Developing Mass Spectrometry for the Quantitative Analysis of Neuropeptides

Christopher S. Sauer[1], **Ashley Phetsanthad**[1], **Olga L. Riusech**[1], **Lingjun Li**[1,2]

[1]Department of Chemistry, University of Wisconsin-Madison, 1101 University Avenue, Madison, WI 53706, USA

[2]School of Pharmacy, University of Wisconsin-Madison, 777 Highland Avenue, Madison, WI 53075, USA

Abstract

Introduction: Neuropeptides are signaling molecules originating in the neuroendocrine system that can act as neurotransmitters and hormones in many biochemical processes. Their exact function is difficult to characterize, however, due to dependence on concentration, post-translational modifications, and the presence of other comodulating neuropeptides. Mass spectrometry enables sensitive, accurate, and global peptidomic analyses that can profile neuropeptide expression changes to understand their roles in many biological problems, such as neurodegenerative disorders and metabolic function.

Areas Covered: We provide a brief overview of the fundamentals of neuropeptidomic research, limitations of existing methods, and recent progress in the field. This review is focused on developments in mass spectrometry and encompasses labeling strategies, post-translational modification analysis, mass spectrometry imaging, and integrated multi-omic workflows, with discussion emphasizing quantitative advancements.

Expert Opinion: Neuropeptidomics is critical for future clinical research with impacts in biomarker discovery, receptor identification, and drug design. While advancements are being made to improve sensitivity and accuracy, there is still room for improvement. Better quantitative strategies are required for clinical analyses, and these methods also need to be amenable to mass spectrometry imaging, post-translational modification analysis, and multi-omics to facilitate understanding and future treatment of many diseases.

Keywords

Correspondence: Lingjun Li, lingjun.li@wisc.edu, Phone: (608) 265-8491, Fax: (608) 262-5345. Mailing Address: 5125 Rennebohm Hall, 777 Highland Avenue, Madison, WI 53706.

1. Introduction:

Regulation of the nervous system is a strictly controlled process influenced by a plethora of signaling peptides, neurotransmitters, hormones, and other modulating molecules [1,2]. These neuromodulators are critical to behavior [3–5], stress responses [6–11], maintaining homeostasis [12,13], and many other biological processes [14–16]. Neuropeptides, signaling peptides originating in the neuroendocrine system, are of particular interest as they have highly diverse function and structure, dynamic expression, and many sites of action [1]. Dysregulation of neuropeptides has been implicated in many diseases and biological states, including heavy metal toxicity [8], hypoxia [7], Alzheimer's disease [17], depression [18], and others. As a result, comprehensive characterization of neuropeptides could have many benefits such as discovering biomarkers or elucidating important biological pathways involved. However, such global analysis is challenging not only due to many neuropeptides having low in vivo abundance and high structural diversity, but also because neuropeptide function is influenced by many factors, such as location, post-translational modifications, and the presence of other co-modulating neuropeptides [1].

This complexity inherent to all neuropeptidomic studies is exacerbated by the difficulties in determining possible neuropeptide sequences. Neuropeptides are produced by the select processing of precursor proteins (i.e., preprohormone) encoded within the genome [19]. These preprohormones contain a signaling sequence and the remaining prohormone. After cleavage of the signaling sequence, the prohormone is selectively and specifically cleaved by endopeptidases, such as various prohormone convertases, to produce several peptide sequences from a single precursor protein [20]. The peptides are then processed further and post-translationally modified to produce the bioactive neuropeptides [20]. The intricate pathways from genome to active neuropeptide, splice variants, and diversity of post-translational modifications lead to many possible peptide forms that are difficult to predict from genomics or even transcriptomics alone. This compounded with the fact that many model organisms do not have a fully sequenced genome to use as a reliable starting point for predicting a full neuropeptide database makes neuropeptide studies even more challenging.

Mass spectrometry (MS)-based approaches have led to enhanced workflows for detecting, characterizing, and quantifying neuropeptides in various samples. MS can provide detailed, high-accuracy information about the intact mass, sequence, modifications, and expression levels of a detected neuropeptide [1]. Furthermore, MS analyses can profile many analytes (neuropeptides) from a sample in a single experiment without requiring extensive *a priori* knowledge. This unbiased, untargeted method facilitates discovery-based neuropeptidomics. As neuropeptide function is influenced by the other neuropeptides present, methods to detect the suite of neuropeptides expressed in a sample are critical to understanding the underlying signaling mechanisms of neuropeptides.

Using various labeling and label-free strategies, MS can further provide useful quantitative information that can be used to determine neuropeptide expression differences between samples [21]. Alternative MS analysis methods, such as data-independent acquisition (DIA)

can enable greater depth of coverage for the neuropeptidome [22]. These quantitative strategies can be applied not only to the detection and identification of neuropeptides, but also to identifying different forms of neuropeptides with post-translational modifications [23]. Additionally, there have been many developments in the field of MS imaging (MSI) to enable the detection of neuropeptides in specific locations of a tissue or tissue section [13,24,25]. This spatial distribution provides an additional level of information for quantitative neuropeptidomics. Furthermore, MS has applications in numerous -omics (e.g., proteomics, metabolomics), that can offer complementary information to the quantitative neuropeptidomic workflows. By combining the structure elucidation, quantitative information, spatial distribution, and analysis of correlated biomolecules, analysis by MS has greatly improved our understanding of neuropeptides and their roles in many biological processes. This review aims to highlight recent developments in the broader field of neuropeptidomics with an emphasis on quantitative strategies.

2. Quantitative Strategies:

As neuropeptide function has some dependence on concentration [26], being able to reliably identify and quantify neuropeptides is necessary for understanding their function, especially as it relates to different biological states. A recent review has been published detailing mass spectrometry strategies applied to functional neuropeptidomics [27]. These functional studies are challenging, however, due to the low concentrations (as low as femtomolar) *in vivo* [26] of neuropeptides, highlighting the continual need for improved quantitative methods. Although MS is not inherently quantitative, its widespread application and growth in the -omic fields has led to the development of various strategies for accurate and sensitive quantitation. The quantification of neuropeptides has been achieved with label-free methods that allow analysis of the neuropeptides without modification and minimal sample loss [28]; a variety of labeling strategies that facilitate more reliable quantitation and greater throughput analyses [29]; and more recently, DIA workflows to increase reproducibility and enable detection of more low abundance analytes and thus deeper profiling of the neuropeptidome [22]. These methods are of course not the only quantitative methods for neuropeptidomics and others have been reported and summarized in other reviews [30,31].

2.1 Label-free Quantitation:

In MS workflows, certain steps, such as desalting, are crucial, but researchers will often forgo extraneous steps to reduce sample loss for low abundance species like many neuropeptides. Label-free quantitation (LFQ) strategies may suffer from reduced throughput, but they often provide the least amount of sample processing (and fewer losses from it). In LFQ methods, samples are analyzed independently of each other, commonly using LC-MS or LC-MS/MS approaches [21,32]. Quantitation is typically performed by comparing signal intensities (e.g., chromatographic peak area). By examining the extracted ion chromatogram (XIC) of analytes, quantitation is achieved by comparing peak areas between sample runs. Controlling for run-to-run variability is critical for reliable quantitation as samples are run separately [28]. Software is typically used to align the XICs by retention time and filter data, such as ensuring the precursor/fragment ions and the charge states match between aligned peaks [33,34]. Additionally, normalization between

analyses needs to be considered to facilitate more accurate comparisons between samples, as variations can arise from instrument calibration, sample preparation, and ambient temperature during analysis. Various methods and software packages for normalization exist and are systematically evaluated in a recent article by Välikangas et al [35]. Ye et al. demonstrated the efficacy of LFQ to quantify neuropeptide expression changes as a result of feeding in a rat model [36]. Anapindi, et al. similarly used XIC analysis to examine neuropeptide expression, but on a much larger scale; over 200 LC-MS runs were performed to identify and quantify over 1500 neuropeptides to examine their effect on chronic migraine and opioid-induced hyperalgesia [4]. Targeted approaches have also proven useful; Salem et al. performed relative quantitation of surrogate neuropeptides and their fragments to characterize the processing of pro-neuropeptides to mature neuropeptides [37]. Frequently, targeted approaches will employ parallel reaction monitoring (PRM) in which a targeted precursor ion and subsequent fragment ion are analyzed. Similarly, multiple reaction monitoring (MRM) analyses a single precursor ion and multiple fragment ions from that precursor ion. As these approaches minimize interference from other matrix components, sensitivity and throughput are greatly enhanced [38]. Although often used for measuring surrogate peptides from a targeted protein, MRM and PRM have seen use in peptidomics workflows, such as the analysis of orexin in mice cerebrospinal fluid [39] and the identification of endogenous signaling peptides in insects [40]. While MRM and PRM have high sensitivity, the simplicity of using XICs for quantitation allows it to be easily applied to novel and/or less developed MS workflows. Bianco et al., for example, used XICs to characterize differences between arginine and lysine vasopressin after analysis by Fourier transform ion cyclotron resonance (FTICR) MS with multiphoton dissociation [41].

LFQ workflows can also employ spectral counting for quantitation. Spectral counting assesses protein abundance by correlating concentration to the number of times a constituent peptide is identified, with the idea being that more abundant proteins will be identified more frequently in a single run [33]. The protein abundance index (PAI) is calculated from the normalized level of observed peptides per protein and its exponential modification (emPAI) is used to estimate protein concentrations [42]. In neuropeptidomic experiments, however, identifications are not made based on constituent peptides, as done in bottom-up proteomics, because the endogenous neuropeptide is generally not digested prior to LC-MS analysis. This greatly reduces the applicability of spectral counting in neuropeptidomic experiments, but it still has gained some use, typically with XIC information used for improved confidence. Southey et al. has shown that spectral counting and spectral indexing (based upon the cumulative intensity of product ions) provide more informative characterization of neuropeptides in the rat suprachiasmatic nucleus over XIC [28]. Other groups have published quantitative methods that utilize both XIC and spectral counting for characterizing endogenous peptides outside of the central nervous system. By comparing the peptidomes of patients with systemic juvenile idiopathic arthritis (SJIA) to healthy patients, LFQ methods enabled the detection of 17 potential biomarkers for SJIA in urine [43]. Similarly, Labas et al. used LFQ methods to characterize peptides in chicken semen to identify key peptides for phenotyping [44]. Although these applications do not address neuropeptides specifically, the peptidomics workflow is translatable and provides future directions for neuropeptide

analyses. Even as other quantitative methods are developed, the scalability and ease of using LFQ will likely ensure its continued use in the future.

2.2 Label-based Strategies:

Although label-free quantitation is adaptable, requires fewer sample processing steps, and is amenable to analyses of many samples, it often requires a greater number of LC-MS runs and can suffer from run-to-run variability. Conversely, many labeling strategies exist that allow samples to be run simultaneously, enabling accurate multiplex quantitation with fewer control samples required, reduced instrument time, and decreased effects due to instrument variation [21]. For neuropeptide applications, most labeling reactions occur post-extraction and create a mass difference between channels via stable isotope incorporation to differentially label samples. Reductive dimethylation is frequently used to label neuropeptides as it targets primary amines (N-termini and lysine residues), common to most neuropeptides. Additionally, this reaction is low cost and easily accessible, requiring only isotopic formaldehyde and a reducing agent such as borane pyridine or cyanoborohydride [45]. Isotopic dimethylation has been used extensively in the Li lab to study neuropeptidomic changes in crustacean models [7,10,11,46,47]. Moreover, the group has shown the method is compatible with both electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) methods to provide complementary coverage of neuropeptides in a single sample [7]. Wilson et al. has developed an online dimethyl labeling system in which neuropeptides are derivatized on-column with either light or heavy reagents [48]. The Fricker lab has also published several articles using isotopic labeling for quantitative peptidomics. They have expanded the reductive dimethyl labeling scheme to an impressive five channels while improving accuracy with isotopic correction calculations [45]. Similarly, the lab has expanded the use of trimethylammoniumbutyryl (TMAB) chemical tags to a 4-plex [49,50]. TMAB tags add a permanent positive charge via a quaternary amine to N-termini and lysine residues of peptides using an amine-reactive NHS ester. These tags have been widely used in peptidomics to study numerous biological processes, including peptide degradation [51], prohormone processing [52], narcotic effects [53], and can be transferred to neuropeptidomic applications. These tags are effective, easily synthesized, and increase signal intensity by incorporating a permanent positive charge. This permanent charge, coming from a charged quaternary amine, does make the peptide more prone to dissociation, thus increasing instability and limiting application.

One of the major limitations with isotopic labeling, as evidenced by Buchberger et al. [7] and others [45], is the added spectral complexity with higher multiplexing. This can lead to difficulties in accurately calculating relative abundances, even with isotopic corrections. Additionally, given the wide dynamic range of neuropeptides, it is possible (and perhaps likely) that peaks corresponding to downregulated neuropeptides could be too low in intensity to be quantified or even be detected. Even analytes of high enough intensity might still not be selected for fragmentation and tandem MS analysis by commonly used data-dependent acquisition (DDA) settings, simply due to the greater number of precursor peaks that come with an isotopic labeling workflow. Isobaric tagging–where neuropeptides are labeled with isotopically encoded tags that add the same mass but produce unique reporter ions upon tandem MS analysis–can offer advantages over conventional isotopic

labeling. Figure 1 depicts the differences between isotopic (**1A**) and isobaric tagging (**1B**). These methods are capable of significantly higher order multiplexing, such as 8-plex with iTRAQ [54], 16-plex with TMTpro [55], or 21-plex with DiLeu [56], because only the MS^2 spectra have added complexity due to the unique reporter ions. Moreover, the tags are strategically designed to create reporter ions in regions of the spectrum that do not contain other useful peaks. Isobaric tags have been used often for proteomics applications, but have had fewer applications with peptides, especially neuropeptides. Dimethylated leucine (DiLeu), for example, has been used to quantify neuropeptides in lobster brains as a function of growth cycle [15], and also as part of a multi-omic profiling of the mouse hypothalamus [57], but the low abundance of many neuropeptides still presents challenges. As quantitation occurs at the MS^2 level, isobaric tagging requires analytes be selected for fragmentation and subsequent tandem MS analysis. Low abundance analytes, like many neuropeptides, are often omitted from tandem MS analysis using conventional DDA strategies. Recently, Sauer and Li have shown that optimization of the DDA parameters can facilitate a greater depth of coverage in the crustacean neuropeptidome. This enabled the characterization of many neuropeptides dysregulated in response to copper toxicity [8]. Similar optimization strategies have been used to improve proteomic coverage [58,59], highlighting a problem not unique to the field of neuropeptidomics. As instrumentation and analytical capabilities improve, it is likely that we will see an increased prevalence of isobaric and isotopic labeling methods reported for neuropeptides and other low abundance analytes.

2.3 Data-Independent Acquisition:

Conventional identification and quantitation of peptides and proteins by mass spectrometry typically involves DDA to trigger and obtain MS^2 fragmentation spectra. Prioritizing fragmentation of the higher signal intensity ions, this acquisition method is biased towards higher abundance and more easily ionizable species. Fragmentation spectra are crucial to obtain confident identification of biomolecules. Neuropeptides are often of low abundance compared to other biological and neurological matrix components [60]; their analysis can benefit greatly by using a less biased fragmentation scheme. DIA methods were developed to overcome the limitations of DDA, by fragmenting all molecules within a desired *m/z* window, not only the highest signal intensity ions [61], as demonstrated in Figure 2. During DIA MS, all molecules within a *m/z* isolation window of user-defined width are fragmented, without a precursor selection to trigger fragmentation. Fragmentation ion spectra are collected from different isolation windows in a cyclic manner, from the beginning of the defined *m/z* survey range to the end before repeating. MS^1 spectra are collected every cycle, or however often is desired. The use of DIA enables the identification, and therefore potential quantitation, of more molecules compared to DDA [61,63–65], including lower abundance molecules such as neuropeptides [22]. As a newer method, DIA MS does not have the breadth of application that DDA does and is not yet commonly applied to neuropeptide analyses. However, the field of neurobiology has seen great benefit from the adoption of quantitative DIA MS, as discussed in a recent review on its application to quantitatively analyze the brain proteome [66].

Although not all quantitative, a few MS analyses using DIA have recently been performed to better improve the characterization of neuropeptides. DIA MS was used in the untargeted

quantification study of neuropeptide expression changes in response to feeding activity in crustacea [67]. The authors were able to detect and quantify 137 peptides directly from microdialysate with minimal sample preparation; no extraction or precipitation steps were required, demonstrating the utility of DIA for the quantification of limited samples [67]. While DIA MS is a powerful technique for untargeted quantification of neuropeptides, Saidi et al. demonstrated its limitations and higher variances during targeted quantification studies of neuropeptides found in animal spinal cord tissues [38]. Delaney and Li also evaluated the utility of DIA MS for the quantification of neuropeptides from crustacean neural tissue. While improving the technical and biological reproducibility of analysis and number of overall neuropeptides identified compared to DDA, the DIA method showed poor quantitative accuracy using a label free approach [22]. To improve LFQ of peptide hormones using DIA MS, a method was developed using an internal standard peptide, enabling accurate quantitation [68]. As the field of DIA MS quantification further advances, new strategies to improve its abilities to characterize neuropeptides will need to be developed.

The utility of DIA searches using a spectral library-free approach and database searches generated from FASTA sequences has been demonstrated for analysis and quantitation. Model spectra generated from these methods are not always as reliable for neuropeptides as the algorithms are often developed for tryptic peptides. PTMs and structural diversity of endogenous peptides complicate these matters, making it difficult to obtain good results without high-quality spectral libraries. Although identification through spectral library searches have been shown to increase identification and quantitation reproducibility [69], spectral library generation, requiring high amounts of starting material, may not always be feasible due to the limited sample concentration of neuropeptides. Database searching and spectral-free methods [70–74] will likely be more beneficial to the future study of neuropeptides by DIA MS.

Overall, DIA has been shown to improve sensitivity of analysis for both identification and LFQ [75], enabling consistent detection with up to a 10-fold increase in sensitivity compared to DDA, and improving the quantitative dynamic range of analysis [61,76]. Notably, the reproducibility and quantitative performance of DIA MS methods was evaluated by 11 sites worldwide, and reproducible quantitation of proteins was observed [76]. Reproducibility is very important for the quantitation of samples; the same analytes must consistently be identified across all conditions for comparison. A large limitation to DIA analysis is the duty cycle for each scan and total time it takes to cycle through the entire desired *m/z* range. Careful consideration is required when choosing parameters such as maximum ion injection time, automatic gain control target, isolation window width, and full *m/z* range, among others, as this may cause analytes to not be fragmented during the appropriate window before fully eluting during the LC gradient [77]. This issue is further exacerbated during DIA MS for quantitation as accurate quantitation requires data to be collected at multiple points across a peak profile. Further complicating quantitation by DIA MS, multiplexed label-based methods increase spectral complexity, although such methods have recently been utilized in a few proteomics experiments [78–81]. Additional information about the utility and considerations of DIA MS for neuropeptide analysis and quantitation, including software resources can be found in a recent comprehensive review detailing advances in

the MS analysis of neuropeptides [manuscript under review]. More general reviews for proteomics also exist [64,72,82–84].

DIA can also be beneficial for the analysis of heavily modified analytes where MS signal intensity is distributed across multiple proteoforms, as this increases the number of precursors needed to be selected for fragmentation using DDA. A limitation to this method is the deconvolution of data; multiple precursor ions are co-fragmented in MS^2 spectra and PTMs can further complicate fragmentation spectra. Although it has been shown to benefit the analysis of glycosylated proteins [85–87], this analysis has not yet been applied to the field of neuropeptidomics. Even with the limitations of DIA, it has been shown to successfully identify more peptides and neuropeptides, with improved reproducibly. With a limit of detection (LOD) in the amol range [61] and improved quantitation capabilities [76], the field of neuropeptidomics would benefit greatly from the adoption of quantitative DIA MS workflows.

3. Post-Translational Modifications:

Neuropeptides and other bioactive peptides are formed after enzymatic cleavage of larger precursors by peptidases. Additional enzymes can alter these peptides with PTMs, altering their structure, function, and stability, among other effects, contributing to the vast diversity of neuropeptides. Neuropeptide modifications can include amidation, phosphorylation, acetylation, glycosylation, and sulfation, to name a few [88]. Identifying and differentiating between these forms is crucial to understand molecular mechanisms in neurobiology, and thus these modified neuropeptides are investigated using MS by a variety of labs [10,89–91], thoroughly summarized in a few recent reviews [92]. The quantification of post-translationally modified neuropeptides faces additional challenges. Labeling approaches often target specific residues and moieties, so post-translational modification of these residues often inhibits quantitation via labeling. For example, many tags target primary amines and are therefore ineffective or less effective for peptides with acetylated N-termini. Further challenging analysis of modified peptides is the decreased ion signal intensity due to poor ionization efficiency of the modified peptides or from distribution of already low abundance neuropeptides across the differentially modified forms. This leads to the need for targeted analyses and enrichment strategies to detect and quantify peptides with PTMs [93–95], especially for those modified by highly dynamic glycosylation [95–98]. As MS considerations are more prominent for peptides modified by glycosylation [94,99,100], we will focus on the discussion of glycosylated peptides.

Estimated to modify potentially 33% of all known human peptide hormones [44], changes in glycosylation have a large impact on the role and efficacy of neuropeptides and other bioactive peptides [101–104]. It is therefore of interest to improve quantification abilities for these lower abundance peptides with decreased ionization efficiency compared to their non-modified counterpart [97]. During a targeted analysis to characterize insulin and other signaling peptides in pancreatic islets, Yu et al. discovered insulin to be glycosylated and found this form to be differentially regulated in mouse models of diabetes [105]. This demonstrates the need for more attention to be given to the analysis and quantification of modified signaling molecules. To this goal, Hansen et al. investigated the potential

presence of glycans on atrial natriuretic peptide (ANP), a peptide hormone with its proteolytic degradation and potency being regulated by glycosylation. They characterized and quantified glycosylated ANPs using a targeted MS approach and demonstrated glycosylation on ANP to impact its stability, circulation time, and receptor activation in rats [106].

To improve the characterization of glycosylated neuropeptides, sensitive and accurate MS methods must be developed. Several dissociation methods have been investigated to better identify glycopeptides, namely collision-based [107–110] and electron-based [107,109,110] dissociation methods. Hybrid methods have also been developed to improve glycopeptide analysis such as electron-transfer/higher-energy collision dissociation (EThcD) [110], higher energy collision dissociation (HCD) product ion-triggered electron transfer dissociation [111], and HCD product ion-triggered EThcD [105]. EThcD has also been shown to be valuable for quantitative proteomics of phosphorylated biomolecules as well [112]. The type of fragmentation scheme used is important to consider during glycopeptide analysis. Riley et al. demonstrated the need for optimizing dissociation methods, finding peptides modified by N-glycans and O-glycans require different dissociation methods for optimal fragmentation [113]. The information-rich fragment ion spectra generated from EThcD, shown in Figure 3, is vital for the confident localization of O-glycans. To characterize glycosylated neuropeptides in crustaceans, Cao et al. utilized HCD triggered EThcD [23]. This study demonstrates the method's utility for sensitive glyconeuropeptide analysis. Though not applied to endogenous peptides, Zhu et al. describe a three-part workflow for the in-depth investigation of proteins and glycoproteins in the central nervous system [114]. More broad strategies for the quantification of glycosylated proteins and digested peptides have been discussed thoroughly in a recent review [115]. Although glycoproteomics and its quantification is of great interest to the scientific community, we can see that there has been a disproportional amount of investigation into glycosylated neuropeptides, and even less with quantitative approaches applied. This is an underdeveloped field but as glycoproteomic strategies improve in the future, we expect to see them applied to the field of neurobiology towards quantifying endogenous peptides more readily.

4. Mass Spectrometry Imaging:

As various modes of MS are being applied to neuropeptide discovery, there has been an increased emphasis in determining the biological relevance through localizing neuropeptides. MSI has been preferentially implemented in these studies due to the unique advantage of permitting targeted and untargeted detection of analytes within a tissue or cell while still providing spatial information [116,117]. To achieve this, many researchers often use MALDI, where the surface area of a tissue is portioned into data-attainable units (pixels) and imaged through several laser ablations. Quantitative MSI of neuropeptides can be achieved in different ways including absolute quantification using labels to create a calibration curve and semiquantitative spiking with an internal standard [116]. Here we discuss recent advances in MSI that enable quantitative and semiquantitative analysis of neuropeptides.

Within the field of neuropeptidomics, relative quantitation is widely practiced due to easier sample preparation and reduced costs. Information obtained by these relative quantitation methods can be increased through biological means such as immunohistology assays and Nissl staining [118,119]. These techniques are applied to tissue after MSI to normalize neuropeptide quantities with respect to number of nerves and free amines present. The relative abundance and spatial information from MSI is valuable, but still comes with challenges such as localized ion suppression, availability of software, duration of data collection, and having a sufficient MSI analyzer [120–123]. These issues are exacerbated by the low concentration of neuropeptides in tissue. There have, however, been advances in desorption electrospray ionization (DESI) and liquid extraction surface analysis (LESA) MSI in lipidomics that may help mitigate these issues [124–126]. Zemaitis et al. demonstrates an increased signal intensity in DESI-MSI compared to MALDI-MSI, leading to higher resolving power for lipids [124]. This can likely be attributed to a lack of matrix clusters, which in MALDI, contribute to a pattern of low ionization efficiency [124,125]. Some imaging techniques seek to circumvent these issues by performing DESI and MALDI on the same tissue, permitting both proteomic/peptidomic and metabolomic analyses [126]. The use of LESA shows increased signal intensity due to larger sampling size, making this method faster and more sensitive at the sacrifice of spatial resolution [126]. Both LESA and DESI come at the expense of spatial resolution; where MALDI can often offer a few micron spatial resolution, DESI is limited to 50–100 micron and LESA images often have pixel sizes of 1000 microns. These techniques can be coupled with subsequent LC-MS/MS to verify trends of relative abundances seen in imaging experiments or XIC for LFQ [117,127].

Practices are being explored in drug MSI for better absolute quantitation using more sensitive mass analyzers, such as FTICR, coupled with an increased number of internal standards as well as adjusting calibration curves to pixel deviation [123,128]. Techniques under development work to decrease the LOD such as those discussed in metabolomics works implementing surface-assisted laser desorption/ionization (SALDI) [129] and other matrix-free MSI methods where nanoparticles are used to coat the tissue to enhance ionization efficiency as well as spatial resolutions (Figure 4) [130,131]. These methods incorporate metals and have been found to retain spatial resolution of MALDI (~2–5 microns) while increasing ionization efficiency for small molecules. They are, however, presently limited to small molecules like drugs and metabolites. Such relative abundance techniques, combined with LFQ via LC-MS/MS back-correlation and software development, could greatly improve the accuracy and performance of quantitative MSI studies. The many forms of MSI complement the diversity of biological problems being studied, and advancements in MSI of neuropeptides will provide insights into the anatomy and physiology of neurological function and regulation. This in turn will help clinicians and other researchers identify and treat various health conditions.

5. Multi-omics:

The exact roles of neuropeptides are often difficult to discern when strictly observing only neuropeptides. Their receptors are typically proteins, such as G protein-coupled receptors (GPCRs) [132]; they regulate biochemical pathways with downstream metabolite products [133]; and are often co-expressed and co-released with small molecule neurotransmitters

[134]. Multi-omic workflows have emerged in which elements of proteomic, peptidomic, metabolomic, etc. experiments are integrated to provide more comprehensive information [135–137]. As a result, multi-omic experiments have seen increased popularity in neuropeptide experiments in recent years.

5.1 Small Molecule Studies:

Characterizing neuropeptides and relevant small molecules in a single experiment is difficult due to stark differences in extraction efficiencies, solubilities, ionization efficiencies, fragmentation patterns, and subsequent data analysis. Specific applications have further constraints, such as matrix compatibilities for MSI. Nevertheless, researchers have made recent advances in combining neuropeptidomics with small molecule analyses (e.g., lipidomics and metabolomics), typically by processing the two separately, then co-analyzing the data. Keller et al. addressed the extraction issues using both acidified methanol and methanol/water/chloroform extractions to efficiently recover proteins, peptides, and metabolites. Two molecular weight cut-off (MWCO) filters were used to separate metabolites (<3 kDa), peptides (3–30 kDa), and proteins (>30 kDa) [137]. Although not specifically studying neuropeptides, the reported extraction methods (i.e., acidified methanol), have been used previously for neuropeptides [7,8,22,46]. Gutierrez et al. reported a similar strategy in which an acetone/chloroform precipitation was used to separate proteins and metabolites [136]. This method, dubbed SPOT (sample preparation for multi-omic technologies), is not described for the study of endogenous peptides, but could be adapted by adding a MWCO step similar to Keller et al. For some approaches, neuropeptides do not necessarily need to be isolated from metabolites prior to LC-MS analysis. Chen et al. used atmospheric pressure (AP) MALDI to study neuropeptides, lipids, and other biomolecules from the same tissue section, but incorporated different ionization methods [138]. This niche strategy eliminates issues with extraction and ionization efficiency differences by collecting data directly from the tissue section. The lack of tandem MS data, however, does require researchers to rely on accurate mass matching, possibly losing confidence in data interpretation

Often co-expressed and released with neuropeptides, neurotransmitters are another target for multi-omics. Wojnicz et al. report a method that combines short neuropeptides (4 residues) and metabolite analyses to study bovine cells without the need for separate extractions. Using synthetic standards, they created a calibration curve that allowed absolute quantitation of both neuropeptides and neurotransmitters [133]. These methods would likely have limited applicability to larger neuropeptides but are important in establishing multi-omic strategies. Similarly, zwitterion exchange has been used for online separation prior to LC-MS to quantify neurotransmitters and select neuropeptides (oxytocin and vasopressin) simultaneously from blood [139]. Alternative separation and sampling methods, like microdialysis coupled to LC-MS, have offered sensitive assays for quantifying both neuropeptides and neurotransmitters, summarized in a review by Zestos and Kennedy [140].

5.2 Proteomics:

Although similar in structure to proteins, neuropeptides require different analytical workflow than proteins largely due to size differences. Most multi-omic workflows that combine

proteomics with the study of endogenous peptides like neuropeptides analyze the two separately and interpret the combined data. For example, Liu et al. describes the use of label-free neuropeptidomics with multiplex DiLeu-labeled neuroproteomics, workflow highlighted in Figure 5, to profile changes in the mouse hypothalamus resulting from the gut microbiome [57]. This study demonstrated the impact of the gut microbiome on neurochemical processes [57]. Similarly, Chen et al. combined label-free and labeled data to interrogate proteomic, metabolomic, and peptidomic (translatable to neuropeptides) dysregulation in metabolic diseases [135]. Neuroproteomics has also revealed dysregulation of neuropeptide and neurotransmitter release with impacts in neuropsychiatric diseases, such as addiction, using LFQ MS [141]. Conversely, Hook et al. characterizes neuropeptide variants to inform neuroproteomics and precursor protein analysis to better understand proteolytic processing and how it relates to cell-cell signaling [88]. The study of endogenous peptides outside of the neuroendocrine system can also provide translatable methods for multi-omic neuropeptide studies. For example, Labas et al. do not specifically study neuropeptides, but report a LFQ assay to combine proteomic and peptidomic data to phenotype chicken semen in a multi-omic experiment using spectral counting and XICs from LC-MS data [44]. By omitting the digestion step typically used to study proteins (bottom-up proteomics), Li et al., describe the use of top-down MS methods to study microproteins and endogenous peptides in mouse brain tissue extracts [142]. Top-down MS methods like these are crucial for studying different protein and peptide forms but need adaptation to studying larger proteins and neuropeptides simultaneously.

While several advances have been made to address MS concerns when studying classes of molecules with distinct chemical properties, such as sequential extractions, methods employing simultaneous co-analysis are far from being common. To fully understand the interplay between different types of biomolecules, improvements into interpreting large amounts of data is also required. As investigations into complicated multi-interaction diseases and biological functions are increasingly being pursued by the scientific community, we expect to see more extensive multi-omics experiments adopted into future workflows.

6. Conclusion:

The field of neuropeptidomics is constantly evolving and recent improvements have led to enhanced detection and quantitation of neuropeptides. Their low *in vivo* abundance, complex functions, and structural diversity make their analysis challenging, but MS analyses have mitigated many of these difficulties [143]. Isotopic labeling strategies, such as dimethyl labeling [45] and TMAB labeling [49], differentiate neuropeptides at the precursor ion level but are only used to compare a few experimental conditions. Enhanced multiplexing can be achieved with the incorporation of isobaric tagging because it does not significantly increase spectral complexity [8]. Labeling strategies do enable higher throughput and typically more reliable quantitation, but additional sample processing steps often cause sample loss which is detrimental for low abundance analytes like many neuropeptides. As a result LFQ methods are still common [141]. These methods are also more compatible with DIA methods, facilitating greater sensitivity and neuropeptidome coverage [22]. Quantitative strategies are also employed in imaging workflows to provide spatial distribution, utilizing a variety of

normalization methods to ensure quantitation is accurate, summarized nicely in a review by Tobias and Hummon [116]. Analysis of PTMs, especially glycosylation, has greatly benefited from improvements in hybrid MS fragmentation method, such as the use of HCD-triggered EThcD to improve fragmentation of glycopeptides (and glyconeuropeptides) [23]. As neuropeptidomic workflows become more common and accessible, their findings can be incorporated into larger multi-omic workflows [57]. There is still much room for improvement in terms of technology and method development, but recent advances in neuropeptidomics have provided much insight into the complex signaling pathways involving neuropeptides, with impacts in the fields of biomarker discovery and drug development.

7. Expert Opinion:

The field of neuropeptidomics is instrumental to our understanding of neuromodulation and signaling. This in turn has applications in clinical settings in the areas of biomarker discovery, drug discovery, and drug action [132,134,144]. Neuropeptide dysregulation has been linked to many biological problems and diseases, such as Alzheimer's disease [145], obesity [146], cancer [147], and depression [148]. The advances in neuropeptidomics have enabled researchers to better understand many of these conditions and the related signaling pathways. This can facilitate the discovery of new, more reliable biomarkers. Additionally, knowledge gained from these signaling pathways may provide new drug action sites or possibly even modifying neuropeptides to act as drugs themselves.

Adoption of neuropeptidomics in a clinical setting does still present challenges, however. As neurochemistry is incredibly complex, most studies are performed in organisms with simpler neuroendocrine systems, including some mammals like mice and rats [149], and many invertebrates, such as crustaceans [150] and nematodes [151]. Translation from these models to humans is difficult and, at the very least, will require more sophisticated, less invasive sampling methods (e.g., microdialysis). Clinical research involving humans also has the added complexity of genetic diversity. Biomarker discovery and genetic risk score assessments have historically been biased, and more and more research is demonstrating the importance of clinical research that accounts for sex, age, and racial diversity [152–159]. Additionally, MS analyses of neuropeptides, while sensitive, accurate, and quantitative, may not offer high enough throughput for clinical applications. Multiplexing techniques, such as the aforementioned DiLeu tags [8,56,160], can greatly improve throughput, but are not commercially available. Conversely, the tags that are commercially available (e.g., TMT) [55] are expensive and do not offer as high a degree of multiplexing. Furthermore, clinical applications could necessitate absolute quantitation, often requiring expensive isotopic peptide standards. The Li Lab has developed chemical tags that enable low cost, absolute quantitation. These isotopic DiLeu (iDiLeu) tags are used to create a calibration curve from a synthesized peptide and compare the target peptide to the calibration curve [161]. Methods like this still require synthetic peptides, albeit much cheaper as the isotopes are incorporated via chemical labeling, not during the synthesis of peptides. When the iDiLeu tags are combined with multiplex isobaric tagging reagents, the throughput of absolute quantification can be greatly enhanced [160], which could be highly beneficial for absolute quantification and biomarker validation with large cohort of clinical specimens.

Advances in quantitation are often aided by chemical labeling methods, but improvements in sample analysis are other avenues to consider. As mass spectrometers become more sophisticated, neuropeptidomic analyses are greatly enhanced. For example, increased scan times enable greater depth of coverage, especially for low level analytes like neuropeptides. Increased resolving power can help differentiate between neuropeptides with similar masses, and can facilitate greater multiplexing, as seen by the incorporation of mass defects in DiLeu 12- and 21-plex tags [56,160]. Newer instruments are also capable of performing alternative fragmentation methods, such as ETD and EThcD, to provide more detailed MS^2 data. This is especially beneficial for analysis of PTMs like glycosylation [23]. DIA experiments have also offered many improvements, especially for low concentration analytes that normally are missed by DDA settings (e.g., neuropeptides) [22], despite being a relatively new method. These benefits are certain to increase as DIA sees more popularity and data analysis/deconvolution software improves. Advances in MALDI-MS have given rise to instruments with high acquisition rates and decreased laser size to generate MSI data with high spatial resolution without requiring extra time [161]. Further improvements in instrumentation will undoubtedly enable more robust neuropeptidomic experiments.

We speculate the field of neuropeptidomics to continue thrive on its current trajectory, but with increased prevalence, informing many biological studies. This is largely due to recent advances in quantitation and the many possibilities of its application for quantitative neuropeptidomics. As instrumentation enables faster analyses, either through increased scan times, or faster separation modes like capillary electrophoresis [162], LFQ could see increased use as throughput improves. Conversely, these advances could also lead to an increase in the use of labeling techniques as instruments are able to resolve miniscule mass differences to enable more accurate and sensitive analyses of neuropeptides. Ultimately these methods will both see continued use depending on application, but it will be interesting to see how they are incorporated into larger experiments. As neuropeptides have profound impacts on many biochemical and physiological processes, their study will undoubtedly be important in larger proteomic and metabolomic experiments, and we predict an increase in multi-omic studies, even at the single-cell level with continued improvements in instrumentation and microscale sample preparation. Advances in neuropeptide analyses are also not limited to quantitation. Spatial distribution information gained from MSI can be used to characterize neuropeptide function, receptors, etc. [118] Additionally, ion mobility MS (IM-MS) is routinely used to provide structural information that can be used to distinguish isobaric neuropeptides and better understand neuropeptide conformation and possibly functional roles dependent on its tertiary/quaternary structure [163,164]. As IM-MS and MSI methods are further developed, our understanding of neuropeptides will greatly improve. Combining the structural and quantitative aspects of mass spectrometry will provide richer characterization of neuropeptides, and thus has the power to lead to better biomarkers and drug design to help combat neurological disorders, obesity, and other common health concerns.

Acknowledgments

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Papers of special note have been denoted as (*) of interest, and (**) of considerable interest.

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Article Highlights:

- Developments in quantitative mass spectrometry have enabled greater sensitivity, higher throughput, and more comprehensive analyses of neuropeptidomics, improving understanding of the signaling pathways involved in many diseases.

- Both isotopic and isobaric labeling strategies have seen increased usage, especially as instrument advancements enable greater multiplexing, and label-free neuropeptidomics remains common due to reduced sample loss and spectral complexity. Recent incorporation of data-independent acquisition strategy has benefits for both labeling and label-free methods.

- Post-translational modification analysis remains challenging, but is in greater demand, especially with the discovery of glycosylated neuropeptides. These analyses have benefited from adapting glycoproteomics methods and improvements in instrumentation, such as the availability of ETD and EThcD for fragmentation.

- Recent advances in normalization methods, matrix development, data analysis, etc., have enabled mass spectrometry imaging to not only be useful for localization, but also for quantitation of neuropeptides.

- Neuropeptides play roles in diverse signaling pathways that involved a suite of co-modulating neuropeptides, proteins, neurotransmitters, and metabolites, highlighting the need for multi-omic workflows. These methods have seen increased use in recent years, facilitated by developments in analyte extraction and separations, differential labeling, and instrumentation.
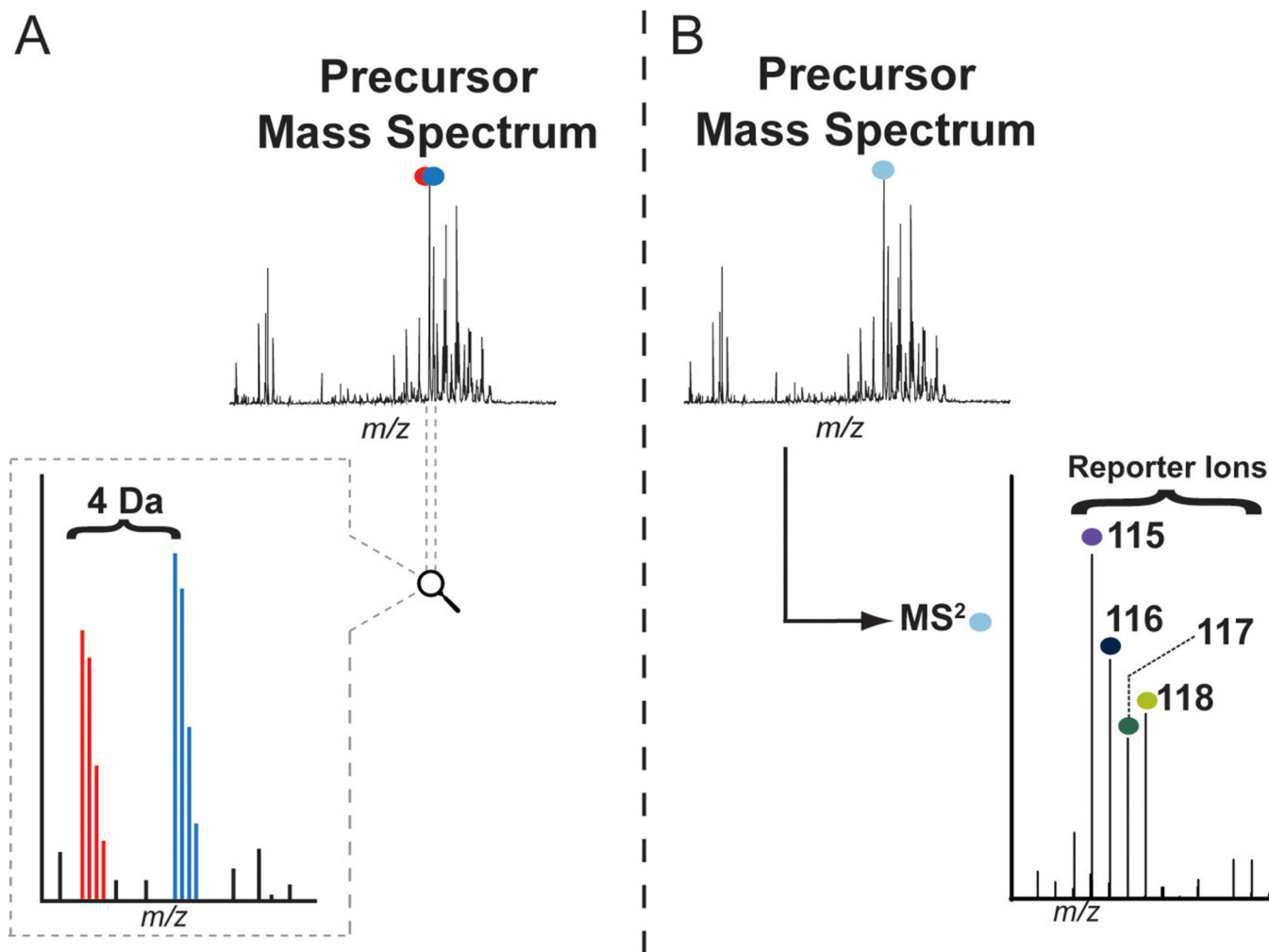
Figure 1:
Comparison between isotopic (A) and isobaric (B) labeling. In isotopic labeling strategies, analytes are differentiated at the precursor mass level due to the incorporation of light and heavy tags. Isobaric workflows result in no differentiation at the precursor mass level, but upon fragmentation, unique reporter ions for each channel form, giving rise to quantitation based on relative intensities of reporter ions.
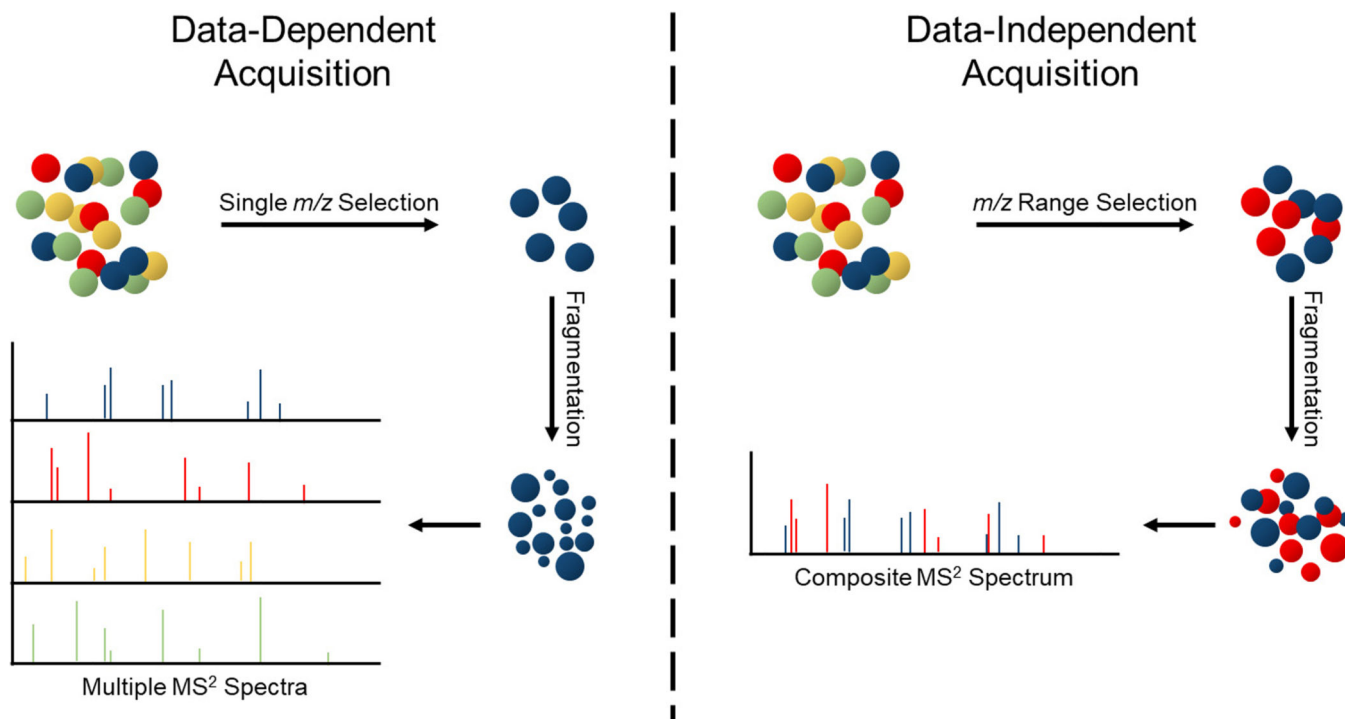
Figure 2:

Depiction of differences between DDA and DIA. In DDA, a single *m/z* is selected at a time for MS/MS. In DIA, several analytes are selected for simultaneous MS/MS across a wide *m/z* window and the composite mass spectrum is deconvoluted during data analysis to discern constituent analytes.
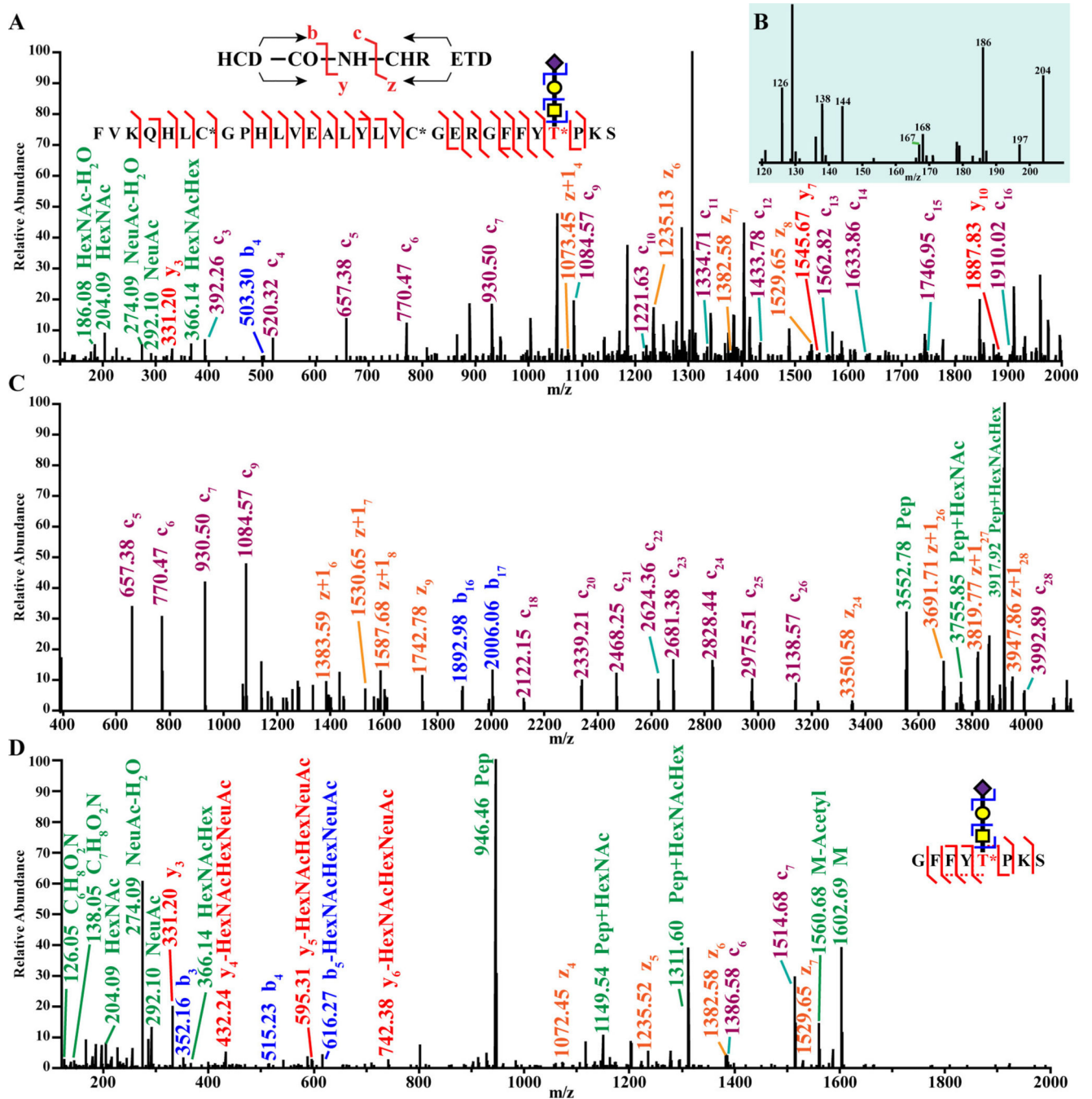
Figure 3:

Sample spectra from mouse insulin. Using EThcD (A) and charge deconvolution (C) resulted in high sequence coverage due to production of both b/y- and c/z-ions. The spectrum in D highlights the glycan localization, and the low mass region is shown enlarged in (B). Figure reprinted with permission from Yu, et al. [105].
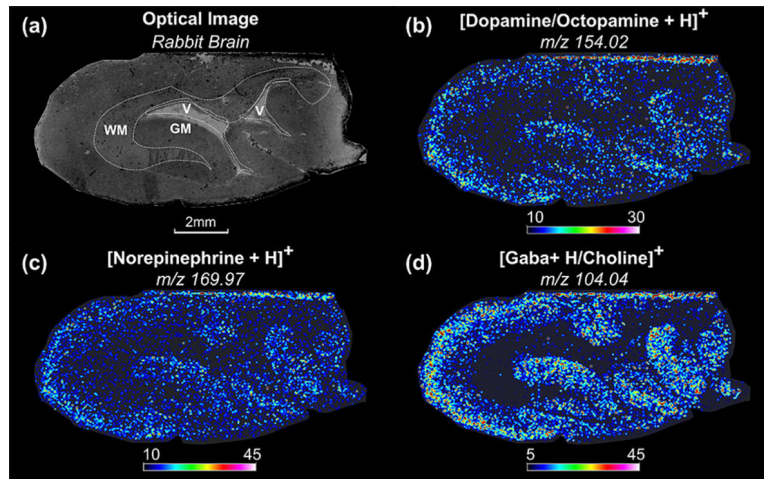
Figure 4:
MSI enhancements from application of gold nanoparticles. Comparison of images from extracted neurotransmitters (b-d) to the optical image (a) shows differential expression based on grey/white matter regions of the brain. Image reprinted with permission from McLaughlin et al. [131]
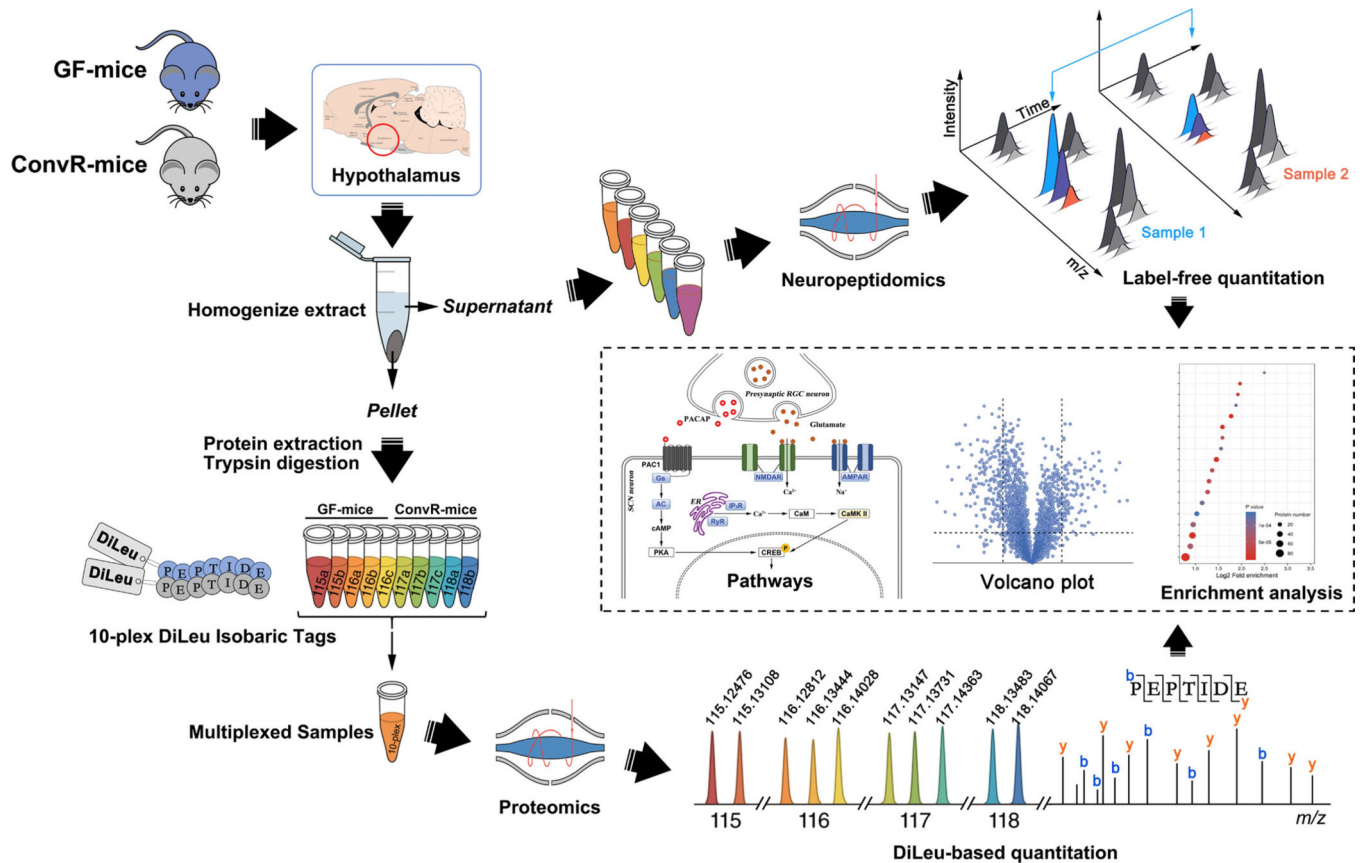
Figure 5:

Multi-omic workflow for studying neuropeptides and proteins. Neuropeptides were extracted from the hypothalamus of germ-free (GF) and conventionally raised (ConvR) mice and analyzed using LFQ. Proteins were pelleted, digested, and differentially labeled with 10-plex DiLeu isobaric tags. The differentially labeled samples were pooled together and analyzed simultaneously to perform relative quantitation based on reporter ion intensities. Adapted from Liu et al. [53] with permission.