Peptides in the Brain: Mass Spectrometry–Based Measurement Approaches and Challenges

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Abstract

The function and activity of almost every circuit in the human brain are modified by the signaling peptides (SPs) surrounding the neurons. As the complement of peptides can vary even in adjacent neurons and their physiological actions can occur over a broad range of concentrations, the required figures of merit for techniques to characterize SPs are surprisingly stringent. In this review, we describe the formation and catabolism of SPs and highlight a range of mass spectrometric techniques used to characterize SPs. Approaches that supply high chemical information content, direct tissue profiling, spatially resolved data, and temporal information on peptide release are also described. Because of advances in measurement technologies, our knowledge of SPs has greatly increased over the last decade, and SP discoveries will continue as the capabilities of modern measurement approaches improve.

1. INTRODUCTION

Signaling peptides (SPs): neuropeptides, hormones, cytokines, trophic factors, and other peptides used in cell-cell signaling

Neuropeptides: endogenous peptides synthesized in nerve cells or peripheral organs that are involved in cell-cell signaling

Single-cell mass spectrometry (MS): accurate mass measurement and/or fragmentation analysis of neuropeptides taken directly from individual nerve cells

Peptidomics: global measurement and identification of the complement of peptides in a cell, tissue, or extract

Animal behaviors are controlled by the actions of the networks of neurons making up their brains. The activity and response of a particular neuron in a network depends not only on the actions of the other cells interacting with that neuron, but also on the neuromodulators surrounding it. The neurons in our brain are bathed in a complex suite of signaling peptides (SPs), which affects the activities of the networks in many profound and subtle ways.

SPs have a variety of functions in the body. They are involved in neuromodulation, neurotransmission, cell outgrowth, cell survival, and hormonal signaling between organs (1–3). SPs contribute to multiple aspects of behavior. For example, SPs have been reported to play important roles in the physiological mechanisms of feeding (neuropeptide Y and galanin) (4, 5), thirst (angiotensin) (6), and pain (enkephalins) (7–9). Improving our understanding of these various physiological processes requires knowledge of the SPs present in the appropriate anatomical regions. The simultaneous detection, identification, and quantitation of the peptide complement (the peptidome) of complex structures such as the brain present a significant challenge that is exacerbated by the inherently small quantities of SPs, their broad dynamic range, and their diverse chemical and physical properties.

Here we explore the analytical methods used to characterize neuropeptides, neurohormones, and other brain peptides (i.e., the SPs), with an emphasis on mass spectrometry (MS)–based approaches. Although our discussion of several of the techniques overlaps with other reviews in this volume, we focus upon what makes the measurement of SPs unique and the analytical requirements for such studies. We also provide an overview of the approaches used to characterize SPs in terms of their structure, function, and dynamics within the brain. Interested readers are encouraged to examine the articles in this volume on high-resolution MS (10), imaging MS (11), and the chemical analysis of single cells (12).

For most animal models studied in neuroscience, dozens to hundreds of distinct brain peptides have been reported. The diversity of peptides characterized is a relatively recent phenomenon; the original peptide experiments involved valiant efforts on sample preparation and characterization. For example, Roger Guillemin required 5 million hypothalamic fragments from sheep, and Andrew Schally used a similar amount of material from pigs; the output of their heroic work was the structure of a doubly modified tripeptide (pyroGlu-His-Pro-NH_2), the thyroid releasing factor (13) (see **http://nobelprize.org/nobel_prizes/medicine/laureates/1977/press.html**). Because of advances in instrumentation, peptides can now be characterized in samples consisting merely of individual neurons, and are routinely characterized in samples nearly 15 orders of magnitude smaller than those used by the original neuropeptide pioneers.

Often, studies are directed toward several previously defined peptides in a sample. Other studies seek to measure the brain peptidome. The recently coined term peptidomics arose simultaneously from three separate groups in 2001 (14–16). Since then, numerous studies characterizing the subset of bioactive peptides related to the brain and other organs have appeared (17–21).

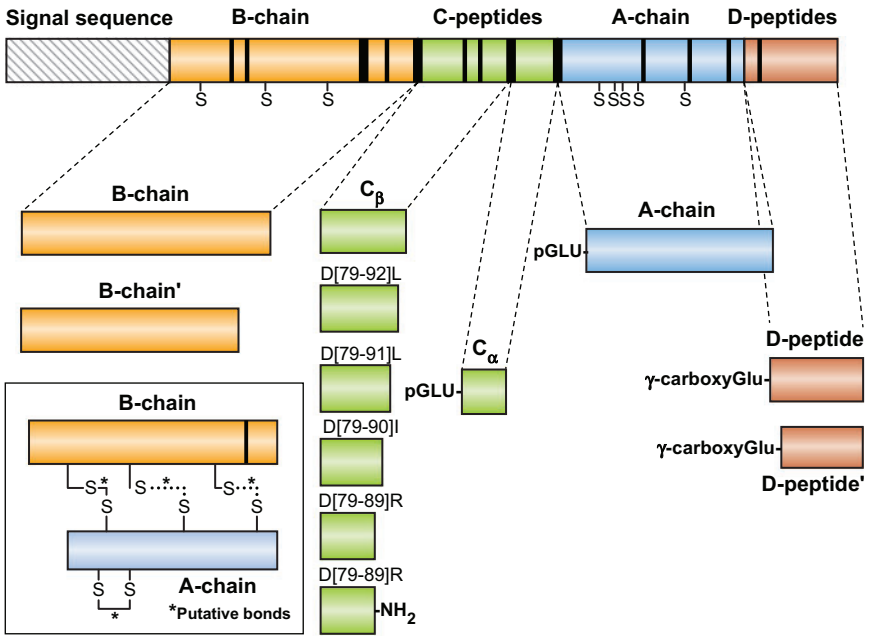


Figure 1

Representation of a single preprohormone for *Aplysia californica* insulin (*long rectangle*); the basic sites that can be cleaved by processing enzymes are shown as vertical black lines. The colored bars represent the partially or fully processed peptides arising from this prohormone; the final products include peptides modified with cleavages, amidation, disulfide bond formation, pyroglutamylation, and gamma-carboxylation (25, 26).

2. WHAT IS A SIGNALING PEPTIDE?

As with all gene products, the creation of a neuropeptide occurs when its RNA is translated into a large protein. In the case of SPs, this protein is a preprohormone that contains a signal sequence. The signal sequence is cleaved from the preprohormone and targets the remaining protein, a prohormone, to the secretory pathway (22, 23). The prohormone is packaged into dense core secretory vesicles, and several processing steps then occur to convert the prohormone into a suite of peptides within the secretory vesicles (24). The prohormone often encodes multiple bioactive neuropeptides within a single vesicle, including replicates of an individual peptide and single copies of other peptides (**Figure 1**) (25, 26). These prohormone cleavages occur under the control of a suite of intracellular prohormone convertases. Because the convertases responsible for the cleavage of the peptides are endogenous, no additional enzymes, such as trypsin, need to be added in order to produce the peptides. As a result, tissues can be dissected from an animal and directly analyzed by MS to observe the final neuropeptides.

Prohormone: gene product packed in dense core vesicles and processed using a suite of intracellular processing enzymes, resulting in a series of peptides

The processing of neuropeptide prohormones into biologically active neuropeptides requires a surprisingly large number of enzymatic processing steps, with the

DCV: dense core vesicle

final products depending on the particular enzymes and the order in which they act. As these steps can occur in a brain region–specific manner, one cannot normally predict the final products without measuring them via MS or another information-rich approach. Whereas such measurements of neuropeptides are critical, there are no experimentally confirmed lists of peptides for most animals—even for animals with genomic information—nor for most brain regions. Genomic information coupled with knowledge of enzymatic processing enables an educated guess as to the resulting SPs from a novel prohormone. For example, cleavage of a prohormone at basic amino acid sites is common; although most of the prohormone cleavages occur at basic amino acid residues (lysine and arginine) (27, 28), only a small percentage of basic sites are actually cleaved (29). Approximately one-third of the ten basic sites found in a survey of mammalian prohormones have been observed to be cleaved during prohormone processing (30). But which of the ten are cleaved? Several research groups have formulated guidelines, based on the frequency of amino acids appearing near cleaved and noncleaved sites, to help determine the location of these basic processing sites (29, 31–33). We describe a statistics-based approach to predicting prohormone processing in Section 8, below.

2.1. Brain Peptides Versus Signaling Peptides

SPs can function as neurotransmitters acting across a synapse, act as hormones with non-neuronal targets, or act at intermediate distant targets by passive diffusion via neuroendocrine pathways. Often, the same peptide can function as both a hormone and as a neurotransmitter at different sites. Peptides can inhibit, excite, or modulate small-molecule transmission. Other functions include cytokine and trophic actions (24). Although neuropeptides can have diverse functions, peptides must have a function associated with cell-cell signaling to be considered neuropeptides—their presence in neural tissue is not enough. Cell-cell signaling is most commonly associated with the site of classical transmitter chemical communication, the synapse. However, neuropeptides are normally packaged into dense core vesicles (DCVs), which lack many of the specialized proteins that cause localized release in classical transmitter-active zones. Thus, neuropeptides can be released anywhere along a nerve terminal membrane; in fact, some peptides are more often released in the soma or dendrites than in axons/synaptic regions (34, 35).

An interesting observation arises when examining most prohormone processing schemes such as the one shown for *Aplysia* insulin (**Figure 1**): A prohormone often encodes multiple peptides. An important question is: Which of the peptides derived from a prohormone are involved in cell-cell signaling? Often, prohormones contain SPs separated by "linker" peptides with no reported function. If these peptides play no role in cell-cell signaling, what is their function? Additional suggested roles for specific peptides or prohormone domains highlight the other functions of these peptide sequences within prohormones. For example, several prohormones have linker peptides that contain a preponderance of acidic residues (36–38); at times these peptides provide charge neutrality during the acidification of the DCVs. In this (nonsignaling) role, the atrial natriuretic peptide's acidic domain affects the size and shape of

the resulting DCVs (39). Also, specific peptides in a prohormone may be required for correct folding and disulfide bond formation, as in the case of the insulin C-peptide, which links the A- and B-chains to promote their effective folding and assembly into insulin and may aid in the solubilization of insulin after vesicular release (40, 41). Thus, the functions of the peptides derived from a single prohormone are diverse, and only a subset of these functions pertain to cell-cell signaling. Although nonsignaling functions are certainly important, it is necessary to develop a more precise nomenclature for brain peptides, as many such peptides are not involved in cell-cell signaling.

Catabolism: extracellular processing and degradation of signaling peptides as a mechanism for peptide deactivation

In addition to the final peptides formed from a prohormone, one can also detect processing intermediates. When preparing a tissue sample, one normally freezes, heats, or chemically denatures the tissue to stop enzymatic processing. Thus, one would expect the sample to contain both final fully processed peptides and prohormone processing intermediates, with relative amounts of each dictated by the enzymatic formation rates and the sampling protocols. Often, these partially processed forms may be difficult to distinguish from final peptides; however, they may contain an intermediate noncleaved basic residue. If these peptides represent processing intermediates, can they be considered bioactive? They may well be: Several SPs interact with their cognate receptor with only a portion of their length; that portion often includes the C-terminal end of the peptide. In such a case, N-terminally extended forms may affect receptor binding, but do not abolish it. For example, FMRFamide peptides can exist in many extended forms in some prohormones; many overlap with regard to receptor binding, and they may have overlapping or distinct functions (42, 43). Peptides can be detected and tested, but without appropriate controls (such as sampling the peptides released after appropriate physiological stimulations), the data obtained might be misleading. Developing and employing appropriate sampling protocols are often key to data collection and interpretation, and thus are highlighted throughout this review.

Another complication in sampling is becoming more important because of the increased performance of modern MS. Many processing enzymes, like other enzymes, may have a high (e.g., 95%) specificity for the correct cleavage but may cleave some sites in error. This may not be an issue if only a small fraction of a prohormone is cleaved incorrectly. However, as the dynamic range and limits of detection for MS techniques improve, more of these peptides can be detected. Using the lower-sensitivity techniques of the past, most of the chemically sequenced peptides were observed to be bioactive. Currently hundreds of peptides can be detected, many of which do not perform easily determined functions.

2.2. Catabolism

So far, our discussion has focused on SP formation (anabolism). After an SP is released, it can be removed or degraded via catabolic processes, such as cellular (re)uptake by target neurons or glia. Degradation of peptides also has profound effects on the ability to measure them. Much of the extracellular degradation is caused by endogenous C-terminal and N-terminal peptidases. Researchers often optimize a technique by maximizing the number of compounds characterized; however, in the case of SPs,

this may create a protocol that allows the characterization of extended extracellular degradation products. Many SPs contain posttranslational modifications (PTMs), such as C-terminal amidation, N-terminal acetylation, or pyroglutamylation, that slow extracellular degradation; such PTMs are often used as hallmarks of bioactivity.

PTM: posttranslational modification

MALDI: matrix-assisted laser desorption/ionization

Whereas extracellular processing of a peptide is normally considered a process of catabolism and peptide deactivation, it has been shown that some of these extracellularly processed peptides become important SPs. For instance, the intracellular processing of the angiotensinogen prohormone produces angiotensinogen. After the angiotensinogen is released from the cell, the enzyme rennin interacts with it to produce angiotensin I. One of the more bioactive forms of angiotensin, angiotensin II, is then produced by the action of angiotensin-converting enzyme (2). These processes can occur hormonally in many locations throughout the body. The brain may use this pathway, but also appears to use other angiontensin processing systems such as alternative processing enzymes and an intracellular rennin/angiotensin-converting enzyme system (44). Thus, the same peptide can follow different routes to formation depending on its specific location, and what may be an extracellular process in one region may be intracellular in others. The complex interplay of SP anabolism and catabolism allows the fine-tuning of the cellular response to the cocktail of SPs that surround a neuron and makes SP measurements critical in determining neuronal function. These overlapping needs for measuring the peptides in the brain require approaches with high chemical, spatial, and dynamic information content, approaches that are not yet available within the context of a single measurement platform.

3. SAMPLE PREPARATION STRATEGIES

Although MS is a useful technique due to its high sensitivity, speed, and chemical specificity, mass spectral characterization of SPs from tissue samples can be complicated by extracellular proteases, high salt content, lipids, and high sample complexity, which may cause ionization suppression and/or problems with dynamic range. Therefore, proper sample handling from animal dissection through analysis is key to obtaining relevant results. Depending on the type of chemical information sought from a given nervous system or neuronal structure, different sample preparation strategies can be employed. **Figure 2** presents three major sample preparation methodologies for the detection of neuropeptides from neural tissues.

Many experiments are performed by direct tissue profiling or by molecular ion imaging from tissue sections using matrix-assisted laser desorption/ionization (MALDI)–based MS. Direct tissue investigation involves less sample preparation and allows comparison among individual samples or animals. The sample preparation can be described as a simple sequence of steps that includes placing the tissue of interest on the MALDI plate, applying a droplet of matrix, and irradiating the cocrystallized tissue to cause desorption/ionization of the analyte for subsequent detection. To obtain quality mass spectra, tissues are commonly rinsed with water (45, 46) or matrix solution (47–49) to prevent interference from the high concentration of salt and lipids in the tissue. This technique is sensitive enough for SP analysis from single organs (50, 51) and even single cells (48, 52–54). In many cases, sufficient signal is obtained for

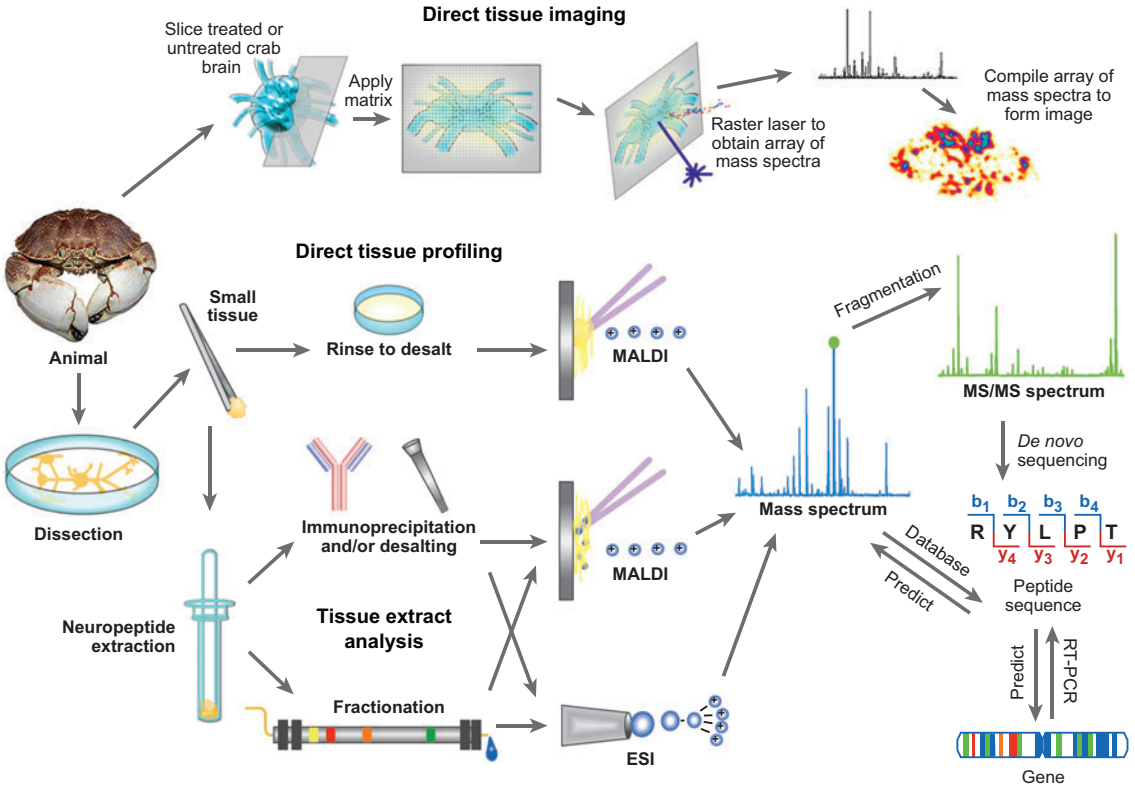


Figure 2

Overview of sample preparation strategies for mass spectrometry (MS)–based neuropeptide analysis. Three major sample preparation approaches are outlined: (1) molecular ion imaging from tissue sections, (2) direct tissue profiling of isolated neuronal tissues and/or clusters of cells, and (3) neuropeptide extraction from the tissue sample. This extract can then be further processed by desalting with C_{18} microcolumns, enriched for a particular peptide family through immunoprecipitation and/or fractionated prior to matrix-assisted laser desorption/ionization (MALDI) or electrospray ionization (ESI) MS analysis. When novel neuropeptide sequences are determined for species with unknown genomic information, the use of reverse transcriptase polymerase chain reaction (RT-PCR) can enable the discovery of novel genes. Adapted and modified from Reference 52 with permission.

peptide fragmentation information to be collected (46, 50, 55–57). Rinsing the tissue with dilute matrix solution and acidified methanol allows the extraction of peptides to the tissue surface and the removal of salts, resulting in improved detection. Direct tissue analysis of neuropeptides has been applied extensively in invertebrate systems (49), and to a lesser extent in more complex vertebrate systems (51, 57). An alternative direct tissue peptide-mapping experiment involves obtaining a collection of molecular ion images across a tissue section by rastering the laser beam at an ordered array of locations from the tissue.

Direct tissue analysis: MALDI MS analysis of freshly dissected and mounted tissue samples with minimal sample preparation

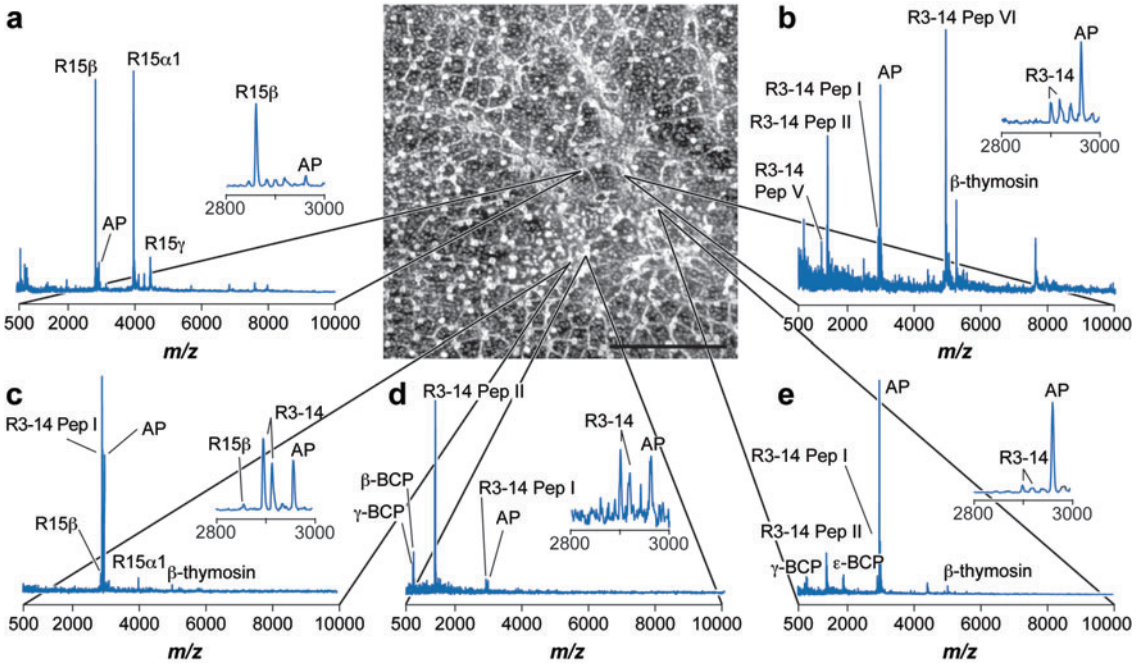


Figure 3

A high-throughput, small-volume sampling approach for direct tissue analysis of neuropeptides. *Center*: Schematic of the massively parallel sample preparation using the stretching sample method. Panels *a–e*: Representative mass spectra obtained from a tissue section showing characteristic peptide profiles at discrete locations. Adapted from Reference 60 with permission.

To improve the sensitivity of in situ peptide analysis, matrix volume must be minimized to avoid dilution of the sample. Although the dried-droplet method of matrix application is sufficient for many applications (49, 58), application of nanoliter volumes of matrix is often desirable when working with smaller samples so as to limit peptide dilution (53, 59). For example, more than 20 FMRFamide-related peptides were detected at nanomolar concentration in single cockroach cells by applying nanoliter volumes of matrix solution (53). Another approach to minimizing peptide dilution and migration is the combined use of an array of glass beads and a stretchable hydrophobic membrane such as Parafilm M®. As shown in **Figure 3**, by adhering a thin tissue section to the glass bead array and then stretching the membrane, one can rapidly create a set of single-cell-sized tissue samples that maximize peptide extraction while limiting analyte diffusion (60). With a precisely controlled micromanipulator and an optimized sampling protocol involving the use of a glycerol cell stabilization technique, single mammalian cells can be investigated for their peptide content by MALDI MS (61, 62).

Although direct tissue experiments allow investigators a quick glimpse at peptide profiles of individual organs or a small cluster of neurons with minimal sample

preparation, a homogenization- and extraction-based strategy offers several advantages for obtaining neuropeptide-rich samples through the pooling of many organs/cells. However, increased sample handling and processing may lead to sample dilution, loss, and/or chemically induced artifacts.

Due to the wide variation in model systems and in the chemical properties of neuropeptides, numerous extraction schemes have been developed. Neuropeptide extraction procedures require the reduction of protease activity. Postmortem protease activity yields protein degradation products that interfere with the identification of SPs. This is especially true for mammalian tissues, which require rapid postmortem protease deactivation to maintain sample integrity. Protease heat deactivation employing microwave irradiation or boiling extraction buffers are common techniques employed to reduce protease activity (20, 63, 64). Microwave irradiation is generally performed on the entire brain and dissection is performed by hand after irradiation. Alternatively, the whole brain can be snap-frozen immediately following sacrifice and subsequently dissected by a combination of cryostat sectioning and tissue punching. In this manner, the tissue is kept frozen during the dissection process. The proteases are deactivated immediately prior to extraction by placing the tissue punches in a boiling extraction buffer (65).

As an alternative to heat deactivation, proteases can also be deactivated using acidified organic extraction buffers such as acidified acetone, ethanol, and methanol. These acidic buffers deactivate proteases via denaturation and precipitation. This technique is more commonly used for invertebrate species but is also effective for mammalian tissues (66–69).

4. QUALITATIVE ANALYSIS

Characterizing the SPs in a sample requires simplifying the chemical complexity of the sample. As described in the previous section, the end result of sample preparation for SPs normally consists of either a small tissue sample (as small as a single neuron) or an extract that is well suited to performing a separation prior to MS. Just as the sampling protocols are distinct for these approaches, so too are their analyses. In this section, we discuss the benefits and drawbacks of each approach.

4.1. Mass Spectrometric Analysis of Tissue Extracts and Extracellular Fluids

After SPs are extracted from tissue samples, the chemical complexity and wide dynamic range of SPs present in the crude extracts require a separation prior to MS detection. Given the low abundance of most endogenous SPs, a capillary-based chromatographic approach for peptide fractionation is often employed (70–72). In this fractionation technique, a nanoflow liquid chromatography (LC) column (e.g., 75-μm internal diameter) is typically connected to a wider-bore precolumn for sample loading and desalting via a column-switching device. This arrangement enables fast sample loading and sample cleanup/concentration prior to eluting on the analytical column that is interfaced to the mass spectrometer. A wide range of peptidomic

applications have been reported to utilize this scheme for effective identification of numerous peptides in a single chromatographic run (66, 67, 73, 74).

ESI: electrospray ionization

To improve the sensitivity for monitoring in vivo peptide secretion, Kennedy and colleagues (75, 76) employed capillary LC columns (25-μm inner diameter) interfaced to a quadrupole ion trap mass spectrometer. The miniaturization of the LC column, coupled with a smaller electrospray emitter and automated two-pump system for high flow-rate sample loading and low flow-rate peptide elution, enabled the detection of attomole-level peptides and the identification of close to 30 SPs in brain extracellular fluid collected in vivo from live rats.

Multidimensional chromatography has been used to reduce sample complexity by improving the resolution of complex mixtures of proteins or peptides (70). A popular two-dimensional LC scheme involves the on-line coupling of strong-cation exchange followed by reverse-phase separation; this technique has been successfully applied to the peptidomic analysis of both *Drosophila melanogaster* and *Caenorhabditis elegans*, which resulted in enhanced detection of low-abundance, novel peptides (69, 77). More recently, an alternative two-dimensional LC scheme employing differential pH selectivity in the first- and second-dimensional reverse-phase separations was used to enhance rat brain neuropeptidome coverage (65).

Although on-line coupling is often preferred to other schemes, LC fractionation off-line coupled to MALDI MS detection offers several unique advantages. These include the flexibility to independently optimize separation and MS detection and the availability of the majority of individual LC fractions for subsequent in-depth chemical and biochemical characterization. Furthermore, compared to electrospray ionization (ESI)–MS detection, which is often coupled on-line to LC separation, MALDI analysis often provides additional information on the peptides in the sample (65, 78). In addition, an array of sample deposition methods ranging from electrospray deposition to a pulsed electric field deposition, in conjunction with modification of the MALDI target surface, has been developed to enable trace-level detection of SPs from brain tissues (21, 79, 80).

Besides coupling to LC separation, MALDI MS has also been coupled with capillary electrophoresis (CE) for SP detection. Due to its characteristic small injection volume, CE is well suited for peptide analysis in single cells. For example, a post-capillary deposition technique involving a dual capillary configuration that mixes the MALDI matrix with CE eluent from the separation capillary outlet enabled single-cell analysis from nanovial preparations (81, 82). To enhance sample concentration detection limits for in vivo analysis, a combined solid-phase preconcentration CE and matrix-precoated membrane target was used to study intracerebral metabolic processing of neuropeptide in rat, with an overall concentration limit of 10 pM reported (83, 84).

4.2. Direct Tissue Profiling

Compared with methods that involve pooling and extracting tissue samples for neuropeptide analysis, direct tissue analysis offers several distinct benefits. This methodology simplifies sample preparation by eliminating the separation step, thereby

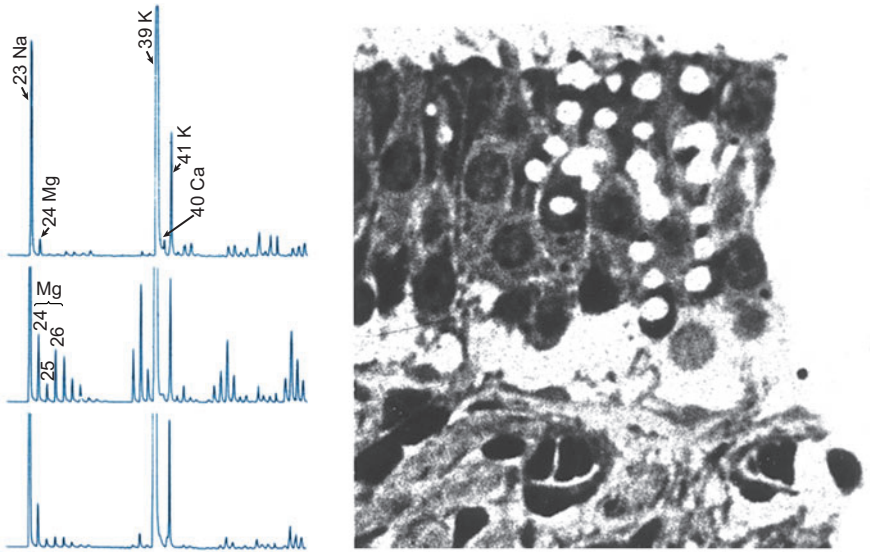


Figure 4

Laser desorption/ionization using a focused laser allows the probing of areas from a tissue section only a few microns in diameter. *Right*: A section of the inner ear used to investigate the homeostasis of the endolymph, with the individual cell outlines and sampling locations clearly visible. *Left*: Several of the associated mass spectra. Adapted with permission from Reference 86 and courtesy of F. Hillenkamp.

minimizing sample preparation artifacts and reducing sample loss and contamination. Perhaps more importantly, direct tissue analysis offers higher sensitivity and can be used with smaller samples such as single brain nuclei and even single cells. Finally, direct tissue analysis provides unique information on the spatial distribution of peptides in a given tissue or cell sample that is often lost in the pooled tissue homogenates.

For peptides that are only made in a rare cell, pooling samples adds chemical complexity but may not result in a greater amount of specific peptides; basically, pooling the sample dilutes the peptides of interest in a complex matrix of the peptides and proteins common to all the cells in the pooled samples. Perhaps one of the more successful advances in neuropeptide research has been the development of single-cell MS. Historically, single-cell MS measurements predate MALDI. Hillenkamp (85, 86) developed the laser microprobe mass analysis technique (LAMMA) that allowed a range of lower-molecular-weight compounds to be detected directly from thin tissue sections. **Figure 4** shows a tissue section with several < 5-μm holes ablated from the tissue by the laser, with each spot producing a mass spectrum (87).

One of the first neuropeptide characterizations using single-cell MALDI MS was described in 1994 and used large neurons from the freshwater invertebrate *Lymnaea stagnalis* (88, 89). Several neurons were examined for peptides encoded by known prohormones. Since then, single-cell MALDI has enabled the characterization of

hundreds of new peptides from a range of animals spanning a number of model organisms (48, 49).

CID: collision-induced dissociation

PSD: postsource decay

4.3. Structurally Characterizing the Signaling Peptide

4.3.1. Mass spectrometry–based neuropeptide identification and characterization. Whether using direct tissue characterization or analyzing separated fractions, characterizing the resulting peptides via MS is of paramount importance. For an organism whose genome has been sequenced and knowledge of neuropeptide prohormone sequence is available, an accurate mass measurement may be employed in conjunction with database searching to identify the amino acid composition of a given neuropeptide (49, 52). However, even with knowledge of the genome sequence, prediction of the final bioactive peptide sequences is often difficult due to extensive PTMs, tissue-specific prohormone processing, and unusual processing sites. Therefore, fragmentation techniques such as collision-induced dissociation (CID) and postsource decay (PSD) are often required to obtain a peptide fingerprint or to discover novel prohormone processing products (52). The combined use of accurate mass measurement and gas-phase fragmentation analysis in conjunction with web-based searching tools has enabled several large-scale peptidomic studies in organisms with sequenced genomes (20, 69, 77, 90, 91).

How does one confirm identifications with direct tissue measurements? One way involves the detection of the multiple peptides that result from processing of a single prohormone (see **Figure 1**). For example, the identification of pro-opiomelanocortin (POMC)-derived peptides from a single pituitary cell was achieved because a dozen peptides from the same prohormone were characterized in every cell measured (61, 62). However, when studying samples with unknown prohormones, additional information is required for structural characterization; unfortunately, the tandem MS (MS/MS) approaches that can be adapted to direct tissue measurements are limited.

The first fragmentation approach applied to single neurons used PSD on identified molluscan neurons (48, 55, 92). More recently, direct single-organ neuropeptide fragmentation was performed by MALDI-time-of-flight (TOF) PSD on cockroach (45) and flesh fly (93). MALDI CID of neuropeptides taken directly from single organs has been performed on lobster and crab by Fourier transform (FT)–MS (50, 94). Furthermore, Neupert et al. (46) recently used a combination of PSD and CID fragmentation to de novo sequence a new insect periviscerokinin peptide in a single insect cell.

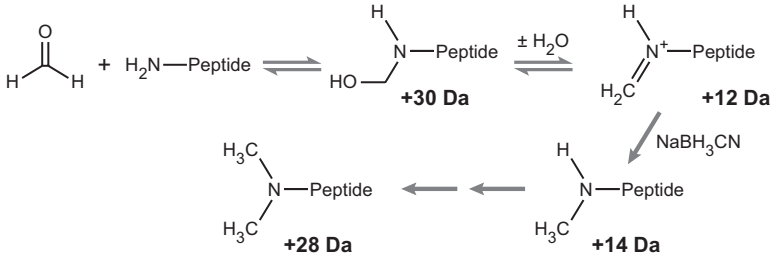
To improve the mass measurement accuracy for peptide identification, studies utilizing direct tissue analysis have employed high-resolution, high-accuracy MALDI-FT-MS (50, 66, 94). Using an in-cell accumulation technique to improve sensitivity, mass measurement accuracy can also be improved to sub-parts-per-million levels by incorporating calibrants on a separate spot from the sample of interest without premixing calibration standards with the sample. This technique is highly beneficial for tissue analysis (50).

4.3.2. De novo sequencing assisted by microscale chemical derivatization. For neuropeptide identification and discovery from organisms lacking genomic information, de novo sequencing is often required; however, it represents a significant analytical challenge. Several chemical derivatization schemes have been developed to facilitate de novo sequencing by introducing a mass shift at either the N terminus (56, 95) or the C terminus (96) of a peptide, thus enabling differentiation between b- and y-type fragment ion series in a complex MS/MS spectrum. Most of these derivatization methods work well for tryptic peptides, but their utility for native neuropeptide sequencing is somewhat problematic and requires further evaluation. For example, acetylation of primary amine groups often changes the ionic states of peptides. This may lead to substantial loss of sensitivity in peptide analysis due to reduced ionization and fragmentation efficiencies for lower charge state ions. Likewise, a C-terminal methyl esterification method would not work for peptides lacking free C termini. Because C-terminal amidation is the most common PTM in SPs, the utility of this labeling approach for SP de novo sequencing is limited. However, a recent MALDI-FT MS/MS study using methyl esterification to characterize acidic amino acid–containing orcokinin peptides in crustacean showed significant improvement in the fragmentation efficiency of derivatized orcokinins due to blockage of the aspartate-selective cleavage pathway of the native orcokinin peptides (97).

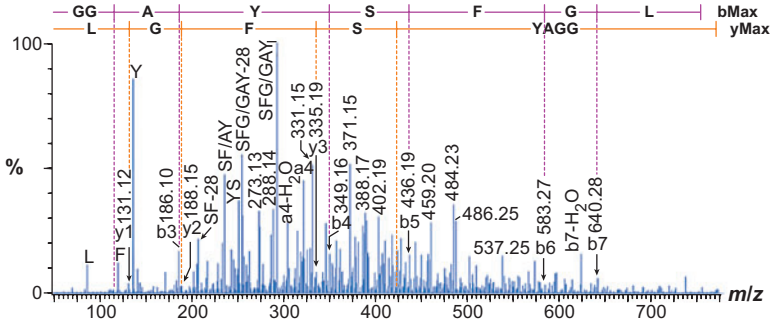
Recently, a reductive methylation-labeling method using formaldehyde has shown great promise for de novo sequencing due to its simplicity and speed (98, 99). This derivatization method labels the N terminus and ε-amino group of lysine (Lys) in peptides through reductive amination and produces peaks differing by 28 Da for each derivatized site. The intensity of the N-terminal fragment a_1 ion is also substantially enhanced upon labeling, which is beneficial for de novo peptide sequencing. Furthermore, the ionic state of the modified peptides is not changed. By incorporating isotopic formaldehyde labeling, several unique features for SP sequencing applications are revealed. For example, reductive methylation enables differentiation of isobaric amino acid residues, such as Lys versus glutamine, due to selective dimethylation of the Lys side chain. Additionally, N-terminal blockage such as pyroglutamate modification can be readily assessed. Perhaps a more significant feature is the simplification of fragmentation pattern for singly charged peptides after dimethylation (**Figure 5**), which is beneficial for SP de novo sequencing because many SPs are singly charged due to the lack of a basic amino acid residue at their C termini. This N-terminal isotopic methylation strategy enabled de novo sequencing of 55 peptides, including 25 novel peptides from a neurohemal organ extract from the crab *Cancer borealis*, an organism whose genomic information remains unknown (99, 100).

Several on-target approaches have been used for tissue measurements. For example, on-target acetylation reacts with primary amine groups and results in a 42-Da mass shift for each added acetyl group. Similarly, treatment of worm head ring ganglion with hydrogen peroxide caused the oxidation of methionine groups to methionine sulfoxide, assayed as a 16-Da mass shift for each incorporation of an oxygen atom. These in situ chemical derivatizations provide a rapid method for confirming the presence of a free amine at the N terminus of a peptide and for counting the

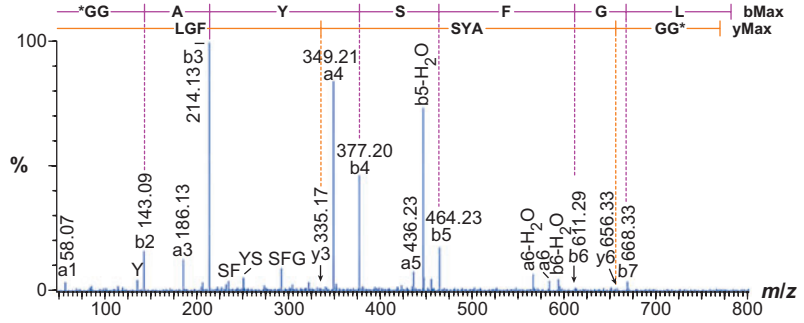
a **Reductive methylation reaction scheme**



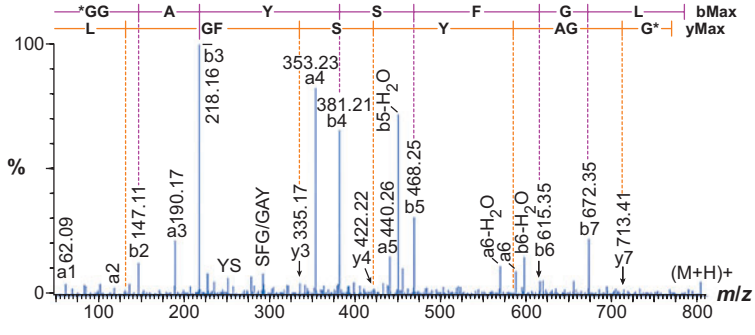
b **GGAYSFGLamide (770.29^{+})**



c **$(CH_3)_2$GGAYSFGLamide (798.45^{+})**



d **$(CHD_2)_2$GGAYSFGLamide (802.51^{+})**



number of Lys or methionine residues in a sequence, thereby serving as an additional constraint for peptide identification with higher confidence (101).

4.3.3. Posttranslational modification characterization. Neuropeptide prohormones undergo extensive posttranslational cleavages and modifications to generate final bioactive peptides. The discovery and characterization of PTMs often rely on the detection of characteristic mass shifts during mass analysis or sequence-specific fragmentation analysis. Sometimes enzymatic treatment can be combined with mass analysis to probe a specific PTM. For example, by comparing the MALDI mass spectra of an *Aplysia californica* nervous tissue homogenate before and after incubation of pyroglutamate aminopeptidase, a peptide with an N-terminal pyroglutamate modification was readily characterized (102). Alternatively, direct multistage MS/MS analysis enabled the discovery of the first carboxylated neuropeptide (26). Furthermore, for several labile PTMs, such as nitrotyrosine-containing SPs, it appears that the choice of MALDI matrix plays a critical role in detection (103). Finally, a global reduction/alkylation procedure followed by mass profiling and sequencing allowed high-throughput characterization of heavily modified conotoxins containing multiple disulfide bonds (104, 105). For a more detailed examination of PTM characterization in SPs, please refer to a previous review (49).

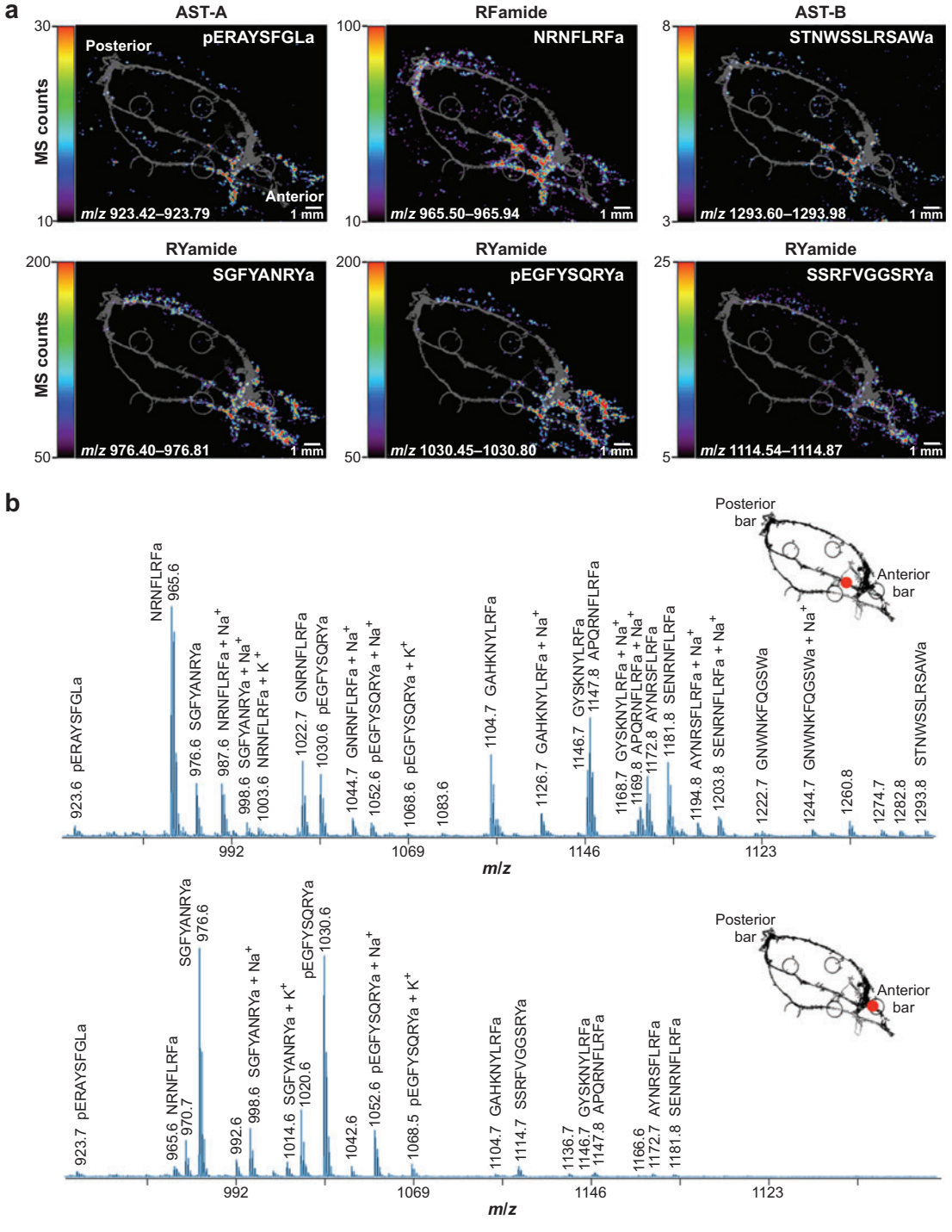
5. IMAGING PEPTIDE DISTRIBUTIONS

The previous sections describe the process of characterizing the peptides in a tissue sample. In addition to knowledge of the chemical form of an SP, an understanding of its specific localization can be important. Several characterization methods, including (perhaps the most obvious method) isolating a particular structure, yield localization information for measurement. Another common approach involves specific stains and microscopy.

For the past century, the ability to image the distribution of a compound within a tissue or single cell has been a powerful tool for scientists, and has often necessitated the development of antibodies or other selective probes. Several MS-based approaches, including secondary ion mass spectrometry (SIMS), have been used for

Figure 5

Reductive methylation via isotopic formaldehyde labeling assists de novo sequencing of neuropeptides by simplifying fragmentation patterns upon derivatization. (*a*) Reductive methylation reaction scheme. (*b–d*) Tandem mass spectrometry (MS/MS) de novo sequencing of GGAYSFGLamide (770.29^{+}). The native peptide displays a highly complex MS/MS spectrum that is difficult to interpret even with the assistance of de novo sequencing software (*b*). However, the formaldehyde labeling results in much cleaner fragmentation spectra (*c* and *d*). By comparing the similar MS/MS fragmentation patterns of the H_2- and D_2-formaldehyde-labeled peptides, the almost-complete a- and b-ion series allowed complete sequencing of the full-length peptide, thereby revealing a new peptide sequence belonging to the A-type allatostatin peptide family. Adapted and modified from Reference 99 with permission.



cellular-level profiling and imaging for decades (106). However, it was MALDI MS imaging (MSI), first demonstrated by Caprioli and advanced by many groups (107–110), that allowed the study of larger peptides and proteins in biological materials. As described by Caprioli et al. (see Reference 11, in this volume), one creates images with MALDI MS by coating the sample with a thin, homogenous layer of matrix prior to the collection of a series of mass spectra from an ordered array of locations across the sample. Following data collection, the intensities of selected signals are plotted for each mass spectrum to produce a series of two-dimensional distribution maps/ion images for each individual selected *m/z*. Because a complete mass spectrum is collected for each location in the sample, literally hundreds of ion images, each of a different compound, are created from each experiment. A recent special issue of the *International Journal of Mass Spectrometry* devoted to MSI highlights the capabilities of this approach (111), and several recent reviews describe the state of the art in greater detail for a range of applications including characterization of peptides (109, 112, 113).

Mass spectrometric imaging (MSI): creates molecular ion images by rastering laser beam across a tissue sample and collecting the resultant mass spectra from an ordered array of locations across the sample

MSI is useful for localizing SPs in brain slices and has produced impressive results from a wide range of samples. MSI has been used to characterize a range of peptides, including endogenous peptides and tryptic fragments of proteins (114, 115). Several earlier MSI studies described the characterization of SPs (116, 117). Because of the cell-cell heterogeneity of peptides, efforts to improve the spatial resolution have also focused on SPs. These include methods to achieve cellular-length scales using stigmatic imaging (112, 118), a spatial resolution at a cellular scale smaller than the laser beam diameter using oversampling (119), and massively parallel cell-sized sampling via the stretched-sample method (60). Besides MALDI MS, SIMS has been used to characterize the distribution of APGWamide, a smaller neuropeptide, in the central nervous system of *L. stagnalis* at high spatial resolution (120). Using *A. californica* as an example, both profiling and imaging of individual neurons have been achieved (121).

The current state of the art for peptide MSI is exemplified by three recent studies that demonstrated MS/MS on selected signals to verify peptide assignments, optimized sample preparation to minimize analyte migration during tissue preparation, and used well-characterized neuronal samples so that the performance could be evaluated. Specifically, Altelaar et al. (112) examined a series of pituitary peptides using stigmatic imaging and obtained an effective resolving power of 4 μm. Combining high-resolution MS with MSI, DeKeyser et al. (122) characterized more than 30 SPs belonging to 10 neuropeptide families, several with distinct spatial patterns (see **Figure 6**). Finally, Verhaert (123) characterized a complex suite of peptides in the brain–corpus cardiacum axis of insects. Future research will lead to improvements in throughput, sample preparation strategies, and spatial resolution of such

Figure 6

Images from the pericardial organ of a crab and the resultant mass spectra showing a differential distribution of peptides from the RYamide and RFamide peptide families. The colors in the images relate to the intensity of the specific mass spectral peak intensities. Adapted from Reference 122 with permission.

neuropeptide MSI experiments, and will also introduce a range of additional applications. We expect a great increase in the knowledge of regional differences in peptide processing as the use of this technology becomes more widespread.

6. QUANTITATION

Quantitative analysis by MS is complicated by the heterogeneity of ionization and the unpredictable discrimination and suppression effects of complex mixtures. Several strategies have been developed to address these issues. An accurate approach employs a corresponding stable isotope–incorporated synthetic peptide for each peptide of interest as the internal standard (124, 125). The drawback of this approach, however, is the need to synthesize isotope-incorporated peptide for each native peptide of interest. For a global-scale survey of relative quantitative changes, numerous isotope-labeling methods have been reported (95, 96, 126, 127). These methodologies produce chemical counterparts of the analytes of interest; these counterparts possess an identical chemical structure but a distinguishable mass difference.

Although the isotope-coded affinity tag (ICAT) approach is widely applicable for protein quantitation, in its original form it is limited to cysteine-containing proteins/peptides. Few SPs contain cysteine residues. Other labeling reagents have been designed for more generic labeling, such as acetylation of the N terminus and the ε-amino group of Lys residues by acetic anhydride (-H_6 and -D_6) (128). Acetylation of basic amino groups, however, may cause a substantial loss of sensitivity for peptides without a C-terminal charge due to the reduction of charge states. An alternative general labeling approach relies on methylation using d0- or d3-methanol (96). This method suffers from the limitation that peptides with C-terminal amidation and without aspartic or glutamic acids will not be labeled. Recently, Fricker and colleagues (129) reported the use of active esters of trimethylammoniumbutyrate containing either nine deuteriums (heavy) or nine hydrogen atoms (light) for quantitative peptidomic applications. The N-hydroxysuccinimide ester of the trimethylammoniumbutyrate reagent reacted with primary amines at the N terminus and Lys side chain. Successful applications of this isotopically encoded reagent enabled the assessment of relative peptidomic changes in response to food intake or drug treatment in mouse models (17, 130, 131).

Another labeling technique is reductive methylation, which targets the N-terminal amine and Lys side chain using isotopically encoded formaldehyde reagents. This dimethyl labeling strategy labels the N terminus and ε-amino group of Lys through reductive amination, producing peaks that differ by 28 Da for each derivatized site and 4 Da (H_2/D_2) for each derivatized isotopic pair (132). As outlined in the de novo sequencing section, isotopic formaldehyde labeling provides both enhanced quantitation and improved fragmentation capability. Of course, this labeling approach would not work for SPs with blocked N termini (pyroglutamate or acetylated amino acid).

Recently, a novel analyte-ion combination scheme that has the potential to enable direct tissue mass spectral quantitation was reported (133). The potential application of this method for quantitative comparison of neuropeptides directly from individual

neurons or tissues isolated from different physiological states will have a great impact in SP research.

7. PEPTIDE FUNCTION: DYNAMIC MEASUREMENTS

Microdialysis: in vivo sampling technique based on size-selective diffusion of the analyte through a semipermeable membrane that collects peptides and/or smaller molecules for further characterization

An SP is not active in cell-cell signaling while inside the cell; it must be released in order to function. Under what conditions and in what chemical form is a specific SP released? Such information is not easy to obtain by directly sampling a brain region, and so a number of approaches have been developed to sample SPs released from neurons. Stated differently, whereas a microliter of brain may contain 100,000 cells and literally tens of thousands of proteins and peptides, only a small fraction of those peptides are released as SPs. How can one selectively measure the release of peptides from a tissue?

Investigations of SP secretion from biological tissues have employed a number of approaches, each with its own advantages and disadvantages (see, e.g., References 134 and 135). Direct detection of SPs can be accomplished by sampling extracellular media such as the blood or the extracellular fluids surrounding neurohemal sites to study hormones.

Preselected peptides in these releasates are often detected via radioimmunoassays. Although it is an exquisitely sensitive technique, radiochemical detection of peptidergic release requires preselection of the peptide of interest, and has a limited ability to monitor multiple peptides during the same experiment. Other approaches, such as amperometric detection (136), offer improved temporal and spatial resolution. The development of approaches that combine release sampling with MS has become particularly important for characterizing unknown SPs.

Both microdialysis and push-pull perfusion are exemplary methods for collecting SP release from localized regions (137–139). These approaches rely on the insertion of a sampling probe into a specific brain region that collects the extracellular peptides. Greater detail on the technology, protocols, and capabilities of microdialysis sampling of SPs can be found in several recent reviews (140–142). Because the peptides are released from a cell/neurohemal release area and are rapidly diluted by diffusion from localized release sites, the concentrations are often in the picomolar-to-nanomolar range, with sample volumes in the nanoliter-to-microliter range. Thus, the collected material is often introduced to capillary-scale separations and requires high-sensitivity MS (75, 76, 140, 143, 144).

What other methods can be used? For brain slices and semi-intact preparations, a number of additional strategies are available for sampling SP release. For example, detection of activity-dependent release of peptides from brain regions as well as from single cultured neurons has been reported using a combination of electrophoretic separation and solid-phase extraction (SPE) (47, 145). Such approaches have been improved by using individual SPE beads placed near the cell or region of interest (146). Because the brain slice is accessible and the bead can be precisely located even over a specific release zone, localized release can be followed, and precise chemical stimulations can be applied while collecting peptide releasate. Using this approach, the release of unexpected peptides has been confirmed for several systems (26, 147).

We expect continued improvement in sampling methods adapted to collecting SP release from the brain. Dynamic information is crucial to understanding peptide function and is among the most difficult types of data to obtain from SP measurements.

8. BIOINFORMATICS

Identification and characterization of neuropeptides can be achieved in several ways, depending on the type of MS data and database availability. The simplest way is by comparing experimental masses against a list of known neuropeptide masses via manual comparison or by automated searching using a database such as SwePep (**http://www.swepep.org/**) (148).

As mentioned previously, a neuropeptide prohormone is processed into multiple gene products by the actions of a number of enzymes. During the past several years, we have created bioinformatics tools to aid in predicting the final peptide forms from a prohormone; these tools use binary logistic regression models, trained on the neuropeptides that we and others have characterized, to identify cleaved basic sites among the many possible cleavage sites in a prohormone (30, 149). Thus, one can make well-informed decisions about the expected neuropeptides from a novel prohormone. These prediction tools are accessible via our NeuroPred suite, available at **http://neuroproteomics.scs.uiuc.edu/neuropred.html** (150). The site also contains lists of prohormones and their associated peptides for a variety of common neuronal models.

As described above, there are two common methods of performing MS analysis of brain tissues: direct profiling via MALDI and ESI-MS/MS of extracts. Direct profiling of a cell or tissue has an advantage in that many peptides from a specific prohormone are detected at the same time. When multiple peptides are detected using accurate mass measurements even without MS/MS, the ability to confidently assign the peaks is enhanced. Single-cell MS is an extreme example where often a significant fraction of a prohormone sequence can be covered with accurate mass MALDI MS measurements.

For extracts or larger brain regions, one gains the benefits of working with a larger amount of material but loses the advantage of prohormone coverage due to greater sample complexity. In such cases, the ability to sequence novel neuropeptides can be critical. Neuropeptide sequencing requires an instrument capable of producing fragment ions (via MS/MS). Depending on instrument availability and the species of interest, a number of different data analysis strategies can be employed for MS/MS neuropeptide identification. If the species of interest has a sequenced genome, a standard protein database search can be performed using proteomic-based search engines such as Mascot (Matrix Science, **http://www.matrixscience.com/**) or SEQUEST (Thermo Corp., **http://www.thermo.com/**) (151). Although these search engines were designed for proteomics applications, they are easily adapted to neuropeptidomics.

Another way to identify neuropeptides using a database is by sequence tag searching. In contrast to Mascot and SEQUEST, which base identifications on parent masses and MS/MS fragmentation patterns, sequence tag searches are performed

using small contiguous strings of identified amino acids (sequence tags) compared against sequences in the database (152). Because sequence tag searching does not rely on parent mass identification, it is a powerful technique for identifying SPs containing PTMs. For example, MS-Seq (Protein Prospector, **http://prospector.ucsf.edu/**) and Mascot offer sequence tag searching for single tag queries. Both interfaces are flexible and powerful but can be time consuming if analyzing a large volume of data.

If the species of interest does not have a sequenced genome, de novo sequencing of the neuropeptide is required. In contrast to database searching, de novo peptide sequencing represents the direct "reading" of the amino acid sequence from the MS/MS spectrum. With a great deal of experience, de novo sequencing can be performed manually. However, this is extremely labor intensive and time consuming. Fortunately, there exist several software packages that can perform de novo sequencing directly on MS/MS data, including LuteFisk (**http://www.hairyfatguy.com/lutefisk/**), PepSeq (packaged with Waters's MassLynx software, **http://www.waters.com/**), Mascot Distiller (Matrix Science), and PEAKS (Bioinformatics Solutions, **http://www.bioinformaticssolutions.com/**). Many of these programs, such as PEAKS and Mascot Distiller, include automated de novo processing to enhance sequencing speed and flexibility.

De novo sequencing does not always produce an accurate sequence for the entire SP. In this case, a BLAST homology search (**http://www.ncbi.nlm.nih.gov/BLAST/**) can be performed. A BLAST search compares a partial SP sequence against the database of a closely related species. Although a homology search rarely yields a complete sequence, it can provide useful information about the evolutionary origins and potential function of the partially sequenced SP. More recently, the use of pattern finding software such as SPIDER (Bioinformatics Solutions) and MEME (**http://meme.sdsc.edu/meme/**) has improved partial sequence homology searches (153). These programs are optimized for MS/MS–derived data and are more tolerant of sequencing errors than BLAST searches. We certainly expect that bioinformatics tools tailored to the unique features of SPs will continue to expand.

9. FUTURE DIRECTIONS

It is evident that the increasing number of SPs described for every animal model reflects significant recent advances in MS. We expect that the number of identified SPs, as well as information on their localization and processing, will continue to increase for all animals. Although peptide characterization by MS is generally a well-developed area, the field of neuropeptidomics requires additional developments in instrumentation and protocol, especially for dynamic and spatially resolved information on very small samples. As the methods of localized sampling and even activity-dependent sampling are further refined, information on dynamics will be combined with information on chemical form and spatial localization to enable a more comprehensive understanding of peptidergic signaling.

It is interesting to speculate whether the vast diversity of peptides found in the brain all contribute to and influence behavior, or whether many of these shortened and processed forms are simply peptides on their way to being catabolized. In other

words, even though many peptides are present, we do not yet know how many of the peptides are actually used by each brain circuit, nor how the circuit activity depends on dynamic changes in the extracellular peptide cocktail. Only with improved analytical measurement tools such as MSI, single-cell MS, and bioinformatics tools can these questions be addressed. Thirty years after the beginning of neuropeptide research, the vast scope of SP complexity is still being investigated.

SUMMARY POINTS

1. MS has become the method of choice for neuropeptide analysis due to its high speed, low detection limits, and the impressive chemical information it provides. The ever-increasing number of new peptides detected in a wide range of animal model systems reflects the advancements of mass spectrometric measurements and improved sampling approaches.
2. No single measurement platform can simultaneously provide chemical, spatial, and temporal information content for probing SPs in the brain. Therefore, a multifaceted approach that combines direct tissue profiling/imaging, peptide sequencing, and dynamic release measurements using a wide array of separation- and MS-based instrumentation is needed to provide a more complete picture of peptide signaling.
3. Given the sensitivity of MS-based approaches and their capability to measure a large number of peptides simultaneously, it is important to develop specialized sample preparation strategies for different types of neural samples in order to obtain biologically relevant results.
4. The development of direct tissue profiling and single-cell MS over the past decade has contributed significantly to neuropeptide research. Further improvements in sampling protocols, mass measurement accuracy, and microscale chemical derivatization will further improve their application to single-cell peptidomics.

FUTURE ISSUES

1. Despite continued development of microscale sampling approaches, dynamic measurement of SP release remains an analytical challenge. Future developments should address issues regarding sensitivity and temporal resolution.
2. MSI has become a powerful tool for exploring the spatial distribution of SPs throughout tissue sections. Future work should focus on the development of improved spatial resolution to enable imaging of peptide distribution at the subcellular level. Instrumentation advancement and data handling capability will be equally important for higher-throughput imaging analysis.

3. Although numerous chemical reagents have been developed for improved de novo sequence analysis of SPs, only a limited range of SPs can be sequenced. Several electron-based MS/MS fragmentation methods have shown great promise for sequencing large peptides and small proteins while preserving labile PTMs. These newer MS/MS techniques, coupled with improvements in instrumentation, will prompt a new generation of studies in peptidomics.
4. Various bioinformatic tools and algorithms have been developed to derive peptide/protein matches via either identity or homology searching. Currently, it is not efficient to query protein databases constructed largely from translated DNA sequences with neuropeptide MS/MS data. New and improved bioinformatics tools tailored to the unique features of SPs are needed and have the potential to accelerate neuropeptide discovery.

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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LITERATURE CITED

1. Sandman CA, Strand FL, Beckwith B, Chronwall BM, Flynn FW, Nachman RJ, eds. 1999. *Neuropeptides: Structure and Function in Biology and Behavior*, Vol. 897. New York: Ann. N.Y. Acad. Sci. 443 pp.
2. Strand FL. 1999. *Neuropeptides: Regulators of Physiological Processes*. Cambridge, MA: MIT Press. 658 pp.
3. Kastin AJ. 2006. *Handbook of Biologically Active Peptides*. Amsterdam/Boston: Academic. 1595 pp.
4. Jensen J. 2001. Regulatory peptides and control of food intake in nonmammalian vertebrates. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 128:471–79
5. Gundlach AL, Burazin TC, Larm JA. 2001. Distribution, regulation and role of hypothalamic galanin systems: renewed interest in a pleiotropic peptide family. *Clin. Exp. Pharmacol. Physiol.* 28:100–5

6. Levin ER, Hu RM, Rossi M, Pickart M. 1992. Arginine vasopressin stimulates atrial natriuretic peptide gene expression and secretion from rat diencephalic neurons. *Endocrinology* 131:1417–23
7. Okada Y, Tsuda Y, Bryant SD, Lazarus LH. 2002. Endomorphins and related opioid peptides. *Vitam. Horm.* 65:257–79
8. Kieffer BL, Gaveriaux-Ruff C. 2002. Exploring the opioid system by gene knockout. *Prog. Neurobiol.* 66:285–306
9. Stefano GB, Fricchione G, Goumon Y, Esch T. 2005. Pain, immunity, opiate and opioid compounds and health. *Med. Sci. Monit.* 11:MS47–53
10. Marshall AG, Hendrickson CL. 2008. High-resolution mass spectrometers. *Annu. Rev. Anal. Chem.* 1:579–99
11. Burnum KE, Frappier SL, Caprioli R. 2008. Matrix-assisted laser desorption/ionization imaging mass spectrometry for the investigation of proteins and peptides. *Annu. Rev. Anal. Chem.* 1:689–705
12. Borland LM, Kottegoda S, Phillips KS, Allbritton NL. 2008. Chemical analysis of single cells. *Annu. Rev. Anal. Chem.* 1:191–227
13. Lindsten J, ed. 1992. *Nobel Lectures, Physiology or Medicine 1971–1980*. Singapore: World Sci. Publ. Co.
14. Clynen E, Baggerman G, Veelaert D, Cerstiaens A, Van der Horst D, et al. 2001. Peptidomics of the pars intercerebralis–corpus cardiacum complex of the migratory locust, *Locusta migratoria*. *Eur. J. Biochem.* 268:1929–39
15. Schulz-Knappe P, Zucht HD, Heine G, Jurgens M, Hess R, Schrader M. 2001. Peptidomics: the comprehensive analysis of peptides in complex biological mixtures. *Comb. Chem. High Throughput Screen.* 4:207–17
16. Verhaert P, Uttenweiler-Joseph S, de Vries M, Loboda A, Ens W, Standing KG. 2001. Matrix-assisted laser desorption/ionization quadrupole time-of-flight mass spectrometry: an elegant tool for peptidomics. *Proteomics* 1:118–31
17. Che FY, Vathy I, Fricker LD. 2006. Quantitative peptidomics in mice: effect of cocaine treatment. *J. Mol. Neurosci.* 28:265–75
18. Jost MM, Budde P, Tammen H, Hess R, Kellmann M, et al. 2005. The concept of functional peptidomics for the discovery of bioactive peptides in cell culture models. *Comb. Chem. High Throughput Screen.* 8:767–73
19. Soloviev M, Finch P. 2006. Peptidomics: bridging the gap between proteome and metabolome. *Proteomics* 6:744–47
20. Svensson M, Skold K, Svenningsson P, Andren PE. 2003. Peptidomics-based discovery of novel neuropeptides. *J. Proteome Res.* 2:213–19
21. Wei H, Nolkrantz K, Parkin MC, Chisolm CN, O'Callaghan JP, Kennedy RT. 2006. Identification and quantification of neuropeptides in brain tissue by capillary liquid chromatography coupled off-line to MALDI-TOF and MALDI-TOF/TOF-MS. *Anal. Chem.* 78:4342–51
22. Emanuelsson O, Brunak S, von Heijne G, Nielsen H. 2007. Locating proteins in the cell using TargetP, SignalP and related tools. *Nat. Protoc.* 2:953–71
23. Emanuelsson O, Nielsen H, Brunak S, von Heijne G. 2000. Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *J. Mol. Biol.* 300:1005–16

24. Kandel E, Schwartz JJ, Jessell TM. 2000. *Principles of Neural Science*. New York: McGraw-Hill
25. Floyd PD, Li L, Rubakhin SS, Sweedler JV, Horn CC, et al. 1999. Insulin prohormone processing, distribution, and relation to metabolism in *Aplysia californica*. *J. Neurosci.* 19:7732–41
26. Jakubowski JA, Hatcher NG, Xie F, Sweedler JV. 2006. The first gamma-carboxyglutamate-containing neuropeptide. *Neurochem. Int.* 49:223–29
27. Seidah NG. 2001. Cellular limited proteolysis of precursor proteins and peptides. In *The Enzymes*, ed. RE Dalbey, DS Sigman, pp. 237–58. San Diego, CA: Academic
28. Steiner D. 2001. The prohormone convertases and precursor processing in protein biosynthesis. In *The Enzymes*, ed. RE Dalby, DS Sigman, pp. 163–98. San Diego, CA: Academic
29. Devi L. 1991. Consensus sequence for processing of peptide precursors at monobasic sites. *FEBS Lett.* 280:189–94
30. Amare A, Hummon AB, Southey BR, Zimmerman TA, Rodriguez-Zas SL, Sweedler JV. 2006. Bridging neuropeptidomics and genomics with bioinformatics: prediction of mammalian neuropeptide prohormone processing. *J. Proteome Res.* 5:1162–67
31. Lindberg I, Hutton JC. 1991. Peptide processing proteinases with selectivity for paired basic residues. In *Peptide Biosynthesis and Processing*, ed. LD Fricker, pp. 141–74. Boca Raton, FL: CRC
32. Rholam M, Brakch N, Germain D, Thomas DY, Fahy C, et al. 1995. Role of amino acid sequences flanking dibasic cleavage sites in precursor proteolytic processing: the importance of the first residue C-terminal of the cleavage site. *Eur. J. Biochem.* 227:707–14
33. Cameron A, Apletalina EV, Lindberg I. 2001. The enzymology of PC1 and PC2. In *The Enzymes*, ed. RE Dalbey, DS Sigman, pp. 291–332. San Diego, CA: Academic
34. Pow DV, Morris JF. 1989. Dendrites of the hypothalamic magnocellular neurons release neurohypophysal peptides by exocytosis. *Neuroscience* 32:435–39
35. Sabatier N, Caquineau C, Douglas AJ, Leng G. 2003. Oxytocin released from magnocellular dendrites. *Ann. N.Y. Acad. Sci.* 994:218–24
36. Fujisawa Y, Furukawa Y, Ohta S, Ellis TA, Dembrow NC, et al. 1999. The *Aplysia mytilus* inhibitory peptide-related peptides: identification, cloning, processing, distribution, and action. *J. Neurosci.* 19:9618–34
37. Furukawa Y, Nakamaru K, Wakayama H, Fujisawa Y, Minakata H, et al. 2001. The enterins: a novel family of neuropeptides isolated from the enteric nervous system and CNS of *Aplysia*. *J. Neurosci.* 21:8247–61
38. Sweedler JV, Li L, Rubakhin SS, Alexeeva V, Dembrow NC, et al. 2002. Identification and characterization of the feeding circuit-activating peptides, a novel neuropeptide family of *Aplysia*. *J. Neurosci.* 22:7797–808
39. Baertschi AJ, Monnier D, Schmidt U, Levitan ES, Fakan S, Roatti A. 2001. Acid prohormone sequence determines size, shape, and docking of secretory vesicles in atrial myocytes. *Circ. Res.* 89:E23–29

40. Shafqat J, Melles E, Sigmundsson K, Johansson BL, Ekberg K, et al. 2006. Proinsulin C-peptide elicits disaggregation of insulin resulting in enhanced physiological insulin effects. *Cell Mol. Life. Sci.* 63:1805–11
41. Steiner DF. 2004. The proinsulin C-peptide: a multirole model. *Exp. Diabesity Res.* 5:7–14
42. Edison AS, Espinoza E, Zachariah C. 1999. Conformational ensembles: the role of neuropeptide structures in receptor binding. *J. Neurosci.* 19:6318–26
43. Santama N, Benjamin PR. 2000. Gene expression and function of FMRFamide-related neuropeptides in the snail *Lymnaea*. *Microsc. Res. Tech.* 49:547–56
44. von Bohlen und Halbach O. 2005. The renin-angiotensin system in the mammalian central nervous system. *Curr. Protein Pept. Sci.* 6:355–71
45. Predel R. 2001. Peptidergic neurohemal system of an insect: mass spectrometric morphology. *J. Comp. Neurol.* 436:363–75
46. Neupert S, Predel R, Russell WK, Davies R, Pietrantonio PV, Nachman RJ. 2005. Identification of tick periviscerokinin, the first neurohormone of *Ixodidae*: single cell analysis by means of MALDI-TOF/TOF mass spectrometry. *Biochem. Biophys. Res. Commun.* 338:1860–64
47. Garden RW, Moroz LL, Moroz TP, Shippy SA, Sweedler JV. 1996. Excess salt removal with matrix rinsing: direct peptide profiling of neurons from marine invertebrates using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J. Mass Spectrom.* 31:1126–30
48. Li L, Garden RW, Sweedler JV. 2000. Single-cell MALDI: a new tool for direct peptide profiling. *Trends Biotechnol.* 18:151–60
49. Hummon AB, Amare A, Sweedler JV. 2006. Discovering new invertebrate neuropeptides using mass spectrometry. *Mass Spectrom. Rev.* 25:77–98
50. Kutz KK, Schmidt JJ, Li L. 2004. In situ tissue analysis of neuropeptides by MALDI FTMS in-cell accumulation. *Anal. Chem.* 76:5630–40
51. Fournier I, Day R, Salzet M. 2003. Direct analysis of neuropeptides by in situ MALDI-TOF mass spectrometry in the rat brain. *Neuro. Endocrinol. Lett.* 24:9–14
52. DeKeyser SS, Li L. 2007. Mass spectrometric charting of neuropeptides in arthropod neurons. *Anal. Bioanal. Chem.* 387:29–35
53. Neupert S, Predel R. 2005. Mass spectrometric analysis of single identified neurons of an insect. *Biochem. Biophys. Res. Commun.* 327:640–45
54. Li L, Romanova EV, Rubakhin SS, Alexeeva V, Weiss KR, et al. 2000. Peptide profiling of cells with multiple gene products: combining immunochemistry and MALDI mass spectrometry with on-plate microextraction. *Anal. Chem.* 72:3867–74
55. Li L, Garden RW, Romanova EV, Sweedler JV. 1999. In situ sequencing of peptides from biological tissues and single cells using MALDI-PSD/CID analysis. *Anal. Chem.* 71:5451–58
56. Yew JY, Dikler S, Stretton AO. 2003. De novo sequencing of novel neuropeptides directly from *Ascaris suum* tissue using matrix-assisted laser desorption/ionization time-of-flight/time-of-flight. *Rapid Commun. Mass Spectrom.* 17:2693–98

57. Jespersen S, Chaurand P, van Strien FJ, Spengler B, van der Greef J. 1999. Direct sequencing of neuropeptides in biological tissue by MALDI-PSD mass spectrometry. *Anal. Chem.* 71:660–66
58. Redeker V, Toullec JY, Vinh J, Rossier J, Soyez D. 1998. Combination of peptide profiling by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and immunodetection on single glands or cells. *Anal. Chem.* 70:1805–11
59. Rubakhin SS, Garden RW, Fuller RR, Sweedler JV. 2000. Measuring the peptides in individual organelles with mass spectrometry. *Nat. Biotechnol.* 18:172–75
60. Monroe EB, Jurchen JC, Koszczuk BA, Losh JL, Rubakhin SS, Sweedler JV. 2006. Massively parallel sample preparation for the MALDI MS analyses of tissues. *Anal. Chem.* 78:6826–32
61. Rubakhin SS, Churchill JD, Greenough WT, Sweedler JV. 2006. Profiling signaling peptides in single mammalian cells using mass spectrometry. *Anal. Chem.* 78:7267–72
62. Rubakhin SS, Sweedler JV. 2007. Characterizing peptides in individual mammalian cells using mass spectrometry. *Nat. Protoc.* 2:1987–97
63. Che FY, Lim J, Pan H, Biswas R, Fricker LD. 2005. Quantitative neuropeptidomics of microwave-irradiated mouse brain and pituitary. *Mol. Cell Proteomics* 4:1391–405
64. Dockray GJ. 1976. Immunochemical evidence of cholecystokinin-like peptides in brain. *Nature* 264:568–70
65. Dowell JA, Vander Heyden W, Li L. 2006. Rat neuropeptidomics by LC-MS/MS and MALDI-FTMS: enhanced dissection and extraction techniques coupled with 2D RP-RP HPLC. *J. Proteome Res.* 5:3368–75
66. Fu Q, Kutz KK, Schmidt JJ, Hsu YW, Messinger DI, et al. 2005. Hormone complement of the *Cancer productus* sinus gland and pericardial organ: an anatomical and mass spectrometric investigation. *J. Comp. Neurol.* 493:607–26
67. Fu Q, Goy MF, Li L. 2005. Identification of neuropeptides from the decapod crustacean sinus glands using nanoscale liquid chromatography tandem mass spectrometry. *Biochem. Biophys. Res. Commun.* 337:765–78
68. Schoofs L, Holman GM, Hayes TK, Nachman RJ, De Loof A. 1991. Isolation, primary structure, and synthesis of locustapyrokinin: a myotropic peptide of *Locusta migratoria*. *Gen. Comp. Endocrinol.* 81:97–104
69. Baggerman G, Boonen K, Verleyen P, De Loof A, Schoofs L. 2005. Peptidomic analysis of the larval *Drosophila melanogaster* central nervous system by two-dimensional capillary liquid chromatography quadrupole time-of-flight mass spectrometry. *J. Mass Spectrom.* 40:250–60
70. Washburn MP, Wolters D, Yates JR 3rd. 2001. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nat. Biotechnol.* 19:242–47
71. Wolters DA, Washburn MP, Yates JR 3rd. 2001. An automated multidimensional protein identification technology for shotgun proteomics. *Anal. Chem.* 73:5683–90
72. Peng J, Gygi SP. 2001. Proteomics: the move to mixtures. *J. Mass Spectrom.* 36:1083–91

73. Baggerman G, Cerstiaens A, De Loof A, Schoofs L. 2002. Peptidomics of the larval *Drosophila melanogaster* central nervous system. *J. Biol. Chem.* 277:40368–74
74. Skold K, Svensson M, Kaplan A, Bjorkesten L, Astrom J, Andren PE. 2002. A neuroproteomic approach to targeting neuropeptides in the brain. *Proteomics* 2:447–54
75. Haskins WE, Wang Z, Watson CJ, Rostand RR, Witowski SR, et al. 2001. Capillary LC-MS2 at the attomole level for monitoring and discovering endogenous peptides in microdialysis samples collected in vivo. *Anal. Chem.* 73:5005–14
76. Haskins WE, Watson CJ, Cellar NA, Powell DH, Kennedy RT. 2004. Discovery and neurochemical screening of peptides in brain extracellular fluid by chemical analysis of in vivo microdialysis samples. *Anal. Chem.* 76:5523–33
77. Husson SJ, Clynen E, Baggerman G, De Loof A, Schoofs L. 2005. Discovering neuropeptides in *Caenorhabditis elegans* by two dimensional liquid chromatography and mass spectrometry. *Biochem. Biophys. Res. Commun.* 335:76–86
78. Floyd PD, Li L, Moroz TP, Sweedler JV. 1999. Characterization of peptides from *Aplysia* using microbore liquid chromatography with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry guided purification. *J. Chromatogr. A* 830:105–13
79. Wei H, Nolkrantz K, Powell DH, Woods JH, Ko MC, Kennedy RT. 2004. Electrospray sample deposition for matrix-assisted laser desorption/ionization (MALDI) and atmospheric pressure MALDI mass spectrometry with attomole detection limits. *Rapid Commun. Mass Spectrom.* 18:1193–200
80. Wei H, Dean SL, Parkin MC, Nolkrantz K, O'Callaghan JP, Kennedy RT. 2005. Microscale sample deposition onto hydrophobic target plates for trace level detection of neuropeptides in brain tissue by MALDI-MS. *J. Mass Spectrom.* 40:1338–46
81. Page JS, Rubakhin SS, Sweedler JV. 2002. Single-neuron analysis using CE combined with MALDI MS and radionuclide detection. *Anal. Chem.* 74:497–503
82. Page JS, Rubakhin SS, Sweedler JV. 2000. Direct cellular assays using off-line capillary electrophoresis with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Analyst* 125:555–62
83. Zhang H, Caprioli RM. 1996. Capillary electrophoresis combined with matrix-assisted laser desorption/ionization mass spectrometry: continuous sample deposition on a matrix-precoated membrane target. *J. Mass Spectrom.* 31:1039–46
84. Zhang H, Stoeckli M, Andren PE, Caprioli RM. 1999. Combining solid-phase preconcentration, capillary electrophoresis and off-line matrix-assisted laser desorption/ionization mass spectrometry: intracerebral metabolic processing of peptide E in vivo. *J. Mass Spectrom.* 34:377–83
85. Kupka KD, Schropp WW, Schiller C, Hillenkamp F. 1980. Laser-micro-mass analysis (LAMMA) of metallic and organic ions in medical samples. *Scan. Electron Microsc.* II: 635–40
86. Meyer zum Gottesberge-Orsulakova A, Kaufmann R. 1985. Recent advances in laser microprobe mass analysis (LAMMA) of inner ear tissue. *Scan. Electron Microsc.* VII:393–405

87. Kaufmann R, Hillenkamp F, Nitsche R, Schurmann M, Wechsung R. 1978. The laser microprobe mass analyser (LAMMA): biomedical applications. *Microsc. Acta* Suppl.(2):297–306
88. Jimenez CR, van Veelen PA, Li KW, Wildering WC, Geraerts WP, et al. 1994. Neuropeptide expression and processing as revealed by direct matrix-assisted laser desorption ionization mass spectrometry of single neurons. *J. Neurochem.* 62:404–7
89. Li KW, Hoek RM, Smith F, Jimenez CR, van der Schors RC, et al. 1994. Direct peptide profiling by mass spectrometry of single identified neurons reveals complex neuropeptide-processing pattern. *J. Biol. Chem.* 269:30288–92
90. Clynen E, De Loof A, Schoofs L. 2003. The use of peptidomics in endocrine research. *Gen. Comp. Endocrinol.* 132:1–9
91. Hummon AB, Richmond TA, Verleyen P, Baggerman G, Huybrechts J, et al. 2006. From the genome to the proteome: uncovering peptides in the *Apis* brain. *Science* 314:647–49
92. Perry SJ, Dobbins AC, Schofield MG, Piper MR, Benjamin PR. 1999. Small cardioactive peptide gene: structure, expression and mass spectrometric analysis reveals a complex pattern of cotransmitters in a snail feeding neuron. *Eur. J. Neurosci.* 11:655–62
93. Predel R, Russell WK, Tichy SE, Russell DH, Nachman RJ. 2003. Mass spectrometric analysis of putative capa-gene products in *Musca domestica* and *Neobellieria bullata*. *Peptides* 24:1487–91
94. Stemmler EA, Provencher HL, Guiney ME, Gardner NP, Dickinson PS. 2005. Matrix-assisted laser desorption/ionization Fourier transform mass spectrometry for the identification of orcokinin neuropeptides in crustaceans using metastable decay and sustained off-resonance irradiation. *Anal. Chem.* 77:3594–606
95. Munchbach M, Quadroni M, Miotto G, James P. 2000. Quantitation and facilitated de novo sequencing of proteins by isotopic N-terminal labeling of peptides with a fragmentation-directing moiety. *Anal. Chem.* 72:4047–57
96. Goodlett DR, Keller A, Watts JD, Newitt R, Yi EC, et al. 2001. Differential stable isotope labeling of peptides for quantitation and de novo sequence derivation. *Rapid Commun. Mass Spectrom.* 15:1214–21
97. Ma M, Kutz-Naber KK, Li L. 2007. Methyl esterification assisted MALDI FTMS characterization of the orcokinin neuropeptide family. *Anal. Chem.* 79:673–81
98. Hsu JL, Huang SY, Shiea JT, Huang WY, Chen SH. 2005. Beyond quantitative proteomics: signal enhancement of the a1 ion as a mass tag for peptide sequencing using dimethyl labeling. *J. Proteome Res.* 4:101–8
99. Fu Q, Li L. 2005. De novo sequencing of neuropeptides using reductive isotopic methylation and investigation of ESI QTOF MS/MS fragmentation pattern of neuropeptides with N-terminal dimethylation. *Anal. Chem.* 77:7783–95
100. Cruz-Bermudez ND, Fu Q, Kutz-Naber KK, Christie AE, Li L, Marder E. 2006. Mass spectrometric characterization and physiological actions of GAHKNYLRFamide, a novel FMRFamide-like peptide from crabs of the genus *Cancer*. *J. Neurochem.* 97:784–99

101. Yew JY, Kutz KK, Dikler S, Messinger L, Li L, Stretton AO. 2005. Mass spectrometric map of neuropeptide expression in *Ascaris suum*. *J. Comp. Neurol.* 488:396–413
102. Garden RW, Moroz TP, Gleeson JM, Floyd PD, Li L, et al. 1999. Formation of N-pyroglutamyl peptides from N-Glu and N-Gln precursors in *Aplysia* neurons. *J. Neurochem.* 72:676–81
103. Sheeley SA, Rubakhin SS, Sweedler JV. 2005. The detection of nitrated tyrosine in neuropeptides: a MALDI matrix-dependent response. *Anal. Bioanal. Chem.* 382:22–27
104. Jakubowski JA, Sweedler JV. 2004. Sequencing and mass profiling highly modified conotoxins using global reduction/alkylation followed by mass spectrometry. *Anal. Chem.* 76:6541–47
105. Jakubowski JA, Keays DA, Kelley WP, Sandall DW, Bingham JP, et al. 2004. Determining sequences and post-translational modifications of novel conotoxins in *Conus victoriae* using cDNA sequencing and mass spectrometry. *J. Mass Spectrom.* 39:548–57
106. Bellhorn MB, Lewis RK. 1976. Localization of ions in retina by secondary ion mass spectrometry. *Exp. Eye Res.* 22:505–18
107. Caprioli RM, Farmer TB, Gile J. 1997. Molecular imaging of biological samples: localization of peptides and proteins using MALDI-TOF MS. *Anal. Chem.* 69:4751–60
108. Chaurand P, Fouchecourt S, DaGue BB, Xu BJ, Reyzer ML, et al. 2003. Profiling and imaging proteins in the mouse epididymis by imaging mass spectrometry. *Proteomics* 3:2221–39
109. Rubakhin SS, Jurchen JC, Monroe EB, Sweedler JV. 2005. Imaging mass spectrometry: fundamentals and applications to drug discovery. *Drug Discov. Today* 10:823–37
110. Stoeckli M, Chaurand P, Hallahan DE, Caprioli RM. 2001. Imaging mass spectrometry: a new technology for the analysis of protein expression in mammalian tissues. *Nat. Med.* 7:493–96
111. Heeren RM, Sweedler JV. 2007. Imaging mass spectrometric imaging. *Int. J. Mass Spectrom.* 260:89
112. Altelaar AF, Luxembourg SL, McDonnell LA, Piersma SR, Heeren RM. 2007. Imaging mass spectrometry at cellular length scales. *Nat. Protoc.* 2:1185–96
113. McDonnell LA, Heeren RM. 2007. Imaging mass spectrometry. *Mass Spectrom. Rev.* 26:606–43
114. Chaurand P, Schwartz SA, Caprioli RM. 2002. Imaging mass spectrometry: a new tool to investigate the spatial organization of peptides and proteins in mammalian tissue sections. *Curr. Opin. Chem. Biol.* 6:676–81
115. Groseclose MR, Andersson M, Hardesty WM, Caprioli RM. 2007. Identification of proteins directly from tissue: in situ tryptic digestions coupled with imaging mass spectrometry. *J. Mass Spectrom.* 42:254–62
116. Garden RW, Sweedler JV. 2000. Heterogeneity within MALDI samples as revealed by mass spectrometric imaging. *Anal. Chem.* 72:30–36
117. Kruse R, Sweedler JV. 2003. Spatial profiling invertebrate ganglia using MALDI MS. *J. Am. Soc. Mass Spectrom.* 14:752–59

118. Altelaar AFM, Taban IM, McDonnell LA, Verhaert PDEM, Lange RPJD, et al. 2007. High-resolution MALDI imaging mass spectrometry allows localization of peptide distributions at cellular length scales in pituitary tissue sections. *Int. J. Mass Spectrom.* 260:203–21
119. Jurchen JC, Rubakhin SS, Sweedler JV. 2005. MALDI-MS imaging of features smaller than the size of the laser beam. *J. Am. Soc. Mass Spectrom.* 16:1654–59
120. Altelaar AF, van Minnen J, Jimenez CR, Heeren RM, Piersma SR. 2005. Direct molecular imaging of *Lymnaea stagnalis* nervous tissue at subcellular spatial resolution by mass spectrometry. *Anal. Chem.* 77:735–41
121. Rubakhin SS, Greenough WT, Sweedler JV. 2003. Spatial profiling with MALDI MS: distribution of neuropeptides within single neurons. *Anal. Chem.* 75:5374–80
122. DeKeyser SS, Kutz-Naber KK, Schmidt JJ, Barrett-Wilt GA, Li L. 2007. Imaging mass spectrometry of neuropeptides in decapod crustacean neuronal tissues. *J. Proteome Res.* 6:1782–91
123. Verhaert PD, Conaway MCP, Pekar TM, Miller K. 2007. Neuropeptide imaging on an LTQ with vMALDI source: the complete "all-in-one" peptidome analysis. *Int. J. Mass Spectrom.* 260:177–84
124. Desiderio DM, Zhu X. 1998. Quantitative analysis of methionine enkephalin and beta-endorphin in the pituitary by liquid secondary ion mass spectrometry and tandem mass spectrometry. *J. Chromatogr. A* 794:85–96
125. Gobom J, Kraeuter KO, Persson R, Steen H, Roepstorff P, Ekman R. 2000. Detection and quantification of neurotensin in human brain tissue by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Anal. Chem.* 72:3320–26
126. Oda Y, Huang K, Cross FR, Cowburn D, Chait BT. 1999. Accurate quantitation of protein expression and site-specific phosphorylation. *Proc. Natl. Acad. Sci. USA* 96:6591–96
127. Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R. 1999. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat. Biotechnol.* 17:994–99
128. Che F-Y, Fricker LD. 2002. Quantitation of neuropeptides in Cpe^{fat}/Cpe^{fat} mice using differential isotopic tags and mass spectrometry. *Anal. Chem.* 74:3190–98
129. Fricker LD, Lim J, Pan H, Che FY. 2006. Peptidomics: identification and quantification of endogenous peptides in neuroendocrine tissues. *Mass Spectrom. Rev.* 25:327–44
130. Che F-Y, Yuan Q, Kalinina E, Fricker LD. 2005. Peptidomics of $Cpe^{fat/fat}$ mouse hypothalamus: effect of food deprivation and exercise on peptide levels. *J. Biol. Chem.* 280:4451–61
131. Decaillot FM, Che FY, Fricker LD, Devi LA. 2006. Peptidomics of $Cpe^{fat/fat}$ mouse hypothalamus and striatum: effect of chronic morphine administration. *J. Mol. Neurosci.* 28:277–84
132. Hsu JL, Huang SY, Chow NH, Chen SH. 2003. Stable-isotope dimethyl labeling for quantitative proteomics. *Anal. Chem.* 75:6843–52

133. DeKeyser SS, Li L. 2006. Matrix-assisted laser desorption/ionization Fourier transform mass spectrometry quantitation via in-cell combination. *Analyst* 131:281–90
134. Anderson L. 1996. Intracellular mechanisms triggering gonadotrophin secretion. *Rev. Reprod.* 1:193–202
135. Lessmann V, Gottmann K, Malcangio M. 2003. Neurotrophin secretion: current facts and future prospects. *Prog. Neurobiol.* 69:341–74
136. Paras CD, Kennedy RT. 1995. Electrochemical detection of exocytosis at single rat melanotrophs. *Anal. Chem.* 67:3633–37
137. Maidment NT, Brumbaugh DR, Rudolph VD, Erdelyi E, Evans CJ. 1989. Microdialysis of extracellular endogenous opioid peptides from rat brain in vivo. *Neuroscience* 33:549–57
138. Ramirez VD, Chen JC, Nduka E, Lin W, Ramirez AD. 1986. Push-pull perfusion of the hypothalamus and the caudate nucleus in conscious, unrestrained animals. *Ann. N.Y. Acad. Sci.* 473:434–48
139. Ungerstedt U, Hallstrom A. 1987. In vivo microdialysis: a new approach to the analysis of neurotransmitters in the brain. *Life Sci.* 41:861–64
140. Andrén PE, Farmer TB, Klintenberg R. 2002. Endogenous release and metabolism of neuropeptides utilizing in vivo microdialysis microelectrospray mass spectrometry. In *Mass Spectrometry and Hyphenated Techniques in Neuropeptide Research*, ed. J Silberring, R Ekman, pp. 193–213. New York: Wiley
141. Horn TF, Engelmann M. 2001. In vivo microdialysis for nonapeptides in rat brain—a practical guide. *Methods* 23:41–53
142. Kennedy RT, Watson CJ, Haskins WE, Powell DH, Strecker RE. 2002. In vivo neurochemical monitoring by microdialysis and capillary separations. *Curr. Opin. Chem. Biol.* 6:659–65
143. Baseski HM, Watson CJ, Cellar NA, Shackman JG, Kennedy RT. 2005. Capillary liquid chromatography with MS3 for the determination of enkephalins in microdialysis samples from the striatum of anesthetized and freely-moving rats. *J. Mass Spectrom.* 40:146–53
144. Jakubowski JA, Hatcher NG, Sweedler JV. 2005. Online microdialysis–dynamic nanoelectrospray ionization–mass spectrometry for monitoring neuropeptide secretion. *J. Mass Spectrom.* 40:924–31
145. Rubakhin SS, Page JS, Monroe BR, Sweedler JV. 2001. Analysis of cellular release using capillary electrophoresis and matrix-assisted laser desorption/ionization–time of flight mass spectrometry. *Electrophoresis* 22:3752–58
146. Hatcher NG, Richmond TA, Rubakhin SS, Sweedler JV. 2005. Monitoring activity-dependent peptide release from the CNS using single-bead solid-phase extraction and MALDI TOF MS detection. *Anal. Chem.* 77:1580–87
147. Jing J, Vilim FS, Horn CC, Alexeeva V, Hatcher NG, et al. 2007. From hunger to satiety: reconfiguration of a feeding network by *Aplysia* neuropeptide Y. *J. Neurosci.* 27:3490–502
148. Falth M, Skold K, Norrman M, Svensson M, Fenyo D, Andren PE. 2006. SwePep, a database designed for endogenous peptides and mass spectrometry. *Mol. Cell Proteomics* 5:998–1005

149. Hummon AB, Hummon NP, Corbin RW, Li L, Vilim FS, et al. 2003. From precursor to final peptides: a statistical sequence–based approach to predicting prohormone processing. *J. Proteome Res.* 2:650–56
150. Southey BR, Amare A, Zimmerman TA, Rodriguez-Zas SL, Sweedler JV. 2006. NeuroPred: a tool to predict cleavage sites in neuropeptide precursors and provide the masses of the resulting peptides. *Nucleic Acids Res.* 34:W267–72
151. Ducret A, Van Oostveen I, Eng JK, Yates JR 3rd, Aebersold R. 1998. High throughput protein characterization by automated reverse-phase chromatography/electrospray tandem mass spectrometry. *Protein Sci.* 7:706–19
152. Mann M, Wilm M. 1994. Error-tolerant identification of peptides in sequence databases by peptide sequence tags. *Anal. Chem.* 66:4390–99
153. Baggerman G, Liu F, Wets G, Schoofs L. 2005. Bioinformatic analysis of peptide precursor proteins. *Ann. N.Y. Acad. Sci.* 1040:59–65

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