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Advances in Mass Spectrometric Tools for Probing Neuropeptides

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Abstract

Neuropeptides are important mediators in the functionality of the brain and other neurological organs. Because neuropeptides exist in a wide range of concentrations, appropriate characterization methods are needed to provide dynamic, chemical, and spatial information. Mass spectrometry and compatible tools have been a popular choice in analyzing neuropeptides. There have been several advances and challenges, both of which are the focus of this review. Discussions range from sample collection to bioinformatic tools, although avenues such as quantitation and imaging are included. Further development of the presented methods for neuropeptidomic mass spectrometric analysis is inevitable, which will lead to a further understanding of the complex interplay of neuropeptides and other signaling molecules in the nervous system.

Keywords

mass spectrometry; neuropeptidomics; microdialysis; quantitation; mass spectrometric imaging; sample preparation

1. INTRODUCTION

Neuromodulation via signaling peptides can initiate a wide variety of responses under numerous conditions including food intake, pain, and other environmental challenges (1–3). One class of increasingly studied signaling peptides is neuropeptides (NPs), for which their structural and functional diversity requires the development of sophisticated analytical tools. NPs are typically short amino acid chains, although they have an unprecedented variety of sizes ranging from 3 to more than 70 residues (1). Their physiological function includes the ability to signal between neurons or neurons and other targets, but even structurally similar NPs can produce very different responses while also possessing conserved functions with

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other family members (4). This diversity has made the global discovery and characterization of NPs challenging.

Anabolism of NPs begins in the neurons, where they are synthesized from RNA chains as a prepropeptide (1). After multiple processing steps, including cleavages and modifications (e.g., C-terminal amidation), the resultant propeptide, which can contain several NPs, is packaged into vesicles along with the processing enzymes to produce final biologically active peptides. Upon stimulation, such as high-frequency firing, the secretory vesicles fuse with the plasma membrane and release the fully processed NPs from the neuron, allowing them to bind to a receiving target (5). Targets can lie nearby within a specific tissue or in a more distant location where the NP has to travel through the circulating fluid. Once at their target, NPs bind with high affinity, and the signal subsides only after peptidases break down the NPs. However, the anabolism and catabolism of an NP can vary dramatically between the location of the neuron, and intracellular processes may occur extracellularly in different regions of the body (6).

Even with complete genomic sequence, given the production's natural complexity, it is difficult to predict the structure and function of a single NP produced. Compared with mammals, the networks of invertebrates are simplified and have been utilized to allow for NP characterization. NP homologs also exist between invertebrates, such as crustaceans, and vertebrates, suggesting a conservation of signaling molecules, pathways, and other complex behaviors across species (7–10). By studying these simplified networks, we can gain a better understanding of a more complex nervous system, such as that of humans.

Measuring NPs requires approaches and platforms that provide sensitive and specific chemical, dynamic, and spatial information. Classical techniques, such as Edman degradation, require lots of sample material, whereas immunoassays (e.g., radioimmunoassay) are often nonspecific, and/or need prior structural knowledge of target NPs (1). Antibody-based immunochemical methods also have difficulty differentiating between isoforms of a NP (1), although this was recently achieved (11).

To compare, mass spectrometry (MS) provides selectivity through accurate mass measurement and MS/MS sequence confirmation without consuming large amounts of sample. As a result, various facets of MS have been used as powerful tools for NP analysis. The small differences between isoforms' mass-to-charge (m/z) ratios measured using high-resolution, accurate-mass MS instrumentation can easily distinguish one isoform from the other (12, 13). In addition to accurate precursor mass measurement, product ion fragmentation mass spectra (MS/MS) enable the discovery of novel NPs from several families through de novo sequencing and BLAST strategies, thereby expanding the neuropeptidome of the corresponding organism (14–17). Furthermore, quantification of NPs, both relative and absolute, has evolved from label-free methods to isotopic labeling strategies, providing a more dynamic view of the NP changes in comparative studies (18). To acquire accurate data, proper handling and separation of the samples are key, especially in specialized MS techniques such as in vivo methods and mass spectrometric imaging (MSI).

The general workflow and major approaches for NP analysis can be readily transferred and utilized across species, although the specific materials used will vary. Figure 1 outlines these strategies using crustacean as a model organism. Throughout this review, these general methodologies are discussed, highlighting the major advances in each area as well as the major discoveries and challenges that still exist.

2. SAMPLE COLLECTION AND PREPARATION

The first major step is collection and proper sample handling of the NP-containing tissues. These samples are well-known for being complex, owing to proteases, lipids, salts, etc., which can create problems with intra/inter-scan dynamic range and ion suppression during MS analysis (1). Therefore, it is critical to reduce chemical complexity of an NP sample before analysis. As shown in Figure 1, several workflow pathways exist depending on the type of information sought from the neuronal sample.

2.1. Extract Versus Direct Profiling

In NP experiments utilizing mechanical/chemical extraction, pooling of several tissues, organs, and cells into one sample is often desirable. This creates a sample with more concentrated NP content that can be used either to discover or to monitor NPs. Depending on the model system used, various schemes for NP extraction have been developed. To break down cellular walls, homogenization using a glass manual homogenizer or a probe sonicator is employed. Released NPs are immediately vulnerable to chemical degradation, and protease activity must be reduced during extraction. Postmortem degradation, which is even more intensified in mammalian tissue, can produce protein fragments that interfere with NP identification (13, 19–21). Commonly, samples are just snap frozen, although this is ineffective and reduces the number of peptides identified compared with other stabilization methods (22, 23). Denaturation and precipitation of proteases can be achieved with acidified organic solvents, such as acidified methanol, acetone, or ethanol, though these extraction solvents may need to be coupled with other methods or each other to increase peptide identification (5). Through combination of these solvent systems, for example, the four-step “mixing on column” extraction method, NP identification rates have been increased by fivefold (i.e., from 100 to more than 500 NPs identified by mixing on columns) (24). Microwave irradiation, boiling in extraction buffers, or heat stabilization are also effective in minimizing postmortem degradation (22–24). By use of the Stabilizer T1 denator, a commercial heat stabilization system, Sturm et al. (23) observed a reduction in interference due to degradation products in crustacean tissues. This method outperformed boiling when directly compared with similar tissues acquired from the same blue crab.

Although technological advances have made MS analysis of single tissue homogenates more accessible, unlike homogenization and extraction, direct tissue analysis is a much simpler sample preparation technique that enables comparison between individual samples or animals. Once dissected out and rinsed with water to desalt, the tissue can be directly placed onto a glass slide or sample plate for analysis. In contrast to pooling tissues for extraction, in situ direct analysis simplifies the workflow; it minimizes artifacts, contamination, and sample loss. Even with smaller sample sizes, high sensitivity can be obtained, as

demonstrated by the studies above. Finally, the spatial information gained from direct tissue analysis is often lost when homogenates are pooled.

2.2. Single Cell Analysis

The use of single cell analysis has allowed for the profiling of rare, low-concentration NPs within a heterogeneous population. Individual cells usually contain distinct isoform(s) of a bioactive molecule, permitting the study of co-transmission at the single-cell level (11, 25–29). In singlecell MS, researchers must carefully consider special strategies and instrumentation, such as the appropriate microscope and capillary for cell transfer. Several sample preparation and technological advances have been made regarding microanalysis of single cells (for a recent review, see 30). Notably, immunohistochemical methods are useful for localizing and identifying clusters, but antibodies can be unspecific, require prior knowledge, and can hinder MS analysis because fixation of the analytes is normal practice. Recently, by using a heating step, investigators reversed these crosslinks and determined that neuron samples produce signals similar to those of freshly dissected cells while causing minimal Schiff base formation (27). Although many NP functions remain elusive, the demonstration of single-cell MS in many organisms is the stepping stone to combining functional studies with neuropeptidomic profiling.

2.3. Liquid Collection Methods: In Vivo Monitoring

Alternatively, liquid-based collection methods can be used to sample NPs from media such as blood or other biofluids. These procedures provide the distinct advantage of determining whether a peptide is secreted while delivering an extract that is less complex than tissue homogenates. Biofluid sample collection is most often done by using a needle attached to a syringe to withdraw a specified volume of liquid that supposedly contains NPs. Although simple, needle sampling can be stressful to the organism, thus producing artificial circulating NPs that can skew results. Biofluids, such as crustacean hemolymph, are also protein abundant, and degradation products lead to the suppression of trace-level NPs (7).

These limitations have led to the development of new sample collection strategies to measure in vivo changes that can further our understanding of important biological questions (31). These techniques target the extracellular space, which enables researchers to monitor secretion and dynamic changes due to stimulus or normal rhythm. Such work provides valuable insights into an NP's possible functional role. The two most common in vivo sampling methods are push-pull perfusion and microdialysis (MD), although MD has acquired the most attention for method development owing to its minimal disturbance to the animal (5, 32–34). Both approaches require the insertion of a sampling probe into a specific region of the brain or circulation system. Many recent reviews include ample discussion about technical considerations involved in MD (7, 8, 33). When coupled to MD techniques, MS offers an attractive tool that can provide sensitivity, aid in identification, and allow for confident quantitation.

Challenges still exist with MD in vivo measurements, specifically whether they provide a balance between both sensitivity and temporal dynamics. Temporal resolution, defined as the shortest time duration over which a dynamic change event can be observed, is required to

understand the possible functionality of NP targets. Short time points intrinsically lead to small volumes and low NP concentrations, and the limited sensitivity provided by MS may often hinder the detection of these low-level signaling molecules. Increasing the collection volume will alleviate this issue but at the cost of temporal resolution. Another strategy is to add a rapid preconcentration step prior to analysis, which Zhou et al.

(35) demonstrated using Sprague-Dawley rats with an optimized system.

One way to increase the amount of NPs in small sample volumes is by improving their recovery rate, which, according to in vitro studies, is approximately 20–30% for NPs (8). One option is to use affinity agents within the probe, such as C18 magnetic micro- or nanoparticles (8). Recovery is enhanced, allowing for the increased sensitivity required for confident MS detection. For example, compared with other affinity agents, antibody-coated nanoparticles provide significantly improved recovery for six NPs in the Jonah crab, *Cancer borealis* (see Figure 2) (8).

3. QUALITATIVE ANALYSIS OF THE NEUROPEPTIDOME

Normally in MS, a top-down approach, or the analysis of intact molecular species, is used for NP detection. By contrast, in a bottom-up approach, samples are subjected to proteolytic digestion prior to analysis. Once prepared, MS investigation requires the ionization of the analytes. Two common ionization methods utilized for NP studies are matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI). Although MALDI-based methods provide high sensitivity, simple sample preparation, and are tolerant to contaminants, this technique preferentially produces singly charged ions. This simplifies the spectra for quantitative analysis, but it can be a problem when the mass range achievable is limited on a high-resolution instrument such as a MALDI Orbitrap system. Fragmentation of singly charged ions is also inefficient, making MALDI-MS alone insufficient for large-scale neuropeptidomic analyses. By contrast, ESI-MS offers greater coverage of the peptidome owing to its ability to produce multiply charged ions and to promote efficient fragmentation for sequence derivation. However, via recent advances in MALDI instruments using laser spray ionization and similar techniques, researchers have produced multiply charged ions under various pressure (e.g., atmospheric) conditions (36–41).

3.1. Separations

Owing to its natural complexity, crude extracts must be simplified prior to MS analysis. Initially, samples can be simplified with a reversed-phase (C18) or strong cation exchange desalting system, such as ZipTip or SepPak, depending on the type and amount of NP material available. When the purpose of the experiment is to investigate a specific NP or family, immunocapture techniques can be utilized to enrich the sample (42). Even with these methods, tissue extracts require chromatographic separation prior to MS analysis to allow for characterization of the wide dynamic range of NPs in a sample (13, 43–46).

Liquid chromatography (LC) is the most common separation method coupled to MS in neuropeptidomic studies. When a sample is injected, it is loaded onto a trap column, which concentrates and desalts the NP sample prior to nano-LC separation and subsequent MS analysis. To improve neuropeptidome coverage, two orthogonal separation methods can be

coupled to provide a multidimensional separation (13, 43, 44). In the past, online reversed-phase liquid chromatography (RPLC) was coupled to an SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis) gel to achieve an orthogonal separation. Unfortunately, manual manipulation (e.g., cutting bands) was required before the samples could be coupled to a mass spectrometer. Today, it is commonplace to couple two different LC stationary phases together, offline or online, to provide enhanced resolution, increased sensitivity, and reduced sample complexity. The first dimension of separation is often strong cation exchange (43) or high-pH reverse phase (13). Recently, an online RPLC/RPLC system, the first dimension containing a C18 column and the second with a polar-RP column, allowed for the accurate and sensitive quantitation of endogenous oxytocin in rat brain and plasma (44).

When the available sample volume is low and sample consumption is a concern, capillary electrophoresis (CE) can provide high-resolution separation for MS analysis. Online coupling is common with ESI sources, whereas MALDI instruments, which require the introduction of matrix, are frequently utilized offline. Several CE-MS interfaces exist; these are nicely reviewed elsewhere (47). One notable highlight is a novel SPE preconcentration method coupled to online CE-ESI-MS, which produced a 5,000-fold improvement of the limit of detection of NPs (48). Although CE-ESI-MS has some limitations, such as nonindependent optimization, intolerance to salts, and MS sampling rate, offline CE-MALDI-MS provides the opposite characteristics. Discrete fractions are usually acquired during separation, which decreases column resolution featured in CE analysis. Collection and detection of a continuous CE trace circumvent this issue. Using MSI, which is discussed below in more detail, to image the entire CE trace, our group (45) successfully separated individual NPs and accurately acquired their relative quantity via isotopic dimethyl labeling.

An MS-separation technology based on the mobility of biomolecules in the gaseous phase, known as ion mobility mass spectrometry (IM-MS), has recently been used in the study of NPs (49–52). Traditional IM-MS separates molecules on the basis of their mass, charge, and different gas-phase conformations, which causes ions to travel at different velocities through a drift gas in the presence of an electrical field (53). Other ion mobility separation modes exist. For example, differential, or field-asymmetrical, ion mobility spectrometry separates ions by measuring their mobility in time-varying electric fields (51, 54). The creation of dimers or molecular complexes also causes mobility differences. For example, NPs were found to interact with amyloid-beta peptides in Alzheimer's disease using IM-MS, leading to the understanding of the disease process and the possible choice of biomarkers (49). Ion mobility detects even minute structural differences within a peptide, such as epimeric differences or post-translational modifications (PTMs), demonstrating the selectivity of this technique (51, 52). Recently, Jia et al. (52) used traveling-wave IM-MS to discern between L- and D-amino-acid-containing peptide fragments.

3.2. Characterization

Once NPs are introduced as gas-phase ions into the mass spectrometer, a precursor scan is performed, from which putative identifications can be made on the basis of the exact mass compared with those in a database. However, many organisms lack genomic information,

and de novo sequencing is required to identify peptides. Initially, product ion scans are acquired for each peptide-like precursor for preliminary sequence information. A precursor is then chosen for fragmentation into characteristic pieces to aid in peptide identification. There are several product ion fragmentation methods, such as collision-induced dissociation (CID), high-energy collisional dissociation (HCD), and electron-transfer dissociation (ETD), each of which provides complementary information for structural elucidation. Although CID has been the predominant technique, it may be biased and promote incorrect sequences via sequence scrambling (50) and PTM removal. HCD and ETD may serve as alternative methods to combat these disadvantages. Although mechanistically similar (i.e., bombardment with neutral ions to produce b and y ions), HCD lacks biases in structural size, amino acid content, and low-molecular-weight cutoff that plague CID. Conversely, ETD utilizes electrons to cause random fragmentation to a target ion to produce orthogonal c and z ions. In combination, the complementary fragmentation methods CID and ETD have provided more complete NP coverage (55).

To facilitate better fragmentation and sequencing, many chemical derivatization schemes have been utilized. By introducing a mass shift at either the C or N terminus, easier differentiation between the b and y ions is readily achieved for de novo peptide sequencing. Owing to the specificity of a label for a particular side group, chemical labeling also determines which amino acids are present in a peptide. Choice of derivatization is important, as some additions may lead to a decrease in ionization and fragmentation frequencies. Successfully developed methods include acetylation (9, 56), methyl esterification (57), and dimethyl labeling (58, 59). In particular, dimethylation, which introduces a mass shift of 28 Da to primary amines (i.e., N terminus and lysine residues), has been utilized to determine amino acid content for mid-sized peptides, such as crustacean hyperglycemic hormone precursor-related peptides (59).

3.3. Bioinformatic Tools

LC-MS/MS is a high-throughput method that produces large data files that require robust bioinformatic tools to parse the MS data to confidently identify and quantify NPs. As stated previously, NPs undergo many processing steps prior to becoming biologically active (1, 5). This limits the amount of information a genetic sequence can provide, but many bioinformatics tools have been created to facilitate prediction of the final peptide forms from the prohormones produced (60–66). One such tool allows for the prediction of prohormones (NeuroPID) and is effective in metazoan proteomes (60). By using several logistic regression models depending on the species of interest, the NeuroPred application suite predicts likely cleavage sites of a prohormone, thus allowing for the discovery of novel NPs and prediction of expected NPs (61). Southey et al. (67) used NeuroPred to locate the prohormone genes and predict cleavage sites to produce a cattle database, which can be useful for comparable species whose complete genome sequences are not available.

Once MS data have been acquired, manual matching or an automated search with a database such as SwePep (62) or Neuropedia is the simplest way to identify NPs (63). However, there is a trade-off in prohormone and sequence coverage when working with small samples, such as single cells or direct profiling, as opposed to tissue-extract analysis. Individual cells are

likely to contain each form of the prohormone that produces biologically active peptides. Yet, peptide sequence coverage is best when NP-rich extracts are used. Although NPs can be identified with mass matching, the use of MS/MS spectral data increases the confidence of assignments and can be critical for novel NP discovery or PTM mapping (68). When a genome is available, standard proteomic-based searches such as Mascot can be utilized. Otherwise, de novo sequencing is required. Manual de novo sequencing is time consuming, but several software packages such as PEAKS facilitate de novo sequencing (64). Although de novo sequencing is a powerful tool, it does not always produce accurate results. Several programs such as SPIDER, BLAST, and MEME can be employed for homology searches. These platforms compare putative peptide sequences against a database of closely related species. This strategy may not provide complete sequence information for the peptide in question; however, they can provide key evolutionary and functional roles of the peptide (65). Several workflows have been developed to use the many available tools, such as BLAST, Uniprot database, and PepNovo, which facilitate peptide identification from complex data sets (66). It is expected that new bioinformatics tools will emerge, providing more confident assignments while increasing the high-throughput nature of MS.

4. NEUROPEPTIDE QUANTITATION

Another aspect in determining NP functions is to assess their quantitative regulation in response to a physiological change or manipulation. However, the heterogeneity of ionization efficiency, unpredictable bias, and suppression effects of complex mixtures may complicate quantitative MS analysis. The necessity for accurate quantitation information has sparked the development of several strategies to address these problems. Currently, lack of standardization exists for aNP-level quantitation strategy. This has led to a growing interest in creating robust MS-based approaches to quantify NPs in targeted and nontargeted workflows.

4.1. Absolute Quantification

There are two main types of MS quantitation strategies: absolute and relative quantitation. Owing to the inherent defects mentioned above, an analyte's intensity or peak area in a spectrum alone is not a reliable indicator of the amount of the analyte in the sample. To determine the absolute amount of an NP in a sample, internal standards, either a homologue or a stable isotope-labeled target peptide, must be included. The abundance of the target peptide is compared with that of the internal standard and back calculated to the initial concentration of the standard using a predetermined standard curve. Initial homologous internal standards suffered from different hydrophobicity, LC elution profiles, and ionization efficiency, resulting in inaccurate quantitation (1,69,70). Subsequent stable isotope-labeled internal standards were introduced for target peptide quantification to improve measurement accuracy and precision. For example, Desiderio & Kai (71) used an O¹⁸ stable isotope-labeled internal standard of methionine enkephalin and leucine enkephalin to quantify target peptides in canine thalamus extract. In this study, both the stable isotope-labeled internal standards and endogenous NPs have indistinguishable physicochemical properties. Thus, the NPs coeluted and were analyzed simultaneously by MS, thereby avoiding inaccuracy caused by different hydrophobicity effects. Subsequent studies used a selected-reaction monitoring

approach to evaluate the amount of methionine-enkephalin (72) and of a larger NP, β -endorphin, in human pituitaries (73, 74) down to picomolar levels. With this approach as the foundation, Gerber et al. (75) later developed the absolute quantification of proteins. In recent years, this method has been modified and applied to measure concentrations of multiple NPs in different chemical environments. Kheterpal et al. (76) employed stable isotopes ^{13}C and ^{15}N on the leucine residue to generate a standard curve for MIF-1 (Pro-Leu-Gly-NH₂), which has potent therapeutic effects in depression and Parkinson's disease, thereby facilitating measurement of its actual concentration in the mouse brain.

4.2. Relative Quantitation

In contrast to absolute quantitation, relative quantitation experiments do not provide information about the actual amount of a specific NP within a sample. Rather, relative quantitation experiments aim to compare the fold change of NPs between multiple samples/treatments and then yield a ratio or relative change. This approach can be broken down into two main categories based on whether the underlying methodology uses a chemical label to modify NPs within a sample: label-free and labeling approaches.

4.2.1. Label-free quantitation.—Label-free quantitative approaches rely on the comparison of different features between independent LC-MS and LC-MS/MS measurements. As such, reproducible chromatograms are key to providing accurate results. These approaches have drawn more attention during the past few years because label-based approaches always cost more and require additional sample preparation steps. Two widely used label-free quantitative methods are spectral counting and peptide peak-intensity/area measurement.

Spectral counting has its roots in bottom-up proteomic experiments and is based on the observation that more abundant proteins have a greater chance or higher frequency to be sampled in tandem MS scans than do low-abundance proteins (77) (Figure 3a). In NP applications, relative quantitation by spectral counting compares the number of identifications of the same peptide between different samples. Using spectral counting, Southey et al. (18) investigated the roles of proprotein convertase subtilisin/kexin type 1 inhibitor peptides and other peptides associated with feeding behavior in the suprachiasmatic nucleus. However, in spectral counting, if peptides are to be identified and thus quantified, they must trigger MS/MS acquisition. Thus, while spectral counting works better for highly abundant NP samples where MS/MS events are readily triggered, this method is less reliable when peptides are present in trace amounts (78). Estimated ratios can be significantly suppressed, and low-abundance NPs may be left unquantified. Spectral counting is also less sensitive toward small-fold changes (<2 orders of magnitude) (79). Finally, each NP elutes at a single time point for MS/MS fragmentation, reducing the sampling depth. As a result, spectral counting is less than ideal for NP quantitation.

An alternative label-free approach to spectral counting is peak-intensity/area measurement, which is illustrated in Figure 3b. This technique measures and compares the chromatographic peak areas of peptide precursor ions from different runs. The theory behind this strategy is that the peak intensity/area of ions after detection correlates with ion

concentrations within a sample. Lee et al. (80) used such an approach to study the circadian rhythms system of rat suprachiasmatic nucleus and found ten endogenous peptides that showed differences between day and night.

Although peak-intensity measurement seems conceptually straightforward, its use requires caution during data processing to ensure reproducible and accurate detection and quantitation between individual sample runs. Concerns may arise when coeluting peptides in a complex mixture having similar m/z values that are overlapped with peptides of interest, especially when using low-resolution MS instruments (81, 82). In such cases, complication of the extracted ion chromatogram and, therefore, quantitation accuracy occurs. Variation in peak intensities, retention times, and m/z values of the same peptide between technical replicates should also be appropriately normalized. Therefore, this label-free technique necessitates computational processing to take into account all these factors. Myriad software solutions for label-free experiments, most of which are designed for proteomic studies, are currently available on the market (83–86). Total ion current normalization (87, 88) as well as normalization to internal standards [e.g., bovine serum albumin (BSA) peptides] (3, 89) are two widely accepted and simple approaches. Several statistical, mathematical methods have been evaluated on endogenous peptide samples (90, 91). Kulima et al. (91) concluded that their novel method, linear regression followed by analysis order normalization (RegrRun), was superior to all the other nine methods compared. Compared to the raw data collected from three different species, RegrRun decreased the median standard deviation by 42–43% between replicates on average, whereas other methods only reduced the median standard deviation by 15–28%. Later on, this approach was employed to investigate the effect of cyanobacterial toxin β -N-methylamino-L-alanine on neurodegenerative disease and several proteins, and peptides were revealed to have dose-dependent responses (92).

4.2.2. Labeling quantitation.—Stable isotopic labeling strategies allow simultaneous comparison of multiple samples by introducing a mass difference tag to the peptide. The technique usually makes use of stable heavy isotopes of ^{13}C , ^{15}N , ^{18}O , and ^2H . Labeling reagents with heavy or light isotopes introduce a mass shift into different samples, and by comparing intensities of massshifted peaks within the same spectrum, relative peptide ratios can be visualized. Mass defect, which exploits the differences between nominal mass and exact mass of peptides, is continually used in quantitative proteomic applications (93), although NP-compatible approaches are currently in development. Overall, the combination of these effects is a powerful way to increase the analytical throughput of quantitation via multiplexing. Experimentally, stable isotope labels can be introduced metabolically or chemically.

4.2.2.1. Metabolic labeling. Metabolic labeling incorporates isotopes into peptides during cell growth and duplication by feeding organisms with a special isotope-enriched medium (94, 95). Ong et al. (96) improved the metabolic labeling approach by inventing stable isotope labeling by amino acids in cell culture (SILAC). SILAC takes advantage of the fact that organisms have to incorporate essential amino acids from the environment for protein synthesis. By providing heavy- or light-labeled essential amino acids in the growth media, usually arginine or lysine, SILAC introduces mass difference tags into target

organisms, ideally with a 100% incorporation efficiency after a few generations. Subsequent pooling of differently labeled samples will help avoid errors from sample preparations. Enzymatic digestion using trypsin produces peptides that contain at least one arginine or lysine residue at a peptide's C terminus, allowing the peptides to be quantified. Multiple proteomic studies have employed SILAC (97–99), whereas hardly any NP work has been reported. Although metabolic labeling of whole animals is a powerful tool, it is both expensive and limited to animals that can be raised in the lab. In addition, the global incorporation of isotopic elements into an animal may lead to different phenotypes. Many groups have already utilized SILAC for global protein quantification in both plants (100) and animals (101, 102), and this powerful tool could be useful for future neuropeptidomic investigations.

4.2.2.2. Chemical labeling.: Relative quantitation via chemical labeling relies on chemical reactions between a labeling reagent and a peptide target to produce a certain mass shift into different biological samples. This can be seen in either the precursor spectrum (mass-difference approaches, e.g., dimethyl labeling) or the product ion fragmentation spectrum [isobaric reagents, e.g., isobaric tags for relative and absolute quantification (iTRAQ), tandem mass tag (TMT), N, N-dimethyl leucine (DiLeu)].

Several chemical labeling strategies have been successfully applied to MS¹ NP quantitation in recent years. Common labeling approaches include succinic anhydride, 4-trimethylammoniumbutyryl (TMAB), and dimethyl labeling, in which primary amines of N termini and ϵ amino groups of lysine residues are chemically derivatized. Duplex succinic anhydride tags with a 4-Da mass difference facilitated the quantification of approximately 50% of known bee brain NPs in the context of foraging. Eight NPs show robust and dynamic regulation during foraging procedure or with different foraging preferences (103).

Developed by Regnier's group, TMAB labels contain a quaternary amine labeled with methyl groups that impart a permanent positive charge on the peptide (104). Originally, only two forms were synthesized (e.g., a heavy form containing nine deuteriums and a light form without deuteriums). However, two additional forms containing three and six deuteriums were later synthesized and tested (105). This scheme features several advantages (low cost, simple synthesis, and labels that differ by 3 Da or more) and eliminates the major limitation of other isotopic labeling reagents (e.g., labeled peptides do not coelute on high-performance LC). This labeling technique has been utilized in a large variety of NP studies that required more than duplex tags to label all samples (15, 106, 107). A drawback of this labeling scheme is that the quaternary amine causes the label to be unstable in many different MS applications (105).

Isotopic formaldehyde labeling is one of the first chemical labeling approaches used for NP quantitation. This labeling scheme adds two methyl groups to any primary amine in the peptide (e.g., N terminus or lysine ϵ amino group). By labeling NPs with light or heavy formaldehyde (CH₂O or CD₂O), a 28-Da or 32-Da mass shift, respectively, will be generated. The 4-Da mass difference between light- and heavy-labeled peptides allows for direct comparison of the same peptide from different samples (108–110). Later, Boersema et al. (111) modified this protocol and successfully introduced triplex formaldehyde reagents

(CH₂O, CD₂O, and ¹³CD₂O), although this increase in multiplexing comes at the cost of increased spectral complexity at the MS¹ level.

Researchers can also generate chemical labeling reagents that allow for highly multiplexed quantification to be performed in product ion MS/MS scans. Tandem MS-level techniques enable simultaneous quantitation and peptide sequencing from a single MS/MS spectrum and allow increased multiplexing without increased mass spectral complexity. Isobaric mass tags, such as TMT (112, 113), iTRAQ (114), and DiLeu (115, 116), allow for multiplexed comparison of samples in parallel (Figure 4). These MS/MS isobaric tags are composed of an amine-reactive group, a mass balance group, and a reporter group. The mass balance group counterbalances the mass difference possessed by the reporter group, which ensures that peptides labeled with different reporter ion channels are detected as a single precursor in the parent scan. Upon MS/MS fragmentation, distinct reporter ions unique to each labeled sample are observed in the low *m/z* region. By comparing reporter ion intensities within the same MS/MS spectra, peptides from different samples can be compared within a single LC run. iTRAQ reagents have been used for peptidomic analysis of the effect of prolyl oligopeptidase inhibition in the rat brain (117). With reduced cost per experiment, DiLeu reagents developed by our group display comparable, if not better, performance compared with that of iTRAQ (115). Recently, DiLeu was used in a study of the neuropeptidomic expression changes in a major neuronal ganglion at multiple developmental stages of the lobster *Homarus americanus* (118).

5. DISTRIBUTION ANALYSIS BY MASS SPECTROMETRIC IMAGING

Although focus has been placed on the chemical identity of NPs, spatial localization can provide important information for understanding functionality, delivering a powerful tool for scientists. Many methods, such as staining or isolation of a neuroendocrine structure, exist, but they can be cumbersome and require antibodies or selective probes. First introduced by Caprioli et al. (119), MSI has been applied to several different tissue types and various molecular sizes over the past several years (120). These data presentations vary from peptide profiling to several clinical applications (89, 121). In a MSI experiment, mass spectra are collected via a predefined grid along an x-y coordinate system on the tissue. Once ions are collected from each position, their intensities can be assigned according to the grid created, and a heat-map display can be generated for every compound detected, producing hundreds of two-dimensional images displaying the spatial localization of any detected ions of interest. Because MSI is not highly quantitative, applications and bioinformatics tools, such as Quantinetix and other novel in-house software (122), are in development to offer quantitative analysis of the MSI data.

5.1. Special Considerations for Mass Spectrometric Imaging

Several MSI methods have been established, although their usage depends on the type of analyte and resolution required. Secondary-ion mass spectrometry provides cellular-level resolution of submicrons but is typically limited to the ionization of small-molecule compounds such as lipids and other metabolites (123). Nanostructure-initiator mass spectrometry, a matrix-free method that performs well when working with low mass-to-

charge (m/z) species such as lipids, is not optimal in imaging NPs (124). Our group and others (120, 123, 125–127) showed that MALDI has enabled the study of large biological peptides and proteins, making it a popular choice for NPs.

Special sample preparation steps must be taken to preserve the tissue and prevent degradation prior to analysis (for a recent summary, see 123). These strategies have allowed for the characterization of a range of peptides and have been successfully applied to studies requiring single-cell resolution (128, 129). Three-dimensional tissue imaging has also been achieved by analyzing consecutive slices of a two-dimensional tissue and in silico combining the images to show the distribution along a three-dimensional z -axis (Figure 5) (130).

5.2. Mass and Space

Three areas of MSI have undergone major development: mass resolution, mass range, and spatial resolution. With the development of MALDI-FTMS (Fourier-transform mass spectrometer) instrumentation, such as the MALDI-FTICR (Fourier-transform ion cyclotron resonance) and Orbitrap technologies, high-resolution accurate mass measurements are achievable. For the American cockroach, the hybrid MALDI-LTQ-Orbitrap XL mass spectrometer provides both high mass accuracy and high mass resolving power of NPs directly from tissue slices (131). Unfortunately, the cost of high-resolution “ion-trap-based” technologies limits the upper mass range to approximately m/z 4,000. Thus, methods to increase the mass range of these instruments have been an important area of development (36–41, 132). High m/z tissue imaging is challenging even for time-of-flight (TOF) instruments, which have a theoretically infinite mass range. Several groups have developed specialized sample preparation techniques (133), equipment (134), and matrices (135) to increase the mass range of detectable ions in TOF instruments. Mainini et al. (135) successfully detected proteins up to 135 kDa using ferulic acid as a MALDI matrix.

As stated previously, MALDI is well-known for primarily producing only singly charged ions. However, several recent studies reported on the production of multiply charged ions on commercially available MALDI instruments by laserspray ionization (36, 38), matrix-assisted inlet ionization (39), or matrix-assisted ionization vacuum (40). By creating multiply charged ions, more efficient tandem MS fragmentation can be achieved when using ETD or CID. Using an intermediate pressure MALDI source with laserspray ionization, the Trimpin group (36) showed the measurement of +12 ubiquitin ions (8.5 kDa). Although MSI is used primarily as a top-down approach, in situ digestion on tissue slices has emerged as another strategy to bring larger NPs into the appropriate mass range of a mass analyzer (136). Instead of directly imaging intact NPs, a bottom-up approach is taken, and the enzymatically digested peptide fragments are visualized and colocalized on the tissue slice. With the appropriate bioinformatics tools (e.g., PEAKS; for more examples, see above), the corresponding large NPs may then be identified. Many factors including the choice of the enzyme, matrix, and instrumentation need to be considered when optimizing in situ digestion (for quality reviews, see 136, 137). Notably, researchers have developed a novel graphene-immobilized trypsin platform for on-tissue digestion that provides more complete sequence coverage compared with on-plate digestion of BSA (77% versus 30%) (138). With

the development of high-resolution and accurate mass measurements, MS/MS (139) and/or orthogonal ESI-MS experiments (140) have successfully imaged and identified larger NPs and neuroproteins.

MSI data relevance depends on the pixel size acquired, which is directly related to the spatial resolution achievable by the experimental setup (125, 141). Spatial resolution is chosen by the step size the plate takes to raster the laser across the tissue, which is primarily defined by the diameter of the laser beam. To increase spatial resolution, investigators have developed several approaches, such as traditional “microprobe” mode, “microscope” mode, oversampling, and parafilm stretching, all of which are summarized elsewhere (129, 142). Recently, the Spengler lab (143) developed a home-built MALDI source paired to the LTQ-Orbitrap that allows for step sizes down to 5 μm . These developments allowed for high-resolution imaging of NPs such as oxytocin and vasopressin in the mouse pituitary gland (143). As spatial resolution continues to improve, single-cell imaging will become more accessible for researchers. Both cell cultures and dissected cells have been used (128, 129, 144), although special consideration must be made to create the images. For example, cells in culture have been stretched prior to placing them on slides and analyzed by MALDI-TOF/TOF, which allowed for the spatial mapping of several ions (129). These methods will become more refined, and efforts will be extended toward subcellular analysis.

5.3. Specialized Applications: Coupling Separation to Mass Spectrometry Imaging

NP MSI has also been used in applications outside of tissue slices, specifically CE fractionation or LC separation (45, 46, 145). Normally, distinct fractions are deposited on MALDI plates as individual spots and analyzed by MS (146, 147). This approach can cause a decrease in separation resolution. To preserve temporal resolution, the separated mixture may be continuously deposited across the MALDI plate surface. Although time-consuming, manual profiling of this continuous trace can be performed. However, MSI enables simplified analysis of the column eluent. MSI has been coupled to both CE-MALDI (45, 46) and LC-MALDI (145) workflows, thus allowing for high-temporal resolution separation of NPs. CE-MSI has led to a four- to six-fold increase in peptide coverage (45). By separating isotopic formaldehyde labeled NPs from a complex mixture of crustacean pericardial organ extract, quantitative information can also be obtained with CE-MSI (Figure 6) (45). With the development of new columns or separation strategies, these successful analysis strategies will be applied to other NP-containing media, such as microdialysates.

6. CONCLUSIONS AND FUTURE DIRECTIONS

As technologies and methodologies are further developed, additional NPs will be discovered and interrogated using a variety of workflows. Although generally well-developed, MS-based NP studies still have many areas that need improvements, especially for low-volume samples. Both qualitative and quantitative characterization strategies have been developed, allowing important biological questions to be answered. The amount of data acquired from a single MS run has only increased, and the bioinformatics tools available will continue to mature to meet these needs. As future technologies enhance the information acquired, we expect to see an expansion of the use of MS for neuropeptidomic analysis.

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Glossary

Signaling peptides

endogenous peptides that are involved in cell-cell signaling (e.g., hormones, neuropeptides, and trophic factors)

Neuropeptides (NPs)

signaling peptides synthesized in the nervous system

Mass spectrometry (MS)

accurate mass-to-charge analysis of an analyte of interest

MS/MS

tandem mass spectrometry

Mass spectrometric imaging (MSI)

technique used to visualize the spatial distribution of an analyte of interest by its mass-to-charge ratio

MALDI

matrix-assisted laser/desorption ionization

ESI

electrospray ionization

LC

liquid chromatography

CE

capillary electrophoresis

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SUMMARY POINTS

1. With its ability to provide maximum information from very small sample volumes, MS has become a powerful tool for the characterization of the neuropeptidome. As instrumentation advances along with innovative sampling strategies, MS-based tools will continue to find widespread utilities in different animal systems.
2. It is clear that not one platform can provide simultaneous chemical, spatial, and temporal information. Thus, the development of tools for better in vivo measurements, MSI, and peptide sequencing should be coupled with new separation or sample preparation steps within new multifaceted approaches.
3. Owing to its high-throughput nature, MS has led to the growth of new characterization techniques and bioinformatics tools. Commercially available instrumentation and downloadable bioinformatics tools make data processing more efficient and much simpler. Yet, new methods will be created.
4. High-resolution instrumentation has provided new depth to NP characterization. Further improvements are surely in development, and new tools (e.g., isobaric quantitative tags that are constructed using subtle mass defects) will be established to utilize their power.

FUTURE ISSUES

1. Although several resources have been established for the quantitation of NPs, the number and availability of such are stunted in comparison to proteomic studies. Nevertheless, further development of cost-effective, multiplexed reagents will be key to allow cross comparisons in high throughput. With the ability to multiplex, absolute quantification using same-run calibration curves will facilitate targeted NP investigations.
2. Although MSI has become a popular tool exploring the spatial distribution of NPs, it still requires further method advancement to better measure NPs. In situ digestion and multiply charged ion production methods are still in their infancy, and focus should be on maturing their use for larger NPs. Furthermore, increasing spatial resolution to visualize subcellular peptide distributions will require innovation in instrumentation and sampling strategies.
3. Although there are hopes of applying MS to more complex systems (i.e., humans), work with MS as a whole is limited to well-characterized model organisms. The global progression of all MS preparatory steps must continue to answer any new and interesting questions related to NP research in these more complex situations.

Figure 1. General overview of sample preparation and data analysis strategies for neuropeptide analysis by MS. Three major sample preparation pathways exist: (a) extract profiling, (b) direct tissue profiling, and (c) direct tissue imaging. Once spectra have been collected, peptides can be identified via database searches, de novo sequencing, and/or prediction algorithms. Abbreviations: ESI, electrospray ionization; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; MSI, MS imaging; RT-PCR, reverse-transcription polymerase chain reaction.

Figure 2.

Recovery rates for several crustacean neuropeptides with different AAs using microdialysis.

Conditions with $p < 0.05$ and compared with No AA are indicated with one asterisk.

Significant differences ($p < 0.05$) for the AbMnP condition are indicated with a lambda.

Adapted with permission from Reference 8. Abbreviations: AbMnP, antibody-coated magnetic nanoparticle; AA, affinity agent; BK, bradykinin; FLP, *Homarus americanus* FMRFamide like peptide; FMRFa, FMRFamide (Phe-Met-Arg-Phe); SMT, Somatostatin-14; SP, substance P.

a Spectral counting

b Peak intensity measurement

Figure 3.

Representation of two label-free relative quantitation strategies. (a) Spectral counting uses the fact that more abundant peptides enable acquisition of more tandem MS scans. (b) Peak-area measurements utilize the chromatogram to provide quantitative information.

Abbreviations: LC, liquid chromatography; MS, mass spectrometry; XIC, extracted ion chromatogram.

Figure 4.

Isobaric chemical labels used for MS/MS quantitation. (a) Structure of DiLeu reagent showing reporter group, balance group, and amine-reactive group. Fragmentation of labeled peptides by CID/HCD generates reporter ions at m/z 115.1, 116.1, 117.1, and 118.1, and the carbonyl balance group is lost as a neutral species. (b) Fourplex DiLeu reporter ion structures. Each colored dot represents a location where a stable isotope has been incorporated, allowing for the mass of each reporter to differ by 1 Da. (c) General workflow for quantitation employing fourplex DiLeu isobaric tags. Initially, four samples containing a peptide of interest are differentially labeled and mixed. During MS analysis, differentially labeled peptides are measured at the same m/z in the parent scan; upon precursor isolation

and fragmentation by CID/HCD in the MS/MS scan, unique reporter ions are generated in the low mass region along with band y-type peptide backbone fragment ions, allowing quantitation and sequence identification of the peptide of interest. (d) Structures of TMT and iTRAQ isobaric tags. Labeling workflows for each are similar to the scheme illustrated in panel c. Abbreviations: CID, collisional-induced dissociation; DiLeu, N,N-dimethyl leucine; HCD, high-energy collisional dissociation; iTRAQ, isobaric tags for relative and absolute quantification; MS, mass spectrometry; TMT, tandem mass tag.

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Figure 5.

Three-dimensional analysis of the crustacean brain to show high spatial information of several neuropeptides of interest. Consecutive sections were analyzed to show the distribution in the z-plane of the tissue. Mass spectrometry (MS) images use an intensity scheme ranging from red, which is considered high (100%), to black/blue, which is considered low (0%). (a) Optical images of each consecutive section from the dorsal to ventral regions of the brain. Several neuropeptides were imaged showing a wide variety of distributions (scale bars = 1mm): (b) CabTRP 1a APSGFLGMRamide (m/z 934.5), (c) Orcokinin NFDEIDRSGFGFA (m/z 1474.1), (d) Orcokinin NFDEIDRTGFGFH (m/z 1554.7), and (e) RFamide SMPSLRLRFa (m/z 1105.6).

Figure 6.

PACE-MSI for quantitative analysis of formaldehyde-labeled NP peak pairs. (a) Representative spectrum of NP extract from crustacean pericardial organ by MALDI-MS highlighting two peak pairs. (b) MS image of the CE trace. (c,d) Mass spectra corresponding to the highlighted regions in the CE trace. Two distinct peak pairs are separated into two color regions for accurate quantitation. Adapted with permission from Reference 45. Abbreviations: CE, capillary electrophoresis; MALDI, matrix-assisted laser desorption/

ionization; MS, mass spectrometry; MSI, MS imaging; NP, neuropeptide; PACE, pressure-assisted capillary electrophoresis.

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