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Pharyngeal Pumping Continues after Laser Killing of the Pharyngeal Nervous System of C. elegans

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Summary

Using a laser microbeam to kill specific subsets of the pharyngeal nervous system of C. elegans, we found that feeding was accomplished by two separately controlled muscle motions, isthmus peristalsis and pumping. The single neuron M4 was necessary and sufficient for isthmus peristalsis. The MC neurons were necessary for normal stimulation of pumping in response to food, but pumping continued and was functional in MC– worms. The remaining 12 neuron types were also unnecessary for functional pumping. No operation we did, including destruction of the entire pharyngeal nervous system, abolished pumping altogether. When we killed all pharyngeal neurons except M4, the worms were viable and fertile, although retarded and starved. Since feeding is one of the few known essential actions controlled by the nervous system, we suggest that most of the C. elegans nervous system is dispensable in hermaphrodites under laboratory conditions. This may explain the ease with which nervous system mutants are isolated and handled in C. elegans.

Introduction

We want to learn how genes determine behavior in the nematode Caenorhabditis elegans. The first step in solving this problem is to identify genes necessary for nervous system development or function. Most of the C. elegans genes known to be needed for nervous system development or function have been found by looking for obviously abnormal behavior, without any advance knowledge of the types of nervous system dysfunction that could cause such abnormal behavior (reviewed by Chalfie and White, 1988). This approach is limited by one's ability to recognize unforeseen behavioral phenotypes. Furthermore, it can be difficult to find out which neurons are defective in the mutants.

Chalfie and Sulston (1981) suggested a more systematic approach to isolating nervous system–defective mutants. Specific neurons that seem likely, on the basis of anatomy, to affect a particular behavior are killed with a laser microbeam (Sulston and White, 1980), the resulting behavioral abnormalities are studied, and then mutants that show these behavioral abnormalities are isolated. Trent et al. (1983) and Desai and Horvitz (1989) later designed and carried out a pharmacological screen for mutants with defective HSN motor neurons by studying the phenotype of worms in which the HSNs had been eliminated by mutations or laser killing. This systematic approach is likely to be more important in the future, since the introduction of nitrogen laser–pumped dye lasers (J. White, personal communication; see also Sulston and Hodgkin, 1988) has improved the ease and precision of laser microsurgery. We show below that as many as 19 identified neurons can be killed in a single worm with little damage to other cells.

C. elegans has two almost independent nervous systems: the extrapharyngeal nervous system, which in the hermaphrodite consists of 282 neurons of 104 anatomical types (White et al., 1986), and the pharyngeal nervous system, which consists of 20 neurons of 14 types and is supposed to be concerned with the regulation of feeding (Albertson and Thomson, 1976). To learn what phenotypes are expected of mutants with specific pharyngeal nervous system defects, we are using laser killing to investigate how feeding behavior depends on the pharyngeal nervous system. As previously reported (Avery and Horvitz, 1987), one pharyngeal neuron, M4, is essential for the contraction of the posterior isthmus muscles of the pharynx and for the growth of the worm. In this paper we show that at least 1 of the remaining 13 types of pharyngeal neurons, MC, is necessary for optimal pharyngeal pumping. However, when any or even all of the 13 types are killed in the same worm, pumping continues and the worm grows and is fertile. We suspect that this result can be generalized: although behavior in C. elegans is regulated by the nervous system, most actions required for viability and reproduction continue in the absence of the nervous system, although incorrectly regulated and at a low level. As a result, mutants with severe and widespread defects in the nervous system are often viable under laboratory conditions.

Results

Background

To help in understanding the results that follow, we begin with a brief review of the normal operation of the pharynx. The following description is based on our observations and on descriptions by Doncaster (1962), Albertson and Thomson (1976), and Seymour et al. (1983).

The pharynx is divided into three functional parts: the corpus, the isthmus, and the terminal bulb (Figure 1). The corpus is further subdivided into procorpus and metacorpus. There are five types of large muscles in the pharynx, arranged from anterior to posterior: m3 in the procorpus, m4 in the metacorpus, m5 in the isthmus, and m6 and m7 in the terminal bulb. (There are also

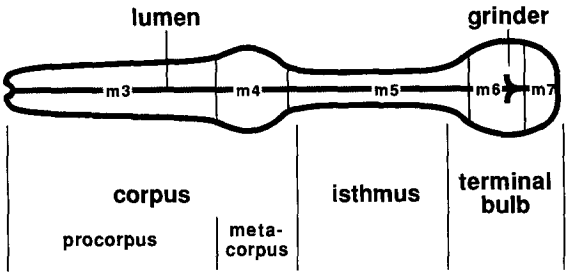

Figure 1. Anatomy of the Pharynx

The pharynx is divided into three functional parts, the corpus, the isthmus, and the terminal bulb. The corpus is further subdivided into the procorpus and metacorpus. There are five types of large muscles in the pharynx, arranged from anterior to posterior: m3 in the procorpus, m4 in the metacorpus, m5 in the isthmus, and m6 and m7 in the terminal bulb. Anterior is to the left.

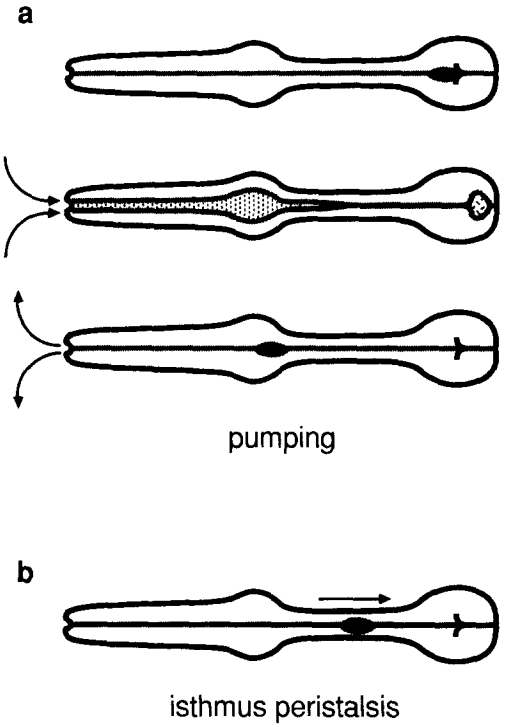

Figure 2. Function of the Normal Pharynx

(a) Pumping: A pump consists of a nearly simultaneous contraction of the corpus, anterior isthmus, and terminal bulb, followed by relaxation. Corpus and isthmus muscles are radially oriented (Albertson and Thomson, 1976), so the lumen opens when they contract, sucking in liquid and suspended bacteria. Terminal bulb muscle contraction inverts the grinder, breaking bacteria that are in front of the grinder and passing the debris back to the intestine (Doncaster, 1962). Relaxation returns the grinder to its relaxed position and allows the lumen of the corpus to close, expelling liquid. Bacteria are trapped in a filter in the back of the corpus (Albertson and Thomson, 1976; Seymour et al., 1983).

(b) Isthmus peristalsis: The feeding cycle is closed by a peristaltic contraction of the posterior isthmus muscles, which carries bacteria from the back of the corpus to the grinder.

three types of small muscles, m1 and m2 at the anterior end of the procorpus and m8 at the posterior end of the terminal bulb, which are not considered here because their contractions are hard to see.)

A pump (Figure 2a) consists of nearly simultaneous contraction of the m3, m4, anterior m5, m6, and m7 muscles, followed by relaxation. The posterior half of the isthmus remains closed during this time. Corpus contraction and relaxation result in accumulation of bacteria in the back of the corpus (Seymour et al., 1983); terminal bulb contraction grinds up bacteria that are in front of the grinder and passes their debris back to the intestine (Doncaster, 1962). Both the contraction and the relaxation are nearly synchronous in all of the muscles. The feeding cycle is closed by a peristaltic contraction of posterior m5, resulting in a wave of contraction that moves posteriorly through the posterior half of the isthmus, carrying bacteria from the back of the corpus to the front of the grinder (Figure 2b). This wave usually occurs just after a pump. However, not all pumps are followed by isthmus peristalsis.

Classification of Pharyngeal Neurons into Three Functional Groups

To determine how the pharyngeal nervous system controls feeding, we killed neurons in newly hatched larvae and looked for effects on feeding behavior. One pharyngeal neuron, M4, was essential for posterior isthmus muscle contraction (Avery and Horvitz, 1987). At least 1 of the remaining 13 types of pharyngeal neurons, MC, was necessary for the normal control of pumping (Table 1).

We began by killing single neuron types, i.e., we killed either one unpaired pharyngeal neuron or both members of a bilaterally symmetric pair (Table 1, experiments 1–14). Only 1 type, the MCs, was necessary for superficially normal pumping in the presence of bacteria (Table 1, experiment 2). When we killed both MC neurons, pumping became slow and irregular compared with that in the wild type. Each individual pump was normal, but the frequency and regularity were decreased. In other respects MC− worms resembled starved worms. Development was retarded: 3 days were required to reach adulthood, rather than the normal 2. The worms looked slightly starved (i.e., smaller and paler than normal) as larvae and young adults and more starved after they began producing eggs. Pumping was stimulated less by bacteria than in intact worms, whether well fed or starved (Table 2). The adults generally retained only a few young eggs (<6, most younger than 80 min), a characteristic of partially starved worms (data not shown). (Intact worms retained up to 30 eggs, which could reach the 150 min stage before being laid.)

Killing any 1 of the remaining 12 pharyngeal neuron types (I1, I2, I3, I4, I5, I6, M1, M2, M3, M5, MI, or NSM) had no obvious effect on isthmus peristalsis or pumping in the presence of bacteria (Table 1, experiments 3–14). (Of the 90 worms shown, 3 became starved adults and 1 was egg-laying defective, due, we assume, to damage sustained during handling.) For convenience, we call these 12 neuron types the GREENs. (This intentionally meaningless name was picked to avoid the danger of choosing a name that might turn out to be misleading.)

Table 1. Pharyngeal Neuron Kills in Young Larvae

Experiment	Neuron(s) Killed	N	Result
1	M4	65[a]	Stuffed, larval arrest
		1[a]	Stuffed, starved adult
2	MC	8	Slow pumping, starved adult
3	I1	9	Normal
4	I2	6	Normal
5	I3	10	Normal
		1	Egg-laying defective
6	I4	10	Normal
7	I5	8	Normal
8	I6	5	Normal
		1	Starved adult
9	M1	9	Normal
10	M2	9	Normal
		1	Starved adult
11	M3	8	Normal
		1	Starved adult
12	M5	11	Normal
13	MI	5	Normal
14	NSM	6	Normal
15	I1 + I2 + I3 + I4 + I5 + I6 + M1 + M2 + M3 + M5 + MI + NSM	1	Slow pumping, starved adult, poor transport
		1	Grinder failed, L3 arrest
16	I2 + I3 + I4 + I5 + I6 + M1 + M2 + M3 + M5 + MI + NSM	2	Slow pumping, starved adult, poor transport
17	MC + M2	3	Slow pumping, starved adult
18	MC + I1 + I2 + I3 + I4 + I5 + I6 + M1 + M2 + M3 + M5 + MI + NSM	7[b]	Slow pumping, starved adult, poor transport
19	M4 + I1 + I2 + I3 + I4 + I5 + I6 + M1 + M2 + M3 + M5 + MI + NSM	1	Stuffed, larval arrest
20	M4 + MC + I1 + I2 + I3 + I4 + I5 + I6 + M1 + M2 + M3 + M5 + MI + NSM	4	Stuffed, larval arrest
21	I2 + I5	3	Normal
		2	Retarded
22	M2 + M3	6	Normal
		2	Retarded
23	MI + I3	8	Normal
24	M2 + M3 + NSM[c]	1	Normal
		8	Variable slow pumping, variably starved adults

The indicated pharyngeal neurons were killed within 4 hr of hatching. Except where noted otherwise, the worms were wild type. I1, I2, M2, M3, MC, and NSM are bilaterally symmetric neuron pairs; in these cases both members of the pair were killed. N is the number of worms. Worms were considered normal if they pumped normally, reached adulthood 2 days after hatching, became large, healthy-looking adults with normal intestinal pigmentation, and produced abundant progeny. Retarded worms took at least 1 day longer than normal to reach adulthood but were otherwise normal. Starved adults were fertile but slightly retarded and were small and pale compared with normal. The egg-laying defective worm was normal except that it failed to lay eggs and turned into a bag of worms (Ferguson and Horvitz, 1985). Stuffed means that the corpus was full of bacteria and that posterior isthmus peristalsis was not seen (Figures 3a and 3b), a symptom of lack of M4 function (Avery and Horvitz, 1987). Pumping continued in stuffed worms. Larval arrest means the worm never reached adulthood or produced progeny. Slow pumping means that the frequency and regularity or pumping were decreased. Poor transport, which correlated with lack of GREEN function, means that bacteria were not efficiently transported posteriorly during pumping. As a result, bacteria accumulated in the distended pharyngeal lumen (Figures 3c and 3d).

[a] These data are reproduced from Avery and Horvitz (1987) for ease of comparison. They include 50 *unc-29(e1072*am) and 10 *unc-54(e190)* worms. Lost worms were not included.

[b] One of these worms was fixed for electron microscopy at the age of 2 days, so we do not know whether it would have reached adulthood or produced progeny.

[c] The genotype of these worms was *unc-29(e1072*am).

Table 2. Bacterial Stimulation of Pumping

Neurons Killed	Pumps per min	
	No Bacteria	Bacteria
None	43 (12-85)	224 (206-308)
None[a]	71 (68-104)	182 (172-188)
MC	30 (19-37)	66 (55-68)
MC + I1 + I2 + I3 + I4 + I5 + I6 + M1 + M2 + M3 + M5 + MI + NSM	16 (13-30)	26 (20-31)

Each row represents a single worm. The pharyngeal contractions were counted in five successive 1 min intervals either in the absence of bacteria or on a dense bacterial lawn. The same two plates were used for all 4 worms. Pumping rates are shown as the median of five 1 min counts, followed by the range.
[a] This worm was starved.

When all 12 (Table 1, experiment 15) or 11 of the 12 (Table 1, experiment 16) were killed in the same worm, bacteria were not efficiently moved posteriorly during pumping and accumulated in the lumen of the corpus and isthmus, as diagrammed in Figures 3c and 3d (referred to as "poor transport" in Table 1). Although this phenotype sounds superficially similar to that of M4- worms (Figures 3a and 3b), the fundamental defect was quite different. In M4- worms, the lumen of the posterior isthmus never opened, and bacteria never entered it. In contrast, the posterior isthmus muscles in GREEN- worms contracted in normal peristaltic waves, and sometimes the posterior isthmus was packed with bacteria. Furthermore, GREEN- worms, unlike M4- worms, did swallow some bacteria and as a result reached adulthood. GREEN- worms, like MC- worms, pumped slowly and irregularly, were retarded, and looked starved.

On the basis of these results, we divide pharyngeal neurons into three functional groups: M4, necessary for isthmus peristalsis; the MCs, necessary for the normal regulation of pumping; and a large group called the GREENs, comprising 12 different neuron types, individually dispensable for superficially normal pumping in the presence of bacteria.

Effects of Killing Combinations of Groups

To determine whether we could detect any interactions among the three functional groups, we killed combinations (Table 1, experiments 17-20). The MCs have gap junctions to the M2s (Albertson and Thomson, 1976). If the MCs acted solely through the M2s, killing both neuron types should have the same effect as killing just M2, i.e., pumping should be normal. In fact, killing the M2s and MCs resulted in animals that behaved like MC- worms (Table 1, experiments 2 and 17), eliminating that possibility. The effect of killing the MCs and the GREENs in the same worm was similar to that of killing just the GREENs: poor transport of bacteria, slow irregular pumping, retardation, and starved appearance (Table 1, experiment 18). Bacteria did not detectably stimulate pumping (Table 2). MC-, GREEN- worms had normal isthmus peristalsis and normal corpus/terminal bulb synchrony, grew to adulthood, and were fertile.

When M4 and the GREENs were killed (Table 1, experiment 19), the effect was similar to that of killing just M4: failure of isthmus peristalsis and consequent failure of growth, along with slow, irregular pumping. Even when the entire pharyngeal nervous system was destroyed (Table 1, experiment 20), pumping continued, and the corpus and terminal bulb still contracted in synchrony.

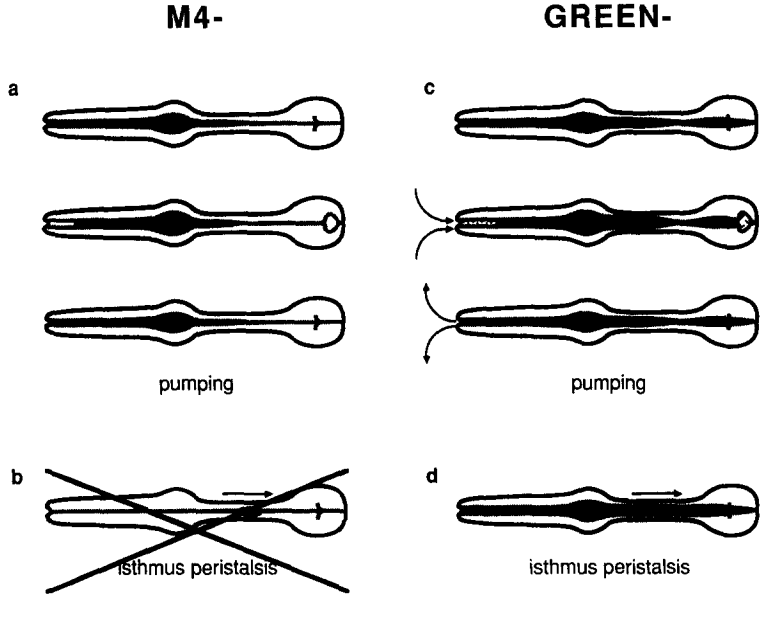

Figure 3. Function of Pharynxes with Defective Nervous Systems

In M4- worms, the contractions of the pharyngeal muscles during pumping appear normal (a). However, isthmus peristalsis does not occur (b), and as a result bacteria taken in during pumping accumulate in the corpus. Because no bacteria reach the grinder or the intestine, these worms starve. This phenotype is referred to in Table 1 as "stuffed."

In GREEN- worms, bacteria are not efficiently transported posteriorly during pumping. When such worms pump in abundant bacteria, bacteria accumulate in the distended pharyngeal lumen (c), instead of being packed into the posterior metacorpus as they are in intact worms (Figure 2a). This phenotype is referred to in the text and Table 1 as "poor transport." Isthmus peristalsis is normal: a posterior-moving wave of contraction in the posterior isthmus follows some but not all pumps (d).

In MC- worms, the frequency and regularity of pumps are decreased. The pumping and isthmus peristalsis that do occur, however, are normal (Figure 2).

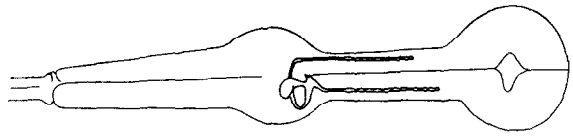

Figure 4. Anatomy of the NSM Neurons

The cell bodies of the NSM neurons are situated one in each subventral nerve cord just anterior to the nerve ring. A process from each cell crosses the ventral side of the nerve ring and exits in the dorsal nerve cord. These two, plus a posterior projection in each subventral nerve cord, run through the isthmus, but terminate before reaching the terminal bulb. Periodically the branches swell to form varicosities filled with large and small vesicles. These cells also have free subcuticular endings in the nerve ring. A very fine projection of this portion of the cell runs into the middle of the terminal bulb, situated under the cuticle between desmosomes that link the subdorsal marginal cells to the subventral muscle cells.

This figure and description are reprinted from Albertson and Thomson (1976).

Controls for Loss of Neuron Function

For at least one type of neuron (Chalfie and Sulston, 1981), and possibly a second (Avery and Horvitz, 1987), neuronal function can persist after laser destruction of the nucleus. Evidence of persistent function is seen only when the neurons are killed at least 13 hr after hatching (Avery and Horvitz, 1987), whereas in the larval kills reported here neurons were always killed within 4 hr of hatching. However, if a low level of neuronal function reliably persisted for several days even after early kills, it might account for the continued slow pumping in worms whose pharyngeal nervous systems were killed (Table 1, experiments 18 and 20). To test this possibility, we looked for evidence of persistent function after early pharyngeal neuron kills. We found none.

First, we were unable to detect serotonin immunocytochemically in a killed neurosecretory motoneuron (NSM). Second, we failed to find any evidence of a persisting NSM process in electron micrographs, and we are certain that in at least some worms in which an NSM was killed, the killed NSM did not have a process that ran the entire length of the isthmus. In serial section reconstructions of pharynxes in which MC and the GREENs had been killed, at least 96% (50/52) of neurons tested lost long-range connections. In at least 2 of the 4 pharynxes reconstructed all long-range connections due to the 19 dispensable pharyngeal neurons were destroyed. There was no evidence that any killed neuron retained function. We detected no damage to cells not purposely killed that could obviously account for the behavioral abnormalities caused by laser killing. Third, embryonic kill experiments provided no evidence for the hypothesis that persistence of pharyngeal function after larval neuron kills is due to persistent neuron function. Readers satisfied with this summary of the control experiments may wish to skip directly to Discussion.

Immunocytochemical Detection of NSMs

The NSMs are a bilaterally symmetric pair of serotonin-containing neurons (Horvitz et al., 1982) that can be detected by staining with anti-serotonin antibodies (Desai et al., 1988; also unpublished data). To determine whether an NSM can contain serotonin after laser killing, we killed NSML (the left NSM) and stained the worms for serotonin. The staining procedure gave variable results, but when we stained worms in which both NSMs were intact, we found that one NSM could serve as a control for the other: 34/59 intact pharynxes examined had visible NSM staining, and in 31/34 both NSMs stained. The staining in the 3 exceptional worms was very faint. In contrast, 18/33 NSML– worms had a single stained NSM, and none had two. In some of these 18 the staining was bright. We conclude that a killed NSML does not contain detectable serotonin.

Electron Microscopy

Each NSM has a dorsal and a subventral process that run most of the length of the isthmus, as shown in Figure 4. We killed NSMR (the right NSM) in each of 5 newly hatched larvae, leaving NSML intact as a control, and allowed the worms to grow for 2 days. During this time, they became stage 4 larvae or young adults. The worms were then prepared for electron microscopy, and a series of about 50 cross sections, each about 50 nm thick, were cut from the anterior third of the isthmus. Figure 5 shows cross sections of left and right subventral regions of the anterior isthmus from one of these worms. Figures 5a and 5b, taken from the control side, show the normal anatomy of this region. The subventral nerve cord consists of the processes of M2, M4, I4, and I5; the NSM process runs along the basal lamina that bounds the pharynx (Albertson and Thomson, 1976). More anteriorly, the process of M3 runs along the basal lamina adjacent to the NSM process.

Series from 2 worms were analyzed in detail. In the first, two processes ran along the basal lamina on the control side for the entire length of the series, presumably NSML and M3L, whereas only one, presumably M3R, could be seen on the experimental side. In the second series NSML, M3L, and M3R were present in the anterior half of the series. M3L and M3R ended approximately halfway through the series, leaving just one process on the control side (Figures 5a and 5c) and none on the experimental side (Figures 5b and 5d). Thus we saw no sign of an NSM process on the experimental side in either of these 2 worms. Furthermore, where the dorsal NSM processes run there was one instead of the usual two. The other 3 worms were not examined as carefully but looked similar: there was always one more process next to the basal lamina on the control side than on the experimental side and always exactly one dorsal NSM process.

Thus, we failed to find any evidence of a persisting NSM process, and we are certain that in at least some worms in which an NSM was killed, the killed NSM did not have a process that ran the entire length of the isthmus.

As a stricter test for persistence of killed neurons, we killed MC and the GREENs in each of 4 worms and fixed them for electron microscopy 5, 2, 3, and 3 days later (worms A, B, C, and D, respectively). (These 4 worms are included in the 7 of experiment 18 in Table 1.) A com-

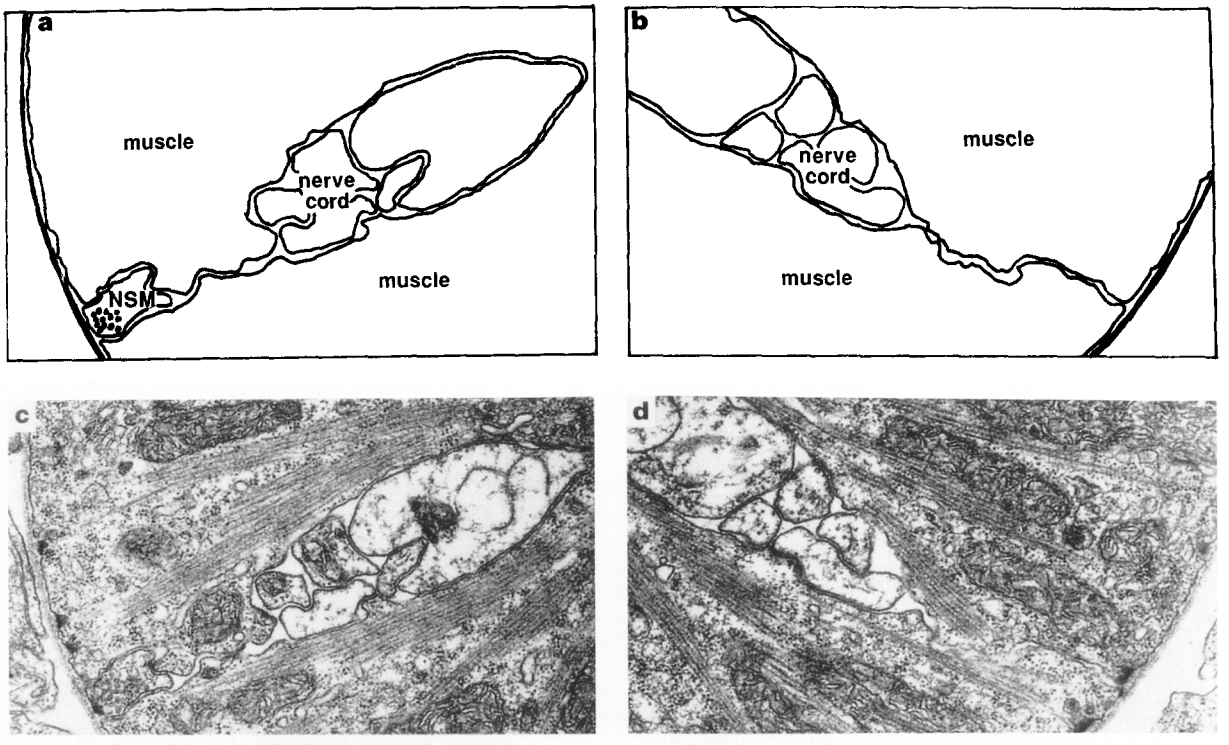

Figure 5. Cross Sections of the Subventral Isthmus in a Pharynx Lacking One NSM Neuron

(a and b) Control side. The subventral nerve cord consists of the processes of M2, M4, I4, and I5; the NSM process runs alone along the basal lamina that bounds the pharynx (Albertson and Thomson, 1976; and our unpublished data). The large process dorso-medial to the nerve cord comes from the gIL gland cell. Muscles m5L and m5VL, fused into a syncytium in a mature pharynx, lie dorsal and ventral to the nerve cords.

(c and d) Experimental side. The nerve cord, gIR process, and m5R and m5VR muscles are normal. The process of NSMR, which was killed shortly after this worm hatched, is absent.

plete series of 50 nm sections that included the entire pharynxes of all 4 worms was cut. We first checked all 4 worms for processes running the length of the isthmus or procorpus. M4, which was not killed, had normal isthmus processes in all. Of the 19 neurons killed in each of these worms, 13 have processes that run the length of the isthmus or procorpus, making a total in the 4 worms of 52. Worm C had a thin process in the ventral right procorpus, and worm A had a thin process in the ventral right isthmus. Each process led back to a cell body remnant containing uniform, moderately electron-dense material. The positions of the cell bodies corresponded to I1R and M2R, respectively. Since 2/52 neurons tested had full-length processes, we estimate that pharyngeal neurons retain full-length processes after laser killing about 4% of the time. Neither of the processes had synapses or other obvious signs of function, so 4% is an upper bound for the proportion of neurons that actually retain connections.

The pharynx of worm D was reconstructed from the anterior end to the anterior terminal bulb. Most of the processes in the pharyngeal nerve ring, the major region of neuropil located in the metacorpus in the intact pharynx, appeared to have been engulfed by marginal cells. However, we cannot say whether all processes were gone, as we were unable to trace even those of M4, which we believe were intact. (The membranes of the M4 processes in this region were wrinkled and not closely apposed to neighboring membranes; this, together with the fact that the plane of section was nearly parallel to the membrane, probably explains our inability to follow the processes.) Several cell remnants were seen: these were smooth, double-membrane-bound, electron-dense spheres or ellipsoids that looked similar to programed cell deaths (see Figure 7 of Robertson and Thomson, 1982). Figure 6 is an example. They were generally found within muscle cells. It was difficult to see internal structure, but the cell remnants in the younger worm B consisted of a cytoplasmic remnant surrounding a nuclear remnant, suggesting that the killed neurons had been engulfed by muscle cells, rather than fusing with them.

One mc2 isthmus marginal cell in worm A looked damaged, and its anterior portion was probably not functional. The only other sign of damage to cells that were not shot at was membrane fusion. The cell bodies of most pharyngeal neurons lie between muscle cell membranes. The membranes often seemed to have fused, leaving behind large flattened vesicles in the muscle cell cytoplasm (Figure 6). In the region we reconstructed, the neurons normally lie in invaginations of the membrane of a syncytial muscle cell, so that the two

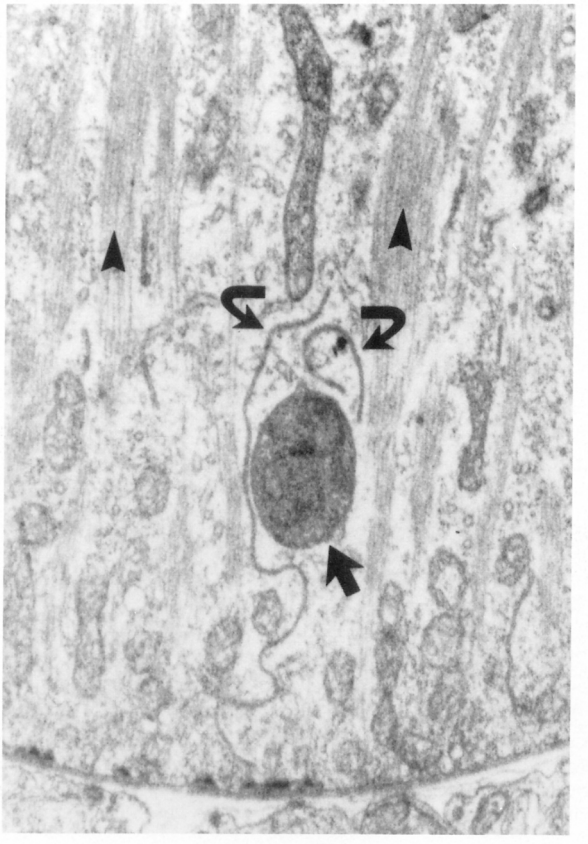

Figure 6. Remnant of a Laser-Killed Neuron

The electron-dense ellipsoidal structure (large arrow) is the remnant of a neuron that was killed 3 days before fixation. The lumen of the pharynx is beyond the top of the figure; the basal lamina that bounds the pharynx can be seen near the bottom. The neuron remnant is embedded in a pharyngeal muscle cell, which can be recognized by the bundles of radial actomyosin filaments (examples indicated by arrowheads). Before laser killing, the neuron was between two apposed muscle cell membranes separating the muscle to its left and right. (These membranes do not extend all the way to the center of the pharynx, so the neuron was not between two muscle cells, but in an invagination of the membrane of a single muscle cell.) The operation appears to have caused these membranes to fuse, leaving behind two elongated flattened vesicles on either side of the neuron, cross sections of which are visible (bent arrows).

membranes between which the neuron lies belong to the same cell and are continuous medial to the neuron. Thus the fusion of the membranes on either side of the neuron did not lead to cell fusion. Muscles of different types along the anterior-posterior axis (m3 in the procorpus, m4 in the metacorpus, and m5 in the isthmus) never fused, i.e., they were separated by membranes.

Two of the three isthmus marginal cells (mc2DL and mc2DR) of worm D were fused to metacorpus muscle (m4) at their anterior ends. Interestingly, the two marginal cells appeared to have become muscle cells; they contained thick filaments rather than the usual intermediate filaments. mc2DR in worm C was similarly transformed to a muscle cell, and when checked, it also appeared to be fused to m4 at its anterior end.

In summary, at least 96% (50/52) of neurons tested lost long-range connections after laser killing. In at least 2 of the 4 pharynxes examined, all long-range connections due to the 19 dispensable pharyngeal neurons were destroyed. There was no evidence that any killed neuron retained function. We detected no damage to cells not purposely killed that could obviously account for the behavioral abnormalities caused by laser killing.

Embryonic Kills

By the time an embryo is 430 min old, all cell divisions have ceased in the pharynx and differentiation is well under way. Neuronal processes are just beginning to grow (Sulston et al., 1983). Since the neurons have not yet made functional processes, persistent neuron function seems less likely after 430 min kills than after larval kills, when neurons are complete and functional. We killed 3 different neurons in the terminal bulb of 430 min embryos: I4, I6, or M5 (Table 3, experiments 1-3). No remnant of the cell was visible by Nomarski microscopy when the worm hatched. The effects of these operations were identical to those of equivalent larval kills (Table 1, experiments 6, 8, and 12): the worms were normal.

In the 430 min kills, the neurons were present in the pharynx before their nuclei were destroyed, and we cannot be sure that no remnant persists. To eliminate this possibility, we did a more extensive series of kills in 200 min embryos. At this stage, the precursors of the anterior ventral pharynx are accessible on the ventral surface of the embryo (Figure 6 in Sulston et al., 1983). Most of these precursors divide twice to produce 4 pharyngeal cells and cell deaths (Sulston et al., 1983). When a precursor is killed, it does not divide and often is excluded from the pharynx (Sulston et al., 1983; also our unpublished data).

Because of the elimination of structural cells (see Experimental Procedures), many of the precursor kills caused defects in growth and development. Therefore we concentrated on the dynamics of feeding. No operation halted pumping. With one possible exception, the dynamic effects of neuron elimination at 200 min were similar to those of killing the neurons in the larva. Precursor kills 5, 6, 8, 9, and 10 (Table 3) can be compared with larval kills 7, 21, 24, 13, and 2 (Table 1). The only apparent conflict is between embryonic and larval MC kills (Table 3, experiment 10; Table 1, experiment 2); 2 worms were normal after the precursor kill. However, the MCL precursor is exceptionally difficult to identify and verify: its position varies from embryo to embryo, and it is hard to determine whether the two nuclei it gives rise to are absent (see Experimental Procedures). We may simply have killed the wrong cell.

In conclusion, embryonic kill experiments provided no evidence for the hypothesis that persistence of pharyngeal function after larval neuron kills is due to persistent neuron function.

Discussion

We can classify the 14 anatomical types of pharyngeal neurons into three functionally distinct groups based on

Table 3. Pharyngeal Neuron Precursor Kills

Experiment	Age (min)	Cells Eliminated	N	Result
1	430	I4	3	Normal
2	430	I6	2	Normal
3	430	M5	3	Normal
4	200	I1L + mc1V + m3VL	1	Normal
5	200	I5 + I2R + arcPV	4	Normal
			1[a]	Normal pumping, egg-laying defective
			1	Normal pumping, starved adult
6	200	I2 + I5 + arcPV + mc3V + e2V	1	Normal pumping, except anterior isthmus stiff, lop-sided grinder, starved adult
			1	Normal pumping, lop-sided grinder, starved adult
			1	Normal pumping, disorganized muscle, starved adult
7	200	I2L + mc3V + e2V	1	Normal pumping, except anterior isthmus stiff, lop-sided grinder, starved adult
8	200	M2 + M3 + NSM + m5R + m5L	2[b]	Isthmus diameter reduced, pumped, L1 arrest
			1[b]	Isthmus shorter than normal, slow pumping, starved adult, egg-laying defective
9	200	MI + m1DR + m2DR + mc2DR	2	Normal
			1	Normal until L3, when M4 died and isthmus peristalsis stopped
10	200	MC[c] + m2L[c] + m2R + m3R	1	Normal
			1[b]	Normal
			1[b]	Slow pumping, starved adult
			1	Deformed buccal cavity, very slow pumping, very starved adult
			1[b]	Pumping, larval arrest

Pharynx precursors were killed in embryos of approximately the given age. Cells Eliminated lists the mature cells normally derived from those killed. (The actual precursors killed are named in Experimental Procedures.) For instance, M2 + M3 + NSM + m5R + m5L were eliminated by killing ABaraapap and ABaraappp. See Albertson and Thomson (1976) for definitions of pharyngeal cells. arcPV is the posterior ventral arcade cell, one of nine extrapharyngeal epithelial cells that form the lips. N is the number of worms. Some of the terms used in the description of results are defined in Table 1.

[a] The genotype of this worm was *fem-1(hc17*ts)/+.

[b] The genotype of this worm was *unc-29(e1072*am).

[c] For technical reasons, we are not certain whether MCL and m2L were removed in all of these worms (see Experimental Procedures).

the behavioral effects of killing them. One group consists of the single neuron M4, which is necessary for normal isthmus peristalsis (Avery and Horvitz, 1987). The second group contains the bilaterally symmetric MC neurons, which were necessary for the normal control of pumping rate. The last group, the GREENs, contains the remaining 12 pharyngeal neuronal types (bilaterally symmetric I1, I2, M2, M3, and NSM and unpaired I3, I4, I5, I6, M1, M5, and MI neurons, a total of 17). None of the GREENs was individually necessary for superficially normal pumping in the presence of bacteria. However, killing individual GREEN types might still have subtle effects on feeding or its regulation.

Although at least the MCs were necessary for the normal control of pumping, pumping per se continued even when all 20 neurons in the pharyngeal nervous system were killed. The extrapharyngeal nervous system has no direct outputs on the nonneural cells of the pharynx (Albertson and Thomson, 1976). Thus pumping may be an intrinsic property of the nonneural cells of the pharynx. We suspect that the role of the nervous system is to regulate the frequency of pumps and precise timing of muscle contractions during a pump in response to environmental, physiological, and proprioceptive cues.

The Control of Pharyngeal Function

Some of our results suggest two ways that pharyngeal muscle contraction is controlled: the MCs regulate the frequency of pumping by controlling the membrane potential of electrically coupled muscles, and M4 regulates posterior isthmus peristalsis by controlling excitation–contraction coupling.

A pump consists of a near-simultaneous contraction of at least the large pharyngeal muscles m3 and m4 (procorpus), m6 and m7 (terminal bulb), and the anterior half of m5 (isthmus), followed by a near-simultaneous relaxation. Pharyngeal contraction and relaxation in Ascaris are caused by depolarization and repolarization of the muscle cell membranes (Byerly and Masuda, 1979); we suspect that this is so in C. elegans as well. The frequency and length of pharyngeal contractions respond to environmental and physiological cues (Avery and Horvitz, 1989), presumably by modulation of depolarization and repolarization. The MCs are implicated in this response, since when the MCs were killed, the frequency of pumping was less dependent than usual on the presence of bacteria. The MCs have possible mechanosensory endings located conveniently for the detection of bacteria in the metacorpus (Albertson and Thom-

son, 1976). The only conspicuous output of the MCs is onto the marginal cells (Albertson and Thomson, 1976); thus they too are implicated in the control of pharyngeal muscle membrane potential.

The posterior half of the isthmus does not contract in synchrony with its anterior half and the other large pharyngeal muscles. Instead it contracts in posterior-moving peristaltic waves just after the pump. This peristaltic contraction occurs after some but not all pumps. (In one videotaped sequence, 32/136 pumps were followed by isthmus peristalsis.) Since each of the m5 muscle cells runs the entire length of the isthmus and the anterior halves contract and relax during the pump, we presume the posterior halves also experience depolarization and repolarization, but respond with a delay or not at all. M4 is implicated in control of this response, since the posterior isthmus muscles do not contract if M4 is killed (Avery and Horvitz, 1987). A plausible hypothesis is that M4 releases a neurotransmitter that enhances excitation–contraction coupling (i.e., the chain of events leading from membrane depolarization to muscle contraction) in posterior m5, perhaps via a second messenger system.

Since the behaviors of the anterior and posterior halves of the m5 muscle cells were different in the absence of the pharyngeal nervous system (Table 1, experiment 20), m5 cells must be regionally specialized. The posterior half is specialized for M4-dependent peristaltic contraction; the anterior is independent of M4.

This model is probably an oversimplification. First, although the contractions of the large pharyngeal muscles look simultaneous to the eye, Seymour et al. (1983) used microcinematography to show that in intact worms contraction and relaxation of the anterior procorpus precede those of the metacorpus by some tens of milliseconds. Our simple model provides no explanation for millisecond differences in the timing of pharyngeal muscle contraction. Furthermore, the model ignores 12 of the 14 types of pharyngeal neurons. It seems unlikely that the GREENs have no function.

Electrical Coupling of Pharyngeal Muscles

With the exception of posterior m5 (discussed above), all of the large muscles of the pharynx contract almost simultaneously. Contraction remained synchronous when all pharyngeal neurons were killed (Table 1, experiment 20). It might be argued that if growth were necessary for loss of neuron function after laser killing, these worms, since they did not grow, could still have had a partially functional pharyngeal nervous system. However, when all neurons except M4 were killed, muscles continued to contract in synchrony for the lifetime of the worm, during which it grew to adulthood (Table 1, experiment 18). We checked 4 such worms by electron microscopy, and at least 2 had lost all long-distance pharyngeal nervous system connections except for M4. It is unlikely that M4 alone synchronized contraction, since its only outputs are synapses to posterior m5 muscle, and its processes do not even reach the procorpus.

Other than nervous system coordination, the only mechanisms we know of that are fast enough to explain the observed synchrony are mechanical forces and electrical coupling. Ascaris body muscles are electrically coupled (deBell et al., 1963), and gap junctions between functionally related muscles are common in C. elegans (White et al., 1986). Unfortunately, gap junctions in the pharynx look similar to desmosomes, which join all pharyngeal muscles, so we do not know whether pharyngeal muscles are joined by gap junctions. No obvious mechanosensory structures are visible in the nonneuronal cells of the pharynx (Albertson and Thomson, 1976). Thus we consider electrical coupling a more plausible explanation for pharyngeal coordination than mechanical communication.

Could it be that muscles are not coupled in intact worms, but were coupled by cell fusion when the neurons were killed? In our electron microscopic reconstructions, different muscle types along the length of the pharynx were clearly separated by membranes. The only fusions between cells in different anterior–posterior positons we saw were those between m4 muscle cells in the metacorpus and mc2 marginal cells in the isthmus. Several of the pharyngeal neurons we killed are clustered around the boundary between these cells (the NSMs, the M3s, and I3), and yet this fusion occurred in only 3/12 possible cases. We feel that fusion of all pharyngeal muscles in all worms in which MC and the GREENs were killed is highly unlikely.

The MCs Regulate Pumping

The conclusion that the MCs regulate pumping is based on larval kill 2 (Table 1) and embryo kill 10 (Table 3), in which the MCs were removed and the worms pumped slowly and irregularly and were less responsive to bacteria. The MCs have free endings in the corpus that could plausibly sense bacteria. The consistent outputs of the MCs are gap junctions to the M2s and chemical synapses to the marginal cells (Albertson and Thomson, 1976). The M2s were not necessary for the effects of the MCs (Table 1, experiments 2, 10, and 17), so we suspect the MCs exert their effects on the marginal cells. We have not tested this suspicion, since the pharynx falls apart when the marginal cells are killed (unpublished data).

Does M4 Regulate Pumping?

When M4 is killed, isthmus peristalsis stops and pumping becomes slow and irregular (Avery and Horvitz, 1987). We suspect that the effect on pumping is an indirect consequence of the failure of isthmus peristalsis. First, M4– worms starve, and starvation affects pumping (Avery and Horvitz, 1989). Second, because bacteria cannot get from the corpus to the terminal bulb, the corpus is continually full of bacteria, and the terminal bulb is continually empty. Neurons MC, M3, and NSM have free endings in the corpus, and I5 and I6 have endings in the terminal bulb (Albertson and Thomson, 1976). If, as our results suggest, the purpose of some of these endings is to sense bacteria, they would not work

properly in an M4− worm. Third, the only output of M4 is onto the posterior m5 muscles of the isthmus (Albertson and Thomson, 1976).

Pumping May Not Depend on the Pharyngeal Nervous System

The most surpising result to us was the apparent lack of any essential role of the pharyngeal nervous system in pumping. Pumping continued even when the entire pharyngeal nervous system was killed (Table 1, experiment 20). When all neurons but M4 (the single neuron essential for isthmus peristalsis) were killed, pumping not only continued, but the worms grew and became fertile adults (Table 1, experiment 18). Electron microscopic analysis of 4 worms from the latter experiment showed that all long-range connections were destroyed in at least 2 pharynxes. Indeed, the results were consistent with the possibility that the function of all neurons except M4 had been destroyed in all 4 pharynxes.

There is nothing fundamentally incredible in this result: spontaneous myogenic activity has long been known (e.g., Eyster and Meek, 1921; deBell, 1965). The only apparent evidence that the nervous system might be essential for pharyngeal pumping comes from a mutation in the structural gene for choline acetyltransferase, *cha-1(m324)*, which almost abolishes pumping (Rand, 1989; Avery and Horvitz, 1989). However, Johnson and Stretton (1985) have shown that Ascaris nonneural cells can contain choline acetyltransferase, so the dependence of pumping on *cha-1* function is consistent with the hypothesis that pharyngeal pumping does not depend on the nervous system.

Our results support a nested series of interpretations of increasing generality and decreasing certainty. First, it seems clear that neuron types I1, I2, I4, I5, I6, M2, M3, M5, MC, MI, and NSM are not individually necessary for functional pumping or growth and development. We removed these cells both by killing a precursor in the embryo and by killing the mature neuron in a young larva, resulting in both experiments in worms able to pump, grow, and produce progeny. (We have not killed I3, M1, or M4 in embryos.) Second, we are almost certain that the nervous system is not necessary for synchronizing pharyngeal muscle contractions, since synchronized contractions occurred in worms that, by electron microscopy, lacked nervous system connections capable of accounting for synchrony. Third, we suspect that if the nervous system does have an essential role, it is merely permissive. That is, while it might be necessary for some neuron remnant to leak out acetylcholine, we doubt that direct nervous system control of the timing or coordination of pumping is essential. Fourth, it is plausible that the nonneural cells of the pharynx can pump with no direction from the nervous system and that no pharyngeal neuron other than M4 is necessary for growth and fertility. This conclusion rests solely on larval kills (Table 1, experiments 15, 16, and 18–20), and therefore on the assumption that neuronal function usually disappears completely after the nucleus of a neuron is destroyed in a newly hatched larva, an assumption supported but not proven by electron microscopy and immunocytochemistry.

Additional laser experiments will not resolve this issue, but genetic analysis might. The prospects for genetic analysis of the neurobiology of feeding are excellent. For instance, we should certainly be able to isolate MC− mutants as viable, fertile homozygotes by screening for slow, irregularly pumping mutants. If M4 is indeed the only essential pharyngeal neurons, we can isolate MC− mutants even if they also lack GREEN neuron function.

Implications for Genetic Analysis of Nervous System Development and Function in C. elegans

Behavioral mutations are very easy to isolate and manipulate in worms, so easy that along with mutations that change the shape of the worm they are the standard markers used for routine genetic manipulations. We think we have stumbled on the reason: most of the nervous system is dispensable in the laboratory. Dozens of types of worm neurons have been eliminated by laser killing or mutation (Chalfie et al., 1981; White et al., 1982; Trent et al., 1983; Chalfie et al., 1985; Avery and Horvitz, 1987; Durbin, 1987; Walthall and Chalfie, 1988; C. Bargmann, G. Garriga, E. Hedgecock, H. R. Horvitz, J. Sulston, J. H. Thomas, personal communication; and this work). So far only 2 have turned out to be essential: the CAN neurons (perhaps necessary for osmoregulation; J. Sulston, personal communication) and M4 (Avery and Horvitz, 1987). Many of the behaviors controlled by the nervous system, for instance egg-laying, chemotaxis, and mating, are not essential for growth and fertility of self-fertilizing hermaphrodites in the laboratory. Moreover, many actions continue in the absence of neurons that control them, although at a low level and without proper control. Examples are egg-laying (Trent et al., 1983), defecation (S. McIntire, personal communication), and feeding. Thus mutant worms with devastating and widespread nervous system defects can be viable and fertile.

Experimental Procedures

General Methods and Strains

Except when noted otherwise, worms were kept at 20°C and handled as described by Sulston and Hodgkin (1988). The wild-type strain was N2 (Brenner, 1974). CB1072 (*unc-29(e1072am) I*; Lewis et al., 1980) was used in some experiments to prevent starved worms from crawling away (Avery and Horvitz, 1987). We also used *fem-1(hc17ts) IV* (Nelson et al., 1978), which is self-fertile at 15°C but does not produce sperm at 25°C, to get synchronized embryos for one embryo kill experiment. Eight spermless *fem-1* females raised at 25°C were mated for 15 min with about 50 virgin adult N2 males (obtained by segregating larval males for a day), then the females were separated from the males. The females were removed 2 hr later, after they had laid a few eggs each. This procedure gives *fem-1/+* embryos with developmental ages within a 30 min range.

Nomenclature

Cell names follow Sulston et al. (1983). Each names consists of a cell type followed by some combination of the letters A, P, V, D, L, and R, standing for anterior, posterior, ventral, dorsal, left, and right, respectively. Sometimes the cell type name is used to refer to all of the cells of that type. For instance, NSMR is the right NSM neuron, and "the NSMs" is short for NSMR and NSML. Capitalized

names refer to neurons. Other cell types are pharyngeal muscles m1 through m8, pharyngeal marginal cells mc1 through mc3, pharyngeal epithelial cells e1 through e3, and extrapharyngeal arcade cells arc, which form the lips (Goldschmidt, 1903).

Laser Killing of Cells

Apparatus

Two different systems were used. The 430 min embryonic kills and most of the larval kills were done on the apparatus we described previously (Avery and Horvitz, 1987). Some of the larval kills and all of the 200 min precursor kills were done using the optical arrangement described by Sulston and White (1980), but the laser they used was replaced by a Photochemical Research Associates (Oak Ridge, TN) LN1000/LN102. The lasers in both systems are nitrogen laser-pumped dye lasers. For the purposes of this paper, there were no important differences between the two systems.

Larval Kills

Mature neurons were killed in newly hatched larvae as described previously (Avery and Horvitz, 1987).

200 min Embryonic Kills

There are two brief periods during which we can do useful operations on the embryonic pharynx. The first is during late gastrulation, from about 180 to 200 min (Figure 6 in Sulston et al., 1983). Grandmothers of cells in the ventral and anterior pharynx form three rows on the ventral anterior surface of the embryo. The beginning of the useful time window is the division of ABarapap, ABarapaa, ABaraapp, ABaraapa, ABalpapp, and ABalpaaa, whose daughters form the three rows. The end of the window is when the precursors enter the interior. There are lots of refractile yolk particles in the embryos, and cells in the interior are hard to see and kill with the laser.

We got young embryos by cutting open gravid adult hermaphrodites in TA buffer, the isotonic buffer used by Sulston et al. (1983) for Turbatrix aceti embryos. (In one experiment, synchronized *fem-1/+* embryos laid by *fem-1* mothers were used instead, as described above.) We put 10–20 embryos on a <10 mm diameter pad of 2% agar in TA buffer, dropped on a 12 mm square coverslip, and added enough TA buffer to surround the pad. Finally, the coverslip was sealed with melted Vaseline. Most embryos mounted in this way survived demounting even after several hours on the pad.

A rough sketch of the embryos was drawn, noting their positions, orientations, and ages. This allowed us to predict which embryos could be operated and when they would be ready. As each embryo became ready, we made a sketch of the pharyngeal precursors, then killed selected ones. Unoperated embryos near operated ones were exploded. The precursors killed were ABalpaaap (MCL + m2L), Abalpappa (I2L + e2V + mc3V), ABalpappp (I1L + mc1V + m3VL), ABaraapap (NSML + m5L + M2L + M3L), ABaraappa (MI + m1DR + m2DR + mc2DR), ABaraappp (NSMR + m5R + M2R + M3R), ABarapaap (m2R + m3R + MCR), ABarapapa (I2R + arcPV + I5), and ABarapapp (I1R + mc2V + m3VR).

These large precursor cells were harder to kill than neurons. The nuclei are large, featureless, low refractive index spheres. When we first fired at them, we saw no effect. However, after about a minute of continuous firing at 5 shots per s, moving the focus of the laser beam around in the nucleus, a refractile clot appeared at the focus and grew. We continued to fire at this clot, looking for the boiling spot described previously (Avery and Horvitz, 1987). Shortly after the boiling spot appeared, the edge of the nucleus became indistinct, and we saw rapid Brownian motion of the yolk particles in the cytoplasm.

When we had operated on as many embryos as we could, we scraped the Vaseline away from one edge of the coverslip and slid the coverslip off the agar. Embryos lay in little dents in the agar surface, so that if the coverslip was removed carefully, the embryos would stay in position on the agar pad and could be identified using the sketch made when we mounted them. They were transferred to individual plates with a platinum pick. Operated embryos were sometimes incubated at 15°C or 25°C to delay or advance hatching until a convenient time.

Unfortunately, pharyngeal neurons and structural cells are closely related by lineage (Sulston et al., 1983), and in only 2 cases (Table 3, experiments 5 and 9) could we eliminate a neuron type by killing a 200 min precursor without eliminating any of the large structural cells of the pharynx (pharyngeal muscles m3–m7 and marginal cells mc1–mc3). If only a few large cells were removed, the remaining structural cells could heal together and produce a small, often deformed, but functional pharynx. The small pharynx was fragile: strong contractions caused it to rip apart in the vicinity of the structural flaw, leading to death of cells near the wound and eventual cessation of pumping. Even kills that did not eliminate any large cells caused local disorganization, so that nuclei that developed from precursors close to the one that was killed were not in their usual positions. These problems complicated the interpretation of the 200 min kills. Precursor kills often caused severe defects in growth and development that were not seen in corresponding larval kills. In 5 cases (experiments 6, 7, and the worm with a deformed buccal cavity in 10) the growth retardation could clearly be explained by gross structural defects. In 4 worms (experiments 5, 9, and the larval arrest in 10) there was no obvious reason for the growth defect. However, since all 200 min operations cause anatomical disorganization, these growth abnormalities may also have been due to structural defects. We presume that the larval arrests in experiment 8 were caused by a failure of the reduced diameter isthmus, which had only four of its normal six m5 muscles. We do not know why the third worm made an isthmus that was short rather than thin.

Verification of 200 min kills was more difficult than that of larval or 430 min kills due to the fragility of pharynxes that lack some of the large structural cells and the anatomical disorganization of the pharynx. We solved the fragility problem by mounting the hatched worms in 1 μl of 1 mM or 5 mM sodium azide on a pad of 2% agar in TA buffer. For all but one of the precursors, anatomical disorganization was a minor problem, since each precursor gives rise to a characteristic set of up to four pharyngeal cells (Sulston et al., 1983) and the combination of missing cells was unmistakable, even if the individuals were not.

The exception was ABalpaaap, which presented a uniquely perverse combination of difficulties. The descendants of ABalpaaap are MCL and m2L (Sulston et al., 1983). m2L fuses with m2VL, and their nuclei come to lie adjacent to each other (J. E. Sulston, personal communication); it was very difficult to be certain that one was missing. (Due to a similar problem in the electron microscopic reconstructions, Albertson and Thomson [1976] saw only three m2 nuclei, although there are actually six [Sulston et al., 1983].) Furthermore, ABalpaaap, unlike the other pharyngeal precursors, varies in position from embryo to embryo (J. Sulston, personal communication). ABalpaaap is born dorsally and moves ventrally to join the other pharyngeal precursors, and since embryos are poorly transparent, it could not easily be identified by determining its mother and following the next cell division. It is possible that MCL was not removed in the 2 worms in experiment 10 (Table 3) that had normal pumping dynamics.

Often embryos failed to hatch after embryonic kills. We examined all such embryos carefully; they always had gross structural defects. Most commonly, the front end of the pharynx was detached from the buccal cavity. Despite these defects, many pumped.

430 min Embryonic Kills

The second time window during which the embryonic pharynx can be operated on occurs at 430 min, shortly after comma stage (Figure 8 in Sulston et al., 1983). Yolk particles have disappeared from most of the embryo, so that interior cells can be seen clearly, and the nuclei begin to look different from one another, making identification easier. Unfortunately, embryos begin to move at about this time (Sulston et al., 1983). We recently discovered that embryos can be anesthetized with 10 mM sodium azide, which may in the future make laser killing easier in late comma stage embryos.

About 100 embryos were picked from a plate and mounted on 5% agar as usual (Sulston and Hodgkin, 1988). (The elaborate mount used for 200 min kills was not necessary, since these embryos were left mounted for only a few minutes.) We searched for one of the correct age and orientation that was not moving very much and was transparent enough to allow pharyngeal neurons to be killed. The same technique was used for killing these neurons as that used for mature neurons in newly hatched larvae. We then used the laser to etch marks in the coverslip pointing to the oper-

ated embryo and exploded other surrounding embryos. The embryo was recovered as usual.

Electron Microscopy

Worms were prepared for electron microscopy, and 50 nm sections were cut as described by White et al. (1986). For the unilateral NSM kills, a series of about 50 sections was cut from the region of the anterior isthmus. All visible sections of two pharynxes were photographed and analyzed. The remaining three pharynxes were inspected briefly using the electron microscope but were not photographed.

For the MC + GREEN kills, a series of 2577 sections, including the entire lengths of the pharynxes of all 4 worms in the block was cut. Approximately every 50th section was photographed. For worm D, which was reconstructed more completely, more photographs were taken: about every 3rd section in the metacorpus, every 25th section in the isthmus, and every 10th section in the terminal bulb. Most analysis was done from the photographs, but specific points were checked by direct examination of the sections.

Immunocytochemistry

Immunocytochemistry was done according to the general methods described by Sulston and Hodgkin (1988). Our serotonin staining method was based in part on one described by Desai et al. (1988). Worms were fixed overnight in 4% formaldehyde (prepared fresh from paraformaldehyde), 0.1 M phosphate buffer (pH 7.2). After fixation, worms were washed twice in distilled water, and about 10 worms were transferred to a slide subbed with 0.5% poly-L-lysine, 0.5% Tween 20. The fixed worms were squashed under a coverslip and immediately frozen on an aluminum block on dry ice. The coverslip was then popped off with a razor blade, and the worms were incubated with 1500 U/ml collagenase (Sigma Type IV), 1 mM $CaCl_2$, 100 mM Tris-HCl (pH 7.4) at 37°C for 3 hr or until most of the cuticle had been removed, leaving isolated pharynxes sometimes attached to intestines. Subsequent incubations were overnight at 4°C with goat anti-serotonin antibody (Incstar, Stillwater, MN) and fluorescein-labeled donkey anti-goat antibody (Jackson Immunoresearch, Westgrove, PA), both diluted 1:100 in PBS + 0.5% Tween 20 with 5% normal donkey serum (Jackson Immunoresearch) added. Incubations were done by placing 10 μl of solution on the worms, then floating an approximately 1 cm^2 piece of Parafilm on the solution. Slides were kept in moisturized chambers during the incubations. Afterward, the Parafilm was removed by flooding the slide gently with PBS + 0.5% Tween 20, and the slide was washed three times in a large volume (about 50 ml for 5 slides) of PBS + 0.5% Tween 20. The washes following the antibody incubations totaled at least 3 hr. Finally the worms were submerged in 25 mg/ml DABCO (diazabicyclo-[2.2.2]octane, Sigma) in buffered glycerol, covered with a coverslip, and examined by epifluorescence microscopy (450–490 excitation filter, 515–565 barrier filter, FT510 beam splitter) on a Zeiss Axioplan microscope or by confocal scanning microscopy using a Bio-Rad Lasersharp confocal scanner mounted on a Nikon fluorescence microscope.

Observation of Pumping

Pumping rates of individual worms were quantitated by counting pharyngeal contractions as described elsewhere (Avery and Horvitz, 1989). We examined the contractions of individual pharyngeal muscles by Nomarski differential interference microscopy. Worms were mounted on 5% agar pads and observed as described by Sulston and Hodgkin (1988). All worms in Table 3 and at least 1 worm (in most cases more) from each experiment of Table 1 were examined by Nomarski microscopy.

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