Peripheral Neurons Depend on CNS-Derived Guidance Cues for Proper Navigation during Leech Development

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In leech, major nerve pathways are pioneered by CNS neurons and evidence from dye-injection and antibody experiments suggest that they may serve as guides for later differentiating neurons. In this study we have directly tested this hypothesis by examining the consequences of CNS ablation on the navigation in the periphery of a well-defined population of afferent sensory neurons. We show that in the absence of CNS-derived axons the axonal growth cones of this population of peripheral neurons extend with little directionality and instead of forming orderly projections, default into forming circular fasciculated pathways with each other. This suggests that CNS-derived guidance cues are absolutely required for the correct navigation of these peripheral sensory neurons. © 1995 Academic Press, Inc.

INTRODUCTION

Precise and stereotyped patterns of connectivity are formed by the specific navigation and correct pathway choices of neuronal growth cones in response to various guidance mechanisms such as selective adhesion, growth cone avoidance, epithelial gradients, guide post cells, and chemotropism (Jessell, 1988; Goodman and Schatz, 1993; Goodman, 1994). In many cases pioneer neurons using different combinations of these pathfinding strategies establish the initial pathways which are then used as a guide by subsequently differentiating neurons that extend growth cones. This kind of strategy is particularly well suited to ensure the formation of common nerve pathways between efferent and afferent projections. However, it has been shown that early pathways are not always obligatory for navigation by the follower neurons, which in many cases still can reach their targets in the absence of the pioneer tracts (Keshishian and Bentley, 1983; Palka, 1986; Pike et al., 1992). In this study we examine the requirement for interactions between CNS outgrowth and navigation in the periphery of a group of late differentiating PNS neurons in the leech embryo.

Process outgrowth and navigation of peripheral neurons in leech provide a particularly well-defined model system in which to study mechanisms of neuronal guidance and their molecular components (Johansen et al., 1994; Jellies and Johansen, 1995). Special advantages of this system are

that probes and techniques are available to assess pathway selections with single cell resolution of every component of both the PNS and the CNS as well as their mutual interactions. These include intracellular dye injection of individual peripheral and central neurons (Jellies et al., 1994, 1995) and a panel of monoclonal antibodies (mAbs) which define highly specific axonal tracts and pathways (Zipser and McKay, 1981; Johansen et al., 1992; Briggs et al., 1993, 1995; Jellies et al., 1995). One of these mAbs, the Lan3-2 antibody, labels the axons of sensillar neurons which project in tightly fasciculated bundles through the periphery into the CNS where they segregate into four stereotypically located fascicles in each of the central connectives (McKay et al., 1983; Johansen et al., 1992). The Lan3-2 antibody also labels a late differentiating population of extrasensillar neurons scattered throughout the skin and body wall which continue to increase in number during the lifespan of the leech (Peinado et al., 1990). These neurons extend growth cones which reach and fasciculate with the Lan3-2-positive sensillar tracts in the major nerve trunks en route to the CNS. Jellies et al. (1995) suggested, based on double-labeling experiments with Lan3-2 antibody and a mAb to acetylated tubulin (ACT) which labels all central efferents, that these neurons extend their growth cones along the CNS efferents, using them as a guide to reach the main nerves.

In the present study we directly test this hypothesis by ablating the CNS ganglionic chain in embryos before the extrasensillar neurons differentiate. We show that in the

absence of CNS-derived guidance cues the growth cones of the sensory neurons appear to lose direction and that their axons default to fasciculation onto each other, often forming circular paths. These results indicate that CNS-derived guidance cues are required for correct navigation and pathway formation of these neurons into the CNS.

MATERIALS AND METHODS

Animals

Leeches *Hirudo medicinalis* were obtained from a laboratory breeding colony. Breeding, maintenance, and staging were as previously described (Fernández and Stent, 1982; Jellies et al., 1987) at 22–25°C, except that embryos were maintained in water that was made as sterile-filtered solutions of 0.0005% sea salt, wt/wt. Cocoons were harvested every other day and opened after 6–8 days. There are about 10–20 embryos in each cocoon and these sibling embryos develop synchronously within a few percent of development. Dissections of embryos were performed in leech saline solutions with the following composition (in mM): 110 NaCl, 4 KCl, 2 CaCl₂, 10 glucose, 10 Hepes, pH. 7.4. In some cases 8% ethanol was added and the saline solution cooled to 4°C to inhibit muscle contractions.

Immunocytochemistry

Two monoclonal antibodies were used in these studies. The Lan3-2 antibody (Zipser and McKay, 1981; McKay et al., 1983) was used to label sensillar and extrasensillar peripheral neurons whereas a monoclonal antibody directed against acetylated tubulin (Sigma) was used to label central neurons as well as a population of nonsensillar peripheral neurons and their axonal projections (Jellies et al., 1995). Lan3-2 is of the immunoglobulin G1 subtype and the ACT antibody of the G2B subtype.

Dissected *Hirudo* embryos were fixed overnight at 4°C in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The embryos were incubated overnight at room temperature with diluted antibody (Lan3-2, 1:75; ACT, 1:1000) in PBS containing 1% Triton X-100, 10% normal goat serum, 0.001% sodium azide, washed in PBS with 0.4% Triton X-100, and incubated with HRP-conjugated goat anti-mouse antibody (Bio-Rad, 1:200 dilution). After washing in PBS the HRP-conjugated antibody complex was visualized by reaction in 3,3'-diaminobenzidine (0.03%) and H₂O₂ (0.01%) for 10 min. The final preparations were dehydrated in alcohol, cleared in xylene or methyl salicylate, and embedded as whole mounts in Depex mountant. Double-labeled preparations were obtained by a subsequent incubation in the other primary antibody and by using fluorescently conjugated subtype-specific secondary antibodies. A rabbit anti-mouse IgG₁ TRITC-conjugated secondary antibody (Cappel) was used for Lan3-2 and a rabbit anti-mouse IgG₂ᵦ FITC-conjugated secondary antibody (Cappel) for the

ACT antibody. Fluorescently labeled preparations were mounted in glycerol with 5% n-propyl gallate.

The preparations were photographed using Ektachrome 100 or 400 HC or Ektar 100 Daylight film. A drawing tube (Leitz) was used to draw some pathways over extensive body wall regions.

CNS Extirpation

Whole embryos (at E9 or early in E10) were anesthetized in embryo water containing 8% ethanol and chains of 3–12 adjacent ganglionic primordia were removed by cutting a small (≈100 μm) hole at the ventral midline over a ganglion, grasping the forming intersegmental connective with fine forceps, and tugging sharply to break the connective at a more posterior location (Fig. 1B). It has previously been shown that the intersegmental connectives between ganglionic primordia form earlier than the peripheral projections into the germinal plate (Jellies et al., 1994, 1995). This makes it possible to slide the ganglionic chain out of the forming ventral sinus that normally encases it. After CNS removal embryos were rinsed through three changes of normal embryo water and reared in individual dishes (35-mm Falcon plastic) until E16. In successfully operated embryos the incision in the germinal plate heals within 2–3 hr. In each experiment embryos from the same cocoon were divided into control and experimental embryos. However, a few embryos were dissected, fixed, and labeled with antibody at the time of the operation to verify the developmental stage of the embryos.

RESULTS

Figure 1A shows a camera lucida drawing of an E10 embryo labeled with the mAb Lan3-2, which recognizes a surface epitope of a membrane-associated antigen expressed by all sensillar and late developing extrasensillar afferents (Johansen et al., 1992). Sensillar neurons arise in the periphery and project axons into the CNS along particular tracts (Johansen et al., 1992; Briggs et al., 1993). Axons from S1 to S5 fasciculate in the periphery along the m.a. nerve, while those from S6 and S7 fasciculate within the developing d.p. nerve (Fig. 2, shown in blue). Once within the CNS different subpopulations of these afferent axons segregate into discrete fascicles the pathway choice of which has been correlated with the expression of particular antigens (Johansen et al., 1994; Jellies and Johansen, 1995). Individual embryos exhibit a rostral–caudal gradient of development having about 2.5 hr between adjacent segments (Jellies and Kristan, 1991). Thus, the chain of 32 segments in each embryo provide a relative sequence of axonal extension (Fig. 1A).

The Effect of CNS Ablation on the Navigation of Peripheral Sensory Neurons

What is the source of the guidance cues in response to which peripheral neurons navigate from the periphery to
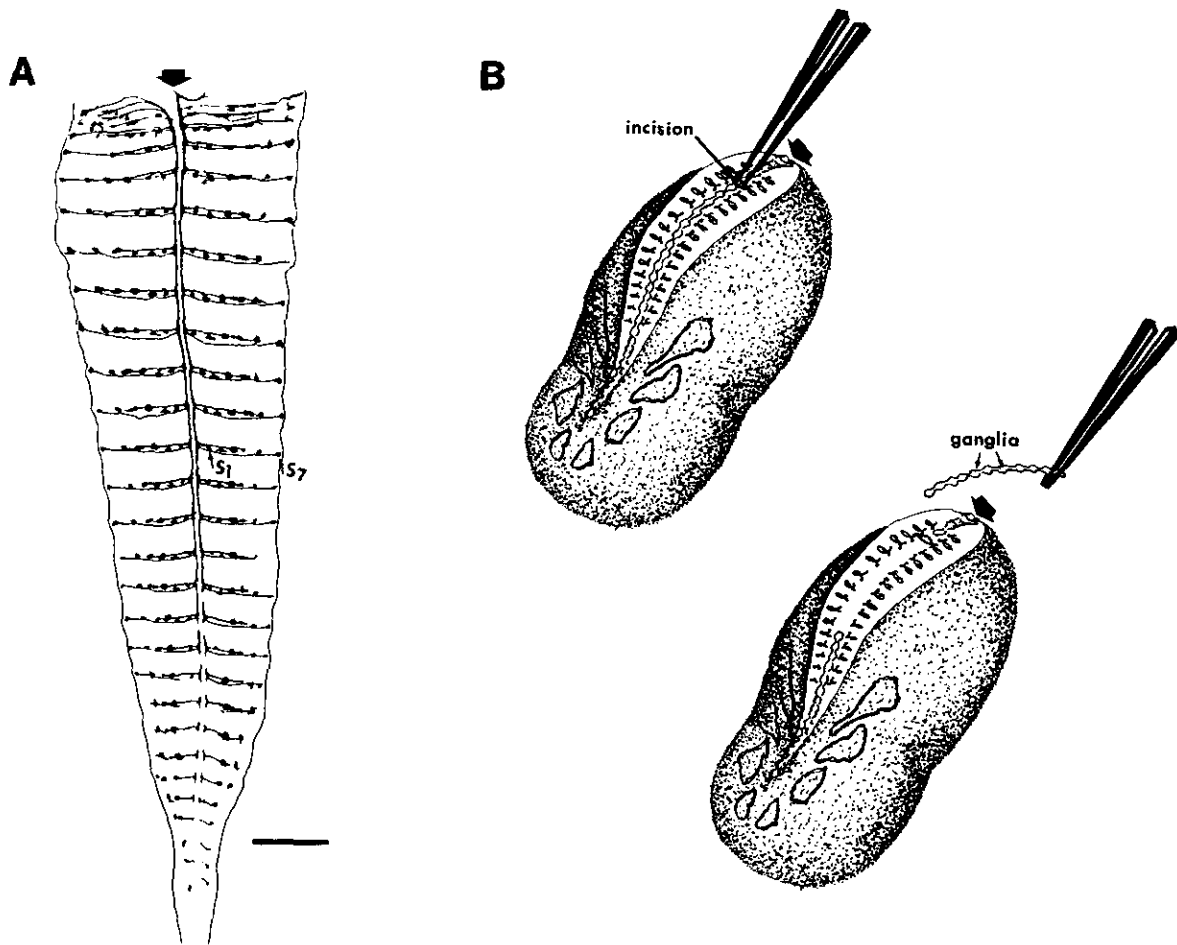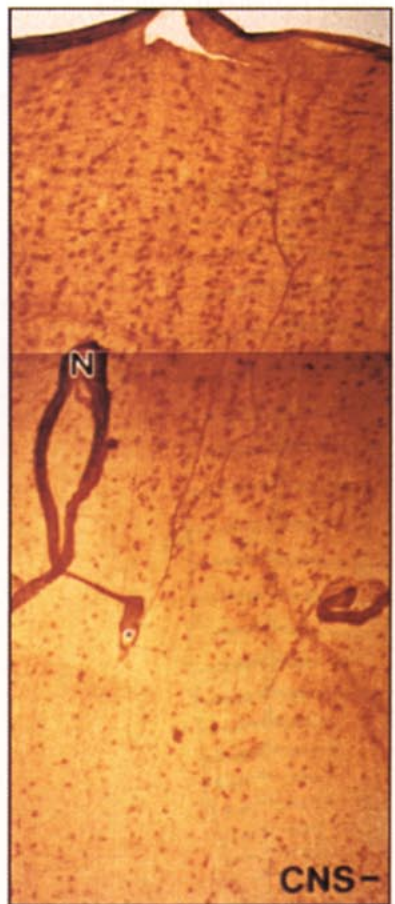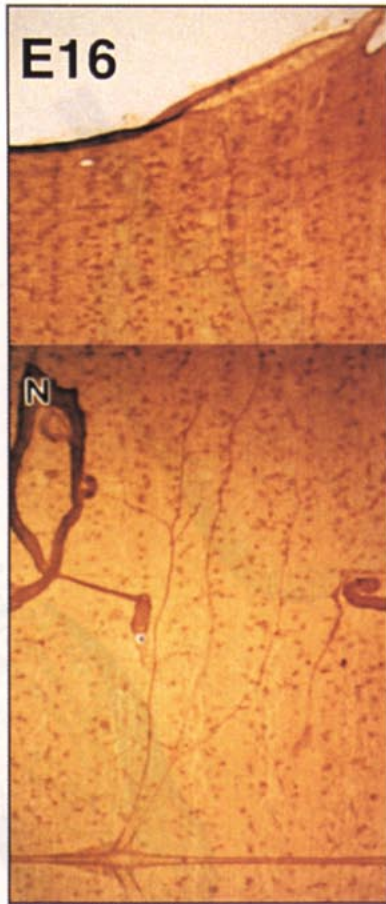
FIG. 1. Early Lan3-2-positive sensillar projections and CNS extirpations. (A) Camera lucida drawing of an E10 *Hirudo* embryo labeled by Lan3-2 showing the differentiation of the sensilla and the formation of the axon fascicles. The expression of the antigen proceeds in a rostrocaudal gradient exhibiting segments in different stages of development. Anterior is upward. The large arrow denotes the position of the CNS and the ventral midline. Dorsal–ventral polarity is determined early and the most dorsal sensillum (S7) has differentiated in the anterior 2/3 of the embryo. Bar, 500 μm. (B) Extirpation of early ganglionic primordia in whole, live embryos was done by grasping the rudimentary CNS through a small incision through the skin and gently pulling consecutive chains of CNS out from the enclosing sinus. For reference, large arrow denotes the same position as shown by a similar arrow in (A).

the CNS in this system? Previous work established that the d.p. nerve in both *Hirudo* (Jellies *et al.*, 1994, 1995) and *Haementeria* (Kuwada, 1985) is pioneered by one of the centrally located pressure-sensitive mechanosensory neurons (P$_D$-cells). Furthermore, it has recently been demonstrated that the correct branching and extension of axons to the periphery of other central neurons is dependent on the dorsal P$_D$-cell (Gan and Macagno, 1995). Thus, since it appears that critical guidance cues are provided by pioneer neurons extending from the CNS we decided to investigate the effect of CNS removal on the navigation of peripheral sensory neurons.

Ganglionic primordia were extirpated at E9 to early E10 by making a small incision into the germinal plate across the ganglionic chain and by grabbing the chain with fine

forceps and gently pulling out from 3 to 12 ganglia (Fig. 1B). The operated embryos and mock-operated controls were then allowed to develop for another 6–8 days before fixation and labeling with antibody. Preparations from such experiments are shown in Fig. 2, where the embryos were operated at E10 and analyzed at E16 after labeling with Lan3-2 antibody. At the embryonic stages of the operations, none of which were done later than the example shown in Fig. 1A, the more anterior segments had established their initial Lan3-2-positive sensillar projections, while the projections in more posterior segments was progressively more rudimentary. However, somewhat surprisingly, the response to CNS ablation in all 110 hemisegments examined (55 segmental ganglia ablated in 8 embryos) was robust and showed no overall segmental differences regardless of the degree of
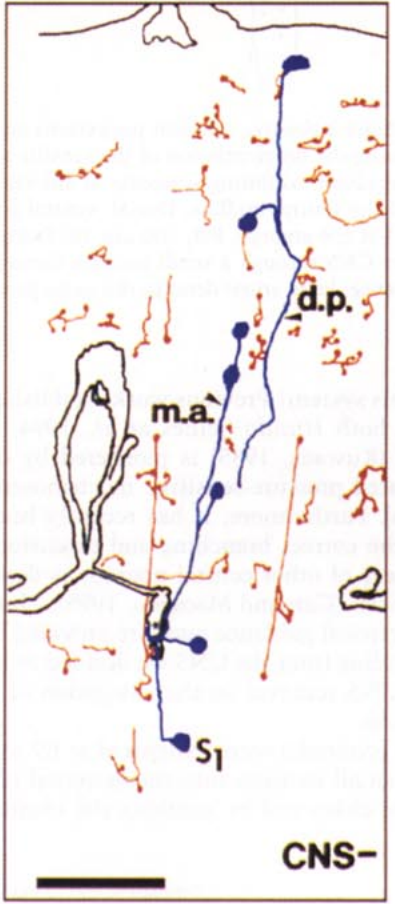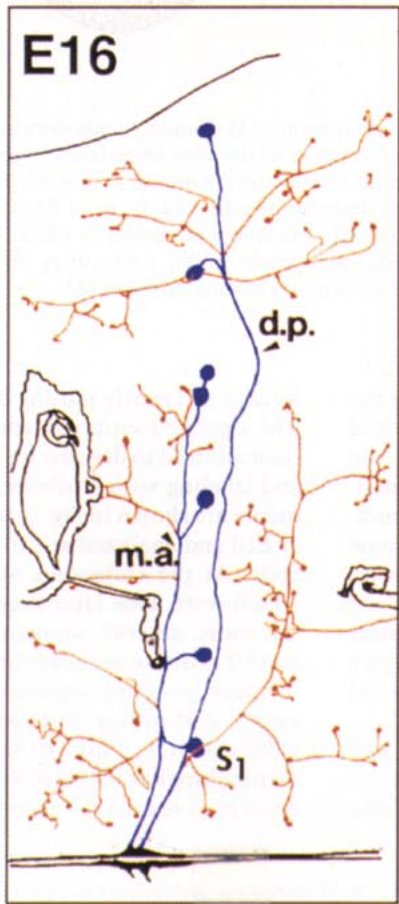
the development of the sensillar projections in these segments, the range of which is apparent in Fig. 1A.

In the absence of the CNS, Lan3-2-positive neurons continued to differentiate and extend axons (Fig. 2, E16). The different panels in the figure are to scale and illustrate the degree of development taking place between E10 and E16; indeed, the most conservative estimate for new axon outgrowth of the sensillar neurons (shown in blue) is in the range of a threefold elongation. In addition to this axonal growth, Lan3-2-positive extrasensillar neurons (Fig. 2, shown in red) arise continually throughout embryonic and postembryonic life. These neurons differentiate no earlier than E14–E16, well after the time of the CNS ablation. Most notably, this population of extrasensillar neurons seems unable to direct their growth cones toward the major Lan3-2-positive fascicles in the absence of CNS-associated cues. Rather, the axons from several adjacent neurons form local tangles of undirected but fasciculated projections (Fig. 2, CNS–). Clearly, the orderly projections of extrasensillar neurons into the CNS along stereotyped pathways seen in the control segment was disrupted in the absence of CNS-derived influence.

Lan3-2-Positive Neurons Fasciculate Together in the Absence of the CNS

The majority of sensillar axons in the absence of the CNS (Fig. 2) continue to fasciculate tightly in the periphery as well as appearing to project toward the ventral midline; however, a closer examination reveals that the latter is not always the case. Notice for example, that axons from the most ventral sensilla (S1), appear to favor fasciculation with other Lan3-2-positive neurons over direction of growth (Fig. 2). Deprived of CNS-associated cues, these axons, which normally project ventrally into the ganglion, instead project 180° in the opposite direction to fasciculate with the other sensillar neurons. Indeed, all the sensillar neurons appear to congregate in the region near the nephridiopore (Fig. 3), suggesting that the default pathway for these neurons is to fasciculate with each other regardless of their normal pathway choices. Thus, in the absence of CNS-associated cues, the Lan3-2-positive axons bundle tightly together and

continue to extend their axons along a circular path (Fig. 3B) that form around the nephridial bladder and pore complex. This complex extends as a pillar through the body wall connecting the internally located nephridium with the outside. Interestingly, when the CNS is present the Lan3-2-positive axons completely ignore this region and project directly past this complex (Fig. 3A).

Although most Lan3-2-positive axons enter the CNS through the m.a. nerve, those from S6 and S7 normally navigate along the previously established d.p. nerve. This nerve does not form within the muscle layers of the body wall as do the other three nerves; rather, it forms along a large bundle of "flattener" muscle that inserts ventrally and dorsally by extending through the body cavity. Thus, if severed near the ganglion (as in our CNS extirpations), this nerve would generally be expected to remain attached dorsally but presumably have a free end that could vary in position. In most cases this resulted in a Lan3-2-positive projection within the d.p. nerve that simply continued to extend but not join other pathways (see Fig. 2). However, in a few cases (4) the remaining free end of the d.p. nerve seemed to come into the vicinity of the axons from S1–S5 sensilla (Fig. 4), with the result that the S6 and S7 sensillar neurons would join and fasciculate with the m.a. pathway. These observations further support the notion that the default condition for CNS-deprived Lan3-2-positive axons in the absence of other cues is to fasciculate with each other, since the S6 and S7 sensillar projections normally would be completely separate from the m.a. pathway.

In a fortuitous observation among our control embryos we encountered a single example of what appeared to be a naturally occurring CNS ablation. In this case, due to an unknown developmental anomaly or genetic defect, the segment's ganglion failed to develop; however, the connectives joining the two neighboring ganglia still formed. Figure 5 shows a camera lucida drawing of this preparation after it was labeled with Lan3-2 antibody. Remarkably, in the segment without a ganglion the sensillar neurons appear to develop normally except that they form a tightly fasciculated ring around the nephridial pore complex as in the case of the surgically performed CNS ablations. This strongly suggests that the formation of circular paths is not an arti-

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FIG. 2. Extrasensillar Lan3-2-positive neurons require CNS-derived cues to navigate. The top panels show whole-mount preparations labeled with Lan3-2 antibody, whereas the bottom panels are corresponding camera lucida drawings of the peripheral projections. The first subpanel shows a hemisegment from an E10 embryo at the time the ablations were made. The middle panel shows a control hemisegment at E16 with the CNS intact and the right panel a hemisegment where the CNS was removed at E10 (CNS–). The panels are to scale, illustrating that the embryo grows normally and that the extrasensillar peripheral neurons do differentiate. However, their pathway formation is perturbed as illustrated in the lower right panel (CNS–) where the projections of sensillar neurons are shown in blue and those of the extrasensillar neurons in red. In the control embryo the projections are orderly and form common pathways (middle panel). In contrast, in the experimental embryo the projections of the extrasensillar neurons, which had not yet arisen before the ablation, do not form organized pathways, only small local fascicles. The sensillar axons (blue) remained fasciculated in the remnants of the m.a. and d.p. nerves as they extended, but were not directed toward the ventral midline. For example, the axons of the S1 sensillum extend 180° in the opposite direction from their normal projection joining the axons from the other sensilla in a tightly bundled ring of axons around the nephridiopore/bladder complex (*). Anterior is to the left and the ventral midline is toward the bottom of each photograph. N denotes the nephridium and the asterisk denotes the nephridial pore/bladder that penetrates the body wall. Bar, 500 μm.
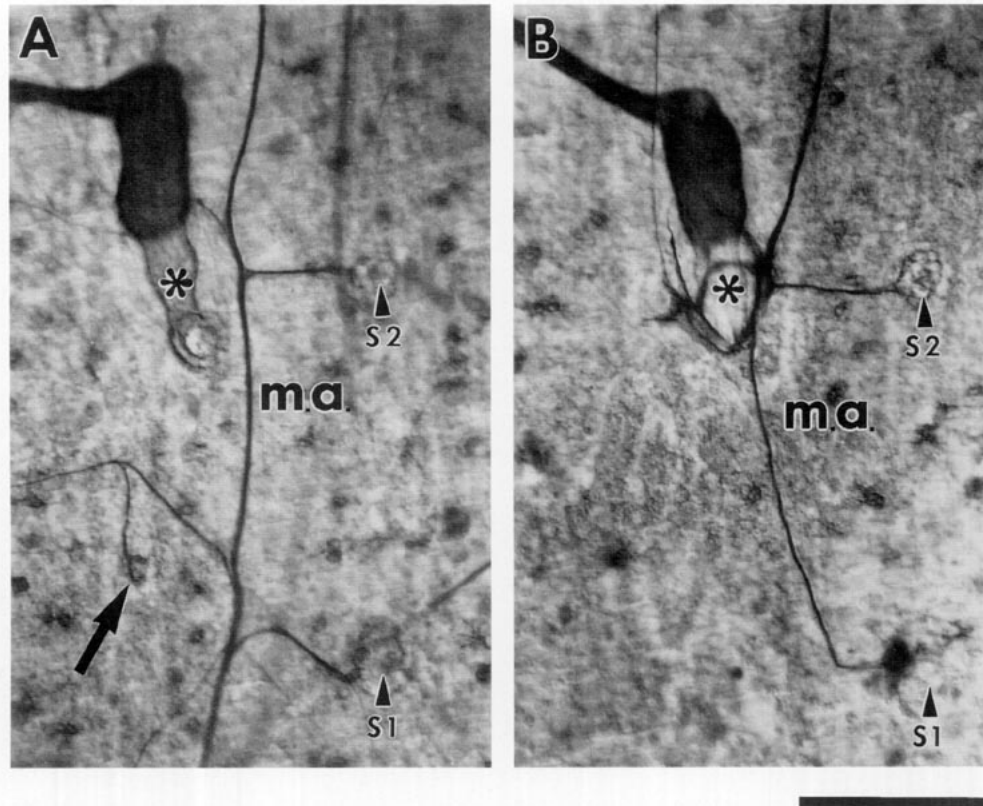
FIG. 3. CNS-deprived peripheral afferents extend in tightly fasciculated circular paths around the nephridiopore. (A) In the normal, unoperated embryo sensillar afferents labeled by the Lan3-2 antibody extend in the m.a. nerve past the nephridiopore/bladder complex (*) and are joined by axons arising from extrasensillar neurons (large arrow) forming orderly projections. (B) Micrograph from a corresponding segment to the one shown in (A) but from a preparation in which the CNS was surgically removed at E10 and the embryo allowed to develop to E16 before dissection and labeling with Lan3-2 antibody. Without the CNS present axons from the sensillar neurons congregate at the nephridia/bladder complex (*), where they fasciculate with each other often forming circular paths. Note that the direction of the projections from the S1 sensillum is reversed. Anterior is to the left and dorsal is up. Bar, 100 μm.

fact of the ablation procedure per se and partially explains why the effect of the CNS ablations appears to be independent of the developmental stage of the segment when the surgery occurs. Notice also that some of the axons from the S1 sensilla in the middle segment have reached the ganglion of the anterior segment. We speculate that this is due to some of the projections from the intact ganglion that are within reach of the growth cones emanating from the S1 sensillum. This interpretation is supported by the observation from CNS ablated preparations labeled with the ACT antibody (see also below) that axons from the immediate adjacent intact segment project numerous ectopic axons which innervate the territory vacated by the CNS removal (data not shown). These projections may then serve as guides for the sensillar neurons to the ectopic neighboring ganglion.

In the absence of such guiding factors it appears that especially the S1 sensillum will extend fasciculating axons in many directions. In most, but not all instances, these reach and fasciculate with other nearby sensillar axons. However, four cases where S1 axons did not join the closest bundle of sensillar afferents are illustrated in Fig. 6. In one case, in a segment which was adjacent to an intact segment (Fig. 6A) the fasciculated S1 axons migrated across the ventral midline and executed a sharp anterior turn to join with the axons of the S2 sensillum in the more anterior segment. We do not know whether these axons encountered CNS-derived axons from this segment, but as discussed above this seems to be the most likely explanation for this part of the anomalous S1 sensillar axon trajectory. In another case (Fig. 6B) the S1 axons encountered the free end of Lan3-2-positive axons in the d.p. pathway, fasciculated with these axons, and then projected anterior. Thus, it seems that there might be no intrinsic obstacle preventing these axons from navigating across the midline or intersegmentally, and indeed, two other cases were encountered where the S1 axon fascicle crossed the ventral midline (Figs. 6C and 6D). In each of these instances, regardless of undirected or anoma-
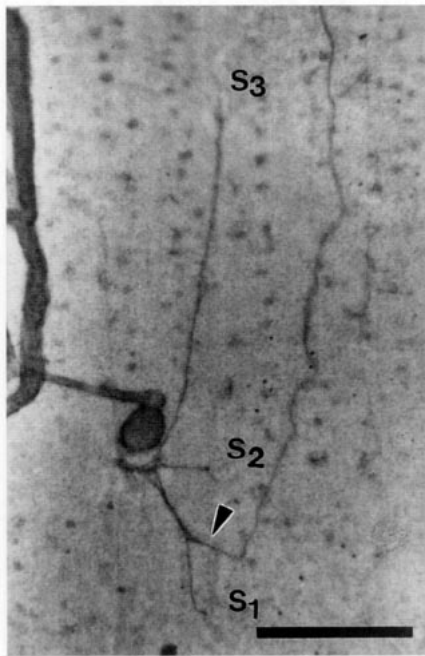
FIG. 4. Normally separate Lan3-2-positive pathways can fasciculate together in the absence of the CNS. CNS-ablated preparation labeled with Lan3-2 antibody where axons from the S6 and S7 sensillar neurons have joined and fasciculated with the axons from the S1–S5 sensilla (arrowhead). Normally, the peripheral pathways of these two groups of sensillar neurons are completely separate. Bar, 250 μm.

lously directed growth in the absence of the CNS, homophilic fasciculation seems to be a common denominator. In all CNS-ablated preparations the tightly bundled circular pathway around the nephridial bladder/pore complex was present and there were numerous examples of other types of circuitous self-fasciculated growth (Fig. 6B, white arrows).

Common Fasciculation of a Different Population of Peripheral Neurons in the Absence of the CNS

Although the focus of this study is directed toward the sensillar and extrasensillar afferents, these are not the full complement of peripheral neurons in *Hirudo* (Jellies *et al.*, 1995; Johansen and Johansen, 1995). Thus, in light of the striking results we obtained with respect to Lan3-2-positive neurons, we wondered about the effect on the pathways chosen by other peripheral neurons in the absence of the CNS. To address this question, as well as to assess the success of CNS extirpation, we labeled several preparations with the ACT-antibody (Fig. 7). This antibody labels the axons of all CNS neurons, as well as all of a population of peripheral neurons which are complimentary to the Lan3-2 antibody-positive neurons and which are not labeled by the Lan3-2 antibody (Jellies *et al.*, 1995). Moreover, the ACT

antibody does not label any of the embryonic Lan3-2-positive population of neurons (Jellies *et al.*, 1995).

Figure 7 shows an experiment where the CNS was removed as previously described; however, in this case the preparations were labeled with the ACT antibody instead
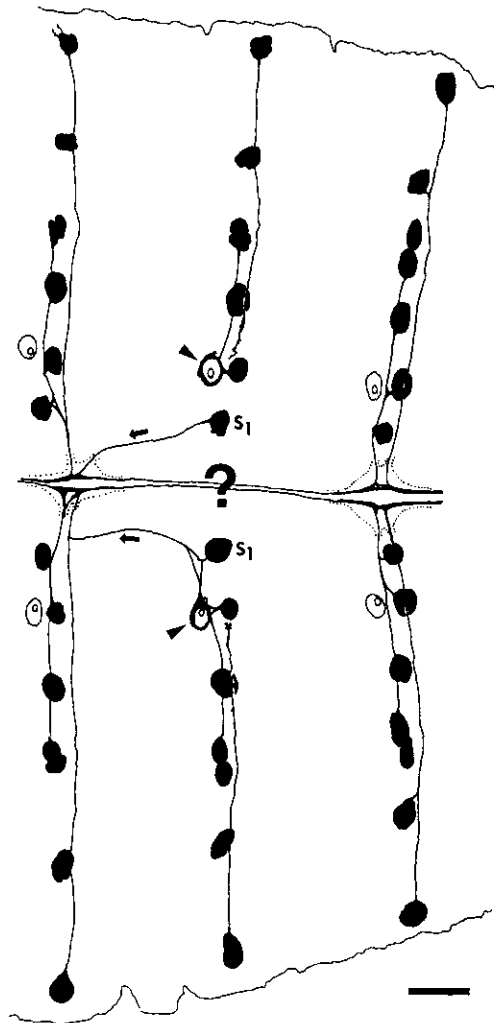

FIG. 5. Sensillar projections in a segment with a naturally occurring CNS ablation. Camera lucida drawing of a segment in a Lan3-2-labeled E15 preparation in which, due to an unknown developmental anomaly, the segment's ganglion (?) failed to develop. The phenotype of the sensillar projections appears very similar to those in embryos where the CNS has been removed surgically. The fasciculated ring of axons formed conspicuously around the nephridiopore/bladder complex (arrowheads). However, in the top hemisegment the S1 axons did not locate the other sensillar axons and instead extended in an abnormal intersegmental pathway (arrows) to the adjacent intact segment, where they fasciculated with the Lan3-2-positive axons there. In the bottom hemisegment the S1 sensillar axons did join the nephridiopore ring but also extended axons to the anterior segment. Anterior is to the left. Bar, 100 μm.
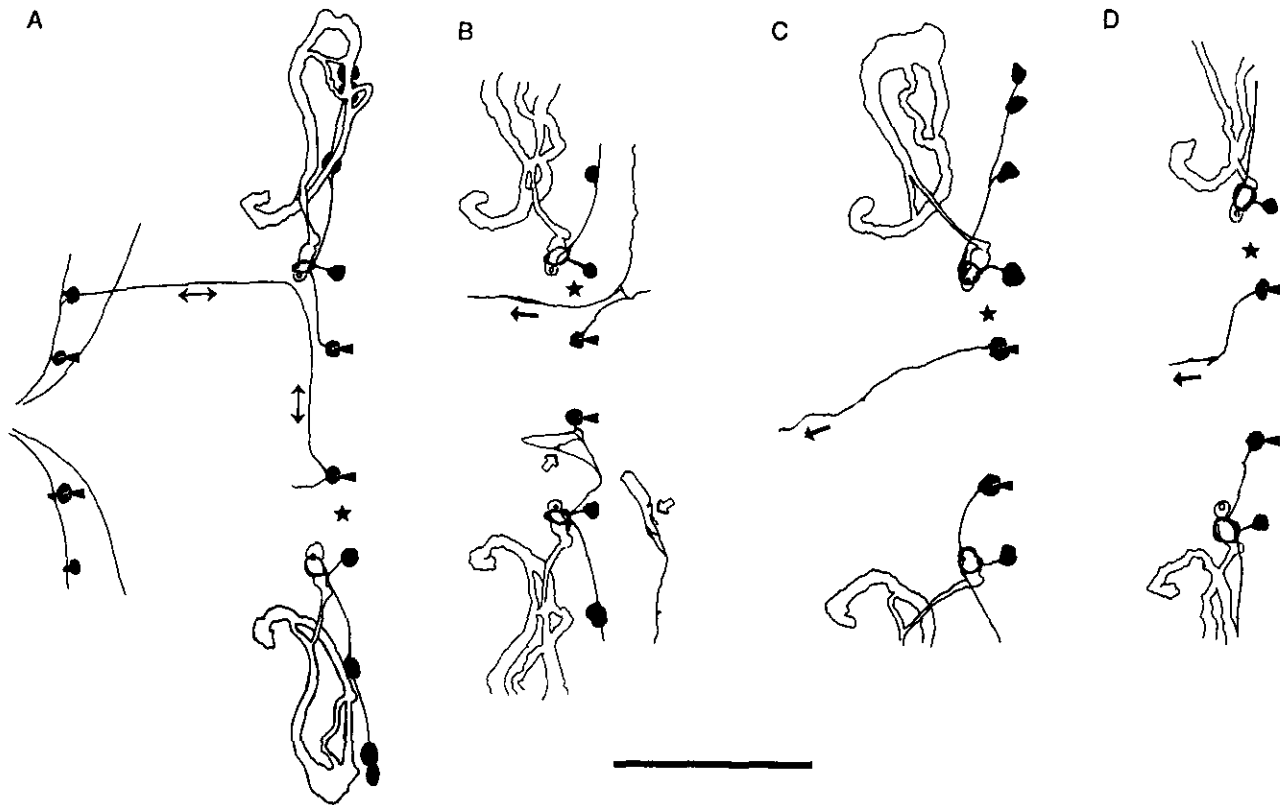
FIG. 6. Sensillar axons can extend along abnormal pathways intersegmentally and bilaterally when not directed by the CNS. Camera lucida drawings from four different preparations labeled with Lan3-2 antibody in which S1 sensillar (arrowheads) axons did not join the ipsilateral circular ring at the nephridiopore (stars). (A) S1 axons navigated across the midline and intersegmentally (double-headed arrows) before fasciculating with Lan3-2-positive axons in the adjacent segment. (B) S1 axons joined those from S6 and S7 and projected anteriorly (black arrow). Hollow arrows on the other side show other roughly circular tracts of fasciculated axons from the S6 and S7 sensillar neurons not associated with the nephridiopore/bladder complex. (C and D) Two different examples (top hemisegments) of S1 axons coursing both across the midline and toward the next anterior segment (black arrows). Anterior is to the left. Bar, 500 µm.

of the Lan3-2 antibody. The general results were strikingly similar to those obtained when we examined Lan3-2-positive neurons alone. For example, fasciculation of axons onto each other also seemed to dominate the behavior of this population of peripheral neurons in the absence of the CNS (Fig. 7B). The peripheral rudiments of all four major nerve pathways were present but consisted only of tight fascicles made up of just a few axons. However, the positions of the four major nerves are largely normal and therefore the basic pattern of primary peripheral nerves does not depend upon the presence of CNS axons. Importantly, in contrast to the extensive projections from the CNS in the control segment (Fig. 7A), there is no indication of any remnants of CNS axons in the CNS ablated segment (Fig. 7B). This strongly suggests that all potentially CNS-derived guidance factors have been successfully removed from the ablated segment.

Interestingly, in the absence of the CNS, the ACT-positive peripheral neurons formed a robust circular pathway around the nephridial bladder/pore complex similar to that formed by the Lan3-2-positive axons. Furthermore, these

neurons also continue to extend axons, suggesting that the CNS is not necessary for their maintenance or general growth. Any directionality in the absence of the CNS seems limited to relatively simple axonal paths in the periphery that are orthogonal to the long axis and that still retain a fasciculated organization. Double labeling of CNS ablated segments with Lan3-2 and ACT antibody shows that the two populations of peripheral neurons form similar but separate circular paths around the nephridia pore complex (data not shown).

DISCUSSION

A major issue concerning peripheral nerve formation relates to the relative temporal contributions from the CNS and PNS in establishing common nerve pathways containing mixed populations of efferent and afferent populations of axons. In leech the four major nerves initially form as discrete, roughly parallel tracts without bifurcation with
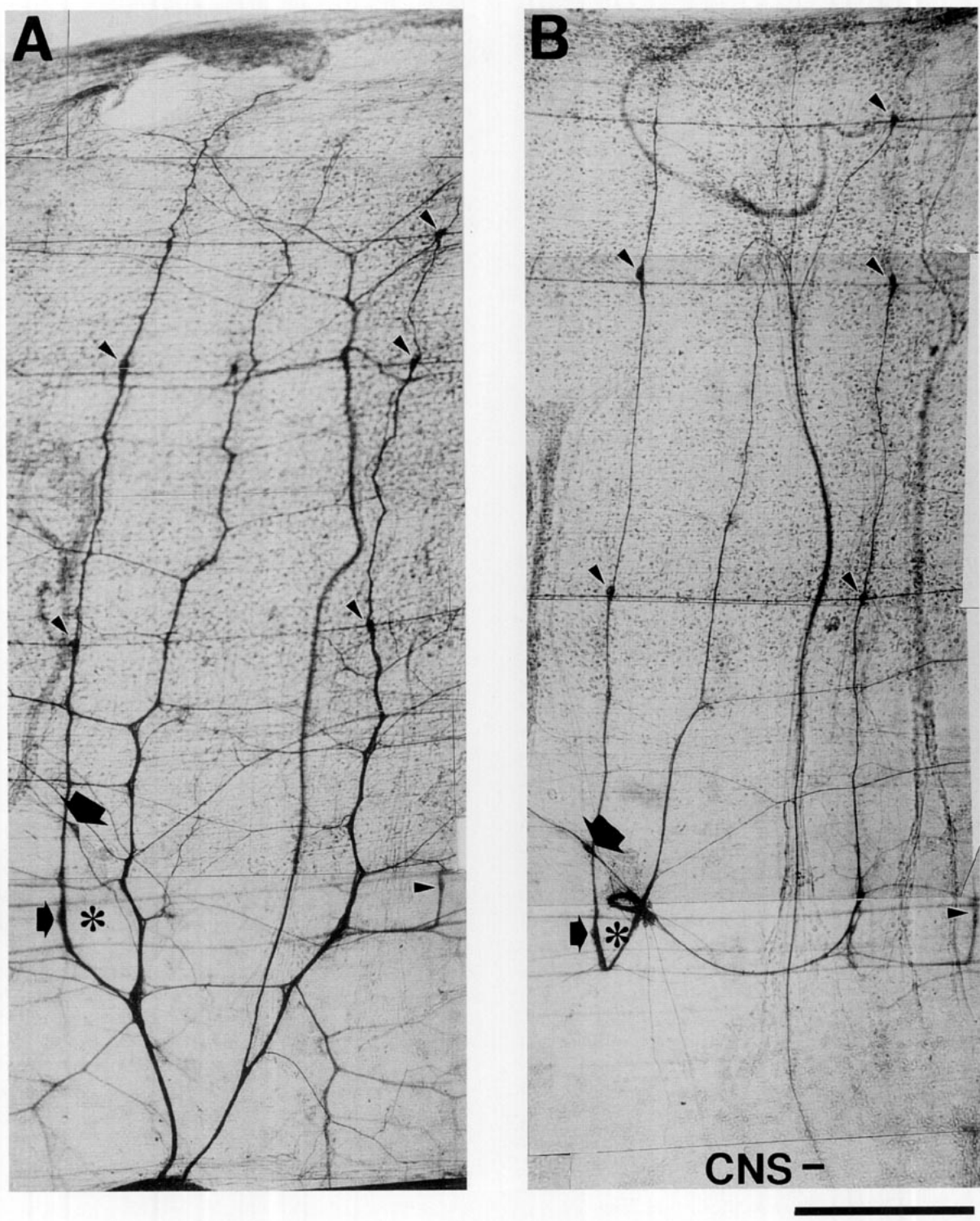
FIG. 7. A Lan3-2-negative population of peripheral neurons also exhibits peripheral fasciculation in the absence of CNS-derived cues. (A) Unoperated and (B) CNS-ablated hemisegments from an E16 embryo labeled with ACT antibody. The arrows and arrowheads denote the positions of peripheral neurons and the asterisk indicates the location of the nephridiopore/bladder complex as in previous figures. Note in the CNS-ablated embryo the tight circular pathway formed by these neurons around the nephridiopore similar to that formed by the Lan3-2-positive peripheral neurons. Comparable locations of reference can be identified in each panel, but because different embryos and even segments of embryos were stretched to different degrees during dissection the two panels do not show alignment in perfect register. Anterior is to the left and dorsal is up. Bar, 100 μm.

the mature branching pattern of the nerve roots generated by a secondary morphogenetic condensation whereby the most proximal portions of already extended axonal tracts coalesce (Jellies et al., 1995). CNS- and PNS-specific antibody double-label experiments and dye injections of pioneer neurons suggest that at least three of these nerves are pioneered by CNS neurons which subsequently may serve as guides for later differentiating CNS and PNS neurons (Jellies et al., 1995; Gan and Macagno, 1995). In this study we have directly tested this hypothesis by examining the consequences of CNS ablation on the navigation of a well-defined population of afferent peripheral sensory neurons. We show that in the absence of CNS-derived axons this population of peripheral neurons still differentiates and extend axons. However, the axonal growth cones extend abnormally and instead of forming orderly projections at best make small local fasciculated tangles. This suggests that CNS-derived guidance cues are absolutely required for the correct navigation of these peripheral sensory neurons. Although our experiments cannot rule out the possibility that there may be a diffusible chemoattractant, perhaps even one produced earlier and imprinted at the midline, the most parsimonious interpretation involves interactions of peripheral sensory neuron growth cones with molecular cues expressed on the surface of the CNS axons. This conclusion is supported by our previous observation that the peripheral sensory neurons normally grow selectively along the CNS efferents and that the genesis of the extrasensillar neurons is developmentally synchronized with the establishment of extensive projections into the periphery, ensuring that the proper guidance cues are in place when the sensory neurons differentiate and extend growth cones (Jellies et al., 1995). The lack of directional outgrowth in the absence of these projections furthermore suggests that the extrasensillar neurons do not have the capacity to respond to guidance cues provided by possible epithelial gradients or guide post cells alone.

Numerous studies in both invertebrates and vertebrates have described pathfinding strategies for nerve formation which relies on pioneer neurons establishing initial pathways that are then utilized by other neurons (Macagno, 1978; Bentley and Keshishian, 1982; Raper et al., 1984; Ghosh et al., 1990; McConnell et al., 1994). For example, in the grasshopper limb bud the majority of the peripheral pathways are established by afferent pioneer neurons (Bentley and Keshishian, 1982; Ho and Goodman, 1982) which navigate in response to epithelial gradients and proximally located guidepost cells (Caudy and Bentley, 1987; Kolodkin et al., 1992). However, while some populations of follower neurons have proven capable of independent pathfinding in the absence of the pioneer neurons (Keshishian and Bentley, 1983; Palka, 1986), others are not (Klose and Bentley, 1989). This has led to the suggestion that the ability of independent pathfinding of the follower neurons is correlated with the degree of complexity of the embryonic landscape when the follower neurons differentiate (Klose and Bentley, 1989). Early on when distances are short and the environment

relatively simple many types of neurons may have the capability for reaching their targets, whereas this is not likely to be the case at later stages. In leech the major peripheral pathways appear to be pioneered by CNS neurons early in development (Kuwada, 1985; Jellies et al., 1994, 1995), whereas the majority of the peripheral afferents are differentiating at considerably later stages when the germinal plate has greatly expanded. Thus, our finding that these neurons use the previously established efferent pathways to reach the CNS may be an adaptation because they arise relatively late in development in a highly complex environment. These results underscore the observation that general mechanisms common to all systems can be utilized in a variety of combinations and strategies depending on differences in the genesis and spatiotemporal relationship of the navigating neurons (Jan et al., 1985).

Another defining feature of the Lan3-2-positive peripheral neurons is their strong tendency to fasciculate with each other. In fact, we show that in the absence of other guidance cues Lan3-2-positive neurons regardless of their normal nerve routes default into forming circular pathways with each other. Especially the sensillar neurons formed a distinct ring of axons growing around the nephridia pore complex. This was a robust finding that was independent of the developmental stage of a given segment when the CNS ablation was performed. However, we do not have any indications of why the axonal rings were consistently formed in this location and not in others; perhaps the complex provides an attractive secondary pathway. We do not think it is an artifact of the ablation procedure itself since an entirely different group of peripheral neurons which are not labeled by the Lan3-2 antibody independently formed a similar fasciculated pathway. Furthermore, the pathway also formed in a case where a segmental ganglion failed to differentiate without surgery. We have previously proposed that the common fasciculation of the sensillar and extrasensillar neurons might involve the Lan3-2 antigen (Johansen et al., 1994; Jellies and Johansen, 1995). This is supported by the demonstration that Fab fragments of Lan3-2 antibody can perturb normal fascicle formation in cultured embryos (Zipser et al., 1989), directly implicating a functional role for this antigen in pathway formation. Furthermore, regeneration experiments show that peripheral axon fascicles indeed carry labels that preferentially guide these axons to extend along the Lan3-2-positive tracts (Peinado et al., 1987). Our present results also suggest that a similar homophilic molecule but of different specificity is expressed on the complementary population of peripheral neurons mediating the common fasciculation of these neurons.

These observations suggest that a sequence of guidance mechanisms are responsible for directing the extrasensillar sensory neurons to the CNS from the periphery. After they differentiate at positions scattered in the superficial body wall we suggest that they extend and retract in random directions until they encounter CNS projections which at this time of development have branched extensively in the periphery. Most if not all extrasensillar neurons are there-

fore likely to be close to such a projection located within the range of its growth cones. We speculate that heterophilic interactions of surface molecules on the peripheral neuron growth cones and CNS-derived axons might be employed to direct the extrasensillar neurons along the CNS towards the major nerve trunks. We have recently identified a 130-kDa protein which is expressed by CNS neurons and efferents and which coimmunopurifies with the Lan3-2 antigen (Y. Huang, J. Jellies, K. M. Johansen, and J. Johansen, unpublished observations). This suggests that an additional function of the Lan3-2 antigen could be to help guide the peripheral sensory neurons to the CNS through heterophilic interactions with this and/or another protein. In this scenario, when the extrasensillar neurons reach the nerves the hypothesized homophilic interactions mediated by the Lan3-2 antigen promote the common fasciculation of all the Lan3-2-positive neurons in the nerves. However, this fasciculation would be superseded by yet another set of heterophilic guidance cues as the Lan3-2-positive neurons segregate into specific tracts in the CNS (Johansen et al., 1992). Since subsets of sensillar neurons selectively express antigens which appear to be correlated with their choices of particular pathways once within the CNS, each of these individual fascicles may be defined by expression of its own distinct set of hierarchically organized guidance molecules (Johansen et al., 1994). Thus, this well-defined population of peripheral sensory neurons in leech promises to be a useful model system for the identification and dissection of hierarchically organized axonal guidance mechanisms.

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