Ectopic CNS Projections Guide Peripheral Neuron Axons along Novel Pathways in Leech Embryos

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Previous studies have indicated that the formation of stereotyped segmental nerves in leech embryos depends on the interactions between CNS projections and ingrowing afferents from peripheral neurons. Especially, CNS-ablation experiments have suggested that CNS-derived guidance cues are required for the correct navigation of several groups of peripheral sensory neurons. In order to directly test this hypothesis we have performed transplantations of CNS ganglia into ectopic sites in segments from which the resident ganglia have been removed. We find that the transplanted ganglia extend numerous axons distributed roughly equally in all directions. When these CNS projections reach and make contact with peripheral sensory axons they are used as guides for peripheral neurons to grow toward and into the ectopic ganglia even when this means following novel pathways that cross the midline and/or segmental boundaries. The peripheral sensory axons turn and grow toward the ectopic ganglia only when in physical contact with CNS axons, suggesting that diffusible chemoattractants are not a factor. These results demonstrate that the guidance cues provided by ectopic CNS projections are both necessary and sufficient to steer peripheral sensory neuron axons into the CNS. © 2000 Academic Press

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INTRODUCTION

A major question concerning how stereotyped common nerve pathways are formed relates to the relative spatial and temporal contributions from mixed neuronal populations. Numerous studies in both invertebrates and vertebrates have described pathfinding strategies for nerve formation which rely on pioneer neurons establishing initial scaffolds of axon tracts that are then utilized by later extending axons as substrates for directed migration (Macagno, 1978; Bentley and Keshishian, 1982; Ho and Goodman, 1983; Raper et al., 1983; Ghosh et al., 1990; McConnell et al., 1994). This kind of strategy is particularly well suited to ensure the formation of common nerve pathways between afferent and efferent projections. We have studied these aspects of nerve development in leech embryos in which the four major segmental nerves per hemisegment contain axons from both peripheral and central neurons (Jellies and Johansen, 1995) and established that three of these nerves (aa, ma, and pp) are pioneered by peripheral neurons, whereas the fourth nerve (dp) is pioneered by central neurons (Jellies et al., 1994, 1995, 1996; Huang et al., 1998).

Ablation studies have further demonstrated that interactions between the central and the peripheral neuron axons are likely to be necessary for correct nerve formation in this system. For example, studies in Helobdella have shown that laser ablation of the peripheral neurons that differentiate in a position to guide outgrowth of ganglionic neurons perturbs the segmental nerve formation (Braun and Stent, 1989a,b). Conversely, CNS projections appear to be required for the correct pathfinding of several populations of late differentiating extrasensillar peripheral sensory neurons (Jellies et al., 1995). In perturbation experiments in which ganglionic primordia were surgically removed from E9 embryos that were allowed to survive for 6–8 days before examination the extrasensillar neurons still differentiate but their axonal outgrowth is stunted, without apparent direction, and at best form small local bundles of axons (Jellies et al., 1995). Thus, these experiments seem to indicate that CNS-derived guidance cues are necessary for correct navigation of these groups of peripheral neurons and
that in the absence of these guidance cues their default option is to fasciculate with each other. In the present study we have performed ectopic transplantations of CNS neurons in order to directly test the hypothesis based on these observations that the projections of central neurons can guide the axons of peripheral neurons. The results show that the guidance cues provided by the ectopic CNS projections are necessary and sufficient to steer peripheral sensory neuron axons into the CNS through novel pathways.

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 a sterile-filtered solution 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 percentages of development. Dissections of embryos were performed in leech saline solution 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. Embryonic day 10 (E10) is characterized by the first sign of a tail sucker, and E30 is the termination of embryogenesis.

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 (ACT) (Sigma) was used to label central neurons as well as a population of nonsensillar peripheral neurons and their axonal projections (Jellies et al., 1996). 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 the HRP-conjugated antibody complex was visualized by incubation overnight at room temperature with diluted anti-mouse IgG1 TRITC-conjugated secondary antibody (Bio-Rad, 1:200 dilution). After the wash 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 fluorescein-conjugated subtype-specific secondary antibodies. A rabbit anti-mouse IgG, TRITC-conjugated secondary antibody (Cappel) was used for Lan3-2 and a rabbit anti-mouse IgG₂a FITC-conjugated secondary antibody (Cappel) for the ACT antibody. Fluorescently labeled preparations were mounted in glycerol with 5% n-propyl gallate. The labeled preparations were photographed on a Zeiss Axioskop using Ektachrome 64T film or Ektachrome 100 daylight film. The color positives were digitized using Adobe Photoshop and a Nikon Coolscan slide scanner. In Photoshop the images were converted to black and white, pseudocolored, and image processed before being imported into Freehand (Macromedia) for composition and labeling. For confocal analysis of double-labeled preparations a separate confocal series of images for each fluorophor was obtained simultaneously with the Leica confocal TCS NT microscope at 1-μm intervals using the krypton and argon laser lines and the appropriate filter sets. An average projection image for each of the image stacks was obtained using the NIH Image software. These were subsequently imported into Photoshop where they were pseudocolored, image processed, and merged. Dynamic 3D-representations as well as stereo pairs of images at ±7.2° and ±7.2° angles, respectively, were generated using the Leica TCS 3D-reconstruction software.

CNS Ablation and Ectopic Transplantation

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. 1A). It has previously been shown that the intersegmental connectives between ganglionic primordia form earlier than the peripheral projections to the germinal plate (Jellies et al., 1994, 1996). This makes it possible to slide the ganglionic chain out of the forming ventral sinus that normally encases it. For transplantation experiments a new incision was made over an area where the CNS had successfully been removed and three or more contiguous ganglia were carefully transferred with forceps through the incision to an ectopic location. After CNS removal and transplantation embryos were rinsed through three changes of normal embryo water and reared in individual dishes (35-mm Falcon plastic) until E14-E15. In successfully operated embryos the incisions in the germinal plate healed within 2–3 h. 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. The results of this paper are based on the examination of more than 100 segments from 20 embryos in the case of CNS ablations and from examination of 7 embryos from which the CNS was removed and in which ectopic CNS transplantation was successfully completed.

RESULTS

In order to follow the early development of central axonal pathways, we used a mAb directed against ACT which labels all known central neurons and their processes in the medicinal leech in addition to a small subpopulation of peripheral neurons (Jellies et al., 1995, 1996). These ACT antibody-positive neurons are referred to as ACT neurons in the following. Figure 1B shows the development of the CNS and its peripheral projections in an E9 embryo labeled with the ACT antibody at the stage at which we performed ectopic transplantations. Each embryo exhibits a rostral-caudal gradient of development having about 3–4 h between adjacent segments (Jellies and Kristan, 1991). Thus, the chain of 32 segments in each embryo provides a relative sequence of axonal extension (Fig. 1B). At E9 in anterior
ganglia the CNS projections were just beginning to join in forming the segmental nerves, whereas in posterior ganglia projections were yet to be extended (Fig. 1B). In contrast, at stage E14–E15 the major nerves (aa, ma, dp, and pp) as well as multiple efferent pathways were fully established in all segments as illustrated in Fig. 1C. For following the extension of peripheral sensory neuron axons we labeled preparations with the mAb Lan3-2, which recognizes a surface glycoepitope on the cell adhesion molecules (CAMs) Tractin and LeechCAM (Huang et al., 1997) that is expressed by all sensillar and late-developing extrasensillar afferents (Johansen et al., 1992; Jellies et al., 1994, 1995). Sensillar neurons (Lan3-2 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–S5 fasciculate in the periphery along the ma nerve while those from S6 and S7 fasciculate within the developing dp nerve. The ACT antibody does not label the Lan3-2 neurons.

Peripheral Neuron Pathway Formation in the Absence of the CNS

We have previously shown that after early CNS ablation all CNS axons atrophy and die (Jellies et al., 1995). How-
ever, all the known groups of peripheral neurons continue to develop and extend axons. In addition to the sensillar and extrasensillar sensory neurons specifically labeled by Lan3-2, the ACT antibody selectively labels 21 peripheral neurons present in each hemisegment (Huang et al., 1998). In this study we have directly examined the interactions between the axons of the two groups of Lan3-2 and ACT antibody-positive peripheral neurons in the absence of the CNS by antibody double-labeling experiments. The most prominent of the ACT-positive peripheral neurons are the nephridial nerve cell (NNC), the eight stretch receptor neurons (HO or “Hoover” cells), and the seven neurons making up the anterior root ganglion (ARG) (Jellies et al., 1995). As shown in Figs. 2A and 2B these neurons were found in their normal positions in an E14 embryo in which the CNS was ablated at E9, and the labeling confirmed that in the absence of the CNS these peripheral neurons are capable of setting up the scaffolding configuring the four peripheral nerves (Jellies et al., 1995). However, without the CNS as a target the axons of neurons in the two posterior peripheral nerves often fasciculate together and form a loop (Fig. 2A, large arrow). Interestingly, even under these conditions the dp nerve, which is normally pioneered by the central Pn neuron (Kuwada, 1985; Jellies et al., 1994; Jellies and Johansen, 1995), still forms along the dorsoventral flattener muscles. This is also the case in posterior segments from which the CNS was removed before any peripheral CNS projections were extended (Fig. 1B). Thus, while the Pn neuron pioneers the dp nerve it is not obligatory for its formation and general trajectory. The axons of neurons from the two anterior nerves also merge and form a ring-like structure close to the nephridiopore (Fig. 2A). This was a robust finding and occurred in all segments examined from which the CNS was removed (n > 200). High-resolution confocal images of double labelings of such a ring structure (Figs. 2B–2D) suggest that the reason a circular pathway was formed at this junction was the close proximity of the NNC, the HO1/LPC cells, and the ARG cells (Huang et al., 1998) (Fig. 2B). These three groups of neurons developed in a triangle and as their projections extended along one another’s axons in the absence of guidance cues from the CNS, they looped around in circles. In Fig. 2B such an axonal loop was about to be completed as indicated by the arrow. As shown in Fig. 2C and in the merged image in Fig. 2D the Lan3-2 group of peripheral sensory neurons in the absence of CNS projections appeared to use ACT axons as guides for directed growth. The sensillar neurons formed coextensive circular pathways with the ACT neurons and even in some cases extended axons toward the nephridia along the NNC cell axon (Fig. 2C, arrow, and Fig. 2D), a completely novel pathway for these neurons. Although the Lan3-2 sensillar neurons and the ACT peripheral neurons extended along each other they formed clearly separate fascicles (Fig. 2D). Additionally, this was verified by generating 3D reconstructions of the images in which the spatial relationship of the fascicles could unambiguously be determined (data not shown).

**Ectopic CNS Projections Guide Sensillar Neurons**

In order to explore the extent to which CNS projections can guide peripheral sensory neurons, we conducted ectopic CNS transplantation studies. The CNS from about 6–10 segments of E9 embryos were removed and three or four ganglia were put back into the germinal plate in an ectopic location. The location of the transplanted ganglia spanned the range from near the ventral midline (n = 3) to near the future dorsal midline (n = 1) with three transplants situated close to the nephridia in the middle of the hemisegments. The embryos were then allowed to develop for 5–6 days until stage E14–E15 when they were dissected, fixed, and labeled with ACT and/or Lan3-2 antibody. Figure 3 shows

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**FIG. 2.** Development and selective fasciculation of peripheral neurons in the absence of the CNS. (A) Superposition of the location of mAb Lan3-2-positive sensillar neurons S1–S6 drawn in red on a composite micrograph of ACT-labeled peripheral neurons in a CNS-ablated hemisegment. Small arrows indicate the locations of the eight stretch receptor neurons (HO or “Hoover” cells), and the seven neurons making up the anterior root ganglion (ARG) (Jellies et al., 1995). Still forms along the dorsoventral flattener muscles. This is also the case in posterior segments from which the CNS was removed before any peripheral CNS projections were extended (Fig. 1B). Thus, while the Pn neuron pioneers the dp nerve it is not obligatory for its formation and general trajectory. The axons of neurons from the two anterior nerves also merge and form a ring-like structure close to the nephridiopore (Fig. 2A). This was a robust finding and occurred in all segments examined from which the CNS was removed (n > 200). High-resolution confocal images of double labelings of such a ring structure (Figs. 2B–2D) suggest that the reason a circular pathway was formed at this junction was the close proximity of the NNC, the HO1/LPC cells, and the ARG cells (Huang et al., 1998) (Fig. 2B). These three groups of neurons developed in a triangle and as their projections extended along one another’s axons in the absence of guidance cues from the CNS, they looped around in circles. In Fig. 2B such an axonal loop was about to be completed as indicated by the arrow. As shown in Fig. 2C and in the merged image in Fig. 2D the Lan3-2 group of peripheral sensory neurons in the absence of CNS projections appeared to use ACT axons as guides for directed growth. The sensillar neurons formed coextensive circular pathways with the ACT neurons and even in some cases extended axons toward the nephridia along the NNC cell axon (Fig. 2C, arrow, and Fig. 2D), a completely novel pathway for these neurons. Although the Lan3-2 sensillar neurons and the ACT peripheral neurons extended along each other they formed clearly separate fascicles (Fig. 2D). Additionally, this was verified by generating 3D reconstructions of the images in which the spatial relationship of the fascicles could unambiguously be determined (data not shown).
two segments from such an embryo labeled with ACT antibody. The three transplanted ectopic ganglia had extended numerous axons roughly equally in all directions. The CNS projections were not confined to the segment of the transplantation but expanded into all centrally denervated segments and would readily cross the ventral midline. This phenotype was found in all seven of the experimental embryos regardless of the specific location of the transplanted ganglia within the hemisegment. In one preparation we observed ectopic innervation spanning at least four segments. As shown by the double labeling in Fig. 4 the ectopic CNS projections were used by the sensillar and extrasensillar sensory neurons as guides to reach the ectopic ganglia. This was the case for sensory neurons located contralaterally with respect to the ectopic ganglia as well as for those located in neighboring segments (Fig. 4). The Lan3-2 neurons in all neighboring segments reached by the ectopic CNS projections were observed to extend axons toward the implanted ganglia in all seven preparations examined. Where the ectopic axons have not yet reached the loops of the sensillar neurons (Fig. 4C, yellow arrow) there was no evidence of directed growth toward the ectopic ganglia. This suggests that guidance of sensillar axon extension into the ectopic ganglia depends on direct physical interactions with the CNS projections and not on diffusible chemoattractants.

DISCUSSION

Previous studies have supported a role for the interdependence of outgrowth from the CNS and ingrowth from peripheral neurons in establishing stereotyped nerves in leech embryos (Braun and Stent, 1989a,b; Jellies et al., 1994, 1995, 1996; Jellies and Johansen, 1995). Especially, CNS ablation studies (Jellies et al., 1995) have suggested that CNS-derived guidance cues are required for the correct navigation of several groups of peripheral sensory neurons. However, these experiments did not directly demonstrate directed guidance of peripheral neurons along CNS projections or examine the possibility of the involvement of diffusible chemoattractants. In this study we have addressed these issues and show by ectopic transplantations of CNS ganglia that CNS projections are necessary and sufficient to guide peripheral sensory neuron axons into the CNS. We find that when ganglia are transplanted into ectopic sites in segments from which the resident ganglion has been removed they extend numerous projections distributed roughly equally in all directions. As these CNS axons reach and make contact with peripheral sensory axons the latter use them as guides to grow toward and into the CNS even when this means following novel pathways that cross the midline and/or segmental boundaries. The peripheral sensory axons turn and grow toward the ectopic ganglia only when in physical contact with CNS projections, suggesting that diffusible chemoattractants are not a significant factor. This result is consistent with recent in vitro experiments that implicate contact-based cues in CNS outgrowth (Harik et al., 1999). Furthermore, these results make it unlikely that there are repulsive epithelial gradients or barriers defining segmental territories for either the CNS or the peripheral projections.

While the dp nerve is pioneered by the central P0 neuron our results indicate that this neuron may not be obligatory for dp nerve formation. The reason for this may be that the dp nerve pathway forms along the dorsoventral flattener muscles which may provide a sufficient substrate for establishing the dp nerve even in the absence of the P0 axon. Comparable nonneuronal cells have been shown to provide the necessary guidance cues for a different segment-specific nerve in leech (Jellies and Kristan, 1988). In contrast, the three remaining peripheral nerves have been shown to be pioneered by peripheral neurons and in the absence of some of these peripheral neurons proper segmental nerve formation is perturbed (Braun and Stent, 1989a,b; Jellies et al., 1994, 1996; Huang et al., 1998). In fact, an interesting finding of this and a previous study (Jellies et al., 1995) is that peripheral neurons develop normally in the absence of the CNS and that they are still capable of establishing the peripheral trajectories of the four segmental nerves. This may reflect the way peripheral neurons differentiate and suggests a developmental sequence for common segmental nerve formation in leech embryos. In this model peripheral neurons differentiate as closely apposed groups of neurons near the ganglionic primordia and develop in the periphery before CNS efferent extension (Huang et al., 1998). As they differentiate the peripheral neurons extend early axons into the ganglion and along each other at this time when distances are short: less than 75 μm from the edge of the germinal plate and to the ganglionic primordium. Subsequently, the peripheral neurons separate and by a combination of germinal plate expansion and migration they reach their final locations setting up a orthogonal grid together with the five ACT antibody-positive longitudinal muscle fibers (Huang et al., 1998). The nerve trajectories set up in this way by the peripheral neurons serve as guides for the initial CNS projections, thus establishing the common segmental nerve pathways. In turn the CNS projections branching in the body wall are used as guides by other groups of peripheral neurons such as the extrasensillar sensory neurons, which start to differentiate relatively late in development at E16 (Johansen et al., 1992; Jellies et al., 1995). These late-differentiating neurons use the peripheral projections of central neurons as a guide to reach the major nerve trunks where they then selectively fasciculate among the sensillar axons in order to reach the CNS (Jellies et al., 1995, 1996). This latter mechanism, the strong tendency of related groups of neurons to fasciculate with each other, is another defining feature of pathway formation in this system. For example, we show here by double-label experiments that the Lan3-2-positive and the ACT-positive groups of peripheral neurons clearly are segregated into different fascicles, and evidence has been provided that
**FIG. 3.** Axon outgrowth from ectopic ganglia. Projections from transplanted ganglia (Eg) labeled with ACT antibody in an E14 embryo from which the resident CNS was removed at E9. Asterisks indicate the location of the nephriodpores in the two segments shown. The stippled lines indicate the approximate positions of the ventral midline and the segmental boundaries. Scale bar, 140 μm.
even within the sensillar neurons there are multiple sub-populations of antigenically distinct neurons the axons of which travel together in separate fascicles (Peinado et al., 1990; Johansen et al., 1992).

In this way relatively simple, but highly temporally coordinated mechanisms can account for common nerve formation of mixed afferents and efferents in leech. We propose that related groups of neurons exhibit distinct surface molecules that through homophilic interactions segregate them into well-defined axon fascicles. Heterophilic interactions between CAMs in turn may allow different groups of neurons to extend axons along one another’s fascicles, providing guidance cues for common nerve formation. The axon outgrowth and novel pathway formation between peripheral neurons and ectopic CNS transplantations in the present study clearly demonstrate that such interactions occur. We further propose that the location and migration of developing peripheral neurons which differentiate prior to the extension of CNS projections define the trajectories of three of the segmental nerves. Interactions of neurons with the dorsoventral flattener muscles are likely to define the trajectory of the fourth segmental nerve. Thus, by providing a relatively simple model system the leech promises to be useful for the further identification and characterization of axonal guidance mechanisms leading to common segmental nerve formation.
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