In situ Hybridization to Study the Origin and Fate of Identified Neurons

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Summary. Egg-laying behavior in *Aplysia* is mediated by a set of peptides, including egg-laying hormone (ELH), which are released by a cluster of identified neurons, the bag cells. A family of neuropeptide genes which includes the gene encoding ELH along with two additional genes encoding the A and B peptides thought to initiate the egg-laying process has been isolated and their nucleotide sequence has been determined. In situ hybridization and immunofluorescence was used to explore the origin and distribution of the neurons that express this family of genes. The ELH genes are expressed, not only in the bag cells, but in an extensive system of neurons distributed in four of the five ganglia of the central nervous system. The genes for ELH are expressed in these cells early in the animal's life cycle. As a result, it was possible to use in situ hybridization to trace the cells expressing ELH to their site of origin. The cells originate outside the central nervous system in the ectoderm of the body wall and appear to migrate to their final locations within the central nervous system by crawling along strands of connective tissue.

The nervous system consists of a vast network of cells, many of which are anatomically and functionally unique. An extreme example of neuronal diversity is seen in the nematode worm *Caenorhabditis elegans*, in which each of the 273 cells in its nervous system differ from each (*1, 2*). But even in the complex nervous systems of vertebrates where there are 10^{12} nerve cells, many cell groups can be distinguished from one another (*3–5*). This diversity suggests that characteristic nerve cells or cell groups express distinct sets of genes not expressed in other nerve cells.

How does one begin to characterize the pattern of gene expression of individual neurons within nervous systems composed of from 10^2 to 10^{12} cells? It is now possible to isolate genes expressed in individual neurons and to relate the activities of specific genes to the particular functions of individual neurons. The expression of these genes can then be explored in both the developing and adult nervous system by hybridization in situ to messenger RNA (mRNA) in tissue sections. We have isolated a gene family encoding the peptides mediating egg-laying behavior in the marine snail, *Aplysia*. In the study described here we have used in situ hybridization to mRNA to trace the developmental origin and ultimate fate of the neurons expressing these genes.

The simple nervous system of the marine mollusc *Aplysia* is particularly suitable for analyzing neuron-specific gene expression because it contains only about 20,000 central nerve cells which are collected into four pairs of symmetric ganglia and a single asymmetric abdominal ganglion. Moreover, many neurons in these ganglia may be recognized by highly reproducible characteristics such as size, shape, position, pigmentation, and function (*6*). These properties have made it possible to relate the function of particular cells to specific patterns of behavior and may permit us to attribute neuronal function to the expression of specific genes. In addition to being few in number, neurons in *Aplysia* can be large, up to 1 millimeter in diameter. Most of these large cells are highly polyploid and contain as much as 2 micrograms of DNA, more than 10^5 times the content of the haploid genome (*7, 8*). Furthermore, our data indicate that the mRNA content is also proportional to cell size, such that the largest of cells contains up to 5 nanograms of mRNA.

Our studies on the relation between the expression of specific genes and the generation of specific behavioral patterns initially focused on egg laying. In *Aplysia*, egg laying consists of a stereotypic fixed action pattern consisting of several behavioral components, aspects of which are understood at both the cellular and molecular level. As the fertilized egg string is extruded, the animal stops walking and feeding, catches the string in its mouth, and waves its head back and forth, depositing the eggs in a folded mass on the ocean floor. The expression of the behavioral sequence is thought to involve the actions of a combination of peptides synthesized and released by the bag cells, two symmetrical clusters of neurons located at the rostral margins of the abdominal ganglion (*9–12*). The bag cells release a 36–amino acid peptide, the egg-laying hormone (ELH), along with other peptides that directly mediate the behavioral components associated with egg laying (*10, 11, 13–15*). Excitation of the bag cells can be elicited in vitro by either one or two related peptides, A or B peptide, released from the atrial gland, an exocrine organ within the large reproductive tract (*16–18*).

Earlier, we isolated and sequenced the three genes that encode the A, B, and ELH peptides (*19, 20*). The three genes are 90 percent homologous in sequence and are representatives of a small multigene family. The genes encode a protein precursor, in which the active peptides are flanked by internal cleavage sites, providing the potential to generate multiple small peptides. Although each of the three genes share significant nucleotide sequence homology, they have diverged so that different member genes express functionally related but nonoverlapping sets of neuroactive peptides in different tissues.

We have investigated the expression of this gene family in the nervous system and peripheral organs of adult and developing *Aplysia*, by means of in situ hybridization to mRNA and application of immunocytochemical techniques of the peptide products of the ELH gene family. As would be expected from the homology shared by the three genes, we detected the mRNA for egg laying in the bag cells and atrial gland. However, we also encountered in the adult an extensive network of additional neurons that express the gene family. Further, we found that the gene family is expressed

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surprisingly early in development in cells of premetamorphic animals. We have therefore used these genes as developmental markers to explore the origin of the cells that express ELH. We found that the ELH-producing nerve cells do not originate in the nervous system but in a proliferative, ectodermal zone of the body wall. They then appear to migrate into the central ganglia along connective tissue fiber tracts that connect the body wall with the nervous system.

Genes Encoding Egg-Laying Peptides

During the egg-laying season, more than half of the protein synthetic machinery of the bag cells is devoted to the production of polypeptide precursors of ELH neuroactive peptide ELH. The bag cells also release several other peptides, and this collection of peptides presumably results in the characteristic and stereotypic behavioral repertoire associated with egg laying. We have determined the nucleotide sequence of the ELH gene as well as the genes encoding the A and B peptides (*19*, *20*). The ELH and the A and B peptide genes (Fig. 1) are all members of a small multigene family in which at least one member encodes a number of different peptides. The sequences of these genes suggests several possible mechanisms whereby nerve cells can utilize polyproteins and give families to generate diverse sets of neuroactive peptides.

First, the gene for ELH encodes a protein consisting of 271 amino acid residues while the ELH peptide itself consists of only 36 amino acids. Thus, ELH is synthesized as part of a larger precursor molecule, and its release requires cleavage at pairs of basic residues that flank the ELH sequence. The precursor, however, contains eight additional pairs of basic residues that may serve as cleavage sites flanking putative neuroactive peptides (Fig. 1). Are these potential sites in fact recognized and cleaved? Are the precursors actually a polyprotein? Three peptides, alpha and beta bag cell factor, as well as the acidic peptide, have been isolated from extracts of bag cells and appear to be coordinately released with ELH (*21*). We have found that each of these peptides is encoded in the ELH precursor and is bounded by cleavage sites. A role for at least two of these peptides, alpha and beta bag cell factor as neurotransmitters altering the activity of specific neurons within the abdominal ganglion, has been demonstrated (*21*).

Furthermore, the ELH gene is only one member of a small multigene family. We have identified two genes expressed in the atrial gland which are 90 percent homologous to the ELH gene but have diverged at essential loci to generate individual, nonoverlapping sets of peptides (Fig. 1). These two genes encode the A and B peptide, which in vitro are capable of initiating the excitation of the bag cells and the release of ELH. Thus these three members of a multigene family are expressed in a tissue-specific manner. The A and B peptide precursors are synthesized in the atrial gland but not the bag cells, while the ELH precursor is expressed in the bag cells but not the atrial gland. These conclusions derive from three sets of observations: (i) Antibody to A peptide does not cross-react with the bag cells; (ii) under stringent conditions of hybridization (*20*), complementary DNA (cDNA) probes from the bag cells do not anneal with the A or B peptide genes and conversely cDNA probes from the atrial gland fail to hybridize with the ELH precursor gene; and (iii) extensive screening of an abdominal ganglion cDNA library has failed to reveal a single cDNA clone encoding A or B peptide under conditions that identify about 100 clones encoding ELH (*22*).

These observations illustrate two important points concerning the evolution of neuropeptides and the generation of diversity in the nervous system. First, the ELH family consists of a minimum of three genes. At least three of the genes diverge in coding regions to generate distinct sets of peptides expressed in different tissues. Each gene encodes a precursor protein consisting of three regions of homology: A or B, ELH, and acidic peptide. Each gene has diverged, presumably satisfying the functional requirements of the tissue in which they are expressed. The homologies among the genes encoding the A, B, and ELH suggest that these genes have arisen from a common ancestor. Although the genes share significant nucleotide homology they have diverged not only by single base changes but also by larger insertions, deletions, and transpositions, so that different member genes express functionally related but not nonoverlapping sets of neuroactive peptides.

Second, the number of possible combinations of egg-laying peptides is made even greater by the fact that these peptides are encoded by a small family of genes in which each member has diverged to generate new peptides. Further, these genes encode polyproteins with the ability to generate a large number of additional combinations of peptides merely by altering the pathway of processing in different cell types or in the same cell in response to different stimuli.

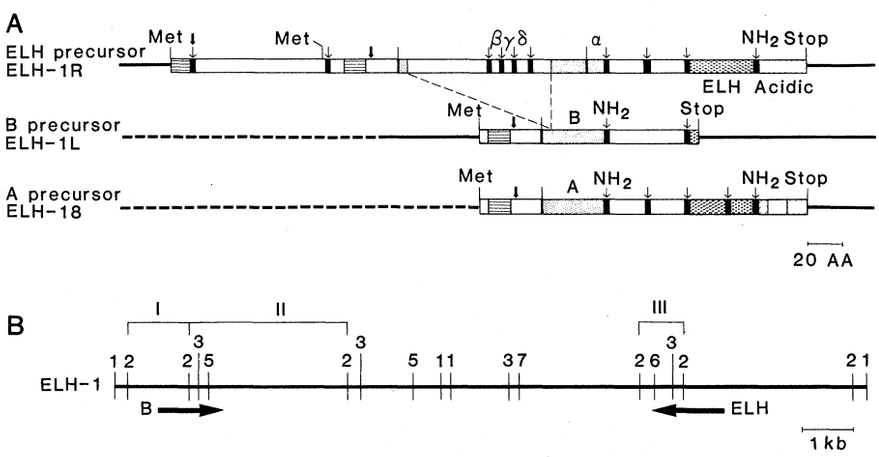

Fig. 1. (A) Comparison of the protein precursors containing the ELH and A and B peptides. Coding regions are derived from the only in-phase amino acid reading frames that match in vitro translation molecular weights. Each of the three proteins is initiated by a methionine followed by a hydrophobic region (horizontal bars). Thick arrows represent the putative site of cleavage of the signal sequence. A line above the sequence represents potential cleavages at single arginine residues (vertical line), while thin arrows represent potential or known cleavages at dibasic, tribasic, or tetrabasic residues. If carboxyl terminal amidation is believed to occur, an NH_2 appears above the arrow. The A or B peptide homology is represented by stippled boxes (dots). The ELH homology is represented by crosshatched boxes (grids). The acidic peptide homology is respresented by parallel lines enclosed in boxes (diagonal bars). Solid lines symbolize sequences noncoding regions, and dotted lines depict regions not sequences. (B) DNA probes for in situ hybridization. The restriction enzyme map of the recombinant phage ELH-l containing linked genes encoding ELH and B peptide (*20*), **1**, Eco RI, **2**, Pst I, **3**, Xho I, **5**, Pvu II, **6**, Hind III, **7**, Bgl II. Arrows indicate the position of mRNA's and point in the direction of transcription. Three Pst I fragments were used as probes in this study. Fragments I and II span the 5′ and 3′ ends of a gene encoding B peptide. Probe III includes the 3′ portion of an ELH gene.

This multiplicity suggests combinatorial mechanisms that may be important in generating some of the morphologic and functional diversity characteristic of the nervous system; for example, the expression of different combinations of ion channels, or the expression of different combinations of recognition molecules. In the specific case of the ELH polyproteins, this diversity may reflect the release of different combinations of peptide transmitters.

A System of ELH-containing Neurons in the Adult

We have demonstrated that different members of the gene family encoding the egg-laying peptides are expressed in the bag cells and atrial gland. To explore systematically the expression of the egg-laying peptides throughout the nervous system, we have examined by in situ hybridization and immunocytochemical methods, sections through ganglia of large mature animals, as well as whole mounts of the entire central nervous system of small animals. The application of these two procedures to serial tissue sections made it possible to determine the site of synthesis of the RNA encoding the egg-laying peptides as well as to demonstrate that this RNA is indeed translated to generate immunologically cross-reactive peptides. These two complementing procedures are important in analyzing gene expression in the nervous system, since the presence of specific proteins in a particular neuron or cluster of neurons need not indicate synthesis at that site but could reflect uptake by the neuron from distant sites of synthesis (*23*).

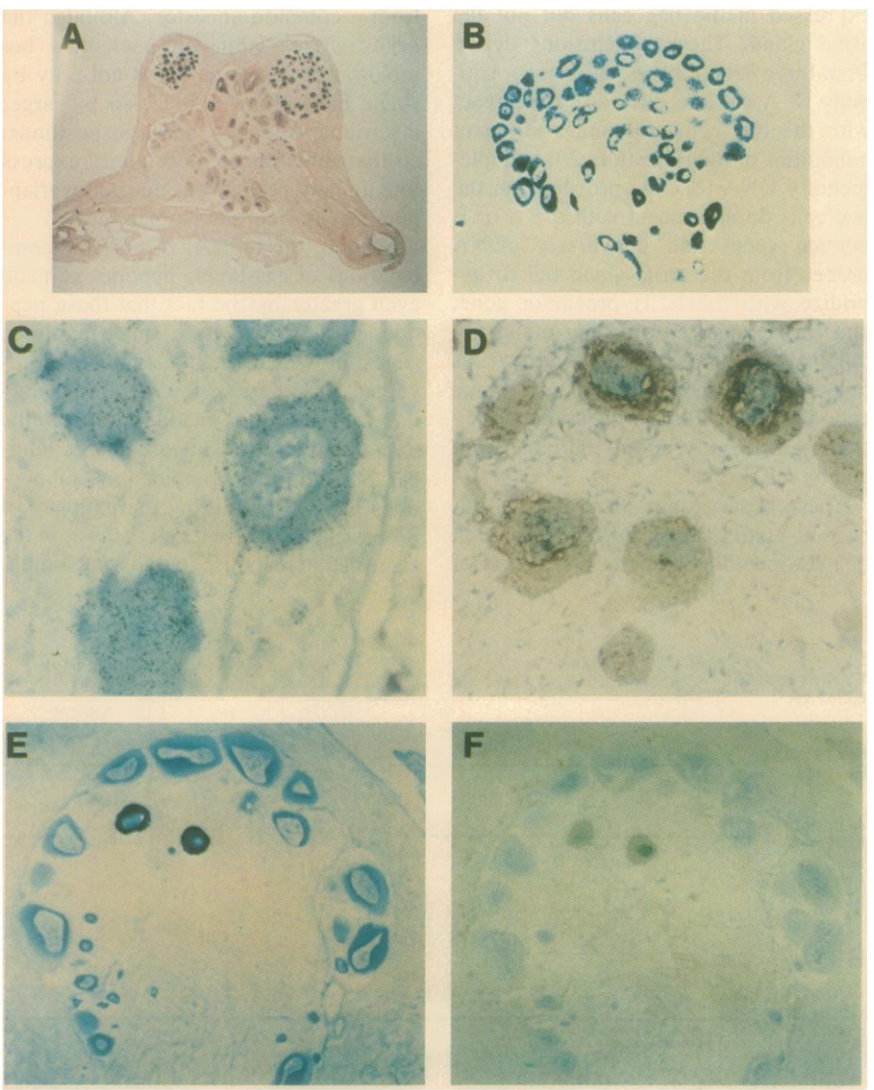

Fig. 2. In situ hybridization and immunocytochemical localizations of ELH expressing cells in the central nervous system of *Aplysia*. (A) The abdominal ganglion from an adult animal was dissected, fixed in Bouin's and sectioned as described (*51*). Iodine-125–labeled nick-translated probes were hybridized in situ to tissue sections according to the condition described (*52*). The bag cells comprise the topmost rounded clusters of neurons on both sides of the ganglion. This photomicrograph shows hybridization to cells in both bag cell clusters as well as to a single isolated neuron in the upper left of the ganglion. The staining of the bag cells reflects the intense collection of grains apparent at higher magnification in (C); magnification is ×15. (B) In situ hybridization to a section through a single bag cell cluster. Neurons are stained with methylene blue; magnification is ×58. (C) In situ hybridization to bag cell neurons at high magnification (×368). Grains are observed in the cytoplasm of the cell bodies as well as in the processes. (D) Immunocytochemistry with antibodies in ELH followed by a second antibody coupled to peroxidase (*53*) (generating a green-brown stain in ELH-positive cells) in bag cell cluster (*53*). Magnification is ×230. (E). In situ hybridization to a section through the pleural ganglia showing a high density of grains in the cytoplasm of two neurons. Magnification is ×92. (F) Immunocytochemistry with the antibody to ELH applied to the section of pleural ganglion contiguous to that shown in (E).

Individual probes consisting of defined sequences from the 3′ or 5′ regions of the genes encoding B peptide or ELH were chosen for hybridization in situ. The regions encompassed by these probes are shown in Fig. 1. Probes 2 and 3 both derive from the 3′ region of the genes encoding B peptide and ELH and include ELH coding sequences. Probe 1 derives from the 5′ portion of the gene encoding B peptide and includes the sequence for this hormone. Under the hybridization conditions used in our experiments, however, each of the individual probes cross-hybridize to mRNA's encoding the precursors for each of the three peptides, A, B, or ELH. Thus, the in situ hybridizations do not permit us to distinguish between mRNA's derived from the different members of the ELH gene family. (For simplicity we use the term ELH-positive cells to describe cells expressing any member of the ELH gene family.) In situ hybridization experiments were complemented by immunocytochemical methods, with affinity-purified antibodies (*24*), directed against either A peptide or ELH.

The bag cells, an electrically interconnected cluster of cells, are the primary site of ELH synthesis. Activation of a single cell within the cluster causes all of the cells to fire in synchrony, leading to the coordinated release of ELH and its companion peptides. In situ hybridization to sections through the bag cells (Fig. 2, A to C) indicates that all of the bag cells contain mRNA transcribed by at least one member of the ELH gene family. It is somewhat surprising that we find mRNA in the axons as well as the cell bodies, since axons are thought to be devoid of ribosomes.

The specificity of hybridization to ELH mRNA is supported by control experiments, which reveal no hybridization when pBR322 plasmid sequences are used as a hybridization probe. Fur-

thermore, hybridization with specific ELH probes is eliminated by treating tissue sections with ribonuclease either before or after the hybridization reaction. Finally, grains are clearly localized to the cytoplasm rather than to the nucleus, an indication that hybridization occurs with mRNA rather than with DNA.

The bag cells and their processes also react with antibody directed against ELH (Fig. 2D). We have previously shown that the precursor protein synthesized in vitro with bag cell mRNA reacts with antibodies to ELH and antibodies to A peptide. However, the bag cells themselves do not react with antibody to A peptide, suggesting that the cross-reactive peptide sequences are rapidly degraded in vivo after cleavage of the precursor protein. The specificity of the ELH antibody was shown by previous in vitro translation experiments in which the antibody immunoprecipitated only the precursors to ELH, A peptides, or B peptides in the midst of a host of other proteins (*20*). Moreover, no staining occurred with serums from nonimmune animals. These observations confirm previous studies demonstrating that the bag cells are a primary site of synthesis of ELH mRNA and peptide (*19, 25, 26*).

The immunofluorescent studies of Chiu and Strumwasser based on serial sections have shown that occasional unidentified neurons located outside the bag cell cluster express ELH-related peptides (*26, 27*). To identify all the cells that constitute the network of neurons expressing members of the ELH gene family, we exposed whole mounts of the total central nervous system taken from young animals as well as tissue sections to DNA and antibody probes. These in situ hybridization and immunofluorescence experiments reveal a network of cells producing both ELH-related RNA and cross-reactive protein in all of the major ganglia of the central nervous system, with the exception of the pedal ganglia (Fig. 3).

Several conclusions may be drawn from examining these ganglia with both in situ hybridization and immunofluorescence (Figs. 3 and 4). First, there exists a network of at least 20 other cells in the central nervous system of *Aplysia* expressing the egg-laying genes in addition to the primary cluster of bag cell neurons (*27*). Second, in the buccal, abdominal, and cerebral ganglia, the individual cells or cell clusters expressing the ELH genes maintain invariant positions (Fig. 3). In contrast, in the pleural ganglion, positive cells appear to vary both in position and number (Fig. 2, E and F). Third, the combination of immunofluorescence and in situ hybridization demonstrates that these cells are the site of both transcription and translation of the egg-laying genes. Fourth, the neurons of the bag cells, as well as the other cells producing ELH or related peptides, send off a complex array of processes readily discernible with antibody to ELH. This network of processes presumably (Fig. 3) allows the egg-laying peptides to be released throughout the central nervous system. The release of ELH-like peptides therefore may not be restricted to the bag cells. Thus, as has been suggested (*27*), ELH may serve a significant role as neurotransmitter in other ganglia.

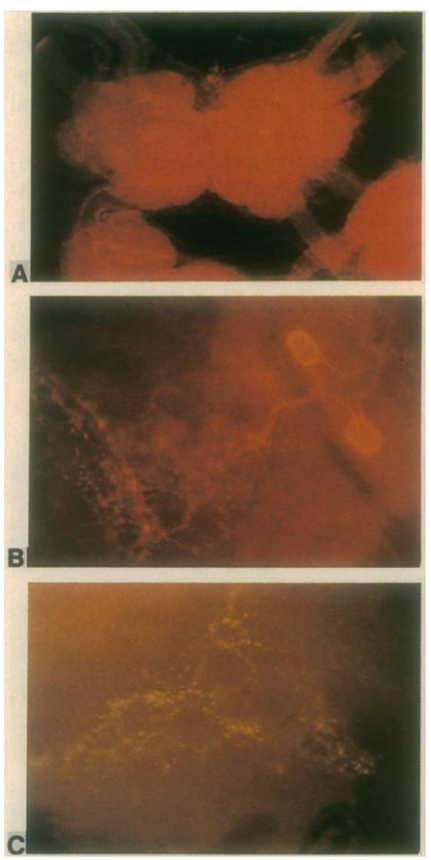

Fig. 3. Indirect immunofluorescence in the central nervous system with antibody to ELH. Whole nervous systems were dissected from 45-day postmetamorphic animals, fixed, and reacted with a rabbit antibody to ELH, followed by goat antibody to serum rabbit coupled to rhodamine (*54*). (A) The buccal ganglia: two large invariant cells that produce ELH lie at the periphery of each ganglion, close to the exit zone of the buccal nerve. In addition, a cluster of four or five smaller cells that produce ELH is always located in the center of the ganglion; magnification is ×95. (B) A pair of invariant bipolar cells producing ELH in the abdominal ganglion; magnification is ×240. (C) The arborization pattern of ELH-containing processes in the abdominal ganglion. The processes of the ELH-containing neurons form a rich arborization pattern that surrounds and appears to make axosomatic contacts with cell bodies in the abdominal ganglion; magnification is ×240.

The Atrial Gland Expresses Egg-Laying Genes

We have also examined nonneural tissue for the expression of egg-laying genes. As noted previously, the atrial gland is the site of synthesis and release of the A and B peptides, which are thought to initiate egg laying. As expected, we detected both the egg-laying peptide and its mRNA in the cells of this gland.

The luminal surface of the common hermaphroditic duct of *Aplysia* is formed by a continuous epithelium composed of three functionally and anatomically discrete segments including the atrial gland, the red hemiduct, and the white hemiduct (Fig. 4, A and B) (*28*). On in situ hybridization, an intense accumulation of grains is observed only in the columnar cells of the atrial gland (Fig. 4B). Immunofluorescence with either antibody to A peptide or antibody to ELH similarly identified the egg-laying peptides only within the atrial gland of the duct (Fig. 4A). The same staining occurs with antibody against A peptide or ELH, since the A peptide precursor encodes a peptide that shares significant homology with ELH. Further, the pattern of antibody staining clearly defines the transitional epithelium demarcating the border of the atrial gland and the red hermaphroditic duct.

Development of the Bag Cells and Other Neurons That Express ELH

When are the ELH genes first expressed during development, and where do the bag cells and other neurons of the ELH system originate? Do they develop in situ within the ganglia, or do the ELH-producing cells develop in a neuronal proliferative zone and then migrate into the ganglia? The use of ELH gene probes for in situ hybridization along with antibodies to ELH permits us to address these questions by analyzing the expression of ELH during the development of the nervous system in *Aplysia*.

Aplysia is an annual organism that undergoes five developmental stages before reaching sexual maturity. The reproductive animal lays long egg strands containing approximately 10^6 fertilized ova packaged in egg cases, each composed of approximately ten eggs (*29*). Egg laying initiates the first, or embryonic, phase of development, which lasts about 10 days. The second, or veliger larval, phase begins when the egg case ruptures, releasing ciliated veliger larvae that feed on unicellular phytoplankton.

After 34 days, the veliger larvae stop swimming and enter the third, or metamorphic, phase. The organism settles on specific species of seaweeds and metamorphoses within 2 to 3 days into a benthic juvenile that crawls and eats microalgae. In the 60 to 90 days of the fourth, juvenile, phase of development, the animal gradually grows into a fifth phase, a much larger, sexually mature adult (*30, 31*).

Substantial anlage of all of the ganglia of the mature central nervous system are present at the beginning of the second, or veliger, larval stage, and these ganglia are quite well developed at metamorphosis. This is consistent with the animal's behavioral repertoire, which, except for reproductive capabilities, is essentially complete at metamorphosis. The bag cells appear rostral to the abdominal ganglion early in juvenile development but do not attain full size until much later (*30, 31*).

We have performed in situ hybridization experiments together with immunocytochemical experiments on sections of the developing organism from the formation of the veliger larvae, through metamorphosis, to the adult. The central ganglia are already present in primitive form in the veliger larvae, although no bag cells are apparent. At this early stage, we detected cells producing ELH or related peptides in a proliferative zone of epidermal cells lining the body wall long before the bag cells were present. In situ hybridization to sections of the veliger larvae 25 days after hatching and 10 days before metamorphosis reveals an array of hybridizing cells distributed throughout the entire length of the inner surface of the body wall, with one particularly dense cluster of cells expressing ELH-related mRNA along the body cavity close to the head ganglia (Fig. 5, A to C). During this early stage in development, this proliferative ectoderm of the body wall is the major site of cells expressing the ELH genes, although occasional positive cells are observed within the central nervous system as well.

ELH-positive cells continue to accumulate in this proliferative zone throughout development. After metamorphosis, small clusters of these cells can be seen extending into the body cavity along what appear to be fibrous connective tissue strands. These strands connect the inner surface of the body wall with the central ganglia and appear to serve as pathways for neurons migrating from the proliferative ectoderm to the central nervous system (Fig. 5, D and E). One particularly clear example is shown in Fig. 5F, in which two strongly hybridizing cells are observed on a connective tissue fiber that has attached to the pleural-abdominal connective. The cells then leave the fiber tracts and appear to migrate along neural connectives to their appropriate location within the central nervous system.

We have focused primarily on the development of the bag cell cluster because it represents a large and clearly delineated neuronal cell group in the adult. The bag cells first appear on the pleuroabdominal connective at about 10 to 20 days after metamorphosis when in situ hybridization reveals a primitive cluster of ten cells some distance from the abdominal ganglion (Fig. 6, A and B). The cluster gradually increases in size over the next 50 days, accumulating about 200 cells, each slightly larger than 20 micrometers. This cluster has now moved much closer to the ganglion and bulges from the connective (Fig. 6, C to F). As the animal, now sexually mature and reproductively capable, continues to grow during the next 100 days, the bag cells increase in size and number until the full complement of 400 cells is reached with each cell more than 50 μm in diameter (*32*). The increase in cell number could be accounted for by continued proliferation of precursors within the ectoderm of the body wall and subsequent migration. We cannot, however, exclude the possibility that cell division is occurring within the primitive bag cell cluster itself.

The proliferative zone of the body wall extends throughout the length of the organism, suggesting that these cells are also precursors of the network of neurons expressing ELH or related peptides in other ganglia as well. Although we have not traced the origins of specific ELH-positive cells within other ganglia, we observe cells migrating along fibers connecting the body wall with other individual ganglia. Figure 6D illustrates a pair of ELH-positive cells positioned along a fiber connecting the body wall to the cerebral ganglia.

The migratory step that is present in the development of ELH-positive cells in the central nervous system sharply contrasts with the development of the cells expressing this gene family in the atrial gland. In the adult, the atrial gland comprises a segment of the luminal surface of the hermaphroditic duct. Hybridization in situ on sections of the her-

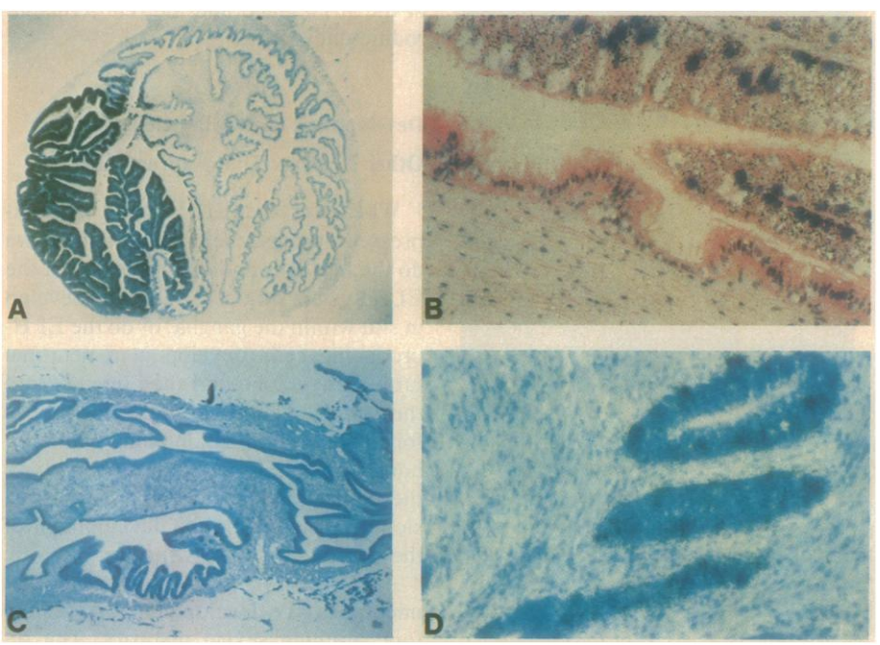

Fig. 4. In situ hybridization and immunocytochemistry to the atrial gland. (A) A section through the large hermaphroditic duct was reacted with antibodies to A peptide followed by a peroxidase-coupled second antibody. The hermaphroditic duct consists of red and white hemiducts on the right and the intensely staining atrial gland on the left; magnification is ×15. (B) In situ hybridization to a magnified section of the atrial and hermaphroditic duct. Grains are evident in the large columnar secretory cells of the atrial gland but diminish at the transitional epithelium bordering the flattened cells of the white hemiduct; magnification is ×370. (C) In situ hybridizations to the developing atrial gland. Sections of tissue from the hermaphroditic ducts of a 67-day postmetamorphic animal. Patchy hybridization with ^{125}I-labeled ELH gene probes is observed in the highly enfolded region destined to become the atrial gland; magnification is ×58. (D) A higher magnification (×230) of in situ hybridization to a developing atrial gland 67 days after metamorphosis.

maphroditic duct in animals ranging from 20 days after metamorphosis to young adults first reveals positive cells only at 40 days after metamorphosis (Fig. 4, C and D). The distribution of ELH-positive cells, present in the thickened proliferative epithelium of the duct, suggests that these cells originate from the epithelial cells of the hermaphroditic duct itself. No positive cells have ever been observed in surrounding tissue migrating to the duct. We presume, then, that ELH-positive cells of the atrial gland develop in situ rather than migrate from distant zones of proliferation.

Neuroactive Peptides and the Generation of Behavior

Peptides can mediate behavior in invertebrates and vertebrates. We can now begin to ask what makes peptides suited to the task. Behavior is produced by the interaction of nerve cells that are interconnected in specific ways. Thus, the behavioral potential of an organism is in part encoded by the specificity of the wiring within its nervous system. By releasing neurotransmitter substances at their synapses, neurons are able to communicate rapidly through point-to-point contact. Like the conventional transmitter substances, neuroactive peptides can act locally as neurotransmitters on neighboring neurons. However, when they are secreted into the circulation, these peptides can also act as neurohormones at distant sites. Both of these functions are carried out by ELH, for example, which serves as a neurotransmitter by specifically altering the properties of individual neurons in the abdominal ganglion (*14, 15*), as well as a neurohormone by acting distantly to cause contraction of the smooth muscle follicles of the ovotestis (*33, 34*). The ability of ELH to exert its different effects on a diverse family of target cells—different central neurons and effector organs—illustrates an important property of neuropeptides—namely, that they are capable of coordinating changes in the nervous system with effector events in order to achieve a common behavioral end.

Coordinate control, but on the molecular level, is also evident in the way in which polyproteins are synthesized. A single promoter element and a single translational initiator can control the simultaneous expression of several different peptides. Moreover, this coordination may extend beyond protein synthesis to the packaging of a set of peptides into single vesicles. Small peptides cleaved from a single precursor may be contained together in the same vesicle and therefore released at the same time by an action potential. In this way, coordinate synthesis may be coupled with coordinate release of companion peptides, on the one hand, and with coordinate control over neural and effector events on the other.

A third property that makes peptides especially good candidates for coordinating functions derives from the nature of their polyprotein precursors. Merely by altering the pathway of processing, many different combinations of peptides can be produced from one precursor. Such alternative processing is illustrated by the precursor of pro-opiomelanocortin (POMC), which undergoes differential processing in the anterior and intermediate lobes of the pituitary and, as a result, generates different peptides in different structures (*35–37*). The purpose of this diversity might be to activate different patterns of behavior by modulating the activity of various combinations of neurons or target organs. The egg-laying peptides have even greater potential for achieving diversity, since the EHL precursor contains ten potential cleavage sites and its constituent peptides are encoded by a small family of genes in which each member has diverged to generate new peptides.

The fact that active peptide sequences are interspersed in the midst of nonfunctional protein sequences enhances the potential for evolutionary change in peptides and therefore in behavior. The functionally inert amino acids that intervene between two peptide sequences offer a natural repository for evolutionary changes in which additional active peptides may be created without interrupting the preexisting set. In this manner, base changes within this intervening protein sequence may create new processing sites. Alternatively, sequences with their own preexisting cleavage sites may be inserted in this region. For example, the ELH precursor contains a 240–base pair (bp) stretch not present in the homologous precursor expressed in the atrial gland. This small insertion encodes three candidate peptides, one of which appears to have arisen from a small internal duplication. Thus, internal duplications within a precursor also pro-

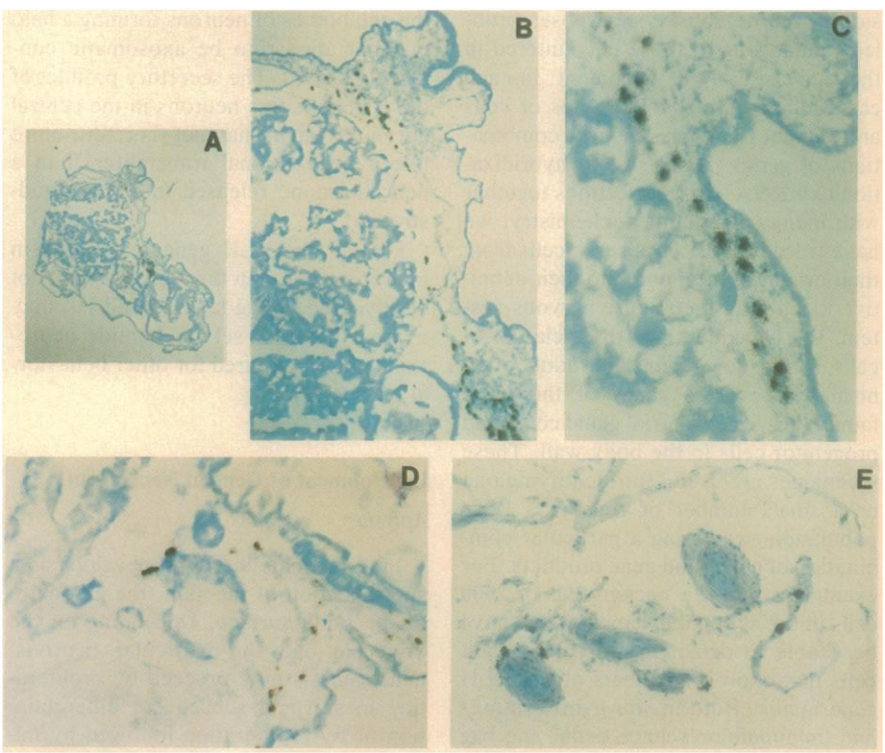

Fig. 5. ELH-positive neurons originate in the body wall and migrate to the central ganglia. (A) In situ hybridization to a section through a whole organism 10-days postmetamorphosis. ELH-positive cells are observed along the length of the body wall; magnification is ×15. (B) As in (A), magnification is ×50. (C) As in A, magnification is ×230. (D) In situ hybridization to developing organisms, 35 days, after metamorphosis. ELH-containing neurons appear to migrate from the body wall along connective tissue fibers to their ultimate position in the ganglia. Cells are shown migrating along fibrous threads to a primitive ganglion in the center of the section. Magnification is ×92. (E) In situ hybridization to a transverse section through a developing pleural abdominal connective showing fibers containing ELH-positive cells; magnification is ×92.

vide a mechanism to test new peptide possibilities without destroying the old. This is also apparent in the family of genes encoding the opioid peptides of vertebrates (*38*). In this manner, the number of peptides expressed by the ELH precursor has expanded without altering the ability of the polypeptide to express active ELH.

Finally, the individual behaviors may now be ascribed to individual peptides or groups of peptides. Different combinations of overlapping peptides could then give rise to different behavioral patterns with overlapping elements. Both egg-laying and feeding behavior in *Aplysia* involves head waving. Common peptides may elicit this activity in both feeding and egg laying in association with other peptides to generate these two distinct fixed action patterns. In this manner, more complex behaviors may be assembled by combining simple units of behavior, each mediated by one or a small number of neuropeptides.

Studying Gene Expression in Nerve Cells by in situ Hybridization

The analysis of specific gene expression in the nervous system poses problems analogous to those encountered in the study of early development. In each case, individual cells or groups of cells are thought to express distinct combinations of genes. Using in situ hybridization to mRNA in tissue sections together with indirect immunocytochemistry, we have traced the ELH-positive cells from their origins in the embryo to their definitive location in the mature nervous system. We have identified three classes of cells in the developing and adult organism that express genes of the ELH family: nerve cells, atrial gland cells, and progenitor cells in the body wall. These techniques are useful for identifying one or a small number of cells in a large population expressing a particular combination of genes and gene products. For example, against a background of 2000 cells in the abdominal ganglion, we have been able to detect the three invariant cells that express members of the ELH gene family. Both in situ hybridizations and immunofluorescence define the bag cells as the major site of synthesis of ELH. Earlier immunocytochemical studies (*26, 27*) first revealed a variable number of ELH-positive cells outside the bag cell clusters. Using whole mounts of the central nervous system taken from young animals, we detected a system of widely ramifying cells that express ELH, with some large and invariant members distributed throughout the CNS. Thus, our data and those of Chiu and Strumwasser (*26*) suggest that ELH may be used extensively as a neurotransmitter throughout the entire central nervous system.

The finding of this extensive system of ELH neurons raises questions as to its function. Do these cells participate with the bag cells to generate the egg-laying repertoire of behaviors? We do not know at present which of the egg-laying peptides are expressed by the individual cells outside of the bag cell cluster. Perhaps these cells release A and B peptides and initiate the discharge of the bag cells. Alternatively, the cells could mediate one or another of the individual behaviors associated with egg laying (such as head waving, grasping the egg strand by the mouth, and inhibition of feeding and walking). Unlike the bag cell processes, which traverse the sheath and release their product diffusely into the hemolymph, the processes of the ELH-containing neurons located in the cerebral, buccal, and pleural ganglia are restricted to the central nervous system and send their processes into the neuropil. In some instances these processes contact the cell bodies of neurons forming a halo of what appear to be axosomatic contacts (Fig. 3C). The secretory product of the ELH-positive neurons in the central nervous system may act therefore more like a conventional transmitter than a neurohormone released into the bloodstream.

Finally, the ELH genes expressed in neurons other than the bag cells may not be involved in egg laying at all but may express different sets of peptide neurotransmitters required for other behavioral processes.

Development of Central Neurons in *Aplysia*

In all animals the brain develops as a specialization of the skin, the ectoderm of the body surface. Depending on the organism and the particular neurons, neurogenesis may proceed by proliferation in situ and subsequent differentiation or by proliferation followed by migration over long distances (*39–41*). In certain invertebrates—including nematode worms, annelid worms, and insects—the ectodermal cells in the body wall give rise to a neural epithelium. Within this neural epithelium primitive neuroblasts lose their contact with the inner and outer surface of the ectoderm, round up, and frequently proliferate in situ giving rise to clones of progeny neurons, a development that typically does not involve migration over any significant distance [for review (*40*); for occasional exceptions (*2*)]. Other neurons, common in the nervous system of vertebrates, develop from the columnar ectodermal cells that withdraw from the mitotic cycle to migrate over varying distances to their definitive locations.

By labeling fertilized eggs with thymidine, Jacob *et al.* (*42*) found that the central neurons in *Aplysia* derive from a proliferative ectodermal zone in the body wall where almost all mitosis occurs. Postmitotic neurons then leave the body wall and migrate to form the central ganglia by crawling along connective tissue strands. We have here provided independent and direct evidence for this mode of development by studying a specific population of neurons that can be identified and marked by its characteristic pattern of gene expression. We have found that, before metamorphosis and through much of juvenile development, ELH-producing cells are present in the body wall and in the body cavity. Some of the cells in the body cavity are directly apposed to ganglia or are located on connective tissue strands that join these ganglia or their connectives to the body wall. Thus, the neurons use what appear to be nonneural connective tissue cells as a migratory path to the nervous system, a mechanism analogous to that of the nerve cells of the cerebral cortex which use radial glial fibers as guides for migration (*43*).

Our data, and those of Jacob, thus suggest an interesting similarity in the development of certain invertebrate neurons and those of vertebrates, particularly the cells of the neural crest (*44, 45*). The occurrence of a migratory step in *Aplysia* makes it likely that migration may also prove a more significant feature in the development of other invertebrate animals than has previously been appreciated.

Consequences of a Migratory Step in the Differentiation of Neurons

Given two modes of neurogenesis, what are the anatomic and functional consequences of each? Our study suggests that cell division followed by migration allows one proliferative zone to seed diverse segments of the nervous system (the bag cells as well as the rest of the central ganglia) with ELH-producing cells. The function of the ELH-pro-

ducing neurons outside of the bag cell cluster is unknown. It is possible that the pattern of gene expression in these various ELH-producing cells may be different. A clone of identical ELH-producing cells arising in a single proliferative zone may therefore diversify during the migration process itself or in response to different environments in which the cells ultimately reside.

We would suggest that in situ neurogenesis optimizes the development of a more precisely and more rigorously preprogrammed nervous system by minimizing extraneous influences and assuring that neurons will undergo the later steps of differentiation in the same microenvironment in which they undergo their final mitotic division. This is consistent with the findings in *C. elegans*, which indicate that much of a nerve cell's fate is programmed and is determined by its lineage (*1, 2*), except when the cell migrates (*46*). Neurogenesis followed by migration may permit the development of a less rigidly determined nervous system (*44, 46*). Migration provides a population of neurons with the additional opportunity to encounter multiple spheres of influences along the course of migration and, more important, with the opportunity to end up in a new microenvironment at their final destination. This is most clearly evident in certain neurons of the neural crest that migrate over considerable distances and whose ultimate choice of transmitter is determined by the local environment of the definitive target (*47*). Nevertheless, mechanisms exist whereby cells can be determined prior to migration and be relatively little influenced by their journey (*48*).

Precocious Expression of ELH

A striking feature of our findings is that the presumptive bag cells express the genes for ELH very early in development. Animals do not begin to release eggs until 60 days after metamorphosis. Yet a full 70 days earlier (10 days before metamorphosis) and well before the cells begin their apparent migration into the nervous system, they express genes encoding ELH. Qualitatively similar findings have been made in certain other migrating vertebrate neurons. Neural crest cells that migrate from the neural tube to form the ganglia of the autonomic nervous system synthesize acetylcholine (or norepinephrine) before they reach their final destination (*44*). On the other hand, cells in the CNS of the grasshopper that differentiate in situ and do not migrate express octopamine and proctolin only after their mature morphology is largely established and just before they begin to form synapses (*49, 50*).

Thus, early in development the bag cell precursors express in abundance a gene product whose function is thought to be required only in the sexually mature adult. Perhaps migrating cells re-

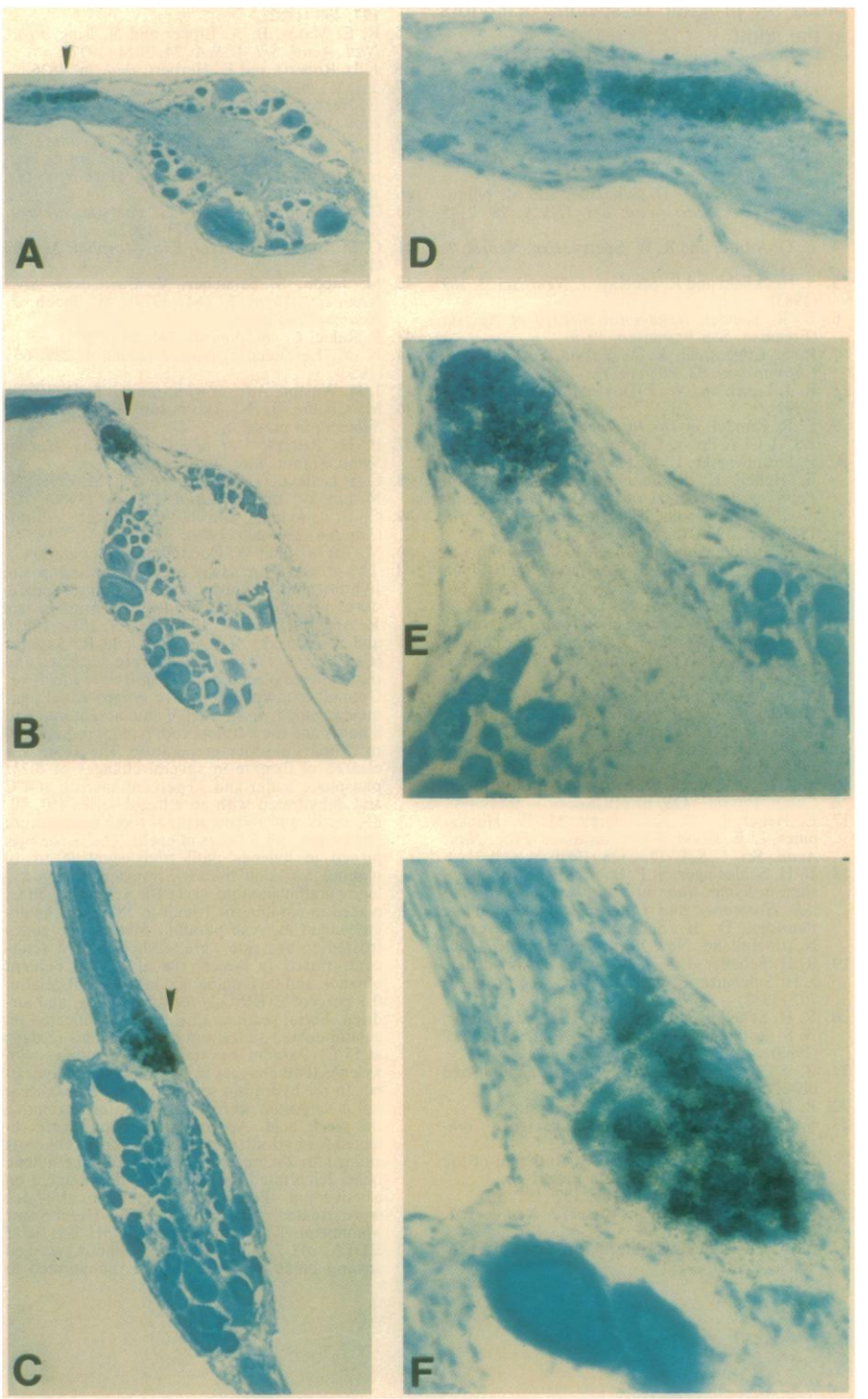

Fig. 6. In situ hybridization to developing bag cells. In situ hybridization experiments were performed on sections through the developing pleuroabdominal connective and abdominal ganglion to show the appearance and migration of the cluster of bag cell neurons. (A) A section 49 days after metamorphosis showing a small cluster of cells along the connective. The diameter of one bag cell is about 5 μm, ten times smaller than that of an adult bag cell; magnification is ×92. (B) A section 58 days after metamorphosis showing that the bag cell cluster has increased in size and has moved closer to the ganglion. Magnification is ×92. (C) A section 67 days after metamorphosis in a sexually mature adult capable of egg laying; magnification is ×92. (D) As in A, magnification is ×368. (E) As in B, magnification is ×368. (F) As in C, magnification is ×368.

quire specific gene products encoded with the ELH polyprotein for pathfinding and other developmental purposes. In this manner specific neurotransmitters or neurohormones may play different roles in different stages of development: an early role in guiding developmental processes and a later role in dictating the program of specific behaviors in the adult.

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51. The protocol for tissue preparation and in situ hybridization involves modification of procedures previously published by L. Angerer and R. Angerer [*Nucleic Acids Res.* **9**, 2819 (1981)] and R. Venezky, L. Angerer, and R. Angerer [*Cell* **24**, 385 (1981)]. Ganglia were dissected and tissues were placed directly in Bouin's fixative. Optimal fixation times were empirically determined; ganglia from a 250-g animal required 6 to 8 hours and the hermaphroditic duct required 10 to 12 hours at room temperature. The tissue was cleared of fixative in several changes of 0.2*M* phosphate buffer and 30 percent sucrose at 4°C and dehydrated with an ethanol series (50, 70, 85, 95, 99, and 100 percent) at room temperature (3 changes, >1.5 hours of each). The tissue was placed in xylenes until translucent (5 to 10 minutes for adult tissues), transferred to a xylene-paraffin mixture (1:1) for 1 hour at 58°C, placed in paraffin for 1 hour at 58°C, and finally embedded in fresh paraffin. Microscope slides frosted on one side were washed in mild detergent, rinsed in water, rinsed in 100 percent ethanol, and then immersed in 5 percent gelatin, 0.1 percent $CrK(SO_4)_2$ for 10 minutes, and air-dried. Serial sections (5 μm) were collected on gelatin-coated slides and fixed to slides (2 days at 55°C). Paraffin was removed from slides with xylenes (two changes in 15 minutes) and hydrated. In the hydration series, the 70 percent ethanol is saturated with $LiCO_4$ in order to remove the picric acid. Appropriate sections were selected for hybridization. Every 20th section was stained for 2 minutes in 0.125 percent methylene blue, dehydrated into xylenes, and mounted on a coverslip (Permount). Experimental slides were treated with proteinase K (Boehringer Mannheim, 1 μg/ml in 0.1*M* tris, *p*H 7.5, .05*M* EDTA, *p*H 7.5) at 37°C for 1/2 hour, washed several times in distilled water, dehydrated to 100 percent ethanol, and used immediately or stored in a desiccator (for a maximum of 24 hours). The control slides, after proteinase K treatment and washing, were treated with ribonuclease (BRL; 100 μg/ml in 0.1*M* tris, *p*H 7.5), for 1 hour at room temperature.
52. Proteinase K–treated, dehydrated slides for in situ hybridization were placed in moist chambers which were equilibrated with the hybridization solution excluding the nucleic acid. ^{125}I-labeled DNA probes were prepared with ^{125}I-labeled CTP as described [Robins *et al.*, *Cell* **23**, 29 (1981)]. The DNA probes were diluted to 400 ng/ml, denatured by boiling at 100°C for 5 minutes, then quenched on ice. The probe was brought to 0.3*M* NaCl, 30 percent formamide, 0.1*M* tris, *p*H 7.5, 4 m*M* EDTA, *p*H 7.5, 2× Denhardts solution, 2 percent dextran sulfate, and pipetted directly onto tissue sections at a concentration of 200 ng/ml. Each section was covered with the smallest possible volume; 30 to 50 μl was necessary to cover a cross section through an adult hermaphroditic duct while only 2 μl was necessary for the ganglia of a very young animal. The slide chambers were wrapped in Parafilm and foil and placed in an oven at 48°C for 12 hours. The hybridized slides were washed twice for 1 hour in 4× SSC, 2× SSC, 1× SSC, 0.5× SSC, and 0.2× SSC + 0.5 percent P_i or PP_i. The 4× SSC rinses the formamide and was conducted at room temperature while the subsequent washes were done at 37°C. Potassium iodide was added to 0.1*M* in the 4×, 2×, and 1× washing solutions. Washed slides were dehydrated, dipped in photographic emulsion, and exposed at 4°C for 1 week.
53. Tissue for immunocytochemistry was processed as described for in situ hybridization with the exception of the protease treatment. The primary antibodies, rabbit antibody to ELH or rabbit IgG to A peptide were kindly provided by E. Mayeri and B. Rothman (*24*). The specificity of these antibodies was established by radioimmune assays with purified ELH and A or B peptides. Immunocytochemistry with peroxidase-conjugated goat antibody to rabbit IgG was performed as described by R. Mesa-Tejada [*J. Histochem. Cytochem.* **26**, 532 (1978)].
54. Indirect immunofluorescence was performed on whole CNS's from 10- to 35-day postmetamorphic animals and on whole premetamorphic animals by a modification of procedures of R. Goldstein, J. Kistler and J. Schwartz (submitted to *Neuroscience*). The whole CNS's from juvenile animals and the whole premetamorphic animals were processed as described above, but for this assay the tissue was rehydrated after fixation, clearing, and dehydration, then blocked 6 to 12 hours in 50 percent normal goat serum, 0.25 percent saponin at 4°C. The tissue was incubated with the primary antibody at 100 μg/ml in 1 percent normal goat serum, 0.25 percent saponin for 3.5 days, washed 6 hours in 0.1*M* phosphate buffer, *p*H 7.4, incubated 3 hours with a rhodamine-conjugated goat antiserum to rabbit IgG at a dilution of 0.1000 in 1 percent normal goat serum and treated with 0.25 percent saponin, washed for 3 hours in phosphate buffer; a coverslip in 66 percent glycerol in phosphate buffer was put in place, and the preparation was visualized and photographed with a Leitz immunofluorescence microscope.

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