Target-induced neurogenesis in the leech CNS involves efferent projections to the target

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**SUMMARY**

During a critical period in leech embryogenesis, the sex nerves that connect the 5th and 6th midbody ganglia (MG5 and MG6) to the primordium of the male sexual organ carry a spatially localized signal that induces the birth of several hundred neurons specific to these ganglia. We examined particular cellular elements (afferents, efferents, non-neuronal components) within these nerves as potential conveyors of the inductive signal. We show that axons of peripheral sensory neurons in the male genitalia travel along the sex nerves and into MG5 and MG6, but reach the CNS after the critical period has elapsed and cannot, therefore, be involved in the induction. Of the six sex nerves, four contain non-neuronal cells that span the entire distance between the male genitalia and the sex ganglia. However, when male genitalia were transplanted to ectopic locations close to MG6, induction occurred frequently but only in MG6, mediated by ectopic nerves that do not contain these cells. Thus, non-neuronal cells specific to the normal sex nerves are not necessary for induction. In addition, dye injections into the target during the critical period failed to reveal migrating cells in the sex nerves that could convey the inductive signal to the CNS. Finally, we show that 11 pairs of central neurons in each ganglion project to the male organ early during the critical period. In the adult, at least 3 additional pairs of neurons in MG6 also innervate this target. We conclude that the only components of the sex nerves that connect the sex ganglia to the target during the critical period that could be associated with induced central mitogenesis are the axons of central neurons that innervate the male genitalia.

Key words: afferents, efferents, sexual organs, peripheral nerves, mitogenesis, *Hirudo medicinalis*

**INTRODUCTION**

The interaction of embryonic neurons with their targets has been shown to regulate adult cell numbers in specific CNS regions in several experimental systems. During vertebrate development, for example, target-derived neurotrophic factors support the survival of innervating neurons (e.g., Davies et al., 1987; Barde, 1989; Oppenheim et al., 1991). The traditional view is that these factors exert their effect retrogradely from the target to the soma of the responsive neuron, but there is also evidence for anterograde effects (reviewed by Korsching, 1993). Recently, a number of neurotrophic factors have been shown to regulate proliferation of neuronal and glial precursors in vitro (e.g., Birren et al., 1993; DiCicco-Bloom et al., 1993; see also Korsching, 1993), but the relationship of the cells that produce these factors and those that respond to them in the intact animal is not well understood. To analyze such inductive interactions, it is important to identify the participating cells and to characterize spatial and temporal patterns of interaction.

In invertebrates, while retrograde trophic dependence for survival does not appear to be a common strategy (e.g., Whitington et al., 1982; Baptista and Macagno, 1988b; but see Campos et al., 1992), anterograde trophic neuron-target interactions do regulate neuronal number (e.g., Power, 1943; LoPresti et al., 1973; Macagno, 1979; Meyerowitz and Kankel, 1987; Fischbach and Technau, 1984; Steller et al., 1987). Notably, retinal afferents from the *Drosophila* eye, upon their arrival in the developing brain, induce proliferation of optic lobe precursors (Selleck and Steller, 1991; Selleck et al., 1992). Control of proliferation of neuronal precursors by afferent fibers has also been proposed in the vertebrate visual system (Kollros and Thiesse, 1988).

In the leech *Hirudo medicinalis*, the birth of a segment-specific population of adult neurons, the peripherally induced central (PIC) neurons of the sex ganglia (Baptista et al., 1990) is controlled by a similar inductive event. Identified nerves from a peripheral organ located in the fifth midbody segment, the male genitalia, carry a signal to midbody ganglia MG5 and MG6 (Baptista and Macagno, 1988a). Induction takes place during a critical period extending from embryonic days E13 to E16 (Becker and Macagno, 1992), and proliferation of PIC neuron precursors can be demonstrated beginning at E16. Induction and hence conveyance of the inductive signal occurs...
independently in MG5 and MG6, suggesting that the inductive signal does not diffuse over great distances.

Here we ask whether induction could be conveyed by (i) afferent axons, (ii) non-neuronal components of the sex nerves, or (iii) efferent axons. We describe the cells that are candidates for conveyance of the inductive signal and that participate in the formation of the sex nerves before and during the inductive period, as well as those that are components of these nerves in adult animals. Our results show that induction can take place in the absence of afferent axons as well as non-neuronal cells that normally participate in the formation of the sex nerves, suggesting that the inductive signal is conveyed from the target organ to the CNS by the axons of central neurons.

MATERIALS AND METHODS

Animals and culture conditions

Leech embryos were obtained from a breeding colony of Hirudo medicinalis maintained at 23°C in our laboratory. Embryos were removed from their cocoons and kept in sterile artificial spring water (0.5 g/l Instant Ocean, Aquarium Systems), supplemented with 2 mg/l quinine hydrochloride (Sigma) to reduce parasitic infections. Embryogenesis lasts about 30 days at this temperature, and embryos were staged by days of development after egg laying (Fernandez and Stent, 1982).

Surgical procedures

Surgical procedures were as described in Becker and Macagno (1992). Transplantations of male genital primordia to the location of the female genitalia were done within the same animal. First, the female organ was ablated in segment 6, then the male organ was removed from segment 5 and transferred to the opening left in segment 6. Both wounds healed within a few hours after the operation and the animals developed otherwise normally.

Dye backfills

Backfills of central neurons were performed in live and fixed animals using the carbocyanine dyes DiI and DiO (Molecular Probes Inc., USA). For backfills in fixed tissue, leeches between E14 and E15 were anesthetized and pinned ventral side down in a Sylgard-coated dish with 8% EtOH in Wenning’s solution (40 mM DL-malic acid, pH 7.4). The yolk was removed and 20-50 nl of a 1% solution of DiI in dimethyl formamide (DMF) were injected into the primordium of the male organ through a broken-tip glass microelectrode attached to a syringe. The embryos were stored in 4% paraformaldehyde in the dark at 37°C for 5 days to allow the dye to diffuse. Embryos were then cleared and mounted in glycerol and labeled nearby terminals of sensory neurons, resulting in false-positive labeling of identified neurons.

Backfills of adult sex nerves with nickel-lysine

Intracellular dye fills

Prior to intracellular dye injection, backfills from the male organ were performed in E12 to E15 embryos as described above with a 1% solution of DiO in methylene chloride. The next day, animals were anesthetized and pinned in a Sylgard dish with 8% EtOH in Wenning’s solution. The ventral aspect of the nervous system was exposed by cutting the body wall tissue and the blood sinus with sharp iridectomy scissors. Electrode tips were bent to a 40˚ angle with a heated Nichrome wire and were filled with a solution of 1% DiI in DMF. Electrode shanks were backfilled with 0.2 M KC1 or 0.2 M LiC1. Using a compound microscope equipped with epifluorescence and a 40x water immersion lens, cells labelled with DiO were injected with DiI using 2.5 nA pulses of a 250 ms duration. Embryos were then stored in 4% paraformaldehyde at 37°C for 5 days, and then were cleared and mounted in glycerol. This method allowed visualisation of the entire arborizations of single neurons and was used to confirm that cells labeled in the backfills were indeed connected to the male organ, as occasionally, during backfills, dye leaked into the body wall and labeled nearby terminals of sensory neurons, resulting in false-positive labeling of identified neurons.

Bromodeoxyuridine labelling

Administration and detection of bromodeoxyuridine to assay for cell proliferation (Gratzner, 1982) have been described in detail previously (Becker and Macagno, 1992).

Immunocytochemistry

Animals were anesthetized, opened along the dorsal midline, pinned out flat in a Sylgard dish and rinsed briefly with leech Ringer’s (Mulder et al., 1981) containing 0.2% Triton-X100 to remove the yolk. Embryos older than E20 were then subjected to a 20 minute treatment with Collagenase type IV (2000 U/ml, Sigma, in leech Ringer’s), whereas younger animals did not require this treatment. Specimens were then fixed overnight in 4% paraformaldehyde in 0.01 M PBS at 4°C, washed 3× 20 minutes with PBT (0.01 M PBS, pH 7.4, with 1% Triton X-100) and incubated with monoclonal antibodies (dilution of supernatants 1:10 in PBT) with 2% donkey serum or, for double stains, in addition with an anti-5HT polyclonal antibody (Insect) both for 24 hours at room temperature. After a 1 hour wash, preparations were incubated with an affinity-purified secondary antibody to mouse immunoglobulins made in donkey diluted 1:50, and, for the double stains, an alkaline phosphatase-conjugated donkey anti-rabbit (both from Jackson ImmunoResearch Inc.) diluted 1:10,000, both at room temperature for 12-20 hours. After another 1 hour wash, tissues were incubated with mouse peroxidase-anti-peroxidase (PAP, Boehringer Mannheim), at a dilution of 1:200, plus 2% donkey serum, for 6-8 hours. After a 1 hour wash, the preparations were reacted with DAB (0.2 mg/ml PBT plus 0.02% H2O2). For detection of alkaline phosphatase, preparations were again washed for 1 hour in PBT, and then 3× 5 minutes in alkaline phosphatase buffer (AP buffer, 10 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl2, 0.2% Triton X-100. 0.5% Levamisol, pH 9.5). The tissues were then reacted with AP staining solution (3.5 µl bromochloroindolyl phosphate and 4.5 µl nitro blue tetrazolium/ml AP buffer, both reagents from the Genius Kit, Boehringer Mannheim) for 60-90 minutes at 18°C. Tissues were then washed for another 20 minutes in PBT and were dehydrated in an Ethanol series, cleared in methylsalicylate, and mounted in Canada Balsam (Fluka).

RESULTS

Nerves in the leech usually contain bundles of afferent and
Peripheral projections and central neurogenesis

Efferent axons, as well as support cells such as glia, muscle fibers or microglia (e.g., Muller et al., 1981). Any of these cellular elements could, in theory, convey developmental signals along the length of the nerve either through extended cell processes (e.g., axons, glial processes) or by means of directed cell migration. To assess which of the components of the sex nerves convey the inductive signal that leads to the generation of the PIC neurons, we examined their development during the critical period for induction. A diagram of the embryonic sex ganglia (MG5 and MG6) and the innervation of the male genitalia is shown in Fig. 1.

**Afferents from the male organ reach the CNS after the critical period**

Since removal of the male sex organ before the critical period results in the absence of PIC neurons (Baptista and Macagno, 1988a), we focused our examination of the afferent projections from sensory neurons that reside within the male genitalia on this period. These neurons have been described in juvenile and adult leeches as projecting along the sex nerves into specific axonal tracts in the neuropil of the sex ganglia (Passani et al., 1991). In dye fills of the adult male sex nerve, afferent fibers projecting into the sex ganglia were regularly observed. In principle, these afferents could mediate the inductive interaction leading to the birth of the PIC neurons. We first asked when these neurons differentiate and when their axons first reach the two sex ganglia.

Afferents from other organs in the body wall are known to reach the CNS before the critical period for PIC neuron induction (e.g., Johansen et al., 1985). Two different monoclonal antibodies, Lan3-2 and Laz6-110a4b4 (both kindly provided by B. Zipser; Zipser and McKay, 1981; Johansen et al., 1985) appear to stain the male afferents at early stages of differentiation. A series of embryos from E10-20 revealed that some cell bodies of the male afferents can first be detected at around E13-14 (see Fig. 2A) and increase in number with time. At about E25, there are several hundred of these neurons surrounding the lumen of the male organ (Fig. 2B). From E13 to E25, increasing numbers of axons from these cells could be seen in the sex nerves, projecting towards MG5 and MG6. Using antibody staining, the earliest stage at which we observed axons of the male afferents reaching the CNS was E19-20 (not shown).

Afferent projections were also revealed by the injection of DiI into the male genitalia in later embryonic stages and postembryonic animals. Injections in postembryonic animals show the expected mature branching patterns (Fig. 2D), while injections at E25 show that projections have just begun to enter the ganglionic neuropil at this stage (Fig. 2C). Since the afferent projections were never seen reaching the CNS in the backfills done at E15 (not shown), we are confident that the results from the antibody stains reflect the actual late growth of these neurons and that, therefore, their projections cannot be the conveyors of the signal that induces the PIC neurons.

**Non-neuronal cells associated with the sex nerves are not necessary for induction**

As a second possibility, the inductive signal could be conveyed to the CNS by cells other than neurons, e.g., cells that migrate along the sex nerves from the male organ or cells that
ensheathe these nerves. To test the first of these alternatives, we injected lipophilic dye into the lumen of the male organs of live embryos. These dyes appear to label most of the cells in the organ as well as cells in the nearby body wall. Since, following removal of the male organ at E14, induction of PIC neurons is nevertheless observed in about 67% of the cases (Becker and Macagno, 1992), we injected live embryos at E12-14 and looked for labeled cells that might be in the process of migrating along the sex nerves in these animals. None were observed in more than 100 animals.

A large glial cell starts to wrap each nerve root in all ganglia around E12 (Macagno et al., 1983), and later also appears to partially ensheathe parts of the sex nerves in MG5 and MG6. However, processes of these glia do not extend the entire length of the sex nerves and hence make no direct contact with the male genitalia, as their processes were never labeled after dye injections into the male organ done in live animals mentioned above, nor after several hundred dye injections performed before the critical period (T. Becker, G. W. M. Bothe, A. J. Berliner, E. R. Macagno, unpublished results), nor in fixed preparations done at E14-15 or at later embryonic stages (see below). It is unlikely, therefore, that they are involved in conveying the inductive signal.

Jellies and Kristan (1988) described a large, conspicuous
Several central neurons innervate the male genitalia prior to the critical period

The central neurons that innervate the male genitalia at E14-15 were first identified by backfilling with DiI in fixed animals (see Methods). Backfilling with DiI usually resulted in the labelling of 1-5 neurons per sex ganglion, presumably because it is impossible to label the organ as a whole, but rather a small part of it. Thus, only neurons that innervate the labeled area can be visualized with the dye. Injection of higher amounts of DiI often resulted in additional labeling of skin tissue nearby, thus labeling neurons that are not connected to the male genitalia. Therefore, to reveal the morphologies of individual neurons, live neurons were first backfilled with DiO at E12 and then injected individually with DiI at E14. All central neurons in any given midbody ganglion are surrounded by 6 large glial cells (packet glia, Kuffler and Potter, 1964). For single cell labeling, therefore, the packet glia has to be penetrated to reach neuronal cell bodies and is often weakly labeled by the dye. This background label, however, was usually much weaker than the staining of the neuron, whose cell body as well as processes within the CNS could be discriminated easily from the background by fluorescence microscopy.

The male genitalia receive innervation from MG5 through two pairs of nerves (SNa[5] and SNP[5]; Fig. 5A) but from MG6 through only one pair (SNa[6]; Fig. 5A; see also schematic in Fig. 1) (Zipser, 1979; Jellies and Kristan, 1988). Branches from the posterior nerve roots of MG6 innervate the female sex organ. All three pairs of male sex nerves are established by E12 (Jellies and Kristan, 1988; Jellies et al., 1993).

Neurons that innervate the male genitalia in the leech appear to fall into two general groups. Neurons in one group innervate the male organ exclusively, and neurons in the other innervate mainly other targets but also have minor projections that contact the male organ. Several neurons of the first group have been described previously. The motor neurons involved in penile eversion, the lateral penile evertor (LPE) and the rostral penile evertor (RPE) motor neurons, were described physiologically as well as anatomically in the hirudinid leech Haemopis by Zipser (1979). More recently, the RPE neurons were shown to innervate the male genitalia by E11-12 in Hirudo (Baptista and Macagno, 1988b). In the present study, we have frequently backfilled both neurons (LPE: n>30; RPE: n>40). The RPE neurons exist as a single pair in the rostral-most aspect of MG6 (Zipser, 1979). They project contralaterally into SNa[6] and also through the connective to MG5, from where they exit through SNa[5] and, in many cases, also SNP[5] toward the target (see, for instance, Fig. 1D in Baptista and Macagno, 1988b; see also Fig. 1). The LPE motor neurons are found dorsolaterally between the two segmental nerve roots. Zipser (1979, 1980) described these cells in Haemopis as one pair in MG6 only, based on their physiology, but found a pair of morphologically similar neurons in the same position in MG5 that also innervated the male organ but were not motor neurons by physiological criteria. Both pairs of neurons, however, stain with the monoclonal antibody Lan3-1 (Zipser, 1982; Macagno et al., 1983). Whether these neurons are lineal homologues with different fates is not known. In Hirudo, we found pairs of LPE neurons with contralateral projections in both MG5 and MG6 that exit only along the anterior nerve roots and innervate the male genitalia (see schematic in Fig. 1).

The serotonergic Retzius (Rz) neurons are found in every midbody ganglion and innervate targets in the body wall (Lent, 1973). In the sex segments, however, these cells innervate the male and female sex organs exclusively (Glover and Mason, 1986; Macagno et al., 1986; Loer et al., 1987; Jellies et al., 1987; Loer and Kristan, 1989; reviewed in French and Kristan, 1992). These neurons (shown schematically in Fig. 1) were routinely backfilled from the male organ at E14-15.

The nociceptive (N) neurons are found as two pairs (lateral and medial) in most midbody ganglia (Nicholls and Baylor, 1968). The sex ganglia, however, contain only the medial pair (Johansen et al., 1984). Like the Rz neurons, the N neurons in MG5 and MG6 (N(5) and N(6), respectively) were shown to innervate the sex organs specifically (Johansen et al., 1984).
N(6), in addition to projecting ipsilaterally into SNa[6], also projects into MG5, from where it exits SNa[5] and SNp[5] to innervate the male genitalia (see Fig. 5B; also Fig. 1), thus innervating the male organ through three axonal branches. In addition, N(6) has a process projecting to the female genitalia and a process in the posterior connective (Fig. 5B). N(5) projects through both roots of MG5 to innervate the target but, although projecting into SNa[6] from MG6, does not always innervate the male genitalia from this ganglion (not shown). The N neurons showed profuse branching over the target, and were the most often backfilled neurons in these experiments (n>60).

Zipser (1980) also described an ipsilateral neuron in the anterior lateral aspect of MG6 in *Haemopis* that backfilled from the adult SNa[6] without connections to adjacent ganglia and showed a characteristic action potential. We saw this cell only rarely (n=5) in all embryonic backfills (n>250), suggesting that, in most cases, this neuron innervates the male genitalia later than E15 or, alternatively, that it has a relatively small arborization in the target at this stage. Following Zipser’s nomenclature, we termed this cell the anterolateral (AL) neuron (shown schematically in Fig. 1; for the adult morphology, see Fig. 7).

DiI backfills revealed that an additional set of 6 bilateral pairs of neurons in both sex ganglia innervate the male organ during the critical period of induction. A pair of medioventral (MC) neurons project to the contralateral anterior root, but...
also have projections that extend into two commissures in the other sex ganglion and contact the male organ from there (Fig. 5C,D; also Fig. 1). One pair of neurons, which innervate the target with only minor branches, have somata located dorsoanterolaterally, and were named the DONUT neurons (Fig. 6A). They project contralaterally and exit through the anterior root, sending a minor axonal branch into SNa[5] or SNa[6] (see also Fig. 1). In addition, we found that a pair of previously identified neurons of unknown function, the NUT neurons (Sargent, 1975), send minor branches into the sex nerves, but mostly innervate the body wall (Fig. 1). A previously unreported paired neuron, similar in position to the lateral N neuron in non-sex ganglia, was termed the mediolateral (ML) neuron. This neuron projects ipsilaterally into the anterior root, from where it exits into the anterior sex nerves, but has no connections to the adjacent ganglia (Fig. 6B). Additional neurons were the contralaterally projecting anterolateral (CPL) and posterocentral (PC) neurons (schematic representation in Fig. 1; see Fig. 7 for the adult morphologies). The CPL neuron was only backfilled very rarely, and the PC neuron, exiting through the posterior roots in both sex ganglia, appears to innervate mainly the blood sinus around the CNS. Since even neurons categorized in the group that have relatively minor branches in the sex nerves (such as NUT, PC, DONUT) were reliably and reproducibly backfilled in these experiments, we are confident that we have mapped the full complement of neurons that innervate the male genitalia around E15. No additional

**Fig. 5.** (A) DiI injection into the male organ (star) of a 50-day-old juvenile results in filling of afferent and efferent axons. MG5 (5) innervates the organ through anterior (SNa[5]) and posterior (SNp[5]) pairs of nerves (arrows). MG6 (6) innervates the male organ only through only an anterior pair of nerves (SNa[6]; arrows). Note the backfilled neuronal cell bodies and afferent fibers (out of focus) in both ganglia. (B) N neuron on the right side of MG6 injected with DiI at E14. In the preparations shown in this panel and in panel C, a small amount of dye that diffused into the glial packet obscures the soma because of over exposure needed to bring out the fine processes. Note the ipsilateral projections of this cell into all three sex nerves (arrows) to the male genitalia (star), as well as a projection to the female genitalia (arrowheads). (C) Right MC neuron in MG5 (5) injected with DiI. Note projection into MG6 (6) and innervation of the male organ (star) from there. Arrows indicate the projections in SNa(5) and SNa(6). (D) Backfilled left MC neuron in MG6 (6) with contralateral branch into SNa[6] (arrow at right) as well as a longitudinal projection along the connective nerve to MG5 (arrowheads). Bar in A corresponds to 250 μm for A, to 200 μm for B and C, and to 85 μm for D.
neurons innervating the male genitalia were found in several hundred live backfills at E12 (T. Becker, G. W. M. Bothe, A. J. Berliner, E. R. Macagno, unpublished results). Nevertheless, we cannot strictly exclude that our techniques failed to identify some neurons. None of the DiI injections at these early stages revealed any afferent axons projecting into the sex ganglia from the male genitalia.

Neurons innervating the male genitalia in the adult
The AL and CPL neurons backfilled only occasionally (AL, \(n=5\); CPL, \(n=4\)) in the experiments described above. The reason for this could be that they have small arborizations in the organ, or, alternatively, that their projections develop later than the stages at which we performed the embryonic experiments. To distinguish between these possibilities, we performed backfills in adult ganglia using nickel-lysine. These experiments were done by cutting the sex nerve and dipping it into the tracer overnight. Because the sex nerves emanating from MG5 are very short, we performed this experiment only in MG6. Both the AL and CPL neurons backfilled consistently in adults (see Fig. 7). In addition, we found three neurons that we never saw in the embryonic backfills (labelled with stars in Fig. 7). It is probable that these neurons reach the male organ later than E15, but we have not tested this by filling them directly in embryos. We do not know whether these neurons also exist in MG5, but if they do (as do all but one of the other neurons described here), there would be a total of 14 pairs of neurons in MG6, and 13 pairs in MG5, that innervate the male genitalia in the adult. Only 9 of these pairs, however, are consistently back-filled at around E15, the middle of the critical period of induction determined earlier (Becker and Macagno, 1992). This population represents the minimal possible complement of CNS neurons that could convey signals related to neurogenesis in these ganglia.

DISCUSSION
During embryogenesis in Hirudo, a signal originating in a peripheral organ, the male genitalia, induces neuronal proliferation in two ganglia of the central nervous system. Nerves connecting the inducing tissue to the CNS are required for this process to occur. In this study, we investigated the cellular components of these nerves during and after the induction takes place, and examined which of these might be a candidate to carry the inductive signal.

In principle, the signal could be carried by any of the three cellular components of these nerves: (i)afferent fibers, (ii) non-neuronal cells and (iii) efferent fibers. We discuss below our present findings in relation to previously established spatiotemporal patterns of induction and cell proliferation in the sex ganglia and conclude that it is most likely that the efferent fibers are the conveyors of the proliferative signal and that they do so in a retrograde direction.

Male afferents
In the fruitfly, ingrowing retinal axons have been shown to cause optic lobe neuroblasts to divide (Selleck and Steller, 1991; Selleck et al., 1992). Therefore, we examined the possi-
bility that, in the leech, the inductive signal might be conveyed by the male afferents reaching the sex ganglia and triggering cell divisions of precursors residing therein. Our findings, however, make this a highly unlikely scenario. First, the projections of the male afferents arrive in the sex ganglia at about E20, as assessed by immunocytochemistry and DiI tracing. This is several days after the inductive period and at a time when proliferation of the PIC neuron precursors is already well underway (Becker and Macagno, 1992; unpublished observations). Dye-filled axons (other than those of the central neurons described here) were never observed to reach the sex ganglia before this time, but were routinely detected when dye injections were performed after E20. Second, as shown by heterochronic transplantations (Becker and Macagno, 1992), the critical period of induction is determined entirely by the CNS, while the age of the inducing male organ, within a broad range, is irrelevant, a result one would not expect if induction depended on the timing of afferent growth.

Non-neuronal cells

One-way components of the sex nerves other than axons could convey a short-range inductive signal such as the one described here would be if they covered most or all of the distance between the inducing tissues and the CNS. The ARC (Jellies and Kristan, 1988) and a similar cell in midbody segment 5 (this study) both extend the full length of the sex nerves that emanate from the anterior roots. These cells are unique to the sex segments, as judged by staining with two monoclonal antibodies (Lan 3-14, Jellies and Kristan, 1988; Laz 10-1, this study; both antibodies from Zipser and McKay, 1981) and by inspecting sex nerves emanating from the posterior roots of MG5 and ectopic sex nerves from the posterior roots of MG6 to a transplanted male organ in the position of the female organ with Nomarski optics. As we demonstrated here, induction can be conveyed by these ectopic sex nerves that do not contain such a cell. Hence, although the ARC is required for formation of the normal sex nerves (Jellies and Kristan, 1988), when mediated by ectopic sex nerves, induction may take place in its absence.

Alternatively, the induction might be carried out by smaller cells that migrate along the sex nerves from the male genitalia. We would expect, however, that such cells would have been labelled by dye injections into the male organ, but none were found, although the tissues were heavily labeled. The fact that induction can be carried out through ectopic nerves that enter the CNS through a different segmental nerve also suggests that non-neuronal cells specific to the sex nerves cannot be directly involved. Although we cannot, at present, completely eliminate the possibility that there are cells other than the ones described here involved in forming normal, as well as ectopic, sex nerves, we found no evidence for the existence of any other cell type in these nerves during the inductive period.

Central neurons

As we have shown previously, the beginning of the critical period during which induction takes place (E13) is determined by the CNS (Becker and Macagno, 1992). We routinely found at least 9 pairs of central neurons to be connected to the male genitalia at this time. An additional 2 pairs of neurons were detected less frequently. Among the first central neurons to reach the target are the Retzius and RPE neurons (Jellies and Kristan, 1988; Baptista and Macagno, 1988b). At E14, and possibly earlier, the full complement of 9 central neurons described here have contacted the target. Target contact by these neurons, therefore, correlates with the beginning of the critical period for induction. If the male genitalia are transplanted to the posterior margin of MG6, at least some of these central neurons alter their projection patterns to innervate the transplanted organ through ectopic nerves, and induction in MG6, but not MG5, is frequently observed (see Fig. 4A). These findings strongly suggest that the axons of at least some of these neurons retrogradely transmit the inductive signal.

It is important to note that induction in MG5 is independent of induction in MG6, as shown by ablation of the sex nerves to MG6 by Baptista and Macagno (1988a). This operation resulted in the generation of PIC neurons only in MG5, the sex ganglion that remained connected to the target. In addition, after early ablation (E13-14) of the male genitalia, proliferation is often seen in only one sex ganglion (Becker and Macagno, 1992), implying that induction can not only occur independently in the two sex ganglia but also at somewhat different times. These results suggest that those neurons described here that are connected to the male genitalia through both ganglia should not participate in the inductive process. The RPE neuron, for example, has connections to the male genitalia through all three sex nerves in both sex ganglia (Baptista and Macagno, 1988b), and hence remains connected to the target if the sex nerves of MG6 are ablated, yet there is no induction in MG6 in these cases (Baptista and Macagno, 1988a). The same argument holds for the N(6) and MC(6) neurons, both of which project through MG5 and innervate the male genitalia from there as well. These observations argue against the RPE, N and MC neurons being involved in the induction. We are currently testing this prediction further by ablating individual identified neurons before the inductive period.

Regardless of which and how many of the central neurons convey the inductive signal, the question remains as to how the signal is picked up by these cells and what kind of connections their axons have with cells in the male genitalia at the time the induction takes place. French et al. (1992) have investigated the contacts of the Rz neurons with the male genitalia at the time their growth cones arrive in the target. Rz growth cones were found to invest into loosely organized mesenchymal tissue without forming any morphologically apparent specialized contacts, such as synapses. If this is also true for the neuron(s) that convey the inductive signal, one would have to assume either that neurons pick up the signal with their growth cones and then transport it to the CNS or, alternatively, that contact with the mesenchymal cells in the male organ triggers the conveying neuron to release, directly or indirectly, a mitogen. The molecular nature of the putative mitogen in this system awaits future elucidation. Some of the growth factors that were originally recognized as survival factors for vertebrate neurons recently have been shown to also promote proliferation of a wide variety of neural precursors (see Korsching, 1993, for a recent review). In addition, neurotrophins have been shown to be transported retrogradely by vertebrate neurons (e.g. DiStefano et al., 1992). Regardless of the particular molecular identity of the factor(s) that trigger induction of PIC neurons, this system allows the investigation of inductive neuron-target interactions by means of single, identified cells
and will, therefore, prove a valuable system for their in vivo study.

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