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Cell-Cell Interactions in the Guidance of Late-Developing Neurons in *Caenorhabditis elegans*

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The initial outgrowth of developing neuronal processes can be affected by a number of extrinsic interactions. Cell-cell interactions are also important in a later stage of neuronal outgrowth when processes grow into the region of their targets. The correct positioning of the process of a postembryonic sensory neuron, the touch cell AVM of the nematode *Caenorhabditis elegans*, at its synaptic targets requires the presence of a pair of embryonic interneurons, the BDU cells. These cells receive synapses from AVM but do not participate in the touch reflex circuit. Therefore, the AVM-BDU synapses may be required to stabilize the association between these cells and assist in the guidance of the AVM processes through a mature neuropil.

Cues provided by neurons (*1, 2*), glia, other associated cells (*1, 3*), and components of the extracellular matrix (*4*) influence the initial outgrowth of neuronal processes (*5*). Much less is known about the final stages of neuronal development, when growing processes must find those regions of neuropil where their targets reside. In this report we examine the growth of a late-developing neuron, the touch receptor AVM, in the nematode *Caenorhabditis elegans*. The AVM cell sends branches into the nerve ring, the major area of neuropil in the animal (*6*), where they synapse to interneurons needed for the touch reflex. Since the nerve ring is essentially complete at hatching, the late-arriving AVM branches must navigate through the mature neuropil of the nerve ring to contact appropriate targets.

Three touch receptors detect gentle touch to the head in *C. elegans* (*7*). Two of these cells arise embryonically, ALMR and ALML (the right and left ALM cells; Fig. 1) (*7*). The third cell, AVM, arises 10 hours after hatching and forms functional connections (via its branch) about 20 hours later (*7, 8*) (complete larval development takes 45 hours at 20°C). All three cells send branches into the nerve ring.

Since the reflex circuit requires the branches of the touch cells, the presence of the cells or their branches can be detected by testing for sensitivity to anterior touch [Table 1 (*7*)]. The bulk of anterior touch sensitivity derives from the ALM cells, since killing AVM with a laser microbeam does not significantly alter sensitivity to anterior touch ($n = 4$) (*7, 9*). When the ALM cells are killed at hatching, animals become only partially touch-sensitive as they mature as a result of the addition of AVM [Table 1 (*7*)].

A second postembryonic cell, PVM, is produced by a lineage homologous to that of AVM (*7, 8*). In adults the PVM cell body is located more posteriorly than AVM, and its process, which terminates before reaching the nerve ring, does not branch. PVM does not normally mediate a touch response. However, if PVM is positioned more anteriorly, as often happens in *mab-5* mutants, then the PVM process reaches the nerve ring, branches, and mediates a partial anterior touch response like AVM (*10*), even when AVM, ALMR, and ALML are absent. Thus, the presence of the branch appears to be position-dependent.

We assessed the effect of a pair of interneurons (the right and left BDU cells), which receive synapses from AVM (*6*), on touch cell development by killing the cells at various times with a laser microbeam and testing the resulting adults for touch sensitivity. Killing the BDU cells in the embryo (*11*) ($n = 3$) or at hatching ($n = 8$) had no effect on touch sensitivity, indicating that the BDU cells are not needed for the connections made by the embryonic ALM cells. Since the presence of the ALM cells would obscure any effect of BDU ablation on AVM development, we also examined animals in which the BDU and ALM cells were killed at hatching. These animals were completely insensitive to touch, despite the presence of AVM (Table 1), indicating that the BDU cells are needed for the AVM-mediated touch response.

The BDU neurons are required only during the early stages of AVM development. Killing the BDU cells 24 hours after hatching did not alter the AVM-mediated response; the resulting adults were partially touch-sensitive (Table 1 and legend to Fig. 2). These results and the fact that the BDU cells do not synapse directly onto any of the interneurons needed for backward movement (*6, 12*) make it unlikely that the BDU cells are a functional part of the AVM-mediated touch-reflex circuit. It seems more likely that the cells act to establish the circuit.

We achieved a genetic equivalent of the above laser ablations by using a temperature-sensitive mutation (*n848*) of the gene *unc-86*. Mutations in *unc-86* affect the lineages of the touch cells and the BDU cells and result in touch-insensitive animals (*13*). Shifting *unc-86(n848)* animals at hatching from the restrictive (25°C) to the permissive temperature (15°C) blocked the embryonic lineage that gives rise to ALM and BDU cells (the cells are sisters) but not the postembryonic lineage producing AVM. The resulting AVM cells, which differentiated in the presence of the undivided ALM-BDU precursor, did not mediate a touch response ($n = 12$).

Similar results were found in experiments with *mab-5* animals. In *mab-5* mutants the PVM cell is variably positioned anteriorly, and its process may or may not reach the nerve ring (*10*). Mutant animals in which the ALM cells were killed at hatching exhib-

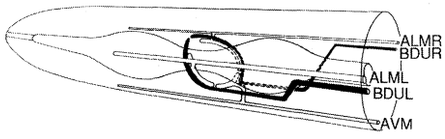

Fig. 1. Schematic diagram of the structural relation between the anterior touch cells and the BDU neurons in the nerve ring derived from a number of serial reconstructions (*6*). The processes of the three touch cells run in fixed positions near the surface of the animal. Each process branches and enters the nerve ring, where synapses are made with interneurons involved in touch-mediated movement as well as with the BDU cells. The anterior processes of the BDU cells are also shown, illustrating their close association with the touch cells in the nerve ring. AVM normally branches between 1 and 2 μm anterior to the excretory pore (not shown); the ALM cells branch at approximately 10 μm anterior to the pore (*6*).

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ited variable touch sensitivity in the head, but this response was stronger than that seen in similarly treated wild types (Table 1). (The enhanced sensitivity is most likely due to input from PVM added to that of AVM. The variability is likely to reflect the variability in the position of the PVM cell or in the extent of its development in these mutants.) Mutants lacking BDU and ALM cells were, with one exception, completely insensitive to head touch. Thus, in *mab-5* animals the formation of functional nerve ring connections for both PVM and AVM is dependent on the BDU cells.

Additional support for a developmental role for the BDU cells was provided by an ultrastructural examination of AVM in the nerve rings of animals in which the BDU cells (but not the ALM cells) were killed at hatching (*14*). The longitudinal processes of all three touch cells and the ALML and ALMR branches were seen in the five animals examined, but the AVM branch was present in only two of the five animals. We were unable to positively identify and follow the AVM cell process once the cells had entered the nerve rings. However, only one of a total of 93 animals without ALM and BDU cells was touch-sensitive (the exception being one of the *mab-5* animals described above); thus some of these animals probably have AVM cells that branch and enter the nerve ring and do not form functional synapses. These data make it unlikely that the BDU cells induce the AVM branch. The BDU cells seem, instead, to guide the AVM branch to its proper synaptic domain and to stabilize its placement. This hypothesis is consistent with the adult neuroanatomy in which AVM branches from its longitudinal process before it contacts the BDU cells (*6*) (Fig. 1).

AVM (as well as ALML and ALMR) makes as many synapses onto the BDU cells as onto any other of its synaptic partners (*7*). Since these connections do not appear to be needed for the reflex circuit, we hypothesize that they serve a role in process guidance, perhaps by providing focal attachment sites. Such synapses could stabilize and reinforce the association between the BDU cells and all three anterior touch cells and may ensure that the AVM cell branches will contact the ALM cells [to which they form gap junctions (*7*)] by growing along the BDU cells. Two other neurons, AVD and PVC, receive significant numbers of synapses from AVM. However, the absence of AVD (*12*) or PVC ($n = 5$) does not alter AVM-mediated touch sensitivity. Thus, of three of the major synaptic targets, only the BDU cells are required during stages when the AVM process is developing.

Such stabilizing synapses may not be unique to *C. elegans*. Chun *et al.* (*15*) have described a population of transient subcortical neurons in the developing visual system of the cat that appears to serve as temporary synaptic targets for ingrowing thalamocortical afferents. These investigators speculated that these subcortical cells may stabilize the incoming afferents via synaptic contacts until their final targets in the cortex mature.

Two other neuronal processes, which derive from either the PVN cells or the PVT cells (there is an ambiguity in their assignment), form significant numbers of synapses and maintain a close association with the BDU cells in the nerve ring (*6*). These cells have little in common with AVM; their synaptic connections suggest that they act as both interneurons and motorneurons. An important feature shared by these cells (whether PVN or PVT) and the AVM cell is that they all extend processes into the nerve ring late in larval development (*6, 8*). These observations suggest the possibility that a major and perhaps primary function of the BDU cells is the guidance and stabilization of a number of late-developing neuronal processes as they grow through the nerve ring.

In their analysis of *C. elegans* circuitry, White *et al.* (*16*) noted that, on the rare occasions when processes were diverted from their normal positions, these processes formed synapses with a novel set of partners. The BDU cells appear to be needed to ensure that AVM, and AVM and PVM in *mab-5* mutants, and possibly other late-arriving processes reach the appropriate synaptic region. Such guidance may be particularly important when late-developing processes must navigate through an essentially established nervous system.

Table 1. Touch sensitivity of laser-treated animals. We assessed the anterior touch sensitivity by touching adults near the pharynx with a fine hair (*8*). Individual animals were tested with a minimum of three trials over a period of 24 hours. Each trial consisted of five touches, each separated by a minimum of 5 seconds. Touch sensitivity was characterized according to the percentage of responses as normal (80 to 100%), almost normal (50 to 80%), partial (20 to 50%), or absent (0 to 20%). Both ALM cells were killed at hatching in all animals. The AVM cell was removed (−) by ablating its precursor QR at hatching. The BDU cells were killed (−) within 4 hours after hatching (early) or at least 24 hours after hatching (late) (*9*). The *mab-5* mutants were strain CB3531 [*mab-5*(*e1239*);*him-5*(*e1490*)].

n	AVM	BDU	Anterior touch sensitivity
		Wild type	
30	+	+	Partial
5	−	+	Absent
35	+	− (early)	Absent
9	+	− (late)	Partial
		mab-5	
15	+*	+	Normal ($n = 3$) Almost normal ($n = 8$) Partial ($n = 4$)
5	+*	− (early)	Absent ($n = 4$) Partial ($n = 1$)

*Plus PVM.

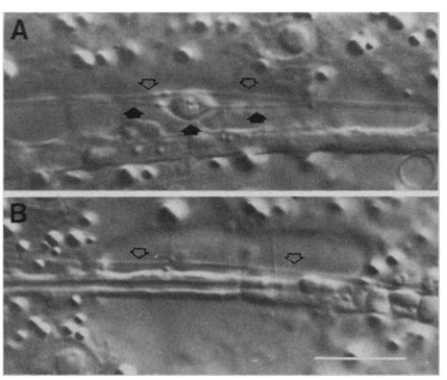

Fig. 2. Differential interference contrast photomicrographs of the right lateral hypodermis in untreated and laser-treated adults (*17*). (**A**) The BDUR cell body and its processes (closed arrowheads) are easily identified in the untreated adult where they associate with the processes from the CANR cell (open arrowheads). (**B**) BDUR is missing and the CANR cell process is unaffected in a similar region of an adult in which BDUR had been ablated in the L3 stage. This picture demonstrates not only the selective removal of the BDUR cell but also that the cell and its processes are completely removed [in previous experiments (*7*) such midlarval ablation of the ALM touch cells did not always result in the loss of the cell processes]. Bar, 10 μm.

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9. In most experiments we used the wild-type strain N2 (*C. elegans* var. Bristol) maintained at 20°C (except where noted) as described by S. Brenner [*Genetics* 77, 71 (1974)]. Laser ablations were essentially as described by J. E. Sulston and J. W. White [*Dev. Biol.* **78**, 577 (1980)]. Before surgery, newly hatched nematodes were anesthetized in 0.5% 1-phenoxy-2-propanol (Pfalz and Bauer) or 0.1% sodium azide (Sigma). The animals were then mounted dorsal side up on a flattened 4% agar pad that was made up with a similar concentration of the anesthetic and covered with a cover slip. Cells were irradiated with the beam from a Candella SLL 500 dye laser with Coumarin 450 dye (Exciton). The

beam was directed into a Zeiss Standard microscope via a sidearm tube and focused onto the specimen with a Plan 100× objective. Each cell to be killed was irradiated a number of times until there was visible bubbling. Animals were examined by Nomarski microscopy on the day after the laser treatment to confirm the loss of the ablated cells.
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11. The procedures used for laser microsurgery on embryos were described in J. E. Sulston, E. Shierenberg, J. G. White, J. N. Thomson, *Dev. Biol.* **100**, 64 (1983). Both of the ALM-BDU precursors divide 430 minutes after the first cleavage of the embryo, approximately the time when the animal begins to move within the egg. These movements make identification and ablation of the BDU cells difficult. To avoid this difficulty, the ALM-BDU precursor on the right side was killed before it divided. The egg was then reoriented so that the left side was uppermost and observed with Nomarski optics. When the ALM-BDU precursor on the left had divided, the posterior daughter (BDUL) was killed. The resulting animals were touch-sensitive upon hatching, an indication that the ALML had formed appropriate synapses in the nerve ring.
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14. Five animals (four adults and one L4 stage larva) in which the BDU cells had been killed at hatching were fixed and embedded for electron microscopy as described in M. Chalfie and J. N. Thomson, *J. Cell Biol.* **82**, 278 (1979). Serial sections (106 to 132) covering the region of the nerve ring from the excretory pore anterior to the branches of the ALM cells (a distance of approximately 10 μm) were examined in a JEOL 1200 microscope. [AVM normally branches 1 to 2 μm anterior to the excretory pore (6).] The AVM and ALM touch cells were identified by their characteristic large-diameter microtubules.
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17. The mutant strain CB3241 [*dr-1 (e1745)*] was used in these experiments. Mutant animals appear to become intrinsically starved in appearance when shifted overnight from 15° to 25°C; under these conditions many neuronal processes are easily seen (E. Hedgecock, personal communication).
18. We thank J. E. Sulston who initiated our thinking on this project, M. Finney for providing us with the *unc-86(n848)* mutant, C. Masuoka and D. Hall for assisting in the electronic microscopy, and E. Berghoz for art work. Supported by a grant from the Muscular Dystrophy Association, a U.S. Public Health Service grant (GM 30997 to M.C.), and an NIH postdoctoral fellowship (W.W.W.).

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The *ras* Oncogenes Increase the Intrinsic Resistance of NIH 3T3 Cells to Ionizing Radiation

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Identification of genes that function to protect cells from radiation damage is an essential step in understanding the molecular mechanisms by which mammalian cells cope with ionizing radiation. The intrinsic radiation resistance (D_0) of NIH 3T3 cells was markedly and significantly increased by transformation with *ras* oncogenes activated by missense mutations. This radiobiologic activity appeared to be a specific consequence of the *ras* mutations rather than of transformation, since revertant cells that contained functional *ras* genes (but were no longer phenotypically transformed) retained their increased D_0's.

CELLS HAVE DEVELOPED MULTIPLE biochemical mechanisms to protect the integrity of their DNA from damage by ionizing radiation, to which they are constantly exposed. While these molecular mechanisms are not well understood in mammalian cells, identification of genes involved in conferring radiation resistance should prove a useful step in understanding the fundamental nature of the radiation response. Increasing environmental exposure and extensive medical uses of radiation in diagnosis and cancer therapy make understanding these processes of considerable medical importance.

Efforts to identify genes affecting radiation response have focused on genes coding for DNA repair enzymes (*1*) because the lethal effects of ionizing radiation in the clinically relevant dose range appear to be due primarily to DNA damage. Genes coding for repair of ultraviolet-induced DNA damage have been isolated and identified (*2*). Our working hypothesis was that variations in expression or structure of genes that directly or indirectly regulate repair and other vital cellular processes may also significantly affect radiation response. Certain oncogenes might affect response to cancer therapeutic agents such as ionizing radiation, since abnormalities of *ras* and *myc* oncogenes have been associated with poor cancer prognosis, and radioresistant cell lines (*3*) and their normal cellular homologs appear to play fundamental roles in regulation of other aspects of cellular growth and proliferation (*4*). Furthermore, a cell line transformed by *ras* was recently shown to have a D_0 greater than that of the parental cell line, although a causative role for the exogenous *ras* gene could not be established (*5*). In the present work, we assessed the role of *ras* genes, the oncogene family most commonly associated with human tumors (*4*), in inducing resistance to ionizing radiation.

To minimize the inherent difficulties of distinguishing the effects of specific genes among different cell lines (*6*), we evaluated the effect of each oncogene by first adding it to the same NIH 3T3 subline by transfection, then determining the radiation survival curve of the transfected cell lines by clonogenic survival curve assays. NIH 3T3 cells were selected because they can be readily transfected and transformed by *ras* and several other oncogenes (*4*) and because they have a radiation survival curve similar to that of many human tumor cell lines (*6*).

An NIH 3T3 cell line, a Kirsten murine sarcoma virus–transformed NIH 3T3 cell line (DT) containing two copies of the virus gene (*7*), and two transformation-revertant cell lines derived from DT (*7*) were obtained from R. Bassin (NIH). The NIH 3T3 cells used for all transfections were obtained from D. Lowy (NIH) as was NN 192, an NIH 3T3 cell line transformed by transfection with an overproducing rat c–H-*ras* protooncogene that had been transformationally activated by linkage with a retroviral long terminal repeat (LTR) [constructed as in (*8*)]. NIH 3T3 cells were transfected by the calcium phosphate precipitation technique (*9, 10*) with either genomic DNA containing human c–H-*ras* [EJ bladder cancer (*11*)] or N-*ras* [Hodgkin's disease (*10*) and HL60 leukemia (*12*)] or cloned oncogenes, including c–H-*ras* (pUCEJ6.6) (*13*), v–H-*ras* (*8*), and v-*fms* (*14*). Transformed cells had previously been cloned and tested for the ability to grow in soft agar and for the presence of the transfected gene by DNA hybridization analysis (*9, 10*). Level of expression was determined by dot-blot analysis (*10*). Detailed descriptions of the transformed cell lines have been presented (*9*). With the exception of the *ras* protooncogene in NN 192, all *ras* genes were activated by missense mutations at codon 12 or 61 (*9–14*).

The effect of *ras* genes bearing missense mutations on radiation survival is shown in Fig. 1 and Table 1. All cell lines transformed with *ras* genes that had been activated by a missense mutation showed a large increase in intrinsic radiation resistance (D_0; the slope of the single dose radiation survival curve as compared to untransformed NIH 3T3). This change was significant at $P < 0.005$ by t test (Table 1).

There were no significant differences among *ras* genes in their effect on D_0 regardless of the type of *ras* gene (H, K, or N; viral or cellular), the site of activating mutation,

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