

Neuropeptides: Mediators of Behavior in *Aplysia*

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Neurons communicate with other neurons and target tissues through various chemical messengers. The modulation of chemical messenger release is thought to play an important role in the modification of behavior through experience (1). During development, the network of interactions mediated by these messengers proliferates and in some cases reorganizes, giving rise to different behaviors (2). In this article, we discuss the regulation of the biogenesis of extracellular messengers, both during development and in the adult nervous system.

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Summary. The *Aplysia* neuroendocrine system is a particularly advantageous model for cellular and molecular studies because of the relatively small number and large size of its component neurons. Recombinant DNA techniques have been used to isolate the genes that encode the precursors of peptides expressed in identified neurons of known function. The organization and developmental expression of these genes have been examined in detail. Several of the genes encode precursors of multiple biologically active peptides that are expressed in cells which also contain classical transmitters. These studies, as well as immunohistochemical studies and the use of intracellular recording and voltage clamp techniques are the first steps toward revealing the mechanisms by which neuropeptides govern simple behaviors.

Chemical messengers can be placed in three overlapping categories according to their mode of action: (i) neurotransmitters, which act quickly and over short distances to alter transiently the excitability of cells; (ii) neuromodulators, which alter the response of a cell to a distinct chemical input; and (iii) neurohormones, which act at a distance from their site of release and give rise to neuronal effects that tend to be slow in onset. The most diverse group of extracellular chemical messengers are the biologically active peptides, which can function as transmitters, modulators, or hormones, and are often localized in neurons that also contain classical transmitters (3). The functional significance of the coexistence of multiple types of messengers is just beginning to emerge.

The gastropod mollusk *Aplysia* is use-

ful for studies of a central nervous system (4). This marine organism can reach 30 to 50 centimeters in length and often attains a mass of more than 1 kilogram. The central nervous system consists of about 20,000 neurons clustered into five major ganglia (buccal, cerebral, pleural, pedal, and abdominal) which are interconnected by an intricate network of connective nerve tracks (Fig. 1A). Many of the neurons have been identified on the basis of their size, shape, color, and endogenous electrical activity. Numerous anatomical and physiological studies

have resulted in the assignment of behavioral roles to many of these identified cells (4, 5). Some *Aplysia* neurons are up to 0.5 mm in diameter and contain about 0.25 μ g of DNA as well as correspondingly large amounts of RNA and protein (6). The large size of these "giant" neurons has not only facilitated physiological studies but more recently has allowed biochemical and molecular genetic analyses of single neurons.

The buccal ganglion, the smallest of the major ganglia, consists of two symmetric hemiganglia joined by a commissure (Fig. 1B). Some buccal neurons innervate tissues that participate in the mechanical aspects of feeding, such as the esophagus and the buccal mass; the latter is a muscular organ that controls biting movements. Other buccal neurons are thought to be involved in motivation-

al aspects of behavior such as food-induced arousal (7). The best studied of the *Aplysia* ganglia is the asymmetric abdominal ganglion (Fig. 1C). Its 2000 neurons mediate reflex and fixed action patterns; these include withdrawal of the gill and mantle organs, as well as inking and egg laying. The abdominal ganglion also governs a number of visceral functions, including excretion, cardiac output, and various aspects of respiration. Many abdominal ganglion neurons use molecules such as acetylcholine, serotonin, and glycine to mediate extracellular communication; however, biologically active peptides most likely provide the largest diversity of chemical messengers. The secretion of peptide messengers into the bloodstream in concert with local activities serves to coordinate physiological and behavioral events.

Neuropeptide Gene Organization and Expression

In vitro translation and immunoprecipitation experiments demonstrate that 20 to 50 percent of the messenger RNA (mRNA) in many *Aplysia* peptidergic neurons encode the precursor for peptides. This has facilitated cloning the corresponding genes (8, 9). The genes are isolated by dissecting homogeneous clusters or individual neurons and extracting the RNA. Approximately 1 μ g of polyadenylated RNA can be prepared from as few as 20 cells that are 300 μ m in diameter. Complementary DNA (cDNA) copies of the mRNA, incorporating radioactively labeled nucleotides, are prepared by reverse transcription. These cDNA's are then used to screen cDNA or genomic libraries, and clones are selected that are complementary to mRNA's specifically expressed in identified neurons. These techniques have been used to isolate cDNA clones encoding neuropeptides from buccal ganglion cells, B1 and B2, and from abdominal ganglion cells L11, R3-14, R15, and the bag cells (Fig. 1, B and C) (10).

The egg-laying hormone (ELH) gene family consists of five members per haploid genome. These genes are expressed specifically in the central nervous system (the ELH gene) and the atrial gland (peptide A and B genes) (Fig. 2). The atrial gland is an exocrine gland situated at the distal end of the hermaphroditic duct near the gonopore (Fig. 1A). Each characterized gene encodes biologically active peptides that are involved in medi-

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ating egg-laying behavior, a classic neuroendocrine fixed action pattern. This family of genes appears to have evolved through initial duplication of a primordial peptide sequence within a single transcription unit, resulting in a precursor that encodes multiple biologically active products—a polypeptide. A multi-gene family arose by duplicating large regions of the chromosome, including complete transcription units. The ELH gene family transcripts have two configurations in the 5' untranslated region. These two forms arise because a 190-nucleotide exon (that is, a coding sequence) has two RNA splicing donor sites, either of which can be joined to a single acceptor site that is contiguous with the coding region. We do not yet know if a single gene is alternately spliced or if independent genes are spliced differently. We do not find any RNA that encodes peptide A or B in the bag cells, nor do we find any RNA that encodes ELH in the atrial gland, suggesting the control of expression is at the level of the RNA.

The gene that encodes peptides expressed in abdominal ganglion neurons R3-14 is present in a single copy per haploid genome (Fig. 2). The transcription unit contains two large introns (non-coding sequences) and spans a region of

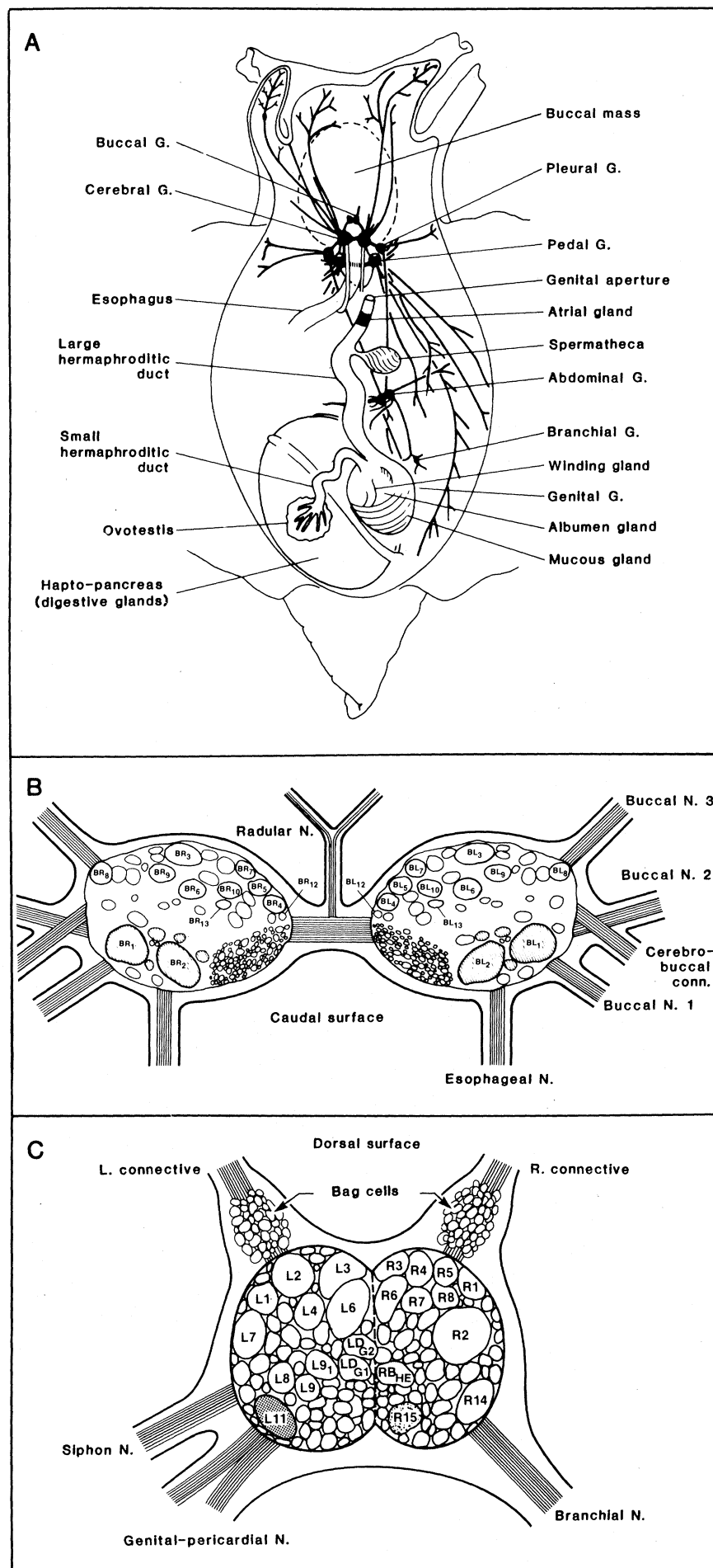


Fig. 1. *Aplysia* reproductive system and central nervous system. (A) Schematic diagram of the *Aplysia californica* body plan. *Aplysia* are non-self-fertilizing hermaphrodites; eggs and sperm originate in the ovotestis and travel through the reproductive system to the large hermaphroditic duct and penis, respectively. After fertilization, which occurs internally, the eggs are released through the gonopore during egg laying. The atrial gland forms a continuous lumen with the large hermaphroditic duct. The head region contains the buccal ganglia situated on the large buccal muscle that governs movement of the mouth. The ring ganglia consist of the pedal, pleural, and cerebral ganglia, which encircle the esophagus. A single asymmetric ganglion is situated in the abdomen. (B) The dorsal surface of the buccal ganglion. Identified neurons are labeled with a *B* (designating the buccal ganglion), an *L* or *R* (designating left or right hemiganglion), and an identifying number. The B1 and B2 cells as well as several less well identified neurons use the small cardioactive peptides SCP_A and SCP_B as extracellular messengers. (C) The dorsal surface of the abdominal ganglion. Representative cells are labeled as above. Neurons that use biologically active peptides are identified by shadings. The bag cells are grouped into two large clusters of about 400 electrically coupled neurons, each on the rostral side of the ganglion. Cells R3-8 and R14 are thought to modulate cardiovascular function. R15 is a neurosecretory cell involved in controlling water and salt balance. L11 is a cholinergic neuron that is also peptidergic.

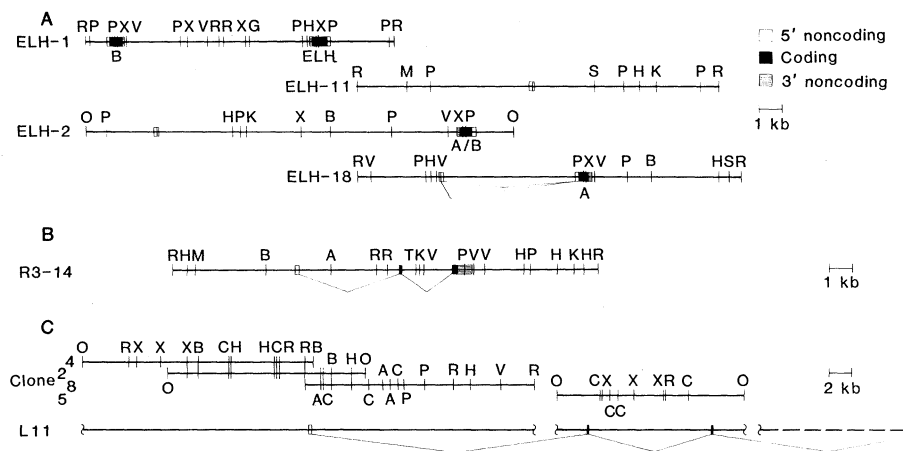


Fig. 2. Recombinant DNA genomic clones containing neuropeptide genes expressed in identified neurons. The letters denote the positions of restriction enzyme sites: E, Eco RI; V, Pvu II; P, Pst I; H, Hind III; X, Xho I; B, Bam HI; S, Sac I; G, Bgl I; A, Sal I; T, Sst I; M, Sma I; O, Mbo II; and C, Xba I. Regions of the mature mRNA molecules are diagrammed: open boxes indicate 5' untranslated regions; closed boxes, coding regions; and hatched boxes, 3' untranslated regions. (A) The ELH gene family. Four nonallelic recombinant clones encoding ELH gene family segments are diagrammed. ELH genes are expressed in the bag cells, and peptide A and B genes are expressed in the atrial gland. Two alternative RNA splicing patterns are indicated in the ELH-18 gene. (B) The R3-14 neuropeptide precursor gene and RNA splicing pattern. (C) Partial characterization of the 5' untranslated and coding region of the L11 peptide precursor gene.

at least 7 kilobases. The first exon is the complete 5' untranslated region of the message (11). The second exon encodes the hydrophobic leader sequence and most of the precursor having a negative charge. The 65 highly basic amino acids at the carboxyl terminal and the 3' untranslated region are encoded by the third exon. In contrast to the gene encoding the ELH precursors, this gene has no internal homologies, suggesting that it may have evolved through recombination events within the introns.

The gene encoding the 151-amino acid L11 precursor appears to be distributed over a minimum of 40 kilobases of genomic DNA (Fig. 2). Although the significance of this organization is still unclear, analysis of a cDNA clone from the head ganglia has revealed a new gene that is transcribed from the opposite DNA strand and overlaps the L11 gene in the 3' untranslated region.

In situ hybridization and immunohistochemistry have been used as complementary techniques to determine the distribution of neurons expressing the ELH gene family (12). In situ hybridization detects cells that are transcribing particular genes by hybridizing radioactively labeled, cloned DNA segments to cellular RNA and using autoradiography to visualize the labeled RNA-DNA hybrids. In Fig. 3, A and B, a single neuron is expressing an ELH gene. Other tissue sections can be treated with antibodies to probe for the presence of the protein product. By these techniques, all major ganglia except the pedal ganglion were shown to contain neurons expressing some member or members of the ELH gene family. All of these ELH-expressing cells are interneurons that arborize extensively throughout the central nervous system, suggesting that they mediate activities of other neurons. Therefore the major role of the bag cells may be to release large amounts of hormones destined for distant targets. Bag cell peptides are known to be released into the circulation and to have actions at a distance, eliciting, for example, contraction of the ovotestis muscle and egg release.

In situ hybridization has also been used to study the developmental expression of the ELH gene family (Fig. 3). Cells of animals at the premetamorphic, or veliger, stage express ELH family genes; however, the small size of these animals has made the precise localization of the cells difficult. The most interesting information has been obtained from studies of postmetamorphic animals. When radiolabeled DNA probes were incubated with consecutive sections through whole animals, numerous

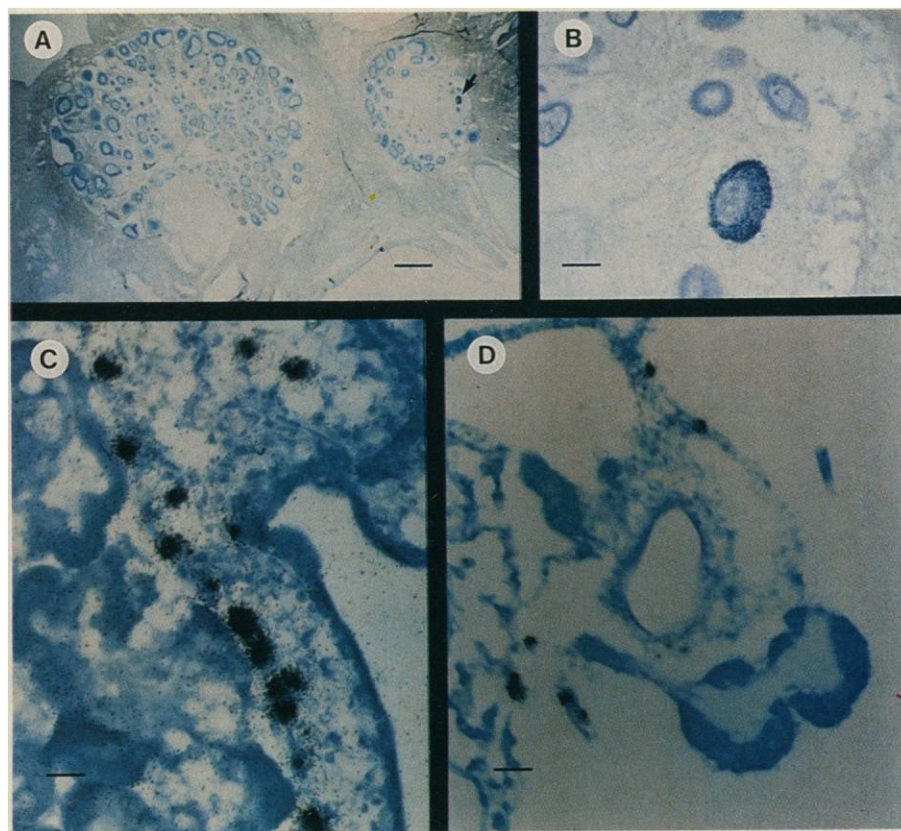


Fig. 3. In situ hybridization as a probe for gene expression in the nervous system. Cloned ELH encoding DNA fragments were labeled with [¹²⁵I]dCTP (dCTP, deoxycytidine triphosphate) and hybridized to sections of fixed tissue. (A) Section through the pleural and cerebral ganglion, showing a single neuron expressing an ELH gene; scale bar, 700 μ m. (B) Higher magnification of (A); scale bar, 50 μ m. The bag cell neurons arise from the body wall and migrate to their adult positions in the central nervous systems. (C) Section through the caudal region of a 10-day postmetamorphic animal. Many cells containing RNA homologous to ELH family genes are observed on the body wall; scale bar, 20 μ m. (D) In situ hybridizing cells in the vicinity of the abdominal ganglion of a juvenile animal. Cells are seen on tissue strands or along the pleural abdominal connective; scale bar, 80 μ m.

cells in the caudal ectoderm hybridized to the ELH gene probe. The largest number of hybridizing cells was found in the tail region; however, other cells expressing ELH genes were seen along the ectoderm leading rostrally. In the region of the abdominal ganglion, hybridizing cells were seen along tissue strands leading from the body wall to the pleural-abdominal connective. These data gave rise to a model in which the bag cells arise from proliferative zones in the caudal ectoderm and their fate is determined early, while they move along the body wall. The experiments further suggest that the cells first migrate along the body wall, then along tissue strands, and finally on the pleural abdominal connective, eventually reaching their destination on the rostral side of the abdominal ganglion. The situation in *Aplysia* is, at least in part, different from other modes of development, such as neuron proliferation

in situ. Furthermore, it does not appear that totipotent or partially restricted cells populate the central nervous system and that positionally dependent inductive events then influence the final state of the cell.

The atrial gland, which expresses the peptide A and B members of the ELH gene family, first becomes apparent 40 to 50 days after metamorphosis, as a highly infolded region of the large hermaphroditic duct. In situ hybridization experiments reveal patchy hybridization in an area of thickened epithelium in the duct. The hybridization becomes more evenly distributed as development continues. In contrast to central neuron development, cells expressing ELH family genes have not been observed outside the region of the gland. This suggests that the atrial gland develops as a specialization of the duct itself. Study of the organization and expression of the ELH gene family

should provide insight into the regulation of tissue-specific gene expression in different states of development.

Precursors of Biologically Active Peptides

All mammalian neuropeptides characterized to date are synthesized as parts of larger precursor proteins, which often contain more than one biologically active component (13). This is also the case for the six precursors characterized in *Aplysia* (Fig. 4). The proteins can be divided into domains based on structure or function. The first domain, common to all the precursors is a "signal" or "leader" sequence, which is necessary for attachment of the nascent protein chain to the rough endoplasmic reticulum. This sequence, which is cleaved from the precursor as the nascent protein chain is

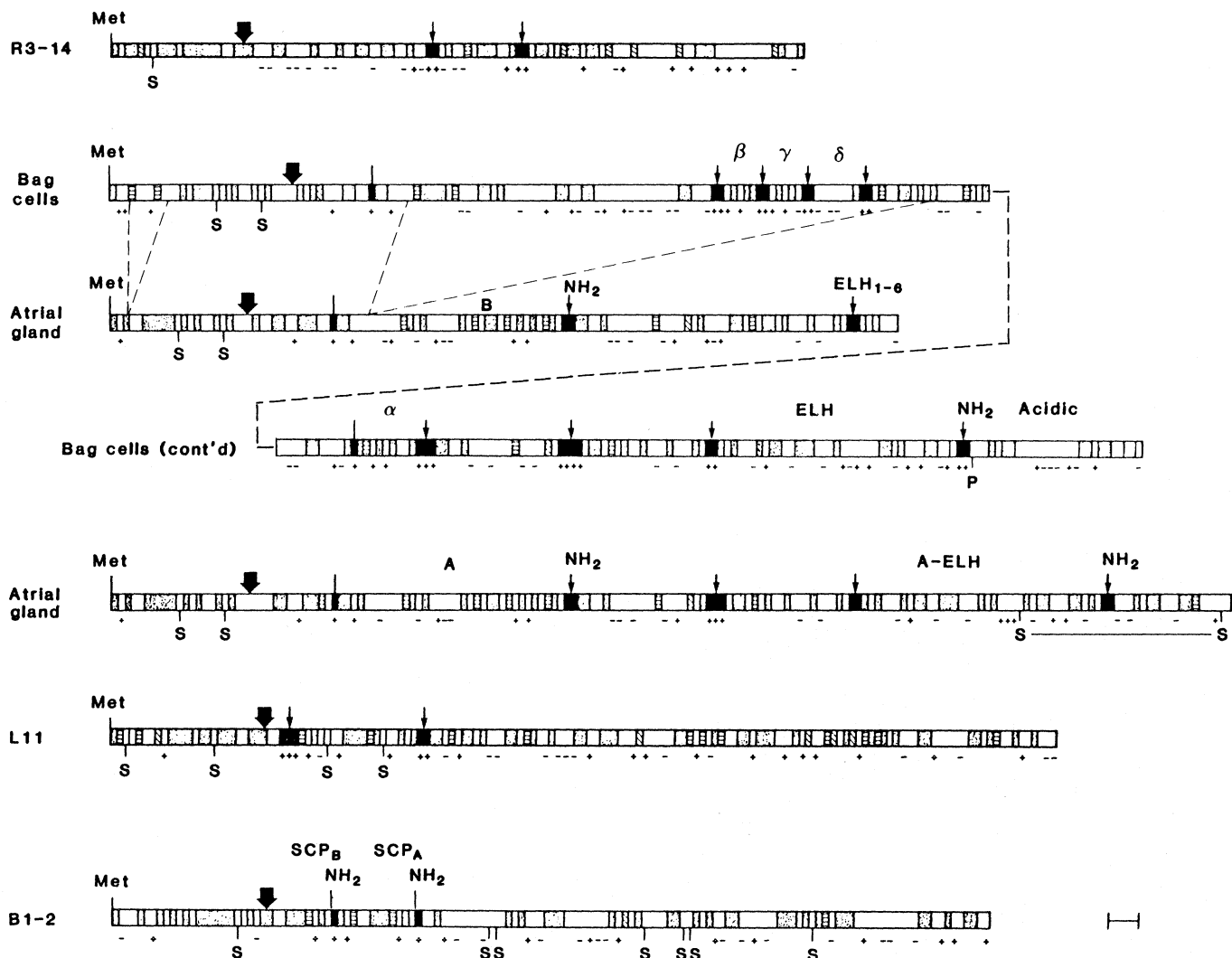


Fig. 4. Schematic representation of neuropeptide precursors found in identified buccal and abdominal ganglion neurons. Known peptides (SCP_A and SCP_B ; α , β , γ , and δ bag cell peptides; peptides A and B; ELH; and the acidic peptide) are indicated. An S below the sequence indicates a cysteine residue. Large arrows are putative sites of signal sequence cleavage. A vertical line above the sequence represents a known or potential cleavage at a single arginine residue, and arrows show the positions of di-, tri-, or tetra-basic cleavage sites. If carboxyl terminal amidation is thought to occur, NH_2 is written above the arrow or line. The bar represents five amino acids.

extended, is essential for insertion of the precursor protein into the lumen of the rough endoplasmic reticulum. In the *Aplysia* precursors, the initiator methionine is normally followed by a stretch of 10 to 20 hydrophobic amino acids, which are flanked on both sides by charged residues.

Other features of the precursors are unique, presumably fitting the functional needs of the cells in which they are expressed. The cDNA clone encoding the precursor for small cardioactive peptides A and B (SCP_A and SCP_B) was isolated by screening a buccal ganglion library with radiolabeled cDNA complementary to RNA from buccal cells B1 and B2 (Fig. 1). The distribution of peptides in the precursor is somewhat unusual in that the known active molecules immediately follow the signal sequence (Fig. 4). The nine-amino acid peptide, SCP_B, has flanking Gly-Arg residues on the carboxyl terminal end, in agreement with the observed amidation of the ultimate methionine residue. Directly following this Gly-Arg is the 11-amino acid peptide SCP_A. The seven residues at the carboxyl terminal of SCP_A and SCP_B are identical, and SCP_A is also flanked by a carboxyl terminal Gly-Arg, leading to amidation of this peptide. The remaining 88 amino acids include six cysteine residues, suggesting a highly bridged structure that does not resemble any previously characterized peptides. Antibodies

to SCP_B reveal intense immunoreactivity in the buccal ganglion, as well as fainter staining of a small set of cells in the other major ganglia. Immunoreactive processes and terminals are seen in the gut, the salivary gland, and the buccal muscles and in the neuropil of all major ganglia. Although the localization of the SCP's in motor neurons that mediate contraction of the radula closer muscle as well as other buccal neurons that modulate contractile activity of the gut establishes a role in feeding behavior, their widespread distribution implies that they have other functions as well.

Three related genes encoding the precursors of peptides that mediate the fixed action pattern of egg laying have been characterized (Fig. 4). The DNA and inferred protein sequences of the three genes are 90 percent homologous. Differences between the genes result in posttranslational processing of the precursors, which generate different sets of peptides in the nervous system and the atrial gland. The gene expressed in the bag cell neurons encodes a 271-amino acid precursor protein (ELH gene in Fig. 2) and is cleaved into at least seven peptides, three of which have biological activity. The atrial gland genes encode precursors of 173 (peptide A) and 122 (peptide B) amino acids. The sequences for peptides A and B are several amino acids beyond the signal sequence. These 234-amino acid peptides elicit egg laying

when they are injected into the animal; the abdominal ganglion is required for this activity of peptides A and B but is not required for ELH activity. These observations, and the fact that peptides A and B depolarize the bag cells, resulting in afterdischarge, are consistent with the concept that the atrial gland products either directly or indirectly activate bag cells. It has been proposed that mechanical stimulation of the atrial gland during copulation results in the release of peptide products that trigger bag cell bursts and result in egg laying (14). However, since the atrial gland is exocrine and situated at the gonopore, the peptide products may act as pheromones, mediating communication between individuals of the species.

The ELH precursor differs from the atrial gland precursors in several ways. First, a gene expressed in the bag cells contains an insertion of 240 nucleotides after the sixth amino acid of peptides A and B (Fig. 4). This segment of the precursor contains four pairs of dibasic residues, flanking three peptides β , γ , and δ bag cell peptides (BCP's). The β - and γ -BCP's are pentamers that differ only in their last amino acid. The four-amino acid sequence common to the β - and γ -BCP's (Arg-Leu-Arg-Phe) is also found in α -BCP and in the carboxyl terminal region of peptides A and B. Another important difference between the ELH and atrial gland precursors is

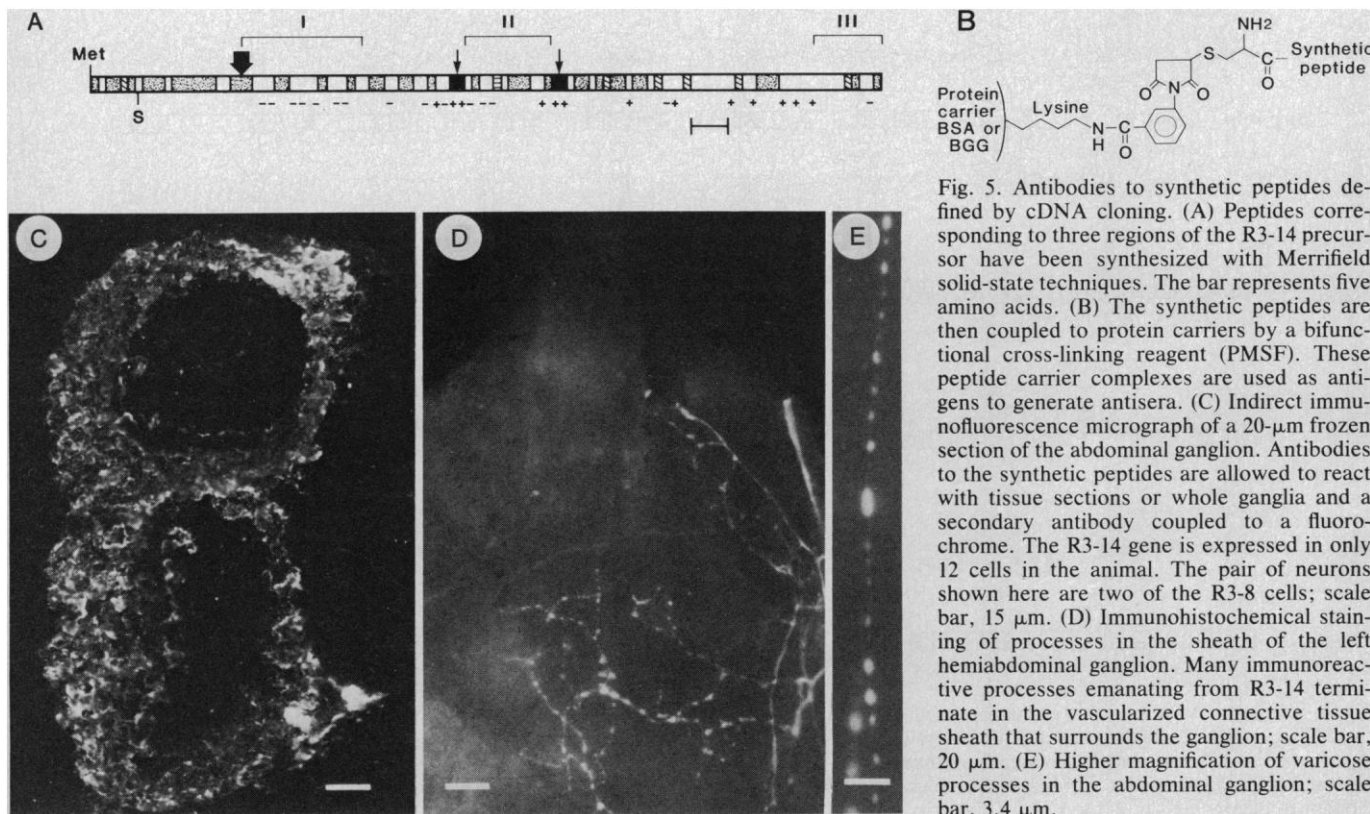


Fig. 5. Antibodies to synthetic peptides defined by cDNA cloning. (A) Peptides corresponding to three regions of the R3-14 precursor have been synthesized with Merrifield solid-state techniques. The bar represents five amino acids. (B) The synthetic peptides are then coupled to protein carriers by a bifunctional cross-linking reagent (PMSF). These peptide carrier complexes are used as antigens to generate antisera. (C) Indirect immunofluorescence micrograph of a 20- μ m frozen section of the abdominal ganglion. Antibodies to the synthetic peptides are allowed to react with tissue sections or whole ganglia and a secondary antibody coupled to a fluorochrome. The R3-14 gene is expressed in only 12 cells in the animal. The pair of neurons shown here are two of the R3-8 cells; scale bar, 15 μ m. (D) Immunohistochemical staining of processes in the sheath of the left hemiabdominal ganglion. Many immunoreactive processes emanating from R3-14 terminate in the vascularized connective tissue sheath that surrounds the ganglion; scale bar, 20 μ m. (E) Higher magnification of varicose processes in the abdominal ganglion; scale bar, 3.4 μ m.

that the carboxyl terminal end of the peptide A and B homologous region encodes the nine-amino acid peptide, α -BCP, in the bag cell precursor. α -BCP is flanked on its amino terminal end by a single arginine residue and on its carboxyl terminus by three basic residues. The glycine codon at the carboxyl terminus of the peptides A and B is, through a single base change, an arginine in the bag cell gene. As a result, α -BCP is not amidated and is therefore less stable than peptides A or B. The sensitivity to protease assures that α -BCP will act only over short distances, whereas peptides A and B remain active some distance from their site of release. In the genes for all three precursors, sequences encoding ELH are found 141 nucleotides from the peptide A and B coding region. In the bag cell gene, the sequence encoding the 36-amino acid amidated peptide ELH is followed by 84 nucleotides that encode the acidic peptide and the stop codon. The amino terminal serine of the acidic peptide is phosphorylated.

The ELH coding region of the genes expressed in the atrial gland is significantly different from the gene expressed

in the bag cells. A single base deletion in the peptide B gene after the sixth amino acid of ELH generates an inframe stop codon at that position. The peptide A gene remains in frame; however, several base changes effect amino acid substitutions, including a transition to a cysteine at position 25 of ELH. The acidic peptide homology remains intact until the 16th amino acid, where a deletion again shifts the frame. The most significant consequence of this frame shift is the coding of a second cysteine one amino acid from the stop codon. This allows the formation of a disulfide linkage between ELH and the acidic peptide region (Fig. 4). These ELH-like peptides are the source of egg-laying activity that can be elicited by injection of whole atrial gland extracts in the absence of the abdominal ganglion.

The precursors of peptides from the R3-14 and L11 neurons contain 108 and 151 amino acids, respectively, and each contains two pairs of basic amino acids (Fig. 4). Since we have little direct information on the peptides used by these cells, the active regions of the precursors are not yet known.

Anatomical Localization of Peptides

Although in situ hybridization is useful for identifying cells transcribing particular genes, immunohistochemical techniques are relied on to detect the eventual gene product. If the peptides have been purified, it is straightforward to prepare rabbit serum antibodies. If the peptides were previously characterized, as is the case with the R3-14 and L11 neurons, we used the method outlined in Fig. 5 (15). Synthetic peptides corresponding to the protein sequence deduced from the nucleic acid sequence of the cDNA clones are synthesized and coupled to larger carrier proteins, such as bovine serum albumin (BSA) or bovine gamma globulin (BGG). Serum antibodies to synthetic peptides from L11 and R3-14, as well as to peptide A, ELH, α -BCP, and SCP_B, have been prepared.

Indirect immunofluorescent micrographs were made by using one of the antibodies against synthetic R3-14 peptides to stain sectioned and whole mount tissue (Fig. 5, C to E). Studies of this type have resulted in several general conclusions. Antibodies directed against

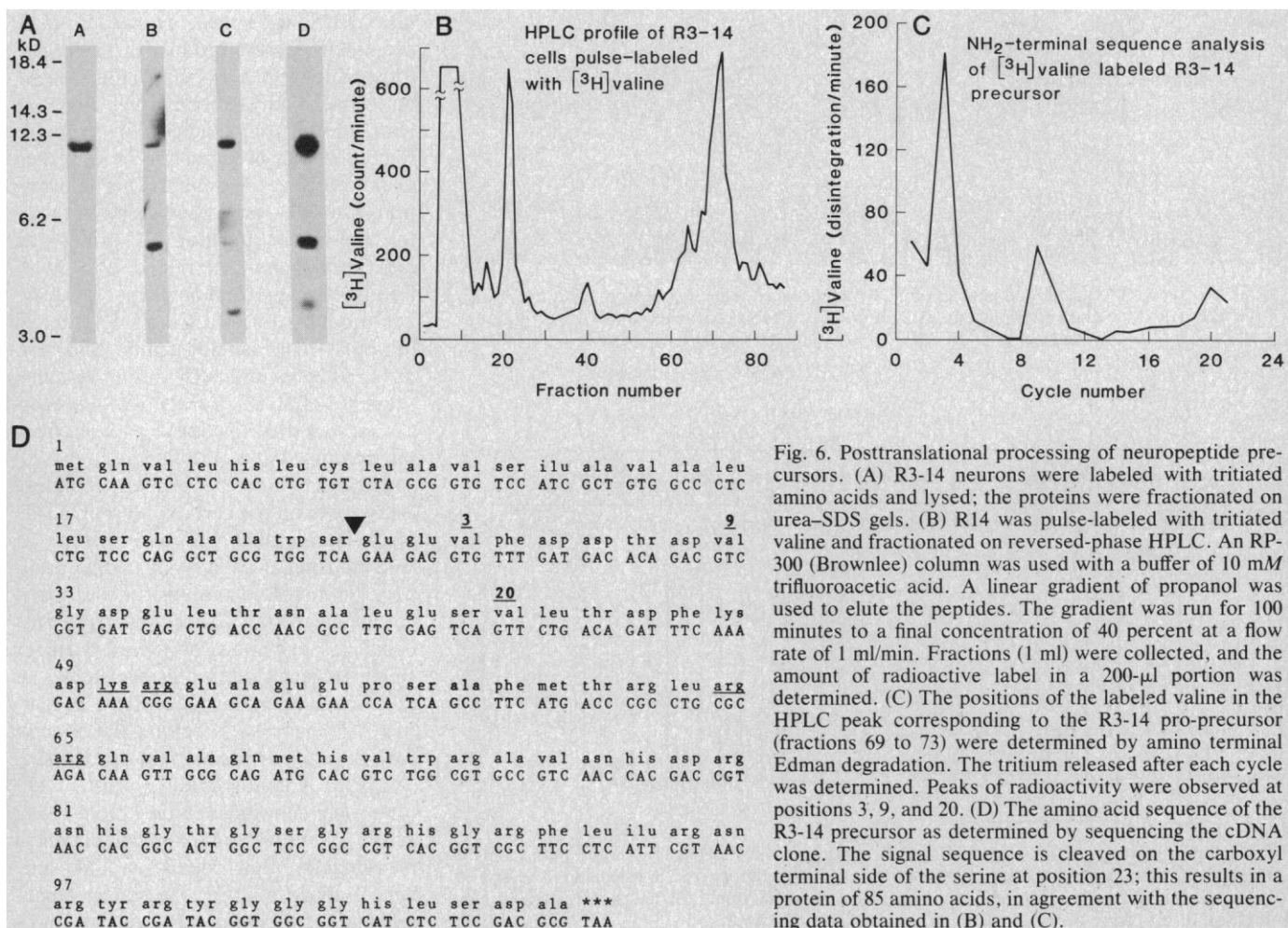


Fig. 6. Posttranslational processing of neuropeptide precursors. (A) R3-14 neurons were labeled with tritiated amino acids and lysed; the proteins were fractionated on urea-SDS gels. (B) R14 was pulse-labeled with tritiated valine and fractionated on reversed-phase HPLC. An RP-300 (Brownlee) column was used with a buffer of 10 mM trifluoroacetic acid. A linear gradient of propanol was used to elute the peptides. The gradient was run for 100 minutes to a final concentration of 40 percent at a flow rate of 1 ml/min. Fractions (1 ml) were collected, and the amount of radioactive label in a 200- μ l portion was determined. (C) The positions of the labeled valine in the HPLC peak corresponding to the R3-14 pro-precursor (fractions 69 to 73) were determined by amino terminal Edman degradation. The tritium released after each cycle was determined. Peaks of radioactivity were observed at positions 3, 9, and 20. (D) The amino acid sequence of the R3-14 precursor as determined by sequencing the cDNA clone. The signal sequence is cleaved on the carboxyl terminal side of the serine at position 23; this results in a protein of 85 amino acids, in agreement with the sequencing data obtained in (B) and (C).

different portions of the same precursor always stain the same cells. In the case of R3-14, double-labeling studies with antibodies to the amino terminal and carboxyl terminal ends of the precursor demonstrate immunoreactivity in the same processes and varicosities. The bag cells and R3-14 have a large number of processes that end in the connective tissue sheath that surrounds the ganglion. Innervation of the sheath is an effective means of delivering the peptides to central neuron targets as well as allowing diffusion into the circulation. The R3-14

cells have additional processes that exit from the ganglion via the branchial nerve and terminate on the efferent vein of the gill at the base of the auricle. The observed distribution of bag cell and R3-14 processes is an anatomically efficient organization for coordinating activities on central and peripheral targets. In contrast, L11 has a single immunoreactive process that is not varicose and travels out of the genital ganglion to the gill. At least the cholinergic component of the messenger system used by this cell is destined for local release.

Processing, Packaging, and Transport of Biologically Active Peptides

Many posttranslational events modify the precursors of biologically active peptides before the generation of a biological response. These modifications, which occur in the rough endoplasmic reticulum, the Golgi complex, and perhaps even the vesicle, include the formation of disulfide bridges, the addition of sugar and phosphate side groups, proteolytic cleavages, carboxyl terminal amidation, and amino terminal acetylation. To develop a complete understanding of the biologically active peptides contained in a precursor, we must determine the structure of the final products released from the neurons. The large size of the neurons and the predominant peptide products synthesized in these cells are also advantageous in this type of study.

The protein products of specific cells can be labeled either by bathing the ganglion in tritiated amino acids or by injecting isotope directly into the neuronal cell body. Individual identified neurons can then be dissected, and the products can be analyzed by gel electrophoresis, high-performance liquid chromatography (HPLC), and microsequencing techniques (Fig. 6). Sodium dodecyl sulfate (SDS)-urea gels reveal a single prominent protein product of 12 kilodaltons, which is processed to smaller products of 6.2 and 3.4 kilodaltons. Labeling with different amino acids allows specific regions of the precursor to be observed in the absence of others. This information, however, is not sufficiently precise for the exact position of proteolytic cleavages to be determined. We therefore isolated purified precursor or cleavage products by HPLC and determined the position of labeled amino acid residues in the peptides by amino terminal sequence analysis. For the R3-14 precursor we found that valine is released from the precursor at positions 3, 12, and 20, suggesting that the signal peptidase cleavage is on the carboxyl terminal side of the serine at amino acid position 25 (Fig. 6). Similar analysis of smaller products has revealed the positions of internal cleavage sites. These cleavage sites occur, as expected, at paired basic or single basic residues. Further studies of the modifications of all of these precursors are necessary before the precise pathway of posttranslational processing can be fully understood.

As was mentioned earlier, biologically active peptides and classical chemical messengers often coexist in the same neuron. Two multiple messenger candidates are the peptidergic neurons R3-14

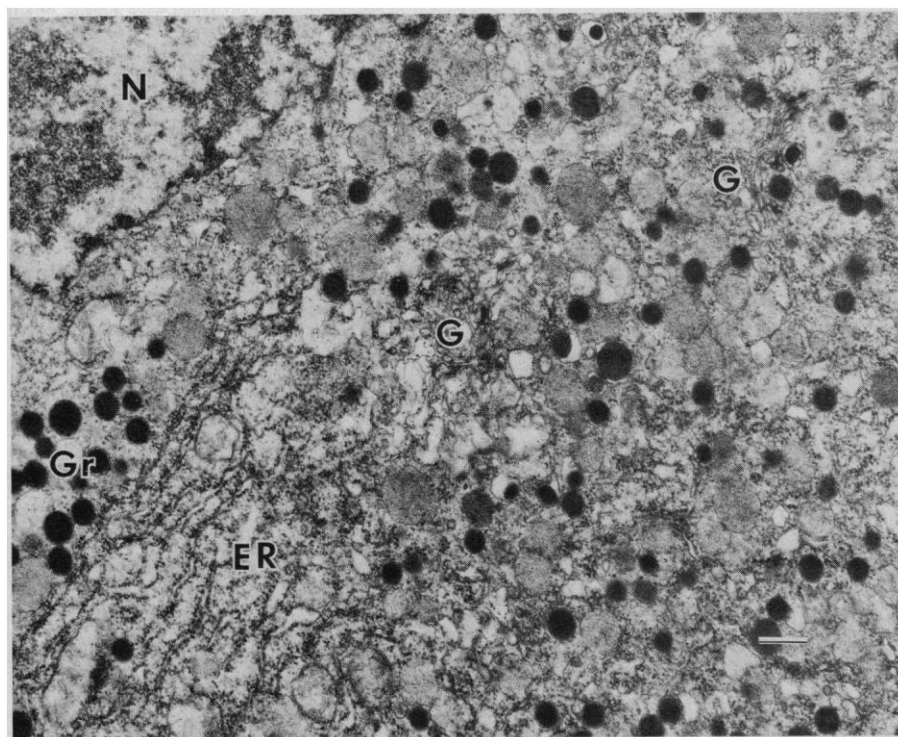


Fig. 7. Electron micrograph of a portion of a bag cell neuron; scale bar, 300 nm. *N* is the nucleus of the cell; *ER*, the rough endoplasmic reticulum; *G*, the Golgi complex; and *Gr*, the granules.

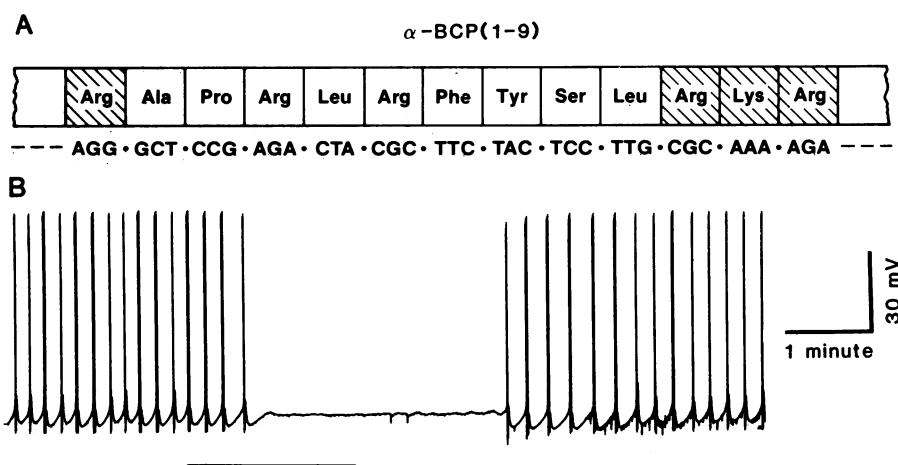


Fig. 8. Physiological activity of α -BCP. (A) A portion of the ELH precursor encoding α -BCP. The shaded amino acids are the proteolytic cleavage sites recognized in releasing α -BCP(1-9). (B) Application of α -BCP to the vicinity of the left upper quadrant neurons results in slow inhibition of the firing of these cells. This recording is from neuron L6.

and L11, which are thought to be glycinergic (16) and cholinergic (17), respectively. The R3-14 neurons have a high-affinity uptake system for the amino acid glycine. Free glycine is packaged into vesicles and transported to terminals. In contrast, cholinergic neurons synthesize choline acetyltransferase in the cell body and then transport the enzyme to the nerve terminals where high-affinity uptake systems for choline are located. Acetylcholine is then synthesized at the terminals. It is of interest to determine whether the different chemical messengers used by a nerve cell are localized at the same terminals, or packaged into the same vesicles, or both. If different vesicle populations exist, the possibility of vesicle queuing or differential release must be considered. Electron microscopic, immunohistochemical, and autoradiographic techniques are being used to investigate these issues. We would eventually like to understand the mechanisms directing chemical messengers to their appropriate locations. These studies depend on being able to follow the flow of information from the nucleus to the rough endoplasmic reticulum, through the Golgi complex, and into secretory vesicles, which are then transported to proper destinations for regulated release (Fig. 7).

Physiological Activities, Ionic Mechanisms, and Behavior

Aplysia neuroactive peptides appear to function during periodic events, such as egg-laying behavior, and during ongoing physiological activities, such as modulation of cardiovascular physiology.

Each of the two *Aplysia* bag cell clusters comprises approximately 400 electrically coupled neurons that are normally silent. An unknown stimulus, possibly the A or B peptide, triggers bag cell afterdischarge, a 20- to 30-minute-long series of action potentials during which a number of peptides are released into the vasculature. The effects of afterdischarge on several identifiable neurons in the abdominal ganglion have been described and placed into four broad categories: slow inhibition, transient excitation, prolonged excitation, and burst augmentation (18). There is evidence that slow inhibition of the left upper quadrant neurons (L2, L3, L4, and L6) is mediated by α -BCP and that the prolonged excitation of the LC cells is mediated by ELH (19) (Fig. 8). Voltage clamp techniques have been used to examine the ionic mechanisms underlying these activities (20). When the nine-amino

acid species of α -BCP [α -BCP(1-9)] is infused over neuron L6 (the overlying sheath of connective tissue is removed in the vicinity of the cell to facilitate access of the peptide), inhibition occurs within several seconds (Fig. 8). The onset, magnitude, and duration of this inhibition is similar to that induced by bag cell afterdischarge. Our preliminary voltage clamp data indicate that at voltage steps ranging from -10 to +10 mV, α -BCP induces a substantial increase in outward current. We attribute this conductance increase to the voltage-dependent delayed potassium current. This current is not responsible for the α -BCP-induced inhibition of L6 because it is activated at voltages well above the resting potential.

Egg laying involves a complex behavioral repertoire including cessation of locomotion, inhibition of feeding, onset of head waving, and enhanced respiratory pumping. Head waving facilitates winding of the egg string as it exits the gonopore. Respiratory pumping is a periodic contraction of the gill and siphon, which clears the mantle cavity of debris and enhances oxygenation of the gills. Demonstration of a link between behavioral and physiological changes associated with egg laying and specific peptides released by identifiable neurons is being approached by studying the neuroendocrine events responsible for enhanced respiratory pumping (21), the cessation of locomotion (22), and the inhibition of feeding (23).

Data on the behavioral impact of bag cell afterdischarge and individual peptides indicate that the mode of action of these peptides is complex. There is a hierarchical structure to the *Aplysia* peptidergic system in which "primary" neurosecretory cells (for example, the bag cells) may alter the activity of "secondary" neurosecretory cells, which in turn release peptides affecting other neurons. A given peptide released from the bag cells triggers a chain of events involving many neurons and the release of numerous peptides and transmitters. For example, bag cell afterdischarge causes slow inhibition of R3-14 neurons, presumably reducing the quantity of glycine and peptides released into the cardiovascular system (16). Afterdischarge also causes a biphasic change in the activity of heart command motor neuron L10; the neuron initially undergoes a slow inhibition, followed by prolonged excitation (18). At the same time, a pericardial motor neuron and abdominal artery vasoconstrictor motor neurons are undergoing prolonged excitation. Furthermore, ELH has direct effects on cardiac output. From these observations, it is apparent

that the activity of the *Aplysia* circulatory system can be changed by numerous factors as a result of an initial neurosecretory event—that is, bag cell afterdischarge.

The action of a recently isolated peptide, small cardioactive peptide B (SCP_B), illustrates how a peptide may function as a neuromodulator in *Aplysia*. SCP_B is present in high concentrations in the buccal ganglia, which control the muscles involved in biting and swallowing. One of these, the accessory radula closer muscle, is stimulated by serotonin, and SCP_B enhances the contractile response of this muscle (24), indicating that parallel peptidergic and serotonergic pathways may mediate similar behavioral events. This response may be mediated by cyclic nucleotides, since serotonin and SCP_B increase adenosine 3',5'-monophosphate concentrations in the accessory radula closer muscle. Buccal cells B1 and B2 innervate the esophagus, and SCP_B directly increases the peristaltic contractions of this muscle. Thus, neuropeptides not only function to induce specialized behavioral events such as egg laying, but may act on a more regular basis to modulate an ongoing activity such as feeding.

Judging by the number of identified neurosecretory cells in the *Aplysia* nervous system, immunohistochemical data, and the genes isolated and characterized to date, we estimate that as many as 40 different neuropeptide precursors and more than 100 biologically active peptides may perform neuroendocrine functions in *Aplysia*. It is clear that neuropeptides are not merely modulators of synaptic interactions mediated by the classical transmitters, but that a complex, peptide-mediated neural circuitry exists.

Describing the *Aplysia* peptidergic neural circuitry may prove to be more challenging than describing the circuitry defined by conventional synaptic interactions, because one cannot rely on the juxtaposition of two interacting neurons as a means of tracing functional connections. This difficulty and the potentially large number of peptides yet undiscovered lead us to believe that we are only in the initial stages of describing the roles of neuroactive peptides in this model neuroendocrine system.

References and Notes

1. E. R. Kandel and J. H. Schwartz, *Science* **218**, 433 (1983).
2. P. H. Patterson and D. Purves, *Readings in Developmental Biology* (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982).
3. D. T. Kreiger, *Science* **222**, 975 (1983); J. M. Lundberg and T. Hokfelt, *Trends Neurosci.* **6**, 325 (1983).

4. E. R. Kandel, *Behavioral Biology of Aplysia* (Freeman, New York, 1979).
5. W. T. Frazier, E. R. Kandel, I. Kupferman, R. Waziri, R. E. Coggeshall, *J. Neurophysiol.* **30**, 1288 (1967).
6. R. E. Coggeshall, B. A. Yaksta, F. J. Swartz, *Chromosoma* **32**, 205 (1971).
7. D. Gardner, *Science* **173**, 550 (1971).
8. D. L. Wilson, *J. Gen. Physiol.* **57**, 26 (1971); Y. P. Loh, Y. Sarne, M. P. Daniels, H. Gainer, *J. Neurochem.* **29**, 135 (1977).
9. R. H. Scheller, J. F. Jackson, L. B. McAllister, J. H. Schwartz, E. R. Kandel, R. Axel, *Cell* **28**, 707 (1982).
10. R. H. Scheller, J. F. Jackson, L. B. McAllister, B. S. Rothman, E. Mayeri, R. Axel, *ibid.* **32**, 7 (1983); J. R. Nambu, R. Taussig, A. C. Mahon, R. H. Scheller, *ibid.* **35**, 47 (1983); R. Taussig, R. R. Kaldany, R. H. Scheller, *Proc. Natl. Acad. Sci. U.S.A.*, in press; A. C. Mahon and R. H. Scheller, in preparation.
11. R. Taussig, M. R. Picciotto, R. H. Scheller, in *Molecular Biology of Development*, E. H. Davidson and R. I. Firtel, Eds. (Liss, New York, in press), vol. 19.
12. L. B. McAllister, R. H. Scheller, E. R. Kandel, R. Axel, *Science* **222**, 800 (1983).
13. E. Herbert and M. Uhler, *Cell* **30**, 1 (1982); E. Herbert *et al.*, *Neurosci. Commentaries* **1**, 16 (1981).
14. F. Strumwasser, L. K. Kaczmarek, A. Y. Chiu, E. Heller, K. R. Jennings, D. P. Viele, in *Peptides: Integrators of Cell and Tissue Function*, F. E. Bloom, Ed. (Raven, New York, 1980), p. 197.
15. T. Kreiner, J. Rothbard, G. K. Schoolnick, R. H. Scheller, *J. Neurosci.*, in press.
16. C. H. Price, D. J. McAdoo, W. Farr, R. Okuda, *J. Neurobiol.* **10**, 551 (1979); C. H. Price and D. J. McAdoo, *Brain Res.* **219**, 307 (1981).
17. E. Giller and J. H. Schwartz, *J. Neurophysiol.* **34**, 93 (1971).
18. E. Mayeri, P. Brownell, W. D. Branton, *ibid.* **42**, 1165 and 1185 (1979).
19. B. S. Rothman, E. Mayeri, R. O. Brown, P. M. Yuan, J. E. Shively, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5753 (1983).
20. M. Schaefer, P. Ruben, S. H. Thompson, R. H. Scheller, unpublished data.
21. P. H. Brownell and M. Schaefer, *Soc. Neurosci. Abstr.* **8**, 736 (1982).
22. S. Mackey and T. J. Carew, *J. Neurosci.* **3**, 1469 (1983).
23. D. K. Stuart and F. J. Strumwasser, *J. Neurophysiol.* **48**, 499 (1980).
24. H. R. Morris, M. Panico, A. Karplus, P. E. Lloyd, B. Riniker, *Nature (London)* **300**, 643 (1982); P. E. Lloyd, I. Kupfermann, K. R. Weiss, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
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Control of Neuronal Gene Expression

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Cellular phenotype is the direct consequence of the concentration of all the proteins expressed by the cell. Although synthesis and accumulation of proteins are sensitive to control at many steps, it is generally accepted that the control of

sets of genes (epigenesis). For genes that are exclusively expressed in one organ, transcriptional initiation is the all-or-none switch. Such genes are likely to give us the clearest insights as to how the differentiation process operates.

Summary. Some 30,000 genes are expressed exclusively in the rat brain, many of which contain a genetic element called an identifier sequence located in at least one of their introns. The identifier sequences are transcribed by RNA polymerase III exclusively in neurons to produce two RNA species, BC1 and BC2, of 160 and 100 to 110 nucleotides. This transcriptional event may define regions of chromatin that contain neuronal-specific genes and may poise these genes for transcription by polymerase II by rendering the gene promoters accessible to soluble *trans*-acting molecules.

transcriptional initiation of messenger RNA (mRNA) is the most consequential of all these steps in determining phenotype. In higher eukaryotes, the ability of cells to form organs or tissues with their own specific functions is the result of these cells producing unique sets of proteins during the course of the developmental program. Because all cell types are believed to contain the same DNA [with the known exception of immunoglobulin (Ig) genes in producer cells], events must occur during development that alter the transcriptional states of

Transcriptional initiation in prokaryotes is well understood. Transcription begins at promoters, specific DNA sequences located 5' to the start of RNA polymerization. These sequences respond to a particular combination of RNA polymerase and so-called sigma or specificity factors. In some cases, promoter selection is influenced by other proteins, such as catabolite activator protein (CAP). In simple prokaryotes such as *Escherichia coli*, there is only one known sigma factor. For bacteria (such as *Bacillus subtilis*) that exhibit

different life forms during processes such as sporulation, the available sigma factors that drive particular genes vary according to life stage. Variable gene regulation in prokaryotic organisms can be simply divided into two classes: positive control, which involves sigma factors and other positive activators (such as CAP) available only in response to certain external conditions, and negative control, which involves repressor molecules that inactivate genes by interacting with their promoters to prevent RNA polymerase from initiating gene expression. At any particular time, all genes are equally available to interact with each positive or negative *trans*-regulatory molecule.

Eukaryotic organisms are obviously more complicated. What formally separates higher eukaryotes from prokaryotes is that the cells of higher eukaryotes must first differentiate into organs, each composed of one or more specific cell types, and then those organs must be able to respond to ambient stimuli reflecting the physiological state of the whole organism. Batteries of genes must be activated in response to certain stimuli, and the required responses to the same stimulus may be different for the cells of different tissues. Actual development of higher animals involves several distinct stages for each differentiating cell type. The cumulative result of these processes is that, in developing and adult organisms, different genes are available for activation in different tissues at different stages. This implies, *a priori*, two distinct levels of gene control, one related to the differentiation state (both temporal and type) of the particular cell and

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