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Commentary

Assaying for peptides in individual *Aplysia* neurons with mass spectrometry

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The set of biological questions that we can answer depends critically on our set of tools. Most traditional analytical methods perform measurements on an ensemble, where information about the individual is buried in averages. To understand biological processes in greater detail, we need tools sensitive enough to allow the study of individual cells or subcellular compartments. The article by Sweedler and coworkers (1) in this issue of *Proceedings* represents an important advance in the development of new tools to monitor biological processes at ever smaller dimensions.

Confocal and two-photon microscopies (2, 3), for example, have provided us with striking images of individual cells at work; in some cases, they have enabled the detection of individual molecules (4–6). Near-field optical microscopy (7), atomic force microscopy (8), and scanning tunneling microscopy (9) permit resolution on the order of molecular dimensions. Although such microscopies can provide remarkable visualization, they have little power to unravel the mysteries of the chemical composition of any cellular or subcellular compartments. To achieve this chemical characterization, we need analytical tools capable of deciphering the chemical nature of the molecules contained in a small biological chamber, such as a single cell. These types of measurements are difficult mainly because of the small volumes that need to be analyzed. For example, the total volume of a 10-μm diameter single mammalian cell is in the picoliter (10^{-12} liters) range. If the molecules of interest are present at millimolar concentrations, only $\approx$1 fmol (10^{-15} mole $= 6 \times 10^8$ molecules) is available for analysis.

Nonetheless, ultrasensitive techniques have been developed to study the contents of individual cells. One such technique is capillary electrophoresis coupled to a sensitive detector; in this way, the contents of individual biological compartments can be separated and analyzed. Indeed, interesting biochemical profiles of individual cells have been obtained with this technique (10–14). These single-cell profiles typically are deduced from detected fluorescence peaks or electrochemical signals present in the electropherograms. Although the measurements contain valuable information, further analyses are often needed to identify the molecules that cause the peaks. MS is one useful and powerful tool that yields precise molecular weight information from which chemical identity can be inferred in favorable cases. It will be a major advance when MS can be adapted to profile the chemical contents of single cells directly in a routine manner. In such a procedure, detailed molecular weight profiles of single-cell contents could be obtained without intermediate steps, thereby revealing information previously buried in statistical averages. The work by Sweedler *et al.* (1) exemplifies the power of such a single-cell molecular weight profiling tool (15–18).

In their study, Sweedler and coworkers (1) adapted matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF) to the analysis of individual peptidergic neurons from the gastropod mollusk *Aplysia californica.* MALDI is a gentle desorption/ionization technique that introduces a sample into the gas phase for mass spectrometric analysis. Because the sample is embedded in a matrix, the energy from the desorption/ionization laser pulse is absorbed partly by this matrix so that a relatively intact sample is sent into the gas phase. Compared with other soft-ionization methods, such as electrospray, MALDI is attractive for single-cell analysis because it tolerates moderate levels of impurities that are inevitably present in tissues and cells. Despite this advantage, the salt concentration present in marine specimens (total ionic strength $\approx$700 mM equivalent of NaCl) is beyond the salt tolerance of MALDI. To study these high salt specimens, Sweedler *et al.* (1) used the simple salt removal technique of Garden *et al.* (18) previously developed in their laboratory.

One important attribute of single-cell MALDI-TOF is the directness of the technique; that is, minimal handling of the sample is required. Because of the neuroactive nature of these peptides, various proteolytic enzymes are present in the extracellular matrix; the enzymes rapidly cleave and degrade the peptides. In the intact system, however, these degradative enzymes are spatially separate from the peptides because the lipid bilayer barrier divides them. Any disruption of this barrier, which unavoidably takes place in the homogenization step of traditional purification procedures, leads to the degradation of the peptides of interest. To overcome this problem, peptidase inhibitors or a low pH environment are often used during the homogenization and purification steps. A much simpler solution is to characterize the peptides without disrupting the bilayer barrier. This solution is offered by single-cell MALDI-TOF.

Another important advantage of single-cell MALDI-TOF is its ability to measure the biochemical contents of individual cells. Conventional methodologies of characterizing peptides are difficult and tedious. Often, cells are isolated and homogenized until enough peptide material can be purified and concentrated for later characterization. Because many cells are needed, such conventional techniques blur the distinctiveness of each individual cell. Although immunohistochemical staining combined with electron microscopy often is used to study, with high spatial resolution, the localization of peptides inside single cells, this method is laborious and can study only a few peptides at a time. In addition, staining techniques require prior knowledge of the peptides to generate the desired antibodies.

In this respect, MALDI-TOF represents a valuable complementary technique to assay single-cell peptides in which the molecular weight of all peptides present can be obtained simultaneously with no prior knowledge of the detected peptides. Although MS generally does not provide unambiguous peptide identification, it offers valuable molecular weight information that is often the first step toward characterization.

Aplysia has been a valuable model organism in neurobiological studies because the simplicity of its nervous system has

Abbreviations: MALDI, matrix-assisted laser desorption/ionization; TOF, time-of-flight; ELH, egg-laying hormone; AP, acidic peptide.

allowed the characterization of neural circuits that govern specific behaviors (19, 20). The most celebrated of these experiments is the study of long term habituation (21), a phenomenon that underlies learning and memory. *Aplysia* also has proved to be an excellent neuroendocrine system for the study of posttranslational processing and packaging of neuropeptides and peptide hormones (22–30). Fig. 1*A* shows the simple neural circuitry and reproductive organs of *Aplysia*. Fig. 1*B* is an expanded view of the abdominal ganglion region, which shows the location of the bag cells studied by Sweedler and coworkers (1). Fig. 1*C* illustrates the previously known posttranslation processing steps of the 271-residue egg-laying hormone (ELH) precursor, which is present in the bag cells shown in Fig. 1*B*.

A thorough characterization of the posttranslational processing steps of peptides is important to gain an understanding of the mechanism by which neuropeptides govern the simple behaviors of an animal. In the case of *Aplysia,* the behavior of egg laying (28) is governed by a family of neuropeptides, including the 36-residue ELH released from bag cells (Fig. 1*C*) and peptides A and B released from the atrial gland (31). Although a battery of peptides are involved in egg laying, they are all derived from and encoded by a small gene family (24). The diversity of these peptides, therefore, arises from the posttranslational processing and cleavage of a few peptide precursors, such as the ELH precursor studied by Sweedler *et al.* (1).

The biological importance of the Sweedler *et al.* article (1) is its elucidation of novel ELH and acidic peptide (AP) processing steps. The posttranslational processing of the 271-residue ELH prohormone has been studied extensively (22, 23). And, the sequence of enzymatic cleavage of the ELH precursor into the various peptides, including bag cell peptides, ELH, and AP, has been investigated in detail (25–27). Fig. 1*C* is a schematic of the previously known cleavage steps. Sweedler and coworkers (1) show that these are incomplete: They report additional processing of both ELH and AP through cleavage of Leu–Leu sites in these peptides. The additional cleavages result in at least five forms of ELH and multiple AP fragments as well. In fact, the reported AP_{9-27} fragment is often the dominant peak in the mass spectra that were obtained. The discovery of these various peptide fragments naturally raises the question: What are the differences in biological activities induced by them? Although the question is not answered in this study, the present report shows that posttranslational processing of the 271-residue ELH prohormone is more complicated than was first thought.

The new findings reported also demonstrate the power of single-cell MALDI-TOF. For example, neuropeptide Y previously was reported in bag cells (29). However, single-cell MALDI-TOF revealed that this peptide is localized instead in the abdominal RG cluster. Sweedler *et al.* (1) detected the intracellular presence of the three forms of α bag cell peptides (α_{1-7}, α_{1-8}, and α_{1-9}) that are derived from the nine-residue α bag cell peptide (Fig. 1*C*). It has been proposed that αBCP is processed intracellularly into the above three forms (30). The data obtained provide direct evidence to support this hypothesis.

The ability of single-cell MALDI-TOF to reveal the uniqueness of each individual cell also is shown; two of the >100

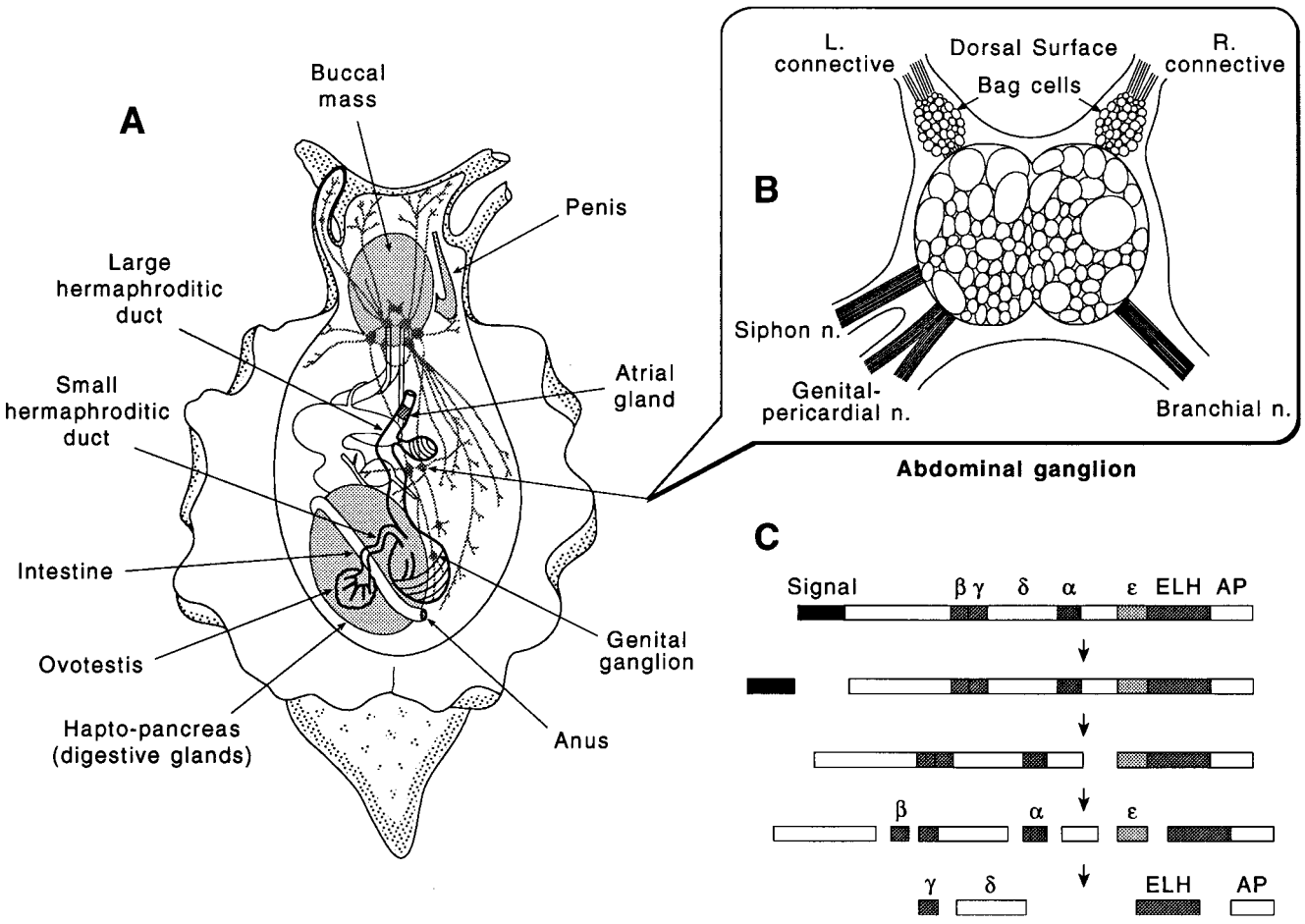

FIG. 1. (*A*) Diagram of *Aplysia*, which depicts the locations of the various ganglia and organs of this animal. (*B*) An expanded view of the abdominal ganglion that shows the location of the bag cells. (*C*) Schematic illustration of the known cleavage steps of the 271-residue ELH prohormone (27), which is synthesized in the bag cells of *B*.

Aplysia studied have been found to contain additional peptides that have not been observed in other animals. These peptides are 30 mass units heavier than the typical AP-related peptides and are thought by Sweedler and coworkers (1) to be a substituted form of AP. These subtle statistical variations are lost easily in traditional techniques that homogenize cells from many animals.

Through the use of single-cell MALDI-TOF, Sweedler *et al.* (1) provided new insight into the posttranslational processing of peptide prohormone in the neuroendocrine system of *Aplysia*. Equally important is their demonstration of the power of MALDI-TOF MS to profile single-cell peptides. This analytical technique opens new possibilities in the study of other important biological problems in which precise molecular weight determinations of the molecular contents of individual cells are needed.

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