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Function-Driven Discovery of Neuropeptides with Mass Spectrometry-Based Tools

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Abstract

A number of unique challenges are inherent to the study of neuropeptides (NPs), both in determining their molecular structure and their function. Traditional studies follow a model in which novel NPs are discovered and identified, then investigated for function. These studies frequently use biochemical techniques that can be imprecise and cumbersome. Mass spectrometry (MS)-based tools are becoming important not only in precisely determining the identity of a NP or quantifying a compound with a known sequence, but also in studies where identity and putative function can be determined simultaneously. Tools based on MS and tandem MS (MS/MS) have been developed, both with isotope labeling strategies and label-free methods, that allow accurate quantitation of NP changes associated with behavior or physiological manipulation, concurrent with identification of sequence. MS and MS/MS have also been implemented with sampling methods that incorporate temporal or spatial information while determining functional role of a NP, such as microdialysis (MD) and imaging mass spectrometry (IMS). These advances in MS and sampling techniques allow investigation of a particular biological phenomenon to guide studies aimed to identify and characterize NPs. Permitting function to drive identification of relevant compounds allows for a broader understanding of the molecular underpinnings of these events. The NPs thus identified can then be validated with more conventional techniques, and successive iterations of identification and function determination will provide rich information about these compounds. This function-driven discovery of NPs using MS-based techniques is an important new approach for their study.

Keywords

Neuropeptides; mass spectrometry; identification; function; quantitation; microdialysis; imaging mass spectrometry

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CONFLICT OF INTEREST

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INTRODUCTION

Neuropeptides (NPs) are a class of neural signaling molecules whose study has greatly expanded in the past several years. The physico-chemical characteristics of NPs relative to classical neurotransmitters require a new set of analytical tools to be developed for their study, but these distinct features also provide greater diversity of NP identity and functional specificity. NPs are short chains of amino acids, typically 4-10 residues in length in invertebrates, or 5-30 in vertebrates (reviewed in [1]). They are also defined by having the ability to signal between neurons, or between neurons and somatic targets. The great structural diversity and common sequence motifs among NPs of the same family allow for each peptide to have distinct and highly specialized functions as well as functions in common with other family members. This great structural diversity has led to challenges, however, in the discovery of NPs and elucidation of their function. NPs are synthesized as prepropeptides in neurons (reviewed in [1]). These long peptides then undergo a number of processing steps, including cleavage into smaller peptides and modification of residues, such as pyroglutamation or amidation of the C-terminus. These processing steps take place as the peptides are packaged into vesicles which are finally sent to the axon terminal for storage prior to release. After release by the neuron, NPs diffuse to the receiving cells, either across the synaptic cleft or after transport to a distant site *via* circulating fluid. NPs in circulation are commonly protected by binding proteins. The peptides then bind to their receptors, typically members of the G-protein coupled receptor superfamily, to transduce the signal to receiving cells.

NEUROPEPTIDE DETECTION AND SEQUENCE DETERMINATION BY CLASSICAL METHODS AND MASS SPECTROMETRY

A variety of experimental strategies have been employed to study the identities and functions of NPs. These encompass both targeted and non-targeted methods, and are illustrated in Fig. 1. Detection of NPs has historically relied heavily upon targeted identification and quantification, frequently with antibody-based techniques. This is problematic due to the high degree of cross-reactivity that most NP antibodies have for multiple NPs, particularly those within the same family. It has recently become clear that most NP families have many members, and not all of these compounds have the same physiological effects, although antibodies cannot distinguish between them (reviewed in [1]). Crossreactivity across NP families is also observed, especially in the case of NPs with common post-translational modifications, such as C-terminal amidation. Antibodies raised against the longer mammalian neuropeptides are frequently more specific owing to greater antigen complexity, although it is often unclear what portion of the sequence the antibody recognizes. This can lead to confusion when different NP isoforms occur and may have different function [2]. Determination of the exact identity of the active NP under this scheme requires purification of a large amount of sample to be subjected to Edman degradation, and more recently, mass spectrometry (MS) and tandem mass spectrometry (MS/MS) for sequencing.

With the advent of soft ionization techniques for MS, the study of NPs has become more feasible and useful (reviewed in [1]). Slight differences in amino acid sequence among NP

isoforms, which can lead to antibody cross-reactivity, create noticeable differences in the mass-to-charge (m/z) ratio observed by MS. MS/MS analysis provides NP sequence information by selecting analytes with a certain m/z in the first dimension of MS and fragmenting them into smaller parts that are then subjected to MS analysis. Confident identification and sequencing of NPs directly from small biological samples is thus achieved with MS and MS/MS tools. MS-based analysis has identified thousands of putative NPs from a wide variety of species (reviewed in [1, 3–7]). However, many studies finding novel NPs by MS can only identify compounds as putative NPs based on sequence similarity to known NPs and/or the organs from which they are extracted. These identifications are either targeted to a particular molecular entity—as in quantification of NPs via antibody or MS-based techniques that require prior knowledge about the compound—or divorced from functional relevance—as in NP surveys that attempt to describe the entire NP complement of an organism via MS-based techniques.

CLASSICAL METHODS OF NP FUNCTION DETERMINATION AND NEW DIRECTIONS

Determination of NP function with classical targeted methods also typically requires prior knowledge about its sequence. As mentioned previously, under the classical definition of a NP, it must have activity on neuronal or target cells. This activity frequently leads to a change in the electrical potential of a cell or the conductivity of ion channels by which this potential is established and maintained. Thus the gold standard for NP function determination is targeted electrophysiological study of isolated organs, neurons, or organ systems upon application of the NP in question (reviewed in [5]). These living cells are removed from animals and kept alive in a preparation, while various techniques are used to determine the electrical potential of one or more cells in the system. The NP is applied to the cells, and changes in the potential are noted. Studies of this type are able to accurately pinpoint the cellular action of NPs and their function in small neural circuits, but are difficult to implement—requiring specialized equipment and expertise—and require a pure sample of known composition. Determination of a NP's function is frequently more complex than determining its sequence. These studies are also targeted to specific putative NPs and particular functions—functional information is only obtained about the compounds applied on the electrical circuits investigated. These targeted techniques for determining NP identity and function provide highly specific detail about compounds for which *a priori* knowledge is available. However, by focusing on a single NP or physiological process, many other important compounds or functions may not be observed. Therefore, there are advantages to a non-targeted approach. One such alternative strategy for non-targeted MS-based NP study uses manipulation of processes known to be regulated by NPs concurrent with MS analysis of putative NPs (examples: [8, 9]), an approach here referred to as function-driven NP discovery. In this strategy, all compounds that appear to be altered in the course of the experimental manipulation can be monitored. Changes in these compounds occurring during the experiment can later be verified with more established methods for neuropeptide function validation, such as *ex vivo* electrophysiology as described above, to confirm its identity and function as a NP under the classical definition with greater confidence. An advantage is that the identity of a NP need not be determined prior to analysis; the use of

MS-based tools allows for discovery and sequence identification of new NPs during the course of the experimental physiological manipulation, and no important compounds are missed by focusing only on a few target NPs. A few candidate compounds discovered in this way can then be the subjects of more targeted investigation with traditional techniques.

Mass spectrometry-based function-driven discovery has expanded in recent years, and it will be the focus of this review. Several studies illustrating the use of many of these tools will be discussed as examples. Particular attention will be paid to studies of decapod crustacean NPs, as this is an area in which NP research has made great headway. The crustacean is an excellent system for NP discovery and functional study, as its nervous system is simple, contains relatively large neurons in well-defined circuits, and is rich in NPs (reviewed in [1, 5, 10]). Validation of the candidate NPs identified in function-driven discovery will also be discussed.

FUNCTION-DRIVEN DISCOVERY OF NEUROPEPTIDES USING MASS SPECTROMETRY

Function-driven discovery of NPs requires the ability to measure and identify putative NP candidates that are differentially expressed or secreted under experimental manipulation, either qualitatively or quantitatively. Although MS is a powerful tool for discovery and accurate identification of NPs in small samples, reliable quantitation using MS can be challenging. Obtaining samples for MS that are correlated with a particular dynamic behavior is also difficult. Toward these ends, a variety of innovative strategies for MS-based quantitation and live-animal sampling have been developed. The MS-based techniques are illustrated in (Fig. 2), and broadly fall into the category of function-driven non-targeted approaches, meaning that they do not require *a priori* information about analytes' identities. These techniques, and MS used in conjunction with novel sampling methods, will be discussed in further detail.

Novel Mass Spectrometry Tools for Quantitation in Studies of Neuropeptide Function

Quantitative analysis by mass spectrometry is complicated by the heterogeneity of ionization and unpredictable discrimination and suppression effects of complex mixtures. Several strategies have been developed to address these problems. Internal standards can be added to samples to account for day-to-day and run-to-run variability. Many of the most reliable MS-based quantitation methods, including selected reaction monitoring/multiple reaction monitoring (SRM/MRM), absolute quantitation (AQUA) by addition of isotopically labeled internal standard, and absolute quantitation calibration curves, fall into the category of targeted analysis and require prior knowledge of the analyte's m/z and/or its fragments' m/z , and these methods are time-consuming to develop (reviewed in [7]). Often times, this prior knowledge is not available in a function-driven discovery study. Of these methods, SRM is more popular for determining NP concentration change as a means to elucidate function. SRM uses a first dimension of MS to isolate a single parent ion. It is then fragmented, as with MS/MS to determine peptide sequence. One daughter ion of this fragmentation is then analyzed by the second dimension of MS. This permits highly accurate quantitation, but only allows a single reaction to be monitored. For MRM, multiple parent-to-daughter

transitions are monitored and quantified. The most salient feature of these quantitation modes is that they require prior knowledge of the analytes' structure and fragmentation patterns, as well as generation of calibration curves to determine the relationship between the instrument response and analyte concentration, and thus can only be used in targeted studies of NP function. For these reasons, function-driven NP discovery can best be achieved with differential display (DD) and relative quantitation (RQ), due to the lack of *a priori* information.

DIFFERENTIAL DISPLAY VS. RELATIVE QUANTITATION

Differential display (DD) and relative quantitation (RQ) are related and often employ the same analysis strategies. Relative quantitation describes changes in the quantity of a compound across different biological samples, often as a fold-change. DD is more often used to describe the all-or-nothing presence of NPs in some samples but not others. This can be of great importance in determining function, because NPs present in some conditions but absent in others can be expected to have an important role in the neurobiology of those conditions. However, since a fold-change cannot be established, it can be difficult to determine the dose-response effect of these NPs. DD data is often displayed as pairs of mass spectra, mass spectra with isotopic peak pairs (described below), Venn diagrams, or tables.

In comparison, relative quantitation (RQ) describes changes in the quantities of NPs across biological samples. Quantity changes are usually expressed as a change from baseline, or fold-change between different states. A variety of MS and MS/MS methods are used in RQ, with important features being the accuracy and reproducibility of quantitation. RQ data allows for determination of the dose-response effects of NPs on progressive behavior; for instance, increases in an anorexigenic NP during feeding may lead to reduced food intake rate, resulting finally in cessation of eating. This allows a more concrete link to be established between the NP and its function. RQ data is usually expressed in bar graphs showing fold-change in different NPs between two states, or line graphs showing fold-change versus time.

MS-BASED QUANTITATION METHODS FOR FUNCTIONAL DISCOVERY OF NPs

RQ and DD data can be obtained using either MS or MS/MS techniques, and these methods can further be characterized by whether or not they employ isotopic labeling (label and label-free). Isotopic labeling is a method to prevent differential MS instrument effects from occurring in different samples. Different isotopes are incorporated into separate biological samples, the samples are mixed, and analysis of the mixed sample allows for determination of NPs present in one condition but not the other (DD) and/or changes in NP concentration (relative quantitation). Because the analytes from different conditions differ only in isotope composition, they experience the same instrumental conditions, and the response received for each can be used as a measure to quantitate them [9].

A number of methods for isotope-based MS quantitation exist. Metabolic labeling of whole animals is an exciting new field. In a scheme similar to stable isotope labeling in cell culture

(SILAC), animals are raised on an isotope-enriched diet, provided by algae or bacteria that are grown in media containing a single isotope of a vital element, usually nitrogen-15 (reviewed in [11]). An additional experimental group is fed the same food source, with the exception that its food source is raised under normal conditions. Each experimental group can be subjected to different conditions expected to lead to changes in NPs (or left unperturbed, as controls), and samples can be collected and combined prior to analysis by MS. To prevent experimental artifacts due to the metabolic labeling, the groups should be switched. The DD of NPs across conditions can be determined by looking for the presence or absence of normal and isotopically-enriched compounds. In addition, relative quantity changes can be determined by comparing the heights of isotopic peaks on a single MS spectrum or the areas under the curve (AUCs) for the extracted ion chromatograms (XICs) for the normal and isotopic NPs if a separation method is coupled to MS, i.e. ultrahigh performance liquid chromatography (UPLC)-MS. Although metabolic labeling of whole animals is a powerful tool, it is expensive, limited to animals that can be raised in the lab, and the global incorporation of isotopic elements into an animal may lead to different phenotypes. Most notably, high-anxiety mice fed ^{15}N diets had greater depression-like behaviors in a recent study [12]. This has profound implications for using metabolic labeling in studies of NP function.

Other methods of isotopic labeling exist for functional discovery of NPs, including several where isotopes are incorporated as part of the sample preparation procedure. Several schemes exist to tag biomolecules with isotopes [13–18]. One scheme that has gained much success is dimethylation with formaldehyde and deuterium formaldehyde [17, 18]. This reagent adds two methyl groups to any primary amine in the peptide, such as the N-terminus or lysine side chain. If deuterium formaldehyde is used, these methyl groups will each contain 2 deuterium atoms, leading to a total mass difference of 4 Da per primary amine. Deuterium formaldehyde is relatively inexpensive and the addition of deuteriums to a molecule usually does not greatly alter its behavior under analytical separation modes, such as liquid chromatography or capillary electrophoresis. As with metabolic labeling, samples of different isotopic composition can then be combined and analyzed concurrently. The ratios of peak heights obtained from a MS spectrum or ratios of AUCs from the XICs if separation is employed prior to MS can then be calculated to yield a fold-change in concentration of that analyte. A schematic illustrating this technique is shown in (Fig. 2) under the heading “Mass Difference Tags.” This figure illustrates the pairs or triplets of peaks observed in such a MS spectrum. Most such tools use only two reagents, but depending on the isotope and label, more reagents may be possible. This technique has successfully been used to quantitate changes in the NP content of several ganglia of the decapod crustacean *Cancer borealis* after feeding [9, 18]. In (Fig. 3), the mass spectrum obtained from the extract of brains of fed and unfed crabs is shown [9]. In this work, 12 NPs exhibited statistically significant ($p < 0.01$) expression level changes after feeding in the brain, and 6 were altered in the pericardial organ (PO). Mass difference tags are a useful tool for global assessment of relative abundance changes of NPs in small samples.

Label-free methods for MS-based quantitation also exist, including standard addition (reviewed in [19]). A standard can be added to samples at the same concentration and the response of analytes relative to that of the ITSD can be determined. This method assumes

that the response of the instrument to the NPs, relative to the response of a standard compound, is linear in the concentration range over which the analysis is run. With most mass spectrometers and typical working ranges, this is usually true. This technique is similar to absolute quantitation by standard addition, but skips the step where a calibration curve for the analyte and internal standard is created. Since many studies quantify hundreds of NPs at once, and the sequences of important NPs are not always known prior to analysis, it is impractical to generate calibration curves for all analytes prior to analysis and standard addition without calibration can identify interesting target compounds for further study. For this type of analysis, ITSD (usually a NP from another species to avoid potential overlap and interference) is added to the sample, the sample is run on chromatography with MS detection, and software is used to create XICs for compounds that are abundant and that show NP-typical chromatographic profiles, characterized by cohesive peak shape and elution around the same time as the NP ITSD. The AUC is calculated for each and divided by the AUC for the ITSD. The concentration changes of these compounds can thus be tracked, and those that change in response to the experimental manipulation can be selected for further study, including determination of the NP sequence. This technique is illustrated in (Fig. 2) under the heading “ITSD/AUC,” and is represented by a series of TICs obtained each of the samples. These TICs would then be extracted and normalized to allow quantitation of changes in NPs over time. Data obtained via standard addition and MS analysis must be treated as semi-quantitative and not definitive, but it is useful for the purpose of functional NP discovery.

MS/MS-BASED QUANTITATION METHODS FOR FUNCTION-DRIVEN DISCOVERY OF NPs

Tandem MS (MS/MS) can also be used for quantitation of NPs as part of a study to determine their function. MS/MS quantitation techniques have the advantage of providing sequence information in the same instrumental run as quantitative information, but they lack the sensitivity of MS-based techniques. As with MS-based techniques, isotopic labels can be incorporated into samples, or label-free methods can be applied. Tandem mass tags (TMTs) are reagents used for MS/MS quantitation of compounds, and these include isobaric tagging for relative and absolute quantitation (iTRAQ), dimethylated leucine-based tags (DiLeu) [18, 20], and others. A full description of the method by which these tags permit quantitation and sequence identification is out of the scope of this paper, and a recent review is referred to for detailed discussion [20]. Briefly, TMTs come in sets of reagents. Each reagent is reacted with a different sample, and the labeled samples are then mixed. The tag adds the same total mass to the peptide for each reagent, but upon MS/MS fragmentation, each tag yields a characteristic low-mass reporter ion due to the incorporation of different numbers of isotopes. The full complement of peptide fragments are also observed, and thus comparing the peak heights of the low-mass reporter ions permits relative quantitation and the remaining sequence-specific fragment peaks allow for sequence identification. A graphical representation of this is shown in (Fig. 2) under the heading “Tandem Mass Tags.” This shows the MS/MS spectrum from a TMT experiment, with fragment ions corresponding to the NP sequence present as well as low mass reporter ions that correspond to each of four samples. iTRAQ has been used for relative quantitation of NPs from distinct neurons in the

F cluster of the cerebral ganglion of *Aplysia californica* [21]. This method is robust and reliable, although, since it relies upon MS/MS; a larger quantity of peptide must be present compared to MS-only techniques.

Label-free techniques also exist for MS/MS-based RQ of NPs in function-based discovery studies as well. Spectral counting is a method that determines the relative abundance of a compound based on the number of MS/MS events for which it is selected. Since ions are selected for MS/MS fragmentation based on their relative intensity, this method is a reasonable estimation of relative abundances of corresponding peptides. A number of additional concerns must be addressed when conducting spectral counting, and these are reviewed in [19]. Data obtained from spectral counting is often used to generate a profile of how NP concentrations change relative to each other at each time point, and this is illustrated in (Fig. 2) under “Spectral Counting.” Although consecutive runs on a single instrument can be compared for quantitative analysis, especially if the spectral counts can be normalized to a peptide/protein that does not change across the time points, it is more reliably used to assess changes in the proportions of NPs that compose each sample. This is shown in the figure by illustrating changes in the relative amounts of NPs of each of several common crustacean NP families. Several MS and MS/MS-based tools have been developed in recent years to enable quantitation of NPs without requiring prior knowledge of these compounds’ sequences. The ability to reliably quantitate and identify NPs that change during an important biological process has been a great boon to function-directed discovery of NPs.

Novel Sampling Methods for MS-Based Functional Discovery of Neuropeptides

In addition to new MS tools for quantification of NPs, different sampling methods for use with MS-based analysis have also been established. These techniques can provide more significant temporal information about changes in NP content, or can be used to determine the localization of NPs within well-defined neural organs or neural circuits that have areas with known function.

LIVE-ANIMAL SAMPLING FOR CORRELATION OF NEUROPEPTIDE CHANGES WITH BEHAVIOR

Many MS-based functional discovery studies rely on the use of tissue-based sampling techniques which typically require sacrificing multiple animals for each biological sample, such as brain or ganglion homogenates. Although these sampling methods have been used to assign function to many NPs, high degrees of inter-animal variability in baseline levels of NPs can obfuscate net changes that may be important to NP function. Obtaining multiple samples from a single animal is thus highly desirable. Bodily fluids may be sampled via needle, but in mammals, the blood-brain-barrier (BBB) prevents changes in many NPs from being reflected in circulating levels of these compounds, and obtaining cerebrospinal fluid (CSF) is invasive. For crustaceans, the BBB is less complex, and sampling from the hemolymph provides greater information about active NPs [22]. However, hemolymph and CSF contain a wide variety of other components, and obtaining a sample of hemolymph from a marine animal *via* conventional syringe withdrawal technique induces a great deal of

stress to the animal. One sampling technique that can be used in both mammals and crustaceans to sample active NPs in a minimally invasive manner is the *in vivo* sampling method of microdialysis (MD).

MD has long been used in neuroscience to sample NPs and neurotransmitters directly from the brains of mammals, but its use in conjunction with MS-based analysis approach has only recently been established (reviewed in [23, 24]). MD employs a probe that is surgically implanted into the tissue of interest. At the tip of this probe is a dialysis membrane, through which molecules from the area outside the probe can diffuse into the probe. Liquid is perfused through the probe at a low flow rate ($\mu\text{L}/\text{min}$), and is collected at the probe outlet. This liquid carries with it molecules that diffuse through the dialysis membrane into the probe according to their concentration gradient. After recovery from the surgical implantation, animals can behave normally, and thus with MD, molecules can be collected from an animal while it is behaving normally with minimal perturbation. This allows maximal correlation of behavior with neurochemistry, and permits multiple samples to be taken from the same animal, to limit the impact of inter-animal variability. The pores of the dialysis membrane also only permit molecules below a certain molecular weight (the molecular weight cutoff (MWCO)) to pass into the probe, thus reducing sample complexity.

METHODS OF ANALYSIS FOR MICRODIALYSATES

Typical methods for quantitative analysis of microdialysates (the liquid collected at the probe outlet) include immunochemistry and chromatographic separations coupled with electrochemical, ultraviolet-visible spectroscopy, photometric, and/or SRM/MRM-based MS detection methods (reviewed in [7, 23]), all of which require *a priori* knowledge of the NP's structure or chromatographic properties. A number of NP MD studies using immunochemical detection have been conducted, however, these analyses often suffer from antibody cross-reactivity with NP of interest. As a result, it is often difficult to clearly assign functions to single molecular entities, and there are instances in which NP isoforms with slight variations in sequence have different biological effects. Although these quantitative analyses of MD samples are often part of studies of NP function, they usually rely upon quantitation that is less specific (antibody-based) and/or require prior knowledge of the NP's structure (targeted MS techniques such as SRM and AQUA), and thus are less well suited for discovery of NPs based on global comparative analysis of NPs from different functional states.

Studies where MS-based techniques are used for discovery of NPs found in dialysate, in contrast, usually do not also include quantitative analysis, and thus provide only minimal functional information [25–28]. A limitation of MS-based functional discovery for NPs is that it is difficult to obtain sequence information about novel NPs concurrent with quantitative analysis methods described previously, except when TMTs or spectral counting methods are used. Most tandem MS instruments are operated in a mode in which they dynamically switch between MS and MS/MS. Sequence information is available only from MS/MS scans, which are added to the duty cycle of the instrument in place of MS scans. This addition to the duty cycle leads to less frequent acquisition of MS data. Therefore, the number of MS data points obtained per peak decreases during the run in data-dependent

manner, and this is not reproducible between runs. Integrating the AUC for the ion of interest is not reproducible for data acquired when dynamic MS to MS/MS switching is used. Separate instrument runs must be conducted for identification based on MS/MS sequence and quantification based on MS, for which ions can be identified by their m/z and chromatographic properties.

QUANTITATION OF NPs IN MD SAMPLES WITH TARGETED MS METHODS FOR ELUCIDATION OF FUNCTION

MS quantitation methods that require prior knowledge of NP sequence to quantitate these compounds in dialysates from mammals have been reported previously [29–33]. MD studies in invertebrates are less common. Several related papers that illustrate such a targeted approach to MS-based quantitation of NPs in MD samples will be highlighted here to demonstrate the potential of MD used in conjunction with MS. Although they are not function-driven experiments, the same sampling techniques could be used with non-targeted MS approaches to conduct function-driven NP discovery. In these studies, capillary LC (capLC)-MS/MS [34] and capLC-MS³ [35–37] using SRM of known fragmentation reactions for selected NPs was conducted to quantitate NPs collected via MD from the basal ganglia of anesthetized and freely-moving rats under baseline and stimulated conditions. As mentioned previously, SRM requires that the structure of the analyte and its daughter ions is known prior to analysis. MS³ for SRM adds another level of MS analysis, in which the daughter ions generated upon MS/MS analysis are selected and fragmented, and a specific grand-daughter ion of this 2nd order fragmentation is monitored. These targeted MS techniques provide stronger evidence for a correlation between NP concentration changes and physiological stimulation, thus could lead to elucidation of a potential function of the target NP.

These studies used two types of stimulation, which provide different amounts of information about the function of the NPs monitored. Several employed K⁺-induced widespread neuronal depolarization in the globus pallidus (GP) [34] and striatum [35, 36] to induce the release of enkephalins [34–36] and dynorphins [36]. Although the non-specific nature of this stimulation does not permit assignment of an exact functional role for these NPs, correlating their release more generally with activity in the basal ganglia may point toward a functional role related to the overall functions of these subnuclei.

The most recent of these studies also employed capLC-MS³ quantitation but used more biologically relevant manipulations concurrent with behavioral monitoring to determine a more specific function for these compounds [37]. This study quantitated enkephalins in GP microdialysate from rats along with locomotor behavioral analysis, monitoring neurotransmitter (NT) concentration changes, and pharmacological manipulations. The SRM capLC-MS³ quantitation system mentioned above was used to quantitate [Met]- and [Leu]-enkephalin (ME and LE) concurrent with administration of drugs that increase dopamine levels (amphetamine) or block specific dopamine receptors 1 and 2 (D₁R and D₂R). The researchers were able to determine that ME and LE increase after administration of amphetamine, and that this effect primarily requires the D₁R. Further monitoring of NT

concentrations and locomotor behavior during administration of D₁R, D₂R, and opioid receptor antagonists permitted the development of a model for the role of ME and LE in locomotor function mediated by the GP. This study is highly significant due to the great specificity with which the authors were able to determine excitatory and inhibitory effects of neurochemicals. This was accomplished through the combined use of MS-based quantitation of NPs, a novel method for NT quantitation, behavioral modeling, and selective pharmacologic agents. This targeted study determined a specific function for ME and LE, based on MS analysis of MD samples.

DISCOVERY OF NPs IN MD WITH MS/MS TOOLS

MS tools can also be used to identify unknown NPs, and a number of such studies have been conducted [27, 38–40]. One such work is complementary to the ME and LE quantitation studies, and consists of MS/MS-based discovery of NPs from the striata of rats under K⁺-stimulated conditions [28]. This study identified 29 peptides from 6 proteins, 25 of which were isoforms that had not previously been observed. The combination of NP identification with the quantitation studies discussed previously comprises an alternative approach to function-driven NP discovery. The decision of which NPs to quantify was based on previously known information about these NPs. A function-driven discovery experiment would instead look for compounds with interesting changes throughout the course of an experimental manipulation, and then attempt to identify them. The power of functional discovery of NPs via MS-based quantitation methods discussed in this article is that the data obtained upon experimental manipulation itself determines which NPs are quantified and subjected to further study.

EXPERIMENTAL DESIGN FOR FUNCTIONAL DISCOVERY OF NPs IN MD SAMPLES

True function-driven discovery of NPs requires functional relevance to guide the choice of which compounds to study further. This can be achieved in a number of ways, but has rarely been done with MD. MS-based relative quantitation of several isoforms of dynorphin A (Dyn A) was conducted on samples collected from rats with a unilateral lesion of the striatum [41]. The identity of the isoforms was then determined by peak mass matching and MS/MS analysis. This study revealed qualitative and quantitative differences in the processing of Dyn A in the lesioned striatum. By quantitating all peaks of interest and later determining their identity, this study conducted function-driven discovery of NPs.

MS/MS-based quantitation techniques for function-driven discovery have been used rarely with MD samples. These techniques permit quantitation and identification in a single run, without prior information, but they often require larger amounts of sample, which makes them unpopular for MD. A study of this type used spectral counting to characterize the NP content of the striatum of the rat brain under baseline and K⁺-stimulated conditions [42], similar to the studies mentioned previously. In this work, 88 proteins and 100 endogenous peptides from 26 protein precursors were identified and quantified. The relative abundances of these compounds in dialysate based on spectral counts were determined, and these relative abundances were observed to change upon K⁺-depolarization. NPs made up a

greater proportion of the components observed in microdialysate after depolarization. In addition, the relative abundances of two NPs, proenkephalin A precursor and a VGF-related peptide, were greater post-depolarization. As the method of stimulation and location of probe placement was similar to previously mentioned studies, the amount and type of functional information obtained from this study is also similar, although additional compounds not previously detected were found and their quantity changes determined. One caveat is that a number of large proteins were detected, including many above the MWCO of probes used. This suggests possible damage to the tissue surrounding the probe causing large-scale protein degradation. However, this study acquired both structural and quantitative information in the same LC-MS/MS run, which is a great boon for function-driven discovery of NPs.

MD has been conducted on alert Jonah crabs (*Cancer borealis*) under baseline conditions with MS and MS/MS analysis of the resulting samples [25, 26]. Although no quantitative results were available in that study, 35 NPs previously identified in *C. borealis* were detected. A major limitation in the application of this scheme for function-related discovery of NPs was the long collection time of MD samples. Collections were at minimum 4 hours, which is not compatible with studying most of dynamic processes thought to be mediated by NPs. This long collection time was required due to the low sensitivity of the systems used. New advances in UPLC-MS and improvements to the MD collection technique termed affinity-enhanced microdialysis have made it possible to follow concentration changes in NPs on a shorter time scale with 30 min sampling intervals [43]. These enhancements have made it possible to determine the concentration changes in NPs induced by feeding in *C. borealis*, and have provided additional evidence that several NPs have feeding-related functions. Further refinement of the techniques used may allow for collection of samples on an even faster time scale, which will provide useful information about the time-resolved changes in these compounds with respect to feeding, and thus aid in elucidating the NPs involved in this important physiological event. MD sampling of alert, behaving animals, combined with MS and MS/MS techniques described previously, allows for tight correlation of NP changes with behavior, and thus is a useful technique.

MASS SPECTROMETRY IMAGING OF TISSUE TO CORRELATE NEUROPEPTIDE LOCATION WITH FUNCTION

Not only do MS-based tools permit NP function to be elucidated by correlating behavior with release over time, they allow determination of NP localization within tissue as a guide for function. Mass spectrometry imaging (MSI) is an emerging technique, in which a tissue of interest is sectioned and analyzed with MS (recent reviews [4, 44–49]). A coordinate system is superimposed on the tissue, and mass spectra are acquired for and registered to positions within this system. The intensity of a given peak in the spectra can be compiled into a heat map image to represent the distribution of that compound throughout the tissue. This technique has been applied with great success to determine the localization of NPs within the brains of several species (see selected reviews and relevant invertebrate papers [3, 44–60]). Previous knowledge about the function of certain brain structures can then be extrapolated to NPs that are heavily concentrated in these structures.

The brain of the decapod crustacean has been studied extensively, and several regions have known functions due to the locations from which they receive inputs and to which they send outputs. For instance, the olfactory neuropil (ON) and antennal lobes (AL) in the brains of decapod crustaceans both receive input from olfactory receptors, although the AL receives higher-order olfactory input—the olfactory signals are relayed through a set of interneurons before they reach the AL—and input from other senses [61]. In a MSI study of the brain of *H. americanus*, a number of patterns of NP expression were determined, including a greater expression of two SIFamide family peptides in the ON, and increased levels of two orcokinin in the AL [58] (shown in Fig. 4). The difference in localization of these two families of peptides may indicate different functional roles, i.e. SIFamides are more important in primary processing of olfactory signals, and orcokinins are involved in higher-order processing, including integration of signals from multiple sensory modalities. MSI is a useful tool that allows assignment of function to a NP based on its localization patterns within nervous structures with known functional areas. In conjunction with novel tools for quantitation with MS, advances in sampling have allowed the identification and assignment of NP function by providing information about their temporal and spatial distribution.

Validation of Potential Function for NPs Discovered via MS-Based Methods

Although function-driven discovery of neuropeptides using MS-based tools provides definitive information about NP identity, the functional information is often less complete; more targeted studies are frequently required for definitive assignment of function. Putative functions identified by MS-based functional discovery of NPs can be further investigated using a variety of targeted tools, including more traditional bioassays, “omics”-based approaches, and some previously mentioned MS techniques. Similarly, many of the methods used for functional discovery of NPs can also be used to determine the identities of compounds with functions already known. Thus, a cycle of identifying compounds and determining their functions forms the basis of neuropeptide research (Fig. 1). Some of the techniques commonly used to validate the putative functions of NPs discovered by non-targeted MS strategies will be described.

FUNCTION VALIDATION BY BIOCHEMICAL TECHNIQUES THAT REQUIRE PRIOR CHEMICAL KNOWLEDGE

Due to the fact that many of the quantitation methods used in function-driven MS study provide relative quantitation rather than absolute quantitation of peptide concentration, validation of these findings by other techniques that require *a priori* chemical knowledge is often desirable. Targeted MS-based techniques mentioned previously can be used to definitively assign function to a known peptide. These most commonly include SRM/MRM and AQUA quantitation. An example of this is provided by the microdialysis studies of enkephalins in the rat brain mentioned above [28, 34, 35, 37, 62]. Peptide quantitation by Western Blot or immunoassay is also often conducted for validation [63]. Another useful method for quantitation of NPs to validate function is mRNA quantitation, typically conducted with Northern Blot or quantitative real-time polymerase chain reaction (RT-PCR) (example [64]). These techniques can also be used to determine NP localization as an indicator of function. These targeted approaches provide valuable information about NPs

that can be used to further validate the functions of these compounds discovered by function-driven MS-based discovery.

Targeted bioassays of NP function are also commonly used to validate putative functions. In a bioassay, the output is not a quantity change over time but a more complex response. These types of studies typically rely upon application or elimination of the compound from a whole animal or isolated tissue, followed by monitoring a behavior of the animal or function of the tissue. For instance, electrophysiological recording from neurons upon application of NPs has been conducted to determine the functions of many peptides on the isolated crustacean stomatogastric nervous system (STNS). In this preparation, the nervous system is dissected from the animal and kept alive in a dish with cold physiological saline. Recording electrodes are placed in specific neurons or on nerves. The STNS of the crustacean has a characteristic firing pattern generated by several sets of ganglia in this nervous system. Several experiments have used this preparation to determine the roles of NPs discovered by MS in modulation of this firing pattern in the animal (examples [65–74], review [75, 76]). This *ex vivo* application of a NP to an isolated nervous system allows the researcher to determine if a putative function determined by non-targeted MS study has physiological action on the isolated neural circuit.

Other bioassays to validate NP function include NP administration to whole animals and knockout (example [77]) or knockdown (interfering RNA [78] or pharmacological manipulation) of NPs or their receptors either in whole animals or tissue preparations. Of these, administration of NP followed by behavioral monitoring is the most straightforward approach to determine a NP's function, and a comprehensive list of such studies cannot be provided here. One such study described the modulation of two RFamide-like peptides on the circadian clock in the cockroach *Leukophaea maderae* [79], and another study in crustaceans (the shrimp *Litopenaeus vannamei*) demonstrated the orexigenic effect of a RFamide-like NP with homology to mammalian neuropeptide F (NPF) [80]. These bioassays and targeted quantitation methods provide further support for postulated NP functions.

PEPTIDOMICS TO GENOMICS: FUNCTION DETERMINATION BY HOMOLOGY SEARCH OF PUBLIC DATABASES

MS-based discovery of NPs can also be combined with homology searches to verify function. The function of many NPs is conserved throughout evolution [1, 81–86]. Recent reviews have discussed the technique of database mining for discovery of novel NPs [5, 87–89], and searching sequences obtained via MS for homologous peptides in other related species follows a similar procedure. A novel NP sequence identified by MS/MS can be searched against the publicly available translated nucleotide database of expressed sequence tags (ESTs) using tblastn. The EST database contains a large number of sequences from many organisms and is constantly being expanded. Protein BLAST is not suitable for most peptides due to their short sequences. Searches of this type may yield a homologous peptide in a different species, for which the function is already known. Due to the conservation of function for many NPs across disparate species, a putative function for a NP can thus be

verified. Many NPs have been predicted from bioinformatics analysis of publicly accessible databases, verified to exist *in vivo* via immunochemical or MS methods, and/or determined to have biological function via bioassays (notable invertebrate examples [64, 85, 88–102]). Homology searching is not only a tool for identification of NPs, but also provides information to their function and can verify the results of a function-driven discovery study.

SUMMARY AND PERSPECTIVE

New tools and approaches that rely upon mass spectrometry and novel sampling methods have been developed that allow researchers to pursue non-targeted function-driven discovery of new compounds. These tools have the unique advantages of allowing functional information about NPs, whether it comes from following concentration changes over time or determining the distribution in a tissue, to be acquired concurrent with or before determination of the sequences of these compounds. The power of this approach is that all NPs with relevance to a particular function can be analyzed; one does not have to determine which compounds will be the focus of an experiment prior to conducting it. The added benefit of MS's ability to distinguish between related NP isoforms increases the power of these analyses and permits knowledge of the exact molecular entity that is active in the biological system. These powerful tools also enable deciphering functional consequences of NP multiplicity and structural diversity. In fact, previously unknown and unpredicted isoforms of mammalian NPs have been observed in MS-based functional discovery studies via the analysis of microdialysate [34, 42]. Previously, a molecular entity had to be characterized prior to determination of its function; now MS-based function-driven discovery can identify NPs with putative functions that can then be validated with more conventional means. In this way, NP identification and functional determination can be accomplished in a cycle (Fig. 1), with multiple iterations providing a more complete picture of both the NPs' identities and functions. By eliminating the need for prior knowledge, new and exciting NPs may be discovered with important functional roles, and vice versa.

In addition, the field of mass spectrometry has experienced constant growth and innovation, and advancements in MS technology improve the sensitivity, speed, dynamic range, and accuracy of analysis on a regular basis. Although the NPs observed most in current MS studies are likely to be the most abundant *in vivo*, future improvements will lead to elucidation of roles for those NPs that are currently below the limits of detection or quantitation for current instruments. By employing MS in function-driven non-targeted NP discovery workflows, even greater information can be obtained as new technology is developed. The types of experiments enabled by future generations of MS instrumentation will further enhance information to be gained about the molecules that mediate intrinsic physiological properties.

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ABBREVIATIONS

AQUA	Absolute quantitation by addition of isotopically labeled internal standard
AL	antennal lobe
AUC	area under the curve
BLAST	basic local alignment and sequencing tool
BBB	blood-brain barrier
CapLC	capillary ultrahigh performance liquid chromatography
CSF	cerebrospinal fluid
DD	differential display
DiLeu	dimethylated leucine isobaric tags
D₁R	dopamine receptor 1
D₂R	dopamine receptor 2
DynA	dynorphin A
EST	expressed sequence tag
ITSD	internal standard
iTRAQ	isobaric tags for relative and absolute quantitation
LE	leucine enkephalin
MS	mass spectrometry
MSI	mass spectrometry imaging
ME	methionine enkephalin
MD	microdialysis
MWCO	molecular weight cutoff
MRM	multiple reaction monitoring
NP	neuropeptide
NPF	neuropeptide F
ON	olfactory neuropil
PO	pericardial organ
RT-PCR	real-time polymerase chain reaction
RQ	relative quantitation

SRM	selected reaction monitoring
STNS	stomatogastric nervous system
MS/MS	tandem mass spectrometry
TMT	tandem mass tag
UPLC	ultrahigh-performance liquid chromatography
VGF	nerve growth factor
XIC	extracted ion chromatogram

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Figure 1.

The role of function-driven NP discovery in the iterative cycle of neuropeptide identity and function determination. A number of techniques exist to determine NP function that require prior identification of the compounds, which was previously done with methods devoid of input from functional studies. Function-driven MS discovery permits discovery of functionally-relevant NPs via comparative peptidomic analysis. The putative functions thus identified can be validated with other tools. Functions previously determined with less specific analytical tools can also be verified and the exact identities of functionally-relevant compounds determined. This permits cyclical study of NPs to better understand their identities and functions.

Figure 2.

Schematic representing the different types of MS-based tools that can be used for function-driven NP discovery, by providing quantitative information about different samples. In this schematic, samples are taken from animals either before and after an experimental manipulation, or several times during the manipulation. MS and MS/MS-based techniques, both those that incorporate isotopic labels and those that do not, are illustrated in the chart below. Further details of these techniques and explanations of abbreviations are found in the text.

Figure 3.

A) Workflow for differential display (DD) of NPs with expression changed after feeding in *C. borealis*. **B)** Representative MALDI-TOF/TOF mass spectrum of labeled mixture of brain extracts from fed and unfed crabs. The heavy labeled peaks (fed crabs, n=3) are indicated with open circles, and the light labeled peaks are indicated with closed circles (unfed crabs, n=3). Several abundant neuropeptides are indicated and labeled with their corresponding amino acid sequences. Adapted with permission from Chen, R., Hui, L., Cape, S. S., Wang, J, and Li, L. *ACS Chem. Neurosci.* **2010**, 1 (3), pp 204-214. Copyright 2010 American Chemical Society. (Ref. 9)

Figure 4.

I) schematic illustrating MALDI imaging workflow. Adapted with permission from Chen, R., Hui, L., Cape, S. S., Wang, J., and Li, L. *ACS Chem. Neurosci.* **2010**, *1* (3), pp 204-214. Copyright 2010 American Chemical Society (Ref. 9). **II)** MALDI imaging of neuropeptide localization in the *H. americanus* brain using both MALDI TOF/TOF and MALDI LTQ Orbitrap mass spectrometers. (a) Schematic drawing of the lobster brain, which contains multiple neuropils, including anterior (AMPN) and posterior medial protocerebral neuropils (PMPN), olfactory lobe (ON), accessory lobe (AL), antenna I neuropil (AnN) and lateral II antenna neuropil (LAN). Ion images of (b) VYRKPPFNGSIFamide (m/z 1423.8) and (c) NFDEIDRSGFGFN (m/z 1517.7) were obtained using MALDI TOF/TOF. Ion images of multiple known neuropeptides were acquired by MALDI LTQ Orbitrap, including: (d) VYRKPPFNGSIFamide (m/z 1423.8); (e) APSGFLGMRamide (m/z 934.5); (f) NFDEIDRSGFGFN (m/z 1517.7); and (g) overlay of the above three neuropeptides. A novel peptide HI/LASLYKPR (m/z 1084.6) in the lobster brain was also mapped using MALDI LTQ Orbitrap instrument by (h) precursor ion scanning of m/z 1084.6 and (i) selected reaction monitoring of transition between m/z 1084.6 and sequence-specific fragment ion m/z 685.4(b6). Adapted with permission from Chen, R., Jiang, X., Conaway, M. C. P., Mohtashemi, I., Hui, L., Viner, R., and Li, L. *J. Proteome Res.* **2010**, *9*(2), pp 818-832. Copyright 2010 American Chemical Society (Ref. 58).