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Dale's hypothesis revisited: Different neuropeptides derived from a common prohormone are targeted to different processes

(protein trafficking/*Aplysia*/vesicles)

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ABSTRACT **In the bag cells of *Aplysia californica*, the prohormone of egg-laying hormone is processed by means of endoproteolytic cleavage into two sets of peptides. The amino-terminal region of the prohormone gives rise to the bag cell peptides (α, β, and γ). These serve an autocrine function; they are autoexcitatory on the bag cells and also act locally to alter the firing patterns of neurons in the abdominal ganglion. The carboxyl-terminal portion of the prohormone gives rise to the egg-laying hormone. This peptide acts as a hormone on nearby neurons and by means of the circulation on peripheral tissues to bring about egg-laying. We have previously reported that the first cleavage of the prohormone, which occurs in the trans-Golgi network, results in two intermediates that are sorted into distinct vesicle classes prior to further processing. Here we show that these distinct vesicles are localized to separate processes, thus spatially segregating autocrine and hormonal release sites. The findings of segregation indicate that neurons need not always release the same set of chemical messengers from all of their endings.**

A key principle in neurobiology identified with Dale states that a neuron is a metabolic unit that extends to all processes (1). The modern version of this idea is that a neuron releases the same set of transmitters from all of its terminals (2). In the course of studying the processing and packaging of the precursor for the egg-laying hormone (ELH) peptide in *Aplysia*, we found that the precursor is processed into peptides that are distributed to different vesicles. We have here asked: Are these vesicles targeted to different parts of the cell?

The bag cell neurons are clustered into two groups of ≈400 electrically coupled cells that fire a synchronous burst of action potentials during each egg-laying episode (3, 4). Each bag cell sends two sets of processes into the vascularized sheath. One set of processes is sent to the sheath surrounding the ganglion and nerve tracts (Fig. 1*A*) (4–6). Release of peptides from these processes delivers the messengers to both neuronal cell bodies and to the circulatory system, facilitating long-distance hormonal interactions (10–12). In addition, the bag cell neurons send a process into the pleural-abdominal tract inside the connective tissue border that surrounds the bundle of axons (Fig. 1*A*) (6). This "cuff" of neurites is the site of electrotonic coupling and the region where the bag cell discharge initiates (13, 14). The autoexcitatory effect of the α-BCP is thought to be localized to the cuff (15–17).

The two sets of peptides derived from the ELH prohormone are differentially packaged (Fig. 1*B*) (7–9, 18). After an initial endoproteolytic cleavage in the trans-Golgi and/or the trans-Golgi network, the resulting intermediates (labeled F2 and I3 in Fig. 1*B*) are packaged into separate vesicles. Peptides derived from the amino-terminal intermediate (the α-, β-, and γ-BCPs) are distributed to two types of vesicles: large (>250 nm) dense core vesicles that are only found in the soma and a smaller (170 nm) class of dense core vesicles that are transported to processes. The carboxyl-terminal portion of the precursor gives rise to three peptides, including the ELH. This hormone is packaged into vesicles that are also transported to processes.

MATERIALS AND METHODS

Generation and Purification of Antisera. Peptide-bovine serum albumin conjugates were injected initially in Freund's complete adjuvant (Sigma) with phosphate-buffered saline (PBS: 0.05 M phosphate/0.17 M NaCl, pH 7.4) at 200 μg of peptide per ml into New Zealand White rabbits (1.5 ml per rabbit) and adult Sprague–Dawley rats (0.5 ml per rat). Four booster injections were administered at biweekly intervals using Freund's incomplete adjuvant (Sigma); bleedings were carried out 7–10 days after booster injections and antiserum was obtained by centrifugation of the blood at 10,000 rpm at 4°C.

Affinity columns were prepared as follows: 3 mg of peptide K and ELH were linked through their amino groups to 1 g of activated CH-Sepharose 4B (Pharmacia) according to the manufacturer's protocol and rinsed with PBS.

Antisera were diluted in PBS and applied to each affinity column at 4°C at a flow rate of 4 ml/hr. Each column was rinsed with 50 ml of 0.1 M PBS (until $OD_{280} < 0.01$) and then 50 ml of each of the following: 2 M NaCl (pH 7.4), 0.1 M borate (pH 9.1), 0.1 M PBS (pH 4.5). The highly specific antibodies were eluted from the column with 20 mM glycine/HCl/0.2 M NaCl, and 0.8-ml fractions were collected in wells containing 0.4 ml of 0.1 M Tris buffer (pH 8.5) with 0.05% sodium azide. The fractions were monitored at OD_{280} and peak fractions were stored at −80°C until use.

Immunocytochemistry at the Light and Electron Microscope Levels. *Aplysia californica* from 500 to 1000 g were obtained from Sea Life Supply (Sand City, CA) and maintained in a seawater tank until use. Bag cell clusters with 0.5 cm of attached connective nerve were removed from animals anesthetized with isotonic $MgCl_2$, fixed in 4% paraformaldehyde, and embedded in Lowicryl K4M resin (Pelco, Redding, CA) as described (9).

Electron Microscopic Immunocytochemistry. Seventy-nanometer sections of Lowicryl-embedded tissue were collected on Formvar-coated nickel slot grids. All grids were rinsed in PBS and floated on the following solutions at 20°C: blocking solution [5% normal goat serum (NGS)/0.2% bovine serum albumin in PBS] for 10 min; primary antibodies (10–20 μg of rabbit or rat antibody in 0.5% NGS/0.2% bovine serum albumin in PBS) for 30 min, washed in PBS three times; blocking solution for 10 min; secondary antibodies [goat anti-rabbit antibody and/or rat antibody conjugated to col-

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Abbreviations: ELH, egg-laying hormone; BCP, bag cell peptide.

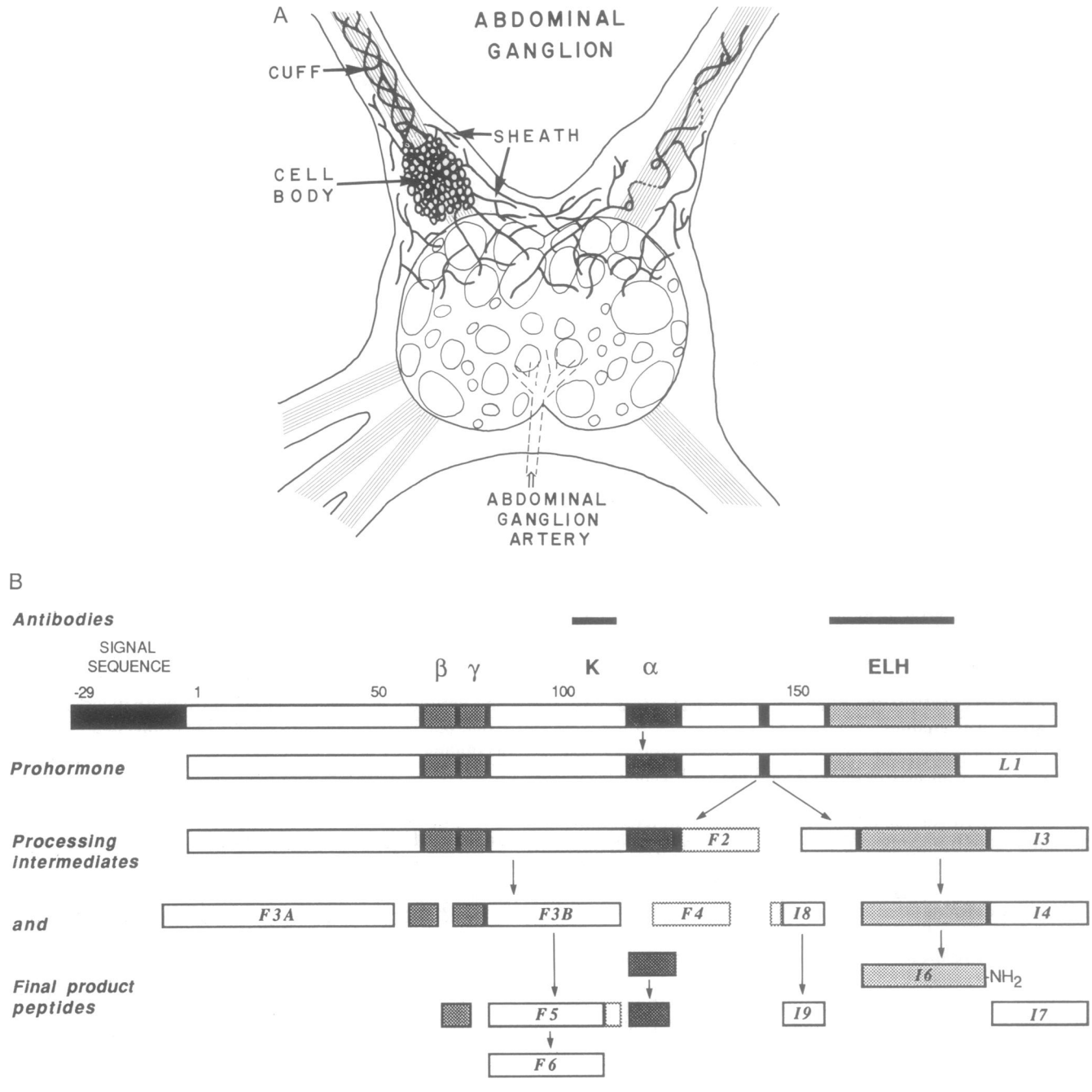

FIG. 1. Bag cell neurons and the ELH prohormone. (*A*) Diagram of the dorsal surface of the abdominal ganglion highlighting the bag cell neurons. On the left, a bag cell cluster is illustrated showing the positions of neuronal cell bodies, processes in the sheath, and processes in the cuff region. The right illustrates the morphology of two individual bag cell neurons. Each cell sends processes to the cuff region and the sheath surrounding the nerve tracks and the ganglion. The abdominal ganglion artery enters the organ along the caudal aspect of the ganglion and ramifies through the interstitial space. Blood flow through the artery actively distributes hormones, including ELH, to peripheral tissues. (*B*) Processing scheme of the ELH precursor (7). The signal sequence is cleaved cotranslationally as the prohormone enters the lumen of the endoplasmic reticulum. The next cleavage occurs in the trans-Golgi/trans-Golgi network, fragmenting the protein into amino-terminal (F2) and carboxyl-terminal (I3) intermediates (8). These intermediates are separately packaged into vesicles followed by cleavage to final product peptides. The positions of peptides used to raise antibodies used in this study (9) are indicated above the precursor. Positions of the known physiologically active α, β, and γ bag cell peptides (BCPs) and the ELH are indicated.

loidal gold (5 or 10 nm, Janssen Pharmaceutica) at OD_{520} = 0.25–0.5] in 0.5% NGS/0.2% bovine serum albumin, PBS for 30 min, washed with PBS twice, washed in water twice, and dried.

Quantitation of Immunoelectron Microscope Micrographs. For the quantitation studies, micrographs were taken randomly (the field of view was chosen without focusing on the gold particles). The photographic prints from the various experiments were then mixed and the labels were masked such that the person quantitating the micrograph did not know which experiment corresponded to which particular micrograph.

For each experiment the average density for each size of gold particle was determined (total number of gold/total area of vesicles). This density was then used to calculate a relative density of label in each vesicle (absolute density of gold in vesicle/average density of gold) for each size of gold. For one experiment, the size of gold particles was switched with no significant effect on the results. Other control experiments have also been described previously (9).

RESULTS

To determine if the two classes of transported vesicles are distributed equally among the bag cell processes, we examined a region immediately around the bag cells (sheath) and a region located ≈2–6 mm up the pleural-abdominal connective (the region labeled cuff in Fig. 1*A*). This upper region was further subdivided into cuff processes that are located within the connective tissue surrounding the bundle of axons and upper sheath processes that are located outside this region. Electron microscopic thin sections from these regions were double-labeled with antibodies directed against the amino-terminal (anti-peptide K, see Fig. 1*B*) and carboxyl-terminal (anti-ELH, see Fig. 1*B*) regions of the ELH prohormone (Fig. 2). As evident in Fig. 2, there are many more vesicles that contain amino-terminal immunoreactivity (small gold) in the cuff than in the sheath. Quantification of vesicle content in the processes and cell bodies from a single individual is shown in Fig. 3 (see also ref. 9). Vesicles in the soma either contain mostly ELH immunoreactivity or mostly amino-terminal immunoreactivity (peptide K), but relatively few vesicles contain both. A very different distribution of vesicles is observed in the processes. Varicosities in the sheath are filled with vesicles that contain mostly ELH immunoreactivity with very few vesicles that contain mostly amino-terminal immunoreactivity. In contrast, the cuff, in addition to containing ELH, contains a much higher percentage of vesicles containing mostly amino-terminal immunoreactivity.

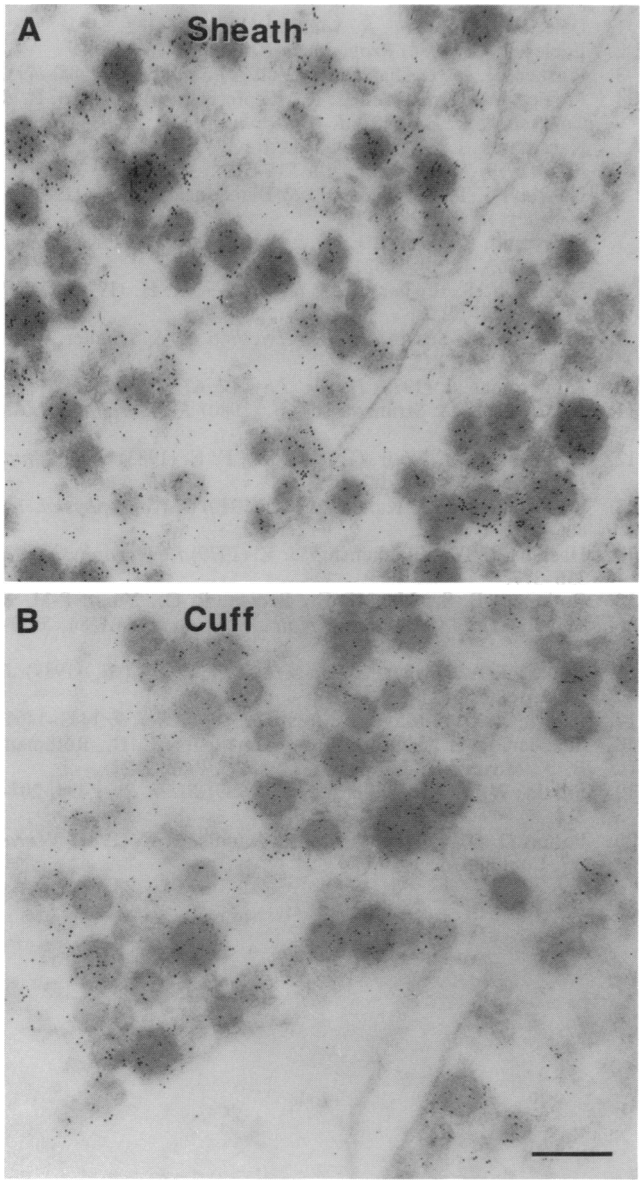

FIG. 2. Immunoelectron microscopy of sheath and cuff processes. (*A*) Micrograph from the sheath region of the bag cells. Small gold particles represent amino-terminal immunoreactivity; large gold particles indicate anti-ELH immunoreactivity. (*B*) Same as *A*, but from the cuff region of the same animal.

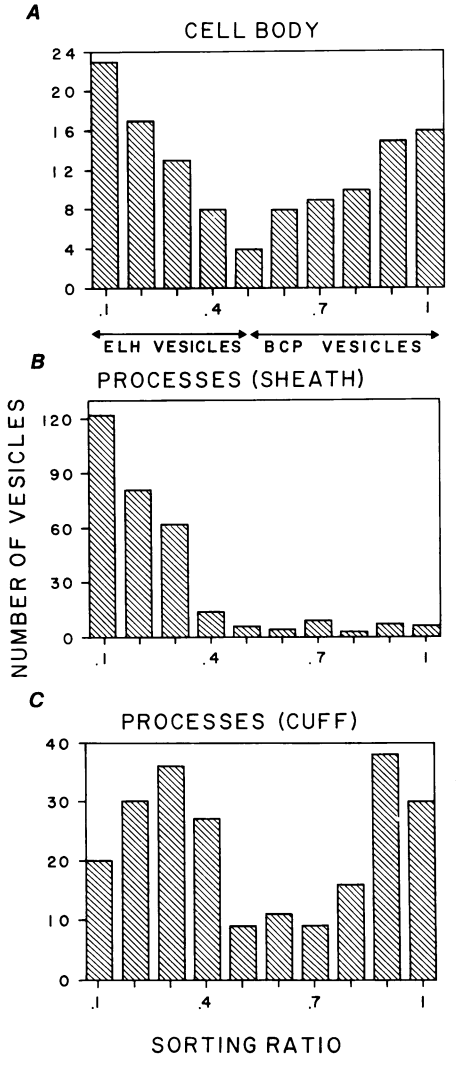

FIG. 3. Bag cell processes contain different vesicle populations. The number of amino-terminal gold particles in each vesicle was counted and divided by the sum of gold particles in the vesicle. This ratio is plotted against the number of vesicles. Thus, values close to 0 reflect vesicles that contain mostly ELH immunoreactivity (labeled ELH vesicles), whereas values close to 1 reflect vesicles that contain mostly peptide K or amino-terminal immunoreactivity (labeled BCP vesicles). (*A*) Quantitation of micrographs from bag cell bodies. (*B*) Quantitation of micrographs from sheath. (*C*) Quantitation of micrographs from cuff. The ELH vesicles in *C* may reflect a distinct population from those observed in the sheath region. Further data will be required to definitively resolve this point.

The summary of our results from three individual animals is shown in Fig. 4. The percentage of vesicles containing mostly amino-terminal immunoreactivity (>70% peptide K immunoreactivity) is much higher in varicosities located in the cuff (30.1%, SEM = 3.7, $n = 19$) than in the sheath (6.4%, SEM = 0.8, $n = 27$, $P < 0.01$). Interestingly, the varicosities located in the upper sheath have more amino-terminal de-

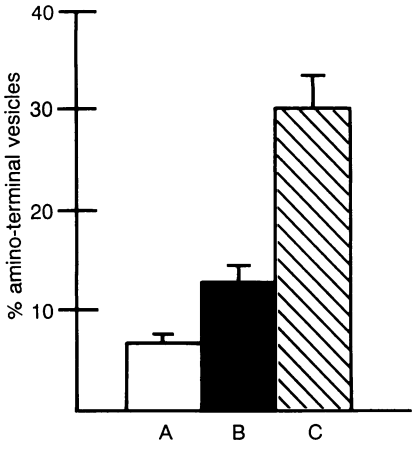

FIG. 4. Varicosities in the cuff are enriched in vesicles containing autocrine peptides. Amino-terminal vesicles are those in the last three bins of the histograms described for Fig. 3 (>70% peptide K gold particles). Error bars are standard error of the mean (SEM). (*A*) Varicosities from sheath (27 varicosities, 1516 vesicles, three animals). (*B*) Varicosities from the upper sheath (32 varicosities, 1663 vesicles, three animals). (*C*) Varicosities from the cuff (19 varicosities, 793 vesicles, two animals).

rived vesicles than the sheath around the bag cells but less than in the cuff (12.7%, SEM = 1.6, $n = 32$, $P < 0.05$). Examination of the data reveals that this is not due to a separate population of varicosities in the upper sheath but rather is due to a mixture of sheath-like varicosities and cuff-like varicosities. Also of interest is that in the cuff there are a small number of sheath-like varicosities (2/19 cuff varicosities have <10% amino-terminal vesicles), whereas there are no cuff-like varicosities in the sheath region (0/27 sheath varicosities have >20% amino-terminal vesicles).

These results demonstrate that the two distinct classes of vesicles produced in the bag cell soma are targeted to distinctly different bag cell processes. If differential localization arises from differential transport, then specifically labeled microtubule tracts may exist in the different types of processes. Alternatively, both vesicle types could be transported equally well, but the amino-terminal peptide-containing vesicles may be specifically retained in the cuff. Either possibility requires proteins, other than the neuropeptides, to be sorted between vesicle types, as has already been demonstrated for one bag cell antigen (19).

Sorting of the autoexcitatory and hormonal substances to different cellular sites may allow for differential release of these substances. Indeed, initiation of the bag cell afterdischarge may arise from excitatory input to the cuff descending from the head ganglia. This is likely to result in an initial release of amino-terminal peptides specifically stored in this region, which will in turn reinforce the depolarizing stimulus. Further autoexcitatory actions of the BCPs may be responsible for maintaining the discharge, allowing sufficient secretion of ELH from processes located in the sheath.

In the 1930s, Dale proposed that the chemical makeup of the cytoplasm of a neuron is identical to that of the terminals (1, 20). More recent discussions of Dale's principle suggest that all of a particular neuron's terminals release the same set of transmitters (2). Our observations may represent an exception to this rule. The ability of a neuron to store and presumably release different sets of transmitters from individual endings expands the array of mechanisms used to generate specific interactions between neurons. The ability of a neuron to selectively localize molecules to different processes may allow the selective alteration of specific terminals during plastic neuronal events.

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