to predict segment-specific differences in the development of the PP nerve. The basis for this prediction lies in the fact that in the O teloblast lineage, only one blast cell (rather than two as in the N lineage) is required to generate a segment's worth of definitive progeny (Zackson, 1984; Weisblat and Shankland, 1985). As the rate of blast cell production is about the same in all lineages, this means that there is segment-specific discrepancy in the clonal ages of consegmental N versus O lineage derivatives. In anterior segments, N-derived and O-derived progeny arise from blast cells born within a few hours of one another, whereas in posterior segments, O-derived progeny arise from blast cells born more than a day before the n blast cells whose progeny occupy the same segment. That is, o blast cell clones for the posterior segments are much older than the consegmental n blast cell clones. As far as we know, cell division patterns and the expression of developmental regulators such as the leech *engrailed*-class gene appear to proceed autonomously within the different blast cell clones (Lans et al., 1993; Seaver and Shankland, 2001). If so, we anticipate that, in posterior segments relative to anterior segments, N-derived signals required to trigger the rearward migration of the P_D neuron arise much later in the terms of the age of the O clone from which the P_D neuron descends, suggesting that P_D migration and subsequent formation of the PP nerve is delayed in posterior segments.

Finally, we note that cells in the ventrolateral stripe of N-derived progeny that appear to interact directly with the O-derived, P_D neuron are those cells which express the leech *engrailed*-class gene in the early N lineage (Wedeen and Weisblat, 1991; Lans et al., 1993). In *Drosophila, engrailed* is expressed in stripes of cells that specify the posterior compartment of each segment (Kornberg, 1981a; Kornberg, 1981b; Lawrence et al., 1999a). By acting upstream of the hedgehog protein, which is secreted by *engrailed*-expressing cells in the posterior compartment (Lee et al., 1992), *engrailed* influences the affinities and positioning of cells in the anterior compartment of *Drosophila* (Lawrence et al., 1999b). By analogy, one could imagine that cells expressing the *engrailed*-class gene in the anterior, ventrolateral stripe of leech may influence local cell-cell affinities that affect the migration of P_D, and thus the position of the posterior nerve root. Whether the expression of the engrailed-class protein is required for the migration of P_D remains to be determined, but these cells do not seem to express the only *hedgehog*-class gene that has been identified in leech (Kang et al., 2003).

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