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47. This work was supported by the Office of Naval Research and the Naval Medical Research and Development Command (contract N00014-88-K-0287). I thank R. Battino, A. Ben-Naim, P. W. Carr, R. Fernández-Prini, and E. Wilhelm for helpful discussions. I especially thank my former students R. Kennan and J. F. Himm for all their explanations and other essential help with this work.

Peptide Processing and Targeting in the Neuronal Secretory Pathway

LINDA J. JUNG AND RICHARD H. SCHELLER

The abdominal ganglion of the marine mollusk *Aplysia* contains a pair of identified neuronal clusters, the bag cells, which control egg laying by means of a number of unique regulatory mechanisms. Each neuron in the bag cell clusters synthesizes several peptides derived from a single prohormone and packages them into separate vesicles. These vesicles are then differentially localized in specific neuronal processes, thus segregating peptides destined for autocrine and hormonal release sites. Therefore in this system, protein trafficking through the secretory pathway organizes multiple peptide neurochemical messengers to efficiently regulate simple behaviors.

UNDERSTANDING THE NEURAL AND ENDOCRINE MECHANISMS that govern animal behavior is a major goal of modern biology. Although considerable information has been amassed in molecular, cellular, physiological, and behavioral studies, it has proven difficult to integrate these various levels of investigation into a unified account of behavior. The primary obstacles to such an integration are the complexity of the behaviors of higher animals and the diversity of the underlying cellular and biochemical mechanisms. In an effort to circumvent some of these obstacles, investigators have turned to invertebrate species, many of which have simpler nervous systems and a limited repertoire of elementary behaviors. This approach has proven fruitful.

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Aplysia as a Behavioral Model

Some molluscan species have distinct advantages for studies of the nervous system, one of which is the fact that many of the nerve cells are very large and reproducibly identifiable among individuals. This characteristic has been exploited in the marine snail *Aplysia californica*, in which both mating and egg laying are highly stereotyped behaviors (Fig. 1) (*1–4*). *Aplysia* are non–self fertilizing hermaphrodites and often mate in groups in which each individual both donates and receives sperm (*5*). The gametes traverse a complex pathway through the reproductive tract where they are fertilized, encapsulated, and finally packaged into a long strand, or cordon (*5, 6*), which contains several million eggs (*1, 6*). The egg strand exits the internal portions of the reproductive tract through a genital aperture in the mantel cavity and then moves along the genital groove toward the head where a series of head waving and tamping movements coil the egg cordon in an irregular compact mass (*2–6*). In this article, we discuss the neural and endocrine factors that govern egg-laying behavior and mating, a social behavior.

Egg-laying behavior is neurally controlled by the bag cells, a set of 800 electrically coupled cells grouped into two clusters along the rostral aspect of the abdominal ganglion (Fig. 5) (*7–12*). When extracts or released material from these neurons are injected into a

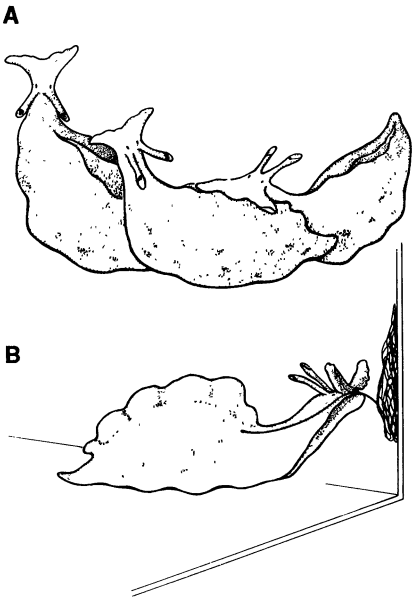

Fig. 1. Reproductive behaviors in *Aplysia*. (**A**) Schematic drawing of animals in the process of initiating mating. This interaction is the highest form of social behavior exhibited by these animals. (**B**) An individual in the process of egg laying. Egg laying comprises a series of simpler behavioral components, including an inhibition of locomotion and feeding. A series of head-waving motions coil the long string of eggs into an irregular pile.

second animal, egg laying ensues within an hour (*11*). Furthermore, in freely moving animals with implanted cuff electrodes around the bag cells, these neurons are active at the beginning of each egg-laying episode (*8*). Each cell is approximately 50 μm in diameter and is filled with many neuropeptide-containing dense-cored vesicles (DCVs) (*9, 10*). The action of the peptides secreted by the bag cells on central neurons and peripheral targets produces the egg-laying behavior (*13, 14*).

A second structure, the atrial gland, is also likely to be important in the reproductive behavior of *Aplysia*; however, its function is less clear. The atrial gland is an exocrine organ (*15*), which forms an integral part of the oviductal portion of the distal large hermaphroditic duct (*16*). Extracts of this tissue will also elicit egg laying (*17*). This effect is due to pharmacologic actions of peptides produced in the atrial gland (see below). In addition, there is a decrease in the latency to a mating event when extracts of this gland are added to the sea water surrounding *Aplysia* (*18*). Because the egg string travels past and is in intimate contact with this gland (*16*), and because animals are often found copulating or laying eggs in groups on or near previously deposited eggs (*4, 19*), this gland may secrete water-borne pheromones that act on groups of *Aplysia* to enhance mating or egg-laying behaviors (*20*).

Genes Governing Reproductive Behavior

The bag cells and the atrial gland were initially found to contain three peptides that generate egg laying. The 36–amino acid egg-laying hormone (ELH) was derived from the bag cell neurons, and the 34 amino acid peptides A and B were purified from the atrial gland (*21*). All of the peptides are amidated at the COOH-terminus and, although peptides A and B are 88% identical to one another, they share no sequence similarity to ELH. Nevertheless, the nucleotide sequences of all three peptide precursors are quite similar. The haploid genome of *A. californica* contains approximately four homologous genes encoding this family of neuropeptides (*22, 23*). One gene encodes ELH and is expressed in the bag cells, while the other three genes are largely expressed in the atrial gland and encode peptides A and B, as well as another homologous molecule in this family called peptide C.

The *A. californica* ELH and peptide A encoding genes are 88% homologous at the DNA level, but most of the nucleotide substitutions are in the first and second codon positions and result in amino acid replacements (*24*). Many of these amino acid substitutions between the ELH and peptide A genes occur in regions of the prohormones that alter the proteolytic processing pathway, thereby generating different sets of peptides from the precursors. Furthermore, the homology is greater in part of the 3′ untranslated region and in one intron than in the coding region. These regions are also the most homologous in the peptide A and B genes of *A. californica*. One explanation for these data (*25*) is that the nucleotide substitutions that result in altered processing pathways may have been selected and fixed in the population while more neutral variations have not yet accumulated in this gene family.

Although the physiological roles of the molecules from the peptide A branch of this gene family are not known, they may mediate interactions between individuals of the species resulting in mating, while the ELH branch of the family coordinates the physiology of individuals to generate the egg-laying behavior.

Processing of the ELH Prohormone

Each of the members of the ELH gene family encodes a precursor protein that is processed by a series of modifications that results in multiple physiologically active products. Such prohormone processing events are highly conserved from yeast to man (*26*). Most neuropeptide precursors have an NH_2-terminal signal sequence that directs the nascent protein into the lumen of the endoplasmic reticulum (ER) (*27*). The resulting prohormone is often further modified by endoproteolytic cleavages at basic residues. These cleavages can occur at mono-, di-, tri-, and tetrabasic sites, but

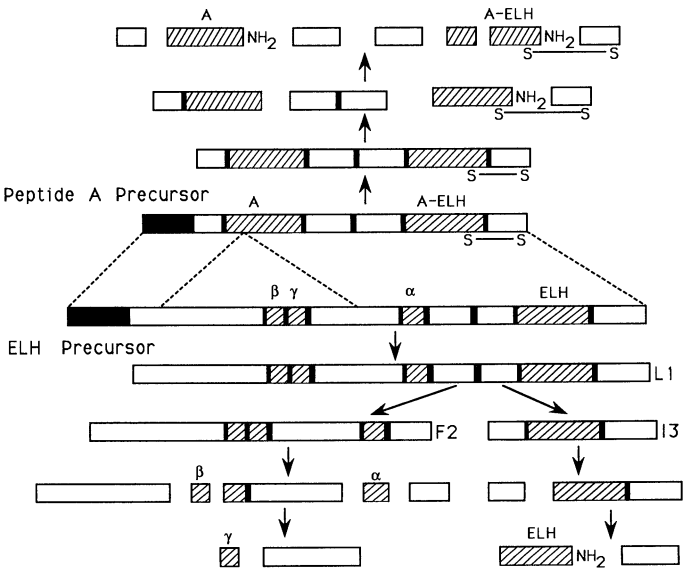

Fig. 2. Processing of the peptide A and egg-laying hormone (ELH) precursors. The ELH and peptide A precursors are predicted from the nucleotide sequences of cDNA clones. Both precursors are initiated by a hydrophobic signal sequence (long black bar). The peptide A prohormone is endoproteolytically cleaved at five sites defined by basic residues (*36*) (short black bar) and the COOH-terminal ends of peptide A and A-ELH are amidated (NH_2). About 10 to 15% of the A-ELH complex is further processed by an endoproteolytic cleavage between a pair of leucine residues. The ELH prohormone (L1) is cleaved at eight internal sites (*34*). The first cleavage of the prohormone occurs at a series of four basic residues resulting in NH_2-terminal (F2) and COOH-terminal (I3) intermediates. The NH_2-terminal intermediate is further processed to at least six peptides, including the physiologically active α, β, and γ bag cell peptides. The COOH-terminal intermediate is processed to the 36–amino acid ELH and two flanking peptides.

dibasic processing sites are most common. In yeast, an enzyme that cleaves at dibasic residues and that is involved in liberating the α-mating factor from its precursor has been isolated and characterized (*28*). The yeast enzyme is the product of the *KEX 2* locus and is homologous to the subtilisin class of bacterial serine proteases. After the endoproteolytic cleavage, a carboxypeptidase, such as carboxypeptidase E (*29*), trims the basic residues from the COOH-terminus of the active sequence. If the ultimate amino acid is a glycine, an enzyme such as peptidyl-glycine-α-amidating monooxygenase (PAM), isolated from porcine brain vesicles, hydrolyzes the terminal peptide bond, leaving a carboxyamide at the terminus of the peptide (*30*). This modification occurs on ELH and is thought to stabilize the peptide from degradation by enzymes that act on the COOH-terminus. Further processing steps include the removal of one or more amino acids from the NH_2- or COOH-termini (*31*).

In pancreatic islet cells, proteolytic processing of proinsulin occurs in the secretory granule subsequent to packaging (*32*). Similarly, some of the endoproteolytic cleavages of pro-opiomelanocortin (POMC) in the mammalian pituitary tumor cell line, AtT-20, occur in the DCVs (*33*). These data suggest that specific processing enzymes are localized in vesicles with the prohormone and that the peptide products from the prohormones are present in stoichiometric amounts.

Studies of the ELH precursor have demonstrated that an ordered series of eight cleavages at mono-, di-, tri-, and tetrabasic sites result in a set of at least nine peptides (Fig. 2) (*34*). The first cleavage is of critical importance and occurs at a set of four basic residues (*22*), Arg-Arg-Lys-Arg, and fragments the prohormone into NH_2-terminal (F2) and COOH-terminal (I3) intermediates. Further processing of the COOH-terminal intermediate produces the mature ELH and other COOH-terminal peptides. Additional physiologically active peptides that arise from proteolytic cleavage of the NH_2-terminal intermediate are the α-, β-, and γ-bag cell peptides (BCPs) (Fig. 2) (*35*). The fact that processing occurs as an ordered series of reactions may reflect the affinity of specifically localized processing enzymes for various cleavage sites, the accessibility of the sites, or both.

Less is known about the processing of the atrial gland prohormones. An 80–amino acid deletion, with respect to the ELH precursor, gives rise to peptide A and removes the β- and γ-BCPs (Fig. 2). This, and other differences between the precursors, alters their processing (*36*). For example, a COOH-terminal Gly-Lys-Arg sequence results in amidation of peptide A, while the Arg-Lys-Arg sequence serves only as a cleavage site for the α-BCP. Further amino acid differences occur at the unique tetrabasic cleavage site in the ELH precursor. Yet another set of differences is observed within the ELH peptide, resulting in a pair of linked cysteines in the peptide A precursor that are not present in the ELH precursor (*36, 37*). Thus, relatively few amino acid differences can have a dramatic effect on the set of peptides derived from precursors.

Sorting and Targeting of Neuropeptides

Intricate trafficking of the ELH prohormone through the secretory pathway results in the independent packaging and localization of peptides derived from the ELH precursor. Products that are on the COOH-terminal side of the first endoproteolytic cleavage are packaged in one set of vesicles, and the products on the NH_2-terminal side of the first cleavage are localized in distinct sets of vesicles (*38–40*).

The discovery of the differential packaging of peptides derived from the single ELH prohormone prompted us to wonder how and why this phenomenon occurs. A model to explain this sorting event is shown in Fig. 3 (*39*). We propose that the prohormone is cotranslationally inserted into the lumen of the ER concurrent with cleavage of the signal sequence. The prohormone is then translocated to the cis-Golgi associated with the membrane. As the precursor enters the trans-Golgi or trans-Golgi network (TGN), the first endoproteolytic cleavage occurs at the tetrabasic site. This site is conserved among species, further supporting the critical importance of this cleavage. After this initial cleavage, the COOH-terminal intermediate dissociates from the membrane and aggregates in the TGN to form the dense core of a future vesicle. Simultaneously, the membrane-bound NH_2-terminal intermediate is transported to a distinct region of the TGN by small clear vesicles, perhaps similar to vesicles that translocate products between other membrane compartments. This step may underlie the sorting of the two regions of the prohormone and the importance of the tetrabasic cleavage site. The transport, as well as the aggregation step that forms the dense core, takes place in a compartment containing a high concentration of membrane-associated acid phosphatase, a lysosomal enzyme not found in mature secretory granules (*41*). Thus, it appears that acid phosphatase is directed to the lysosomes after the sorting of the two intermediates. The NH_2- and COOH-terminal intermediates are then further processed to form the secreted peptides in the vesicles after their final budding from the TGN. Thus, in the bag cells, the order of endoproteolytic cleavages is critical for the sorting and packaging scheme that occurs in these neurons.

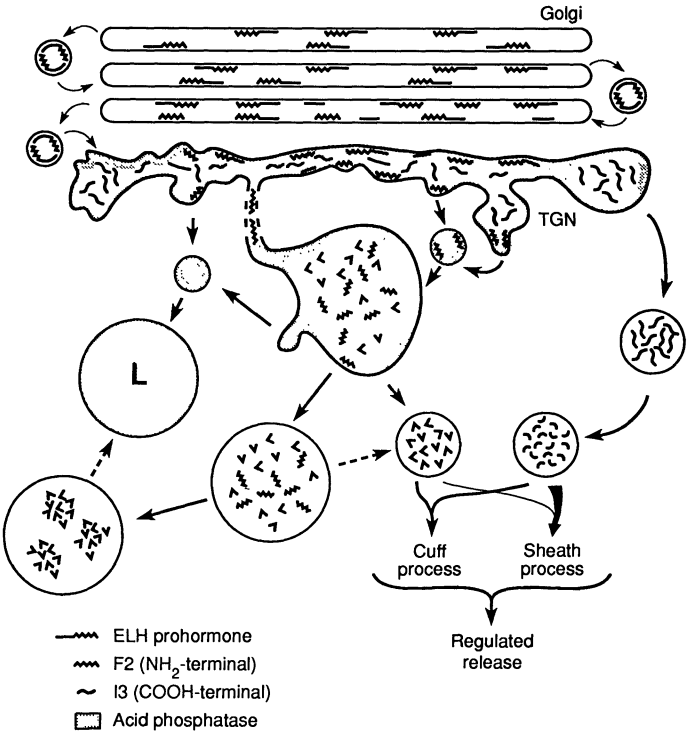

Fig. 3. Model for sorting and trafficking of the ELH prohormone in the secretory pathway. The ELH prohormone is membrane associated in the cis-Golgi. In the trans-Golgi network (TGN) the first endoproteolytic cleavage occurs, generating the NH_2-terminal (F2) and COOH-terminal (I3) intermediates. The TGN–immature vesicle compartment contains acid phosphatase on its membrane. Cleavage of the intermediates (F2 and I3) takes place within the secretory vesicles. The NH_2-terminal intermediate is packaged into two types of vesicles. The large vesicles turn over their contents, perhaps by shunting to the lysosomes (L). Neuronal processes that ramify through the connective tissue sheath largely contain vesicles loaded with COOH-terminal peptides. Processes that are in the cuff region contain equal mixtures of vesicles loaded with NH_2-terminal– and COOH-terminal–derived peptides.

The various peptides derived from the NH_2-terminus are present at three to eight times lower concentrations than are the products from the COOH-terminus (*38*). About 80% of the material from the NH_2-terminus is sorted into morphologically distinct "large vesicles," which are localized in the cell body and not in the bag cell terminals. The contents of these large organelles are metabolized by a degradative pathway that may involve lysosomes (*39*). This pathway may account for the differences in the steady-state amounts of the various peptides, which are initially synthesized at equivalent levels.

To understand the molecular aspects of this sorting process, we placed a cDNA encoding the ELH precursor behind the RSV-LTR promoter and transfected it into AtT-20 cells (*42*). These cells are derived from a mouse pituitary tumor and normally express, process, package, and sort peptides derived from the endogenous POMC precursor, including adrenocorticotropic hormone (ACTH) and β-lipotropin (β-LPH) to the regulated secretory pathway. In AtT-20 cells there are two pathways of protein secretion, constitutive and regulated (*43, 44*). The rate of protein secretion in the constitutive pathway is constant and does not depend on external stimuli. In contrast, in the regulated pathway proteins are stored and are released only upon the appropriate stimulus.

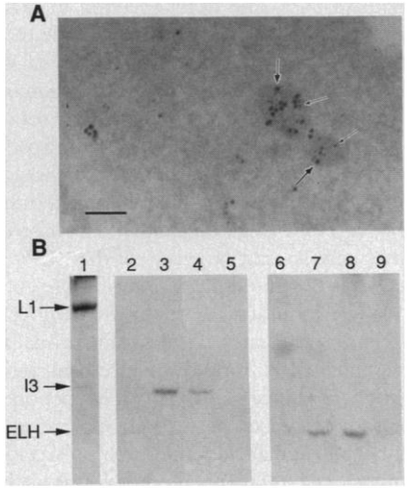

Fig. 4. Analysis of the ELH precursor expressed in AtT-20 cells. (**A**) Colocalization of ELH and ACTH in transfected cells. Small gold beads (5 nm) (small arrows) indicate ELH immunoreactivity, and large gold beads (10 nm) (large arrows) ACTH immunoreactivity. Bar, 90 nm. Cells were grown on Permanox slides for 48 hours and fixed in 4% paraformaldehyde in 0.1 M $NaPO_4$ for 1 hour at room temperature. Cells were then washed three times, dehydrated in ethanol, and then infiltrated and embedded in Lowicryl K4M at −20°C. Eighty-nanometer sections were stained first with affinity-purified rabbit antibody to ELH (25 ug/ml) (*38*) and then with 5-nm gold beads conjugated to goat antiserum to rabbit immunoglobulin G (*12, 49*). The same grids were then stained with polyclonal rabbit serum against ACTH (1:25 dilution) (Chemicon) and then with 10-nm gold beads conjugated to goat serum directed against rabbit immunoglobulin G. Staining was according to (*38*) but with a blocking solution of 10% normal goat serum and 1% bovine serum albumin in phosphate-buffered saline. (**B**) Processing and packaging of the ELH precursor into the regulated secretory pathway in AtT-20 cells. Samples were analyzed as in (*66*). (Lane 1) Cells were incubated with 0.2 mCi of [^{35}S]cysteine for 3 hours at 15°C and the major intracellular product was the prohormone L1; (lane 2) cells were incubated for 30 minutes with 0.25 mCi of [^{3}H]leucine and chased for 2 hours with unlabeled leucine at 37°C and the major product was mature ELH (also see lane 9); (lane 3) constitutive secretion of I3 into the medium; (lane 4) the major intracellular product in the presence of monensin; (lane 5) constitutive secretion in the presence of monensin. Cells were preincubated with 10 μM monensin for 30 minutes at 37°C, 0.25 mCi of [^{3}H]leucine was then added, and cells were incubated for 3 hours at 37°C. (Lane 6) The major product in the medium without stimulation; (lane 7) the major product in the medium after addition of 8-br–cAMP; (lane 8) the major intracellular product without stimulation; (lane 9) the major intracellular product after stimulation with 8-br–cAMP. For this experiment, two identical plates of the ELH-transfected cell line were incubated for 16 hours in 1 mCi of [^{3}H]leucine. The medium was changed, and the cells were incubated for 1 hour with 0.1 mCi of [^{3}H]leucine. Cells were then rinsed, chased for 5 hours, and then 5 mM 8-br–cAMP was added to one dish. Three hours later, the cells and media from both dishes were harvested. As a control, a plate of untransfected AtT-20 cells was also treated in the same fashion for stimulation of secretion.

Analysis of this ELH-transfected cell line is shown in Fig. 4. ELH and ACTH were packaged in the same DCVs, which are concentrated in the ends of processes, consistent with the light microscopy studies. In these transfected AtT-20 cells, unlike the bag cells, no staining was observed by light or electron microscopy with any of the antibodies that recognize NH_2-terminal regions of the precursor. Because some of the antibodies raised against the NH_2-terminal region react with the precursor and intermediates in the processing scheme, we hypothesize that the NH_2-terminal products are being degraded or constitutively released, while ELH, which is derived from the COOH-terminal end of the peptide, is processed, packaged, and stored.

The results from a series of biosynthesis studies on this ELH-transfected cell line were consistent with this hypothesis. Transfected AtT-20 cells were incubated at 15°C with radioactive amino acids and then with unlabeled amino acids; cellular proteins were immunoprecipitated with the ELH antibody. At this temperature, transport of secretory products from the ER is greatly reduced or blocked (*45*) and, as expected, the dominant product was the ELH prohormone (Fig. 2, L1, and Fig. 4B). After longer chase periods with unlabeled amino acids, mature ELH was the major stored product in these transfected cells, and some of the COOH-terminal intermediate was constitutively secreted into the media. Constitutive secretion of the intermediate was similar to what is seen in AtT-20 cells transfected with cDNAs that encode the insulin precursor. In these experiments, 26% of the proinsulin is processed and stored, while the remainder is constitutively secreted (*43*).

The carboxylic ionophore monensin disrupts the ionic gradient across Golgi membranes, resulting in a transport block beyond the Golgi apparatus (*46*). When ELH-transfected AtT-20 cells were treated with monensin, the initial cleavage of the prohormone at the tetrabasic site was accomplished, but further cleavages of the COOH-terminal intermediate were blocked. Thus, the COOH-terminal intermediate, not the mature ELH, was the major stored product in monensin-treated cells (Fig. 4B). This is the same result as that observed in native bag cell neurons (*38, 46*). According to the model (Fig. 3), the first cleavage of the ELH prohormone occurs in the Golgi-TGN compartment and is not affected by monensin in either ELH-transfected cells or the bag cells. The latter cleavages that occur in vesicles after the peptides pass through the Golgi are blocked by monensin in both systems. This result suggests that, like the bag cells, AtT-20 cells have the capacity to initiate endoproteolytic processing of prohormones in the Golgi-TGN compartment prior to packaging in mature vesicles. These data imply the existence of at least two distinct enzymes, a membrane-associated protease, which may reside in the Golgi-TGN, and one or more soluble endoproteases, which may be copackaged with the precursors or processing intermediates (*47*).

In AtT-20 cells, secretion of stored products in the regulated secretory pathway can be stimulated by the addition of 8-bromo-cAMP (*48*). Similarly, in ELH-transfected AtT-20 cells this treatment stimulated the release of ELH into the culture media, producing a concurrent decrease in the intracellular store of the peptide (Fig. 4B).

Although the metabolism of the ELH prohormone in transfected AtT-20 cells is not identical to that in bag cell neurons, it is similar. The first cleavage of the ELH prohormone occurs at the same position in the bag cell neurons and in AtT-20 cells, and the COOH-terminal products are packaged and stored in the regulated secretory pathway in both systems. Although some of the NH_2-terminal products are stored in the bag cells, a substantial amount is degraded. This is also true in the AtT-20 cells, as we could not detect NH_2-terminal products, which suggests that they are degraded or

constitutively released. Thus, although the bag cells sort a fraction of the NH_2-terminal peptides to the regulated secretory pathway, much of the information required for correct processing and sorting is contained within the structure of the ELH precursor protein. This information can be correctly utilized by processing and secretory machinery common to other cells.

In addition to independently regulating the steady-state amounts of the peptides, as described above, the bag cell neurons also localize the different vesicles to distinct sites in the cell. The bag cells send processes into two anatomically distinct regions called the cuff and the sheath (Fig. 5). The cuff neurites are found inside the connective tissue that surrounds the nerve track leading to the head, while the sheath neurites are found in the interstitial space between the outer connective tissue and the abdominal ganglion neurons (*9, 12, 49*). There is a high concentration of ELH-containing vesicles in the sheath processes and very few BCP- or NH_2-terminal peptide-containing vesicles. In contrast, processes in the cuff contain approximately equal numbers of ELH- and BCP-containing vesicles (*50*). Thus, bag cells can sort the two vesicle types. This suggests that the contents of each class of vesicles must be matched with specific surface markers. This localization may be accomplished by either differential transport or variable stabilization at particular sites. These results are an exception to the proposal by Dale (*51*) that a nerve cell will always release the same chemical messengers from all of its terminals.

These studies on the processing and sorting of the peptides derived from the ELH prohormone define a series of novel regulatory roles for the secretory pathway and highlight the fact that prohormone organization is an effective means of co-expressing a set of modulatory substances.

Regulation of Behavior

The information available concerning the physiology of bag cell activation and the activities of the secreted products allows one to put the gene structure and cell biology of the ELH prohormone into a biological context. In this section we will discuss the physiological relevance of (i) separating the peptides derived from a single prohormone into different vesicle classes, (ii) spatially separating these vesicle classes, and (iii) regulating the amounts of these peptides.

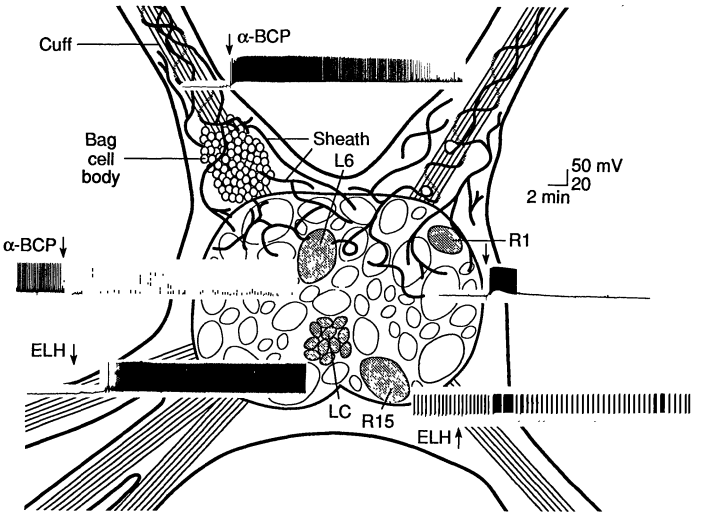

Fig. 5. The abdominal ganglion and electrophysiological activity of the bag cells, L6, LC, R1, and R15 during egg laying. The dorsal surface of the abdominal ganglion is illustrated, including a bag cell cluster on the left and two individual bag cells on the right. Processes in the sheath contain almost entirely vesicles with COOH-terminal–derived peptides, while processes in the cuff contain about equal numbers of vesicles with COOH-terminal– and NH_2-terminal–derived peptides. Intracellular recordings are shown from the bag cells and four abdominal ganglion neurons that alter their firing patterns in response to bag cell activity.

The precise physiological stimulus that activates the bag cells is not known; however, developmental, circadian, thermal, nutritional, and other factors may be important in regulating their activity (*52, 53*). Upon activation, both clusters of electrically coupled cells fire a burst of action potentials called the afterdischarge. This activity usually lasts approximately 20 minutes, is irreversible, and is followed by a refractory period of several hours wherein the neurons are not excitable (*54*). The onset of electrical activity in the cells is accompanied by an increase in cAMP, which in turn stimulates a cAMP-dependent protein kinase, resulting in phosphorylation of a variety of substrates (*55*). Activation also leads to phosphoinositide hydrolysis, which in other systems leads to the activation of additional kinases (*56*).

The bag cell afterdischarge initiates in the neurites at the cuff and travels down the pleural abdominal connective to the bag cell somata (*57*) (Fig. 5). At the cuff, the afterdischarge stimulates secretion of vesicles containing peptides derived from the NH_2-terminal intermediate (*50*). The BCPs secreted from these neurites, particularly the α BCPs, act in an autocrine mode to regulate the excitability of the bag cells (*35, 58*). The details of this process remain to be elucidated, particularly the contribution of the physiological state of the animal, which may influence the role of the BCPs in regulating the afterdischarge (*35, 58, 59*). One hypothesis is that the BCPs are auto-excitatory early in the afterdischarge, when they reinforce the burst, and subsequently inhibitory, terminating electrical activity in the bag cells. Regardless of their precise roles in regulating bag cell firing, the specific targeting of the BCPs to the cuff region spatially localizes the autocrine action of these compounds.

While local actions of the BCPs govern the bag cell discharge, ELH acts on a large number of neuronal and peripheral targets. For example, ELH modulates electrical activity in many abdominal ganglion neurons (Fig. 5). The peptide modulates the bursting pattern of neuron R15, both increasing the number of spikes per burst and enhancing the hyperpolarization during the interburst interval (*14, 60*). This modulation is likely to increase the secretion of peptides from R15 that mediate water and salt balance (*61*). Application of ELH also raises the levels of cAMP in R15 and increases two currents, a voltage-sensitive calcium current and an inwardly rectifying potassium current (*62*). ELH also excites neurons in the left lower quadrant, both the LC and the RB clusters of cells, which have unknown specific functions (*14*). It is these actions of ELH on central neurons that are thought to generate the egg-laying behavior. ELH also acts hormonally from its site of release to cause contraction of the smooth muscle of the ovotestis (*12, 63*). Accordingly, release of ELH in the vascularized sheath region of the abdominal ganglion effectively delivers the peptide to the circulation, thereby coordinating the egg-laying behavior with egg deposition.

The bag cell peptides are not limited to autocrine activities, but also act on abdominal ganglion cells. The α-BCP mimics the slow inhibition seen in cells L2 to L6 in the left upper quadrant (Fig. 5) (*35, 59, 64*). The transient excitation of neuron R1 during a bag cell discharge may be due to the actions of the β-BCP; however this activity has been difficult to reproduce with the synthetic peptide (*64*). It is interesting that some of the neurons that respond to ELH also have receptors for α-BCP and appear to sum the effects of the two modulators (*64*). Thus, the net response of these cells to the various peptides released during the bag cell afterdischarge will depend on the turnover times of the peptides, the quantities

released, as well as the distribution of release sites. One could imagine biphasic responses arising from actions of independent modulators. For instance, the smaller and less stable BCPs turn over considerably faster than the larger amidated ELH, providing a mechanism for temporal regulation of their modulatory actions (*63*).

The set of peptides is secreted from the bag cells at ratios similar to those measured in the cluster. Furthermore, the ratios of the various released products do not dramatically change throughout the discharge, suggesting that the different vesicle types are not differentially secreted during a bag cell burst (*65*). The relatively low amounts of the BCPs that are released are sufficient to generate adequate concentrations of peptides necessary for local interactions, while the endocrine functions of ELH account for the large concentration of ELH secreted by the neurons.

Egg laying in *Aplysia* requires the coordinated action of a set of neuropeptides to modulate the physiology of a series of cells and tissues. The group of peptides is synthesized as an ensemble, but then the peptides are independently packaged and targeted to suit the signalling requirement of the neurons.

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42. AtT-20 cells were grown in DME H-21 supplemented with 10% fetal calf serum and penicillin-streptomycin (100 U/ml) at 12.5% CO_2 and 37°C. The plasmid, pRSV-ELH was constructed by replacing a DNA fragment between the Hind III–Bgl II sites of the plasmid pRSV-TG [H.-P. H. Moore and R. B. Kelly, *J. Cell Biol.* **101**, 1773 (1985)] with a Hind III–Bgl II fragment containing an ELH cDNA (clone U) (*16*). Cells were transfected by the calcium phosphate procedure (*35*) with 103 μg of pRSV-ELH and 17 μg of the plasmid pSV2neo to confer neomycin resistance. Stable clones were selected with the antibiotic G418 (0.25 mg/ml active G418) and screened by light level immunocytochemistry and Northern (RNA) analysis. The analyses were from selected clone B4(7).
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66. In these experiments, 10-cm plates of clone B4(7) were grown to 80% confluency. Labeling with radioactive amino acids (Amersham) was done in the presence of DME H-21 plus 2% dialyzed fetal calf serum without the relevant amino acid. Cells were then washed in media, harvested, and resuspended in 1.5 ml of ice-cold lysis buffer (50 mM Hepes, pH 7.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride), and homogenized. The homogenate was clarified, and the supernatant was incubated with Sepharose CL-6B hydrated in lysis buffer. The Sepharose was removed by centrifugation, and the supernatant was incubated overnight at 4°C with protein A–Sepharose that had been incubated with an excess of affinity-purified rabbit serum against ELH (30). Collected beads were washed four times in 1.0 ml of 20 mM Hepes, pH 7.0, 200 mM NaCl, 0.1% Triton X-100. After the wash, the beads were collected and electrophoresed on a SDS-urea polyacrylamide gel (15% acrylamide) (31). The gel was then fixed and prepared for fluorography. Media from the cells was immunoprecipitated in a similar manner.

67. We thank H. McGee, D. Payan, E. Kandel, and C. Stevens for useful comments on this manuscript, and H.-P. H. Moore for the pRSV-TG plasmid. L.J.J. is supported by a National Institutes of Health Postdoctoral Fellowship. This work is supported by the National Institutes of Mental Health.