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## Quantitative neuropeptide analysis by mass spectrometry: advancing methodologies for biological discovery

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Neuropeptides are critical endogenous signaling molecules involved in a wide range of biological processes, including neurotransmission, hormonal regulation, immune responses, and stress management. Despite their importance, the field of neuropeptide research has been historically hampered by significant technical challenges. These include their low abundance in biological systems, diverse and complex post-translational modifications, dynamic expression patterns, and susceptibility to degradation. As such, traditional proteomics approaches often fall short of accurately characterizing neuropeptides, underscoring the need for specialized methodologies to unlock their biological and translational potential. This review evaluates state-of-the-art quantitative mass spectrometry (MS)-based peptidomics, emphasizing their impact on neuropeptide analysis. We highlight how strategies in label-free and label-based quantitation, tandem MS acquisition, and mass spectrometry imaging provide unprecedented sensitivity and throughput for capturing the landscape of neuropeptides and their modifications. Importantly, the review bridges technological innovation with practical applications, highlighting how these approaches have been utilized to uncover novel neuropeptides and elucidate their roles in systems biology and disease pathways.

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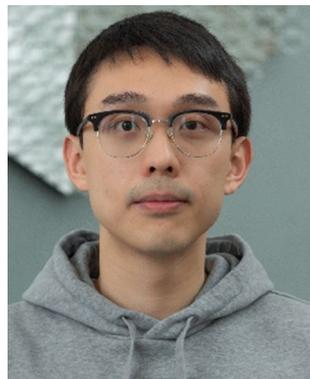
graduate work centers on the development of advanced high throughput mass spectrometry methods, including DDA-MS, DIA-MS and MALDI imaging, to explore neuropeptidomics, glycoproteomics, and small molecule analysis.



## Introduction

Neuropeptides are essential regulators for numerous cellular and physiological processes, acting as key neurotransmitters and neuromodulators. They are synthesized in neurons primarily through mRNA translation on ribosomes and undergo extensive post-translational processing, including proteolytic cleavage from larger precursor proteins.<sup>1</sup> These bioactive peptides, typically composed of 3 to 50 amino acids, range in molecular weight from 200 Da to 10 kDa. Their amino acid

sequence and composition largely determine their function, while post-translational modifications (PTMs) further refine their physicochemical properties, influencing their solubility, receptor binding, and stability.<sup>2–4</sup> This enables neuropeptides to participate in diverse neuromodulatory functions beyond being mere byproducts of protein degradation. Neuropeptides play critical roles in synaptic transmission, neuroinflammation, brain development, stress responses, pain perception, appetite regulation, and psychiatric behavior.<sup>5–11</sup> Some also exhibit antimicrobial properties, contributing to neuroimmune



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interactions that help regulate inflammation and host defense mechanisms.<sup>12,13</sup> The regulation of neuropeptide biosynthesis, processing, and secretion is critical for maintaining neuronal homeostasis, as dysregulation can lead to neuropsychiatric disorders, neurodegenerative diseases, and other pathological conditions.

The interdependent functions of many neuropeptides collectively regulate diverse physiological and neural processes, making studies that focus on a single neuropeptide inadequate for capturing their full scope of influence. Neuropeptide co-transmission, along with classical neurotransmitters, further complicates the isolation of their specific contributions to neuronal signaling, while their rapid degradation, low *in vivo* concentrations, and diffusion in the extracellular space present additional challenges for detection and analysis.<sup>14,15</sup> Neuropeptide analysis by mass spectrometry (MS)-based techniques, termed neuropeptidomics, has supplanted classical methods such as radioimmunoassays and Edman degradation due to its ability to handle complex samples while providing detailed chemical information.<sup>16</sup> Therefore, MS-based techniques have become the premier method for neuropeptide analysis by providing invaluable information on peptide sequence, relative abundance, PTM characterization, and spatially resolved localization.

Neuropeptidomics involves the direct analysis of endogenous peptides without relying on enzymatic digestion and does not require prior knowledge of peptides present in a sample.<sup>17,18</sup> These peptidomic methods stem from a branch of proteomics that similarly aims to characterize protein compositions. So-called “bottom-up” proteomics is based on the controlled proteolysis of proteins into sets of cleaved peptide fragments followed by liquid chromatography-tandem mass spectrometry analysis (LC-MS/MS).<sup>19–22</sup> However, several neuropeptides are endogenously produced fragments derived from larger prohormones with more variable peptide lengths than tryptic fragments. Since trypsin digestion produces a highly complex mixture of peptides, these may mask observations of some other low-abundance short peptides.<sup>23</sup> By forgoing the use of enzymatic proteolysis, native peptide sequences are preserved, allowing for the identification of physiological cleavage patterns without introducing artificial peptide fragments. This enables the detection of low-abundance short neuropeptides in their naturally processed, cleaved form.

Electrospray ionization (ESI) is a soft ionization technique that facilitates the transition into the gas phase with minimal degradation and is readily coupled to online reversed-phase LC separation, enabling high sensitivity and quantitative capabilities in peptide analysis.<sup>24,25</sup> Peptides partially elute according to their size and hydrophobicity and the analyte is continuously sprayed into and measured by the mass analyzer. MS first measures the mass-to-charge ratios ( $m/z$ ) of intact peptide “parent” ions in the gas phase ( $MS^1$ ). These precursor ions are then selected for further fragmentation ( $MS^2$  or  $MS/MS$ ) to reveal characteristic peptide fragmentation patterns, allowing for peptide characterization and PTM differentiation.

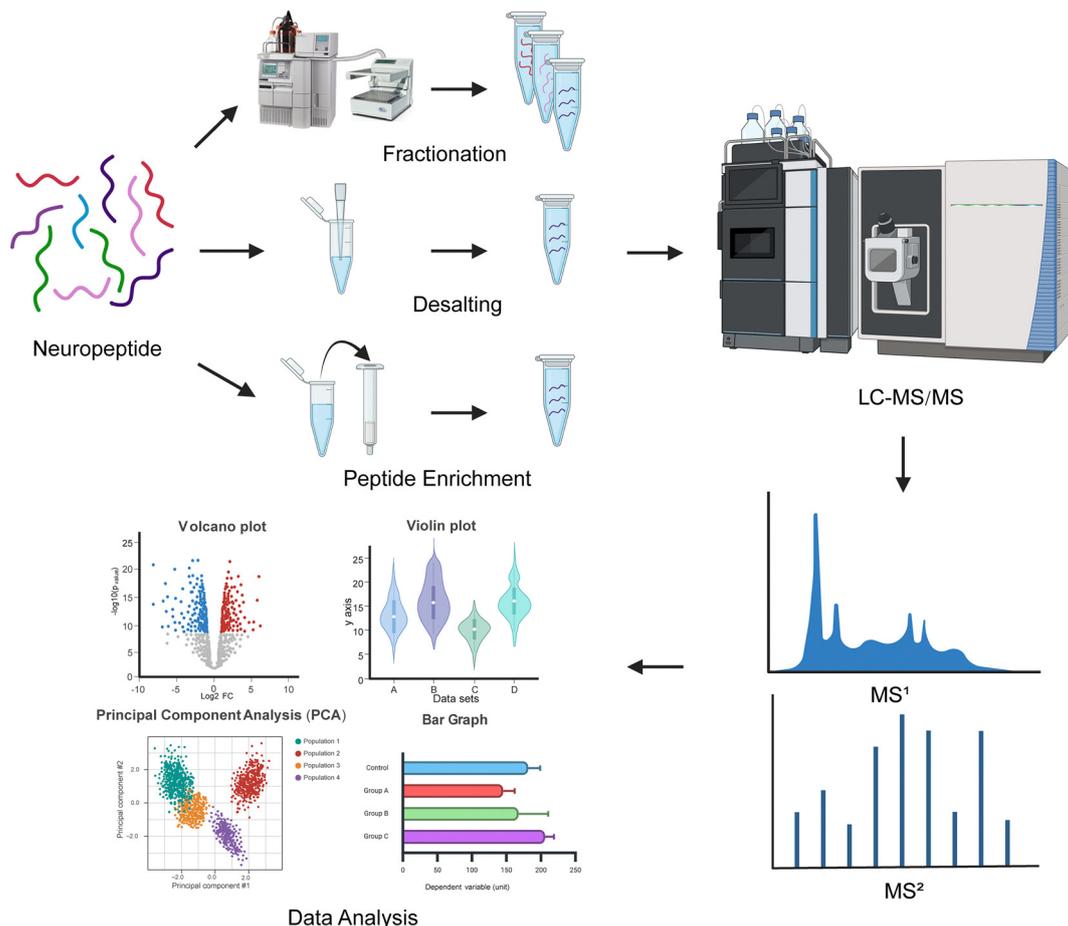
In MS-based peptide analysis, fragment ions are categorized as  $a/x$ -,  $b/y$ -, and  $c/z$ -type, depending on which peptide backbone bond is broken, and peptide sequences are then determined from the  $MS^2$  fragment ion series following an initial  $MS^1$  selection.<sup>26,27</sup> Commonly employed fragmentation strategies, such as high-energy collision-induced dissociation (HCD) and electron transfer dissociation (ETD) methods, produce  $MS^2$  spectra that contain critical fragment ion information for identifying peptides.<sup>28–30</sup> In proteomics, these spectra are often searched against an *in silico*-generated library of theoretical spectra obtained from predicted proteins with predictable cleavage sites from enzymatic proteolysis. Tryptic peptide fragments thus serve as proxies for the protein they originated from, and quantification is performed by aggregating peptides into a single value at the protein level.<sup>31–33</sup> Whereas bottom-up proteomics attempts to piece together tryptic peptide fragments to inform protein compositions and their isoforms, peptidomics forgoes the use of proteolytic enzymes to directly measure naturally occurring endogenous peptides. Specifically, quantitative peptidomics refers to the systematic characterization and quantification of endogenous peptides present in a biological sample through label-based and label-free methods for relative and absolute quantification.<sup>34,35</sup>

There is no one-size-fits-all approach for endogenous peptide quantitation by MS-based methods. Label-based quantitative techniques provide increased accuracy and reproducibility with high multiplexing capabilities. In contrast, label-free quantitation can accommodate large sample sets due to straightforward, cost-effective sample preparation and LC-MS/MS analysis. These approaches vary depending on whether the study is discovery-based (untargeted) or if specific peptides (targeted) are being explored. Further, peptides may exert their function based on their PTMs and cellular/tissue localization. Therefore, quantitative MS-based techniques are powerful and multifaceted approaches to probe peptide organization, structure, localization, PTMs, and overall distribution. By bridging technological innovations with practical applications, we aim to demonstrate how these techniques have been utilized to identify novel peptides, unravel their functions in complex systems, and uncover their relevance to clinical medicine.

## General considerations for peptide analysis and quantitation

Peptidomic workflows can be divided into four stages: sample preparation, chromatographic separation, mass spectral acquisition, and data processing and analysis (Fig. 1). Each stage introduces variation that can affect quantitation downstream. Careful experimental design is necessary to reduce experimental variation to ensure optimal peptide coverage and quantification, which have been discussed previously for peptidomic and proteomic workflows in detail.<sup>17,35,36</sup> Nevertheless, achieving reliable, high-quality quantitation by MS can be complicated due to biological factors underlying neuropeptide biology and challenges inherent to MS.





**Fig. 1** Experimental mass spectrometry-based neuropeptidomic workflow. Peptides are extracted from neural tissue or circulating biofluids and forego the use of enzymatic digestion, as commonly applied to bottom-up proteomic workflows. Neuropeptides are processed and cleaned for downstream LC-MS/MS analysis. MS<sup>1</sup> precursor ions are then isolated and fragmented into smaller fragment ions (MS<sup>2</sup>), where different fragment ions correspond to specific peptide bonds. MS<sup>2</sup> spectra are then analyzed *via* database searching and *de novo* sequencing for quantitative data analysis of neuropeptides.

Sampling neuropeptides from neural tissue is difficult as these are often expressed in very low *in vivo* concentrations (pM–nM) and prone to proteolytic processing.<sup>37</sup> The high sample complexity of neural tissue may introduce interference from high salt content and lipids. In addition, many neuropeptides require PTMs in order to become bioactive such as C-terminal amidation and N-terminal pyroglutamate formation, which cannot be predicted from genomic information.<sup>38</sup> This results in dynamic levels of chemical diversity of neuropeptides that require analytical methods with high resolution and sensitivity.

Preventing degradation must begin at harvest to mitigate enzymatic proteolysis and preserve the *in vivo* peptide sequence and PTMs. Endogenous bioactive peptides are more susceptible to degradation in biological matrices than proteins, as various proteases and peptidases can readily target them.<sup>39–42</sup> Their rapid turnover is essential for timely peptide signaling and regulation in order to maintain homeostasis and prevent prolonged bioactivity. Further, endogenous peptides generally lack tertiary structures and are shorter in length than larger folded proteins, making their cleavage sites accessible to proteolytic enzymes in these biological environments. The

proteolytic background in the mass spectra can mask the true peptidome due to peptide artifacts resulting from proteolytic degradation. This susceptibility to degradation poses challenges for accurately detecting and quantifying endogenous peptides in peptidomics studies, as it often necessitates using protease inhibitors and rapid sample processing.<sup>42–47</sup> Several techniques have been developed to minimize peptide degradation. These include protease inhibitor cocktails to inhibit a variety of endogenous peptidases and heat stabilization through tissue boiling and microwave irradiation.<sup>48–54</sup> The concentration of endogenous peptides also presents a concern in quantitation capabilities. Their sparse cellular localization, tight regulation, and PTMs often result in a wide range of peptide concentrations, with some falling outside the linear dynamic range of high-resolution mass spectrometers.<sup>55,56</sup>

Although LC-MS/MS approaches are among the most sensitive and selective analytical techniques, matrix effects are a significant issue affecting peptide quantitation.<sup>57–59</sup> Biological samples are inherently complex matrices possessing interferences from salts, lipids, abundant proteins, and metabolites. Matrix effects cause alterations in the ionization efficiencies of analytes and differences in LC retention time due to co-eluting



compounds from the same sample.<sup>59–63</sup> This can lead to signal suppression or enhancement that may result in dramatic differences in analytical performance. Several strategies have been proposed that mitigate matrix effects in quantitative MS-based analysis.<sup>57,58</sup> Matrix interferences can be mitigated in peptidomics by employing isotopically labeled internal standards or offline peptide fractionation to reduce interference between peptides, enhance signal-to-noise ratios, and boost peptidome coverage.<sup>63–65</sup> Acidified methanol (90:9:1 (v/v/v) MeOH:H<sub>2</sub>O:glacial acetic acid) is commonly used for neuropeptide extraction due to its ability to simultaneously precipitate proteins, inhibit protease activity, and solubilize peptides.<sup>66</sup> Molecular-weight cutoff (MWCO) devices can help further concentrate neuropeptides while removing abundant, larger proteins and are featured in common neuropeptidomic workflows.<sup>67,68</sup>

## Quantitation techniques

Neuropeptides can be abundantly expressed in tissues or synthesized at trace levels.<sup>69</sup> MS-based methods can provide quantitative information on the abundance of expressed neuropeptides due to their broad dynamic range capabilities across several orders of magnitude.<sup>70–73</sup> Mass spectrometry is inherently nonquantitative due to several factors that affect the relationship between analyte concentration and the signal intensity following the ionization process.<sup>74,75</sup> The ionization efficiency varies significantly between endogenous peptides under similar analytical parameters, depending on their physicochemical properties, including size, charge, and polarity, meaning analytes at the same concentration can produce vastly different signals.<sup>76–78</sup> Instrument factors such as variability in optics, ion transmission, and detector sensitivity introduce inconsistencies unrelated to the analyte concentration.<sup>79,80</sup> Nonlinear detector response, particularly at high ion counts where signal saturation occurs, reduces reliability for highly abundant analytes.<sup>81</sup> Additionally, fragmentation efficiency in tandem MS can vary between precursors, affecting the intensity of peptide fragment ions used for quantitation.<sup>82</sup> Matrix effects further complicate quantitation, as other components in the sample can suppress or enhance ionization, skewing signal intensities.<sup>83,84</sup> To overcome these challenges, quantitative MS for endogenous peptide analysis relies on techniques like isotopic labeling, internal standards, and carefully constructed quality control metrics to correct for variability and improve accuracy.<sup>85–87</sup> Quantitation of endogenous peptides can be categorized as label-based or label-free method performed by either evaluating the area under the curve of the chromatogram (signal intensity) of precursor ions, the signal intensity of diagnostic reporter ions following peptide fragmentation (MS<sup>2</sup>) or counting the number of MS/MS spectra assigned to a peptide (Fig. 2).<sup>88,89</sup>

### Label-based quantification

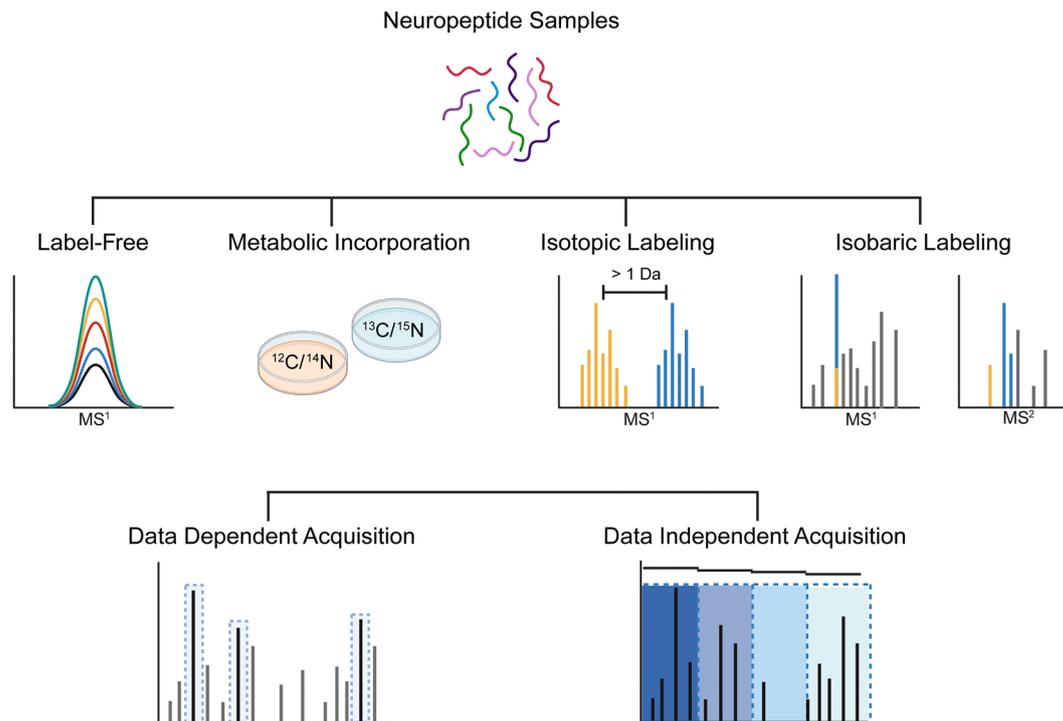
Label-based methods incorporate stable isotopes in order to obtain quantitative ions at the MS<sup>1</sup> or MS<sup>2</sup> level. Stable isotopes enable accurate quantitation in LC-MS/MS by serving as

internal standards that closely mimic the physicochemical behavior of natural peptides. Labeled peptides are chemically identical to their natural counterparts, ensuring they co-elute during chromatography and share similar ionization efficiency, while their distinct mass shift allows for spectral differentiation.<sup>90</sup> By comparing the intensity ratio of labeled and unlabeled peptides, relative or absolute quantification is achieved, with calibration curves enabling precise measurements of peptide concentrations.

Stable isotopes can be introduced through metabolic, enzymatic, or chemical methods. Metabolic labeling was considered the gold standard for quantitative proteomics as isotopically labeled amino acids can be introduced *in vivo* and metabolically encoded into the proteome.<sup>91–93</sup> This early-stage labeling enhances quantitative accuracy and reduces variability from sample preparation or enzymatic proteolysis that can propagate downstream. SILAC, stable isotope labeling with amino acids in cell culture, is the most popular approach and typically uses <sup>13</sup>C or <sup>15</sup>N-labeled arginine and lysine in the culture media.<sup>93–96</sup> Briefly, two isotopically distinct culture mediums are used, with one containing the light medium of the natural amino acids and the other heavy medium containing the stable isotope-labeled residues. Following a sufficient number of cell incorporations to complete labeling ( $\geq 95\%$  labeling efficiency), the two cell populations are then pooled together in equal concentrations before injection into the mass spectrometer. It is demonstrated that SILAC bolsters reproducibility and quantification accuracy due to its ease of implementation and is especially powerful at probing intracellular signaling.<sup>97–101</sup> With SILAC, quantitation is determined from the extracted ion chromatograms of peptide precursor ions from both the heavy and light peptides, where small mass differences are distinguishable at the MS<sup>1</sup> level. Despite its benefits, SILAC is limited almost exclusively to cell cultures, and attempts at multiplexing may cause overlapping isotope clusters at the MS<sup>1</sup> level, which increases spectral complexity. If deuterium is used, this may also alter chromatographic retention times. Nevertheless, though introduced over two decades ago, continuous innovations in SILAC technology such as pulsed SILAC, super SILAC, and spike-in SILAC, among others, are becoming amenable towards spatiotemporal analysis of the dynamic peptidome along with integration of select model organisms.<sup>101–104</sup>

Chemical and enzymatic labeling are typically done *in vitro* with samples from biological extracts such as biological fluids and tissue samples, including cell cultures. This type of chemical labeling can be performed with either isotopic or isobaric tags; however, isobaric tagging is more commonly used in quantitative peptidomic workflows and will be the focus of this discussion.<sup>105,106</sup> In general, precursor ion-based quantification methods with SILAC or isotopic tags suffer from isotopic envelope interference, thus requiring a mass difference of  $> 4$  Da.<sup>107,108</sup> Isotopic envelope interference occurs when overlapping signals from isotopic variants of different peptides distort the intensity of a target precursor ion, leading to inaccurate quantification. This is particularly problematic in complex





**Fig. 2** Overview of mass spectrometry-based strategies for neuropeptide quantification. Label-free quantification measures the intensity of peptide ions at the  $MS^1$ -level over an elution time. The area under the curve correlates with peptide abundance. Stable isotopes can be incorporated into peptides through isotopic labeling or metabolic incorporation (e.g. SILAC). Labeled and unlabeled samples are mixed, and the ratio intensities of labeled to unlabeled peptides are measured. Isobaric labeling incorporates stable isotopes where labeled peptides are indistinguishable at the  $MS^1$ -level. Upon fragmentation, reporter ions are generated, and their  $MS^2$  intensities are used for quantification, enabling multiplexing of samples. Data-dependent acquisition (DDA) and data-independent acquisition (DIA) are commonly employed for neuropeptide analysis, with DDA enabling targeted fragmentation of the most abundant precursor ions for detailed sequencing. DIA systematically fragments all ions within a mass range, providing comprehensive coverage and improved detection of low-abundance neuropeptides.

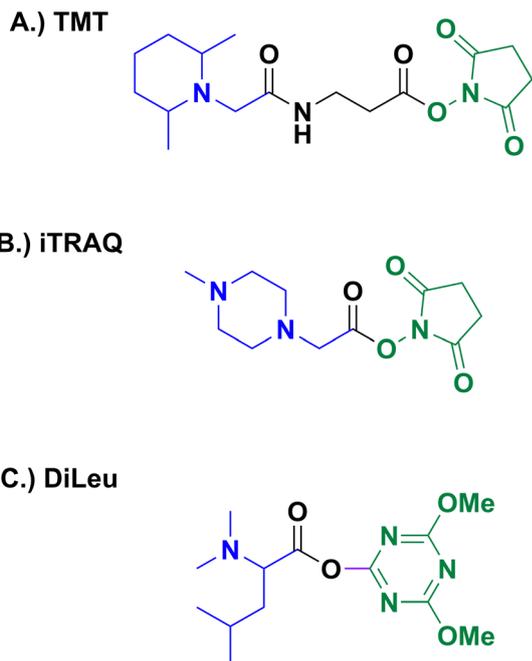
samples where isotopic peaks from multiple peptides overlap in the same  $m/z$  range.<sup>109</sup>

Isobaric tags overcome challenges faced in  $MS^1$ -based quantitation rising from isotope-induced spectral complexity by shifting quantitation to the  $MS^2$  level.<sup>82</sup> These labels incorporate isotopically encoded tags with the same nominal mass addition and structure that generate reporter ions of differing masses upon  $MS^2$  fragmentation. Regardless of isotopic distribution along the tag, labeled peptides co-elute as a single composite peak during the precursor scan with a modest increase in spectra complexity at the  $MS^2$ -level. This enables increased multiplexing capabilities as the  $MS^1$  spectral complexity does not increase with the number of samples.<sup>110</sup>

Isobaric tags such as tandem mass tags (TMT), isobaric tags for absolute and relative quantification (iTRAQ), and *N,N*-dimethyl leucine (DiLeu) follow a common structure motif consisting of a reactive group, a balancing group, and a reporter ion group (Fig. 3).<sup>111–113</sup> The reactive group selectively reacts with specific functional groups or residues on peptides, typically targeting amine groups located on the N-terminus and the  $\epsilon$ -amine in lysine. The balancing group functions as a mass normalization *via* encoded isotopes. The reporter ion group contains discrete isotopes for different ion channels to generate

diagnostic ions with different masses for quantitation. This unique structural configuration allows for the synthesis of significantly higher-order multiplexed tags. For instance, whereas SILAC-based quantitation can modestly achieve up to 4-plex multiplexing, isobaric tags can readily achieve up to 8-plex with iTRAQ, 21-plex with DiLeu, or 35-plex with TMTpro.<sup>101,114–118</sup> These amine-reactive tags, however, require at least one primary amine; therefore, endogenous peptides with PTMs altering the amino groups or those lacking a primary amine will require appropriate adjustments, such as using cysteine-reactive tags.<sup>119–121</sup> Further, bolstering the multiplexing capacity of these tags can lead to decreased quantitative accuracy in the  $MS^2$  scan due to chemical noise, signal interference, false positives, and ratio distortion.<sup>110,122–124</sup> Analytes with similar  $m/z$  values to the tagged peptide can be co-isolated during the initial  $MS^1$  scan; the isobaric reporter ion signal can be masked by the contaminating peptide, leading to distorted quantitation. Nevertheless, sample multiplexing enabled by isobaric tags at the  $MS^2$  level drastically reduces sample analysis time and significantly boosts throughput capacities without compromising the high-quality quantitation of endogenous peptides. Lastly, the additional steps in sample preparation and high-cost reagents are a significant limitation of label-based approaches, deterring the widespread use and





**Fig. 3** Chemical structures of common isobaric tags used in quantitative peptidomics. Isobaric tags follow a common structure motif consisting of an amine-reactive group (green), a mass balancing group (black), and a reporter ion group (blue). (A) Tandem mass tags (TMT), (B) isobaric tag for relative and absolute quantification (iTRAQ), and (C) *N,N*-dimethyl leucine.

adoption of these techniques when sampling low-abundance, precious peptides.

### Label-free quantification

Label-free quantification (LFQ) forgoes the use of stable-isotope labels, providing several experimental advantages, including large-scale cohort analysis, inexpensive and cleaner sample processing, and amenability to any sample matrix.<sup>125,126</sup> LFQ analysis is performed by aligning multiple data sets using *m/z* and LC retention times and correlating peptide abundance to the number of MS/MS spectra or precursor signal intensities.<sup>127,128</sup> A relatively straightforward method for LFQ analysis involves integrating the chromatographic peak areas for a given peptide at the MS<sup>1</sup>-level (MS<sup>1</sup>-AUC).<sup>129</sup> The area under the curve of the extracted ion chromatogram (XIC), which measures the signal intensity of a peptide as it elutes from a chromatographic column, is observed to be linearly related to its concentration across very broad dynamic ranges.<sup>129,130</sup> Therefore, by comparing the peak areas between samples, relative quantification of peptides can be achieved. Conversely, spectrum count approaches infer peptide quantity indirectly from the total number of MS/MS spectra acquired.<sup>127,131</sup> The spectral counts of peptides are reproducibly observed to be linearly correlated with protein abundance over a linear dynamic range of over 2 orders of magnitude.<sup>127,132</sup> For both strategies, high-quality LFQ necessitates narrow LC peak widths to enhance the signal-to-noise ratios of peptide ions while extending the dynamic range of

peptide quantitation to account for low-abundance ions. While isotopic labeling methods can accommodate reasonably sized sample cohorts, the number of samples that can be quantitatively assayed is limited to the multiplexing capacity of the chemical tag. When combined with costly tagging reagents, LFQ methods offer flexible sample preparation steps, rendering them attractive options for large-scale analyses.<sup>133,134</sup>

Common challenges in LFQ analysis stem from limitations of LC-MS/MS, referred to as batch effects, which are the systematic differences in measurements caused by technical factors.<sup>135</sup> For example, run-to-run variability in peptide elution time differences due to difficulties obtaining stable chromatography complicates the alignment of RT and *m/z* values.<sup>136</sup> Multiple sample injections into the same LC column can lead to chromatographic shifts where, even within the same sample, peptides may exhibit different responses across injections, resulting in variations in peak intensity and elution between runs.<sup>137</sup> This fluctuation in reproducibility often compromises accuracy and precision. Isobaric peptides with higher precursor intensities can co-elute, making it difficult to identify, much less quantify, these peptides. Various normalization strategies have been proposed to mitigate systemic bias, employing novel algorithms and benchmarks to account for the variation introduced by batch and matrix effects.<sup>138–141</sup>

## Data acquisition methods

When considering ways to achieve high-quality LC-MS/MS quantitation, the type of study must be delineated. Peptidomics research can be categorized into two groups: discovery-based (untargeted) and targeted. Discovery-based platforms aim to maximize overall peptide coverage and identification by exploring “all” peptides across a broad dynamic range – known, novel, or unexpected. These untargeted approaches ultimately generate hypotheses and focus on peptide screening. Here, only relative quantification can be achieved, yet this remains a powerful method for biomarker discovery and understanding cellular mechanisms in disease.<sup>142–144</sup> Targeted platforms are hypothesis-driven and limit peptide coverage to fully exploit the analytical capabilities of high-resolution MS. Selected or multiple reaction monitoring (SRM) and parallel reaction monitoring (PRM) are employed as they enhance quantitative capacity by boosting sensitivity and reproducibility with rapid detection due to a predefined set of peptides.<sup>145–147</sup>

Following LC separation, the MS detects the signal intensities of the ionized peptides and quickly acquires mass spectra. Two broad approaches to generating tandem MS spectra exist: data-dependent acquisition (DDA) and data-independent acquisition (DIA). DDA is the conventional approach for quantitative peptidomics; however, rapid advancements in DIA have also demonstrated robust and reproducible peptide quantitation.<sup>148</sup> DDA selects the most abundant precursor ions, top *N*, for MS<sup>2</sup> fragmentation. Recently fragmented precursor ions are excluded to enhance peptide coverage for low-abundance ions and prevent redundant fragmentation.<sup>149</sup> However, DDA is



inherently stochastic as ion selection in complex mixtures is nontrivial due to interfering artifacts obstructing peptide MS<sup>1</sup> selection, MS scanning speeds, exclusion efficiency, and separation resolution.<sup>127</sup> Yet, since only the top N precursors are selected, this typically results in MS/MS spectra with numerous data points, allowing for sensitive and accurate quantitation. Given the stochastic nature of DDA, run-to-run variability, and inherently low abundance of endogenous peptides, this may result in missing peptides lacking sufficient data points for sensitive and accurate quantification. On the other hand, DIA overcomes the stochastic nature and bias selection of DDA wherein MS/MS spectra are collected systemically and independently of precursor ion information.<sup>150,151</sup> In DIA mode, the instrument accumulates and fragments all precursor ions within a user-defined *m/z* isolation window and proceeds iteratively, typically within a mass range of *m/z* 400–1200.<sup>152–154</sup> In addition to its increased specificity and greater reproducibility, DIA can detect low-abundance peptides owing to its ability to analyze the whole mass range. Since biologically active endogenous peptides are typically present in the low mass range and may not produce enough of a response to surpass the signal threshold in DDA, DIA is ideally suited for global discovery-based experiments for increased coverage and relative quantification.<sup>88</sup> For instance, DIA has made major advancements in large-scale *de novo* sequencing of neuropeptides possible with improved neuroptidome coverage.<sup>155,156</sup> These have detected nearly twice as many neuropeptides than traditional data-dependent acquisition (DDA) techniques, many of which were detected for the first time.<sup>157–159</sup> However, because DIA methods generate significantly more data compared to DDA and all MS<sup>1</sup> precursor ions are selected for fragmentation, advanced deconvolution, and processing tools are necessary to reduce spectral complexity.<sup>160</sup>

In bottom-up proteomics approaches, which generate various peptide fragments from protein precursors, the sum of all peptide-spectrum matches (PSMs) derived from a protein can be used to quantify protein abundance.<sup>161</sup> It is assumed that in DDA mode, the more abundant peptides selected for fragmentation produce a larger number of MS/MS spectra. Quantitative DDA approaches may face substantial missing data and values, a challenge that is amplified with larger sample sets.<sup>148,162</sup> Therefore, accurate and sensitive quantification by spectral count requires large numbers of tandem spectra, whereby abundant peptides result in more PSM data points than less abundant proteins and may result in detector saturation, compromising quantitative accuracy.<sup>163</sup> MS<sup>1</sup>-AUC quantifies peptides by leveraging precursor ion signals, making it suitable for peptides that may not generate sufficient fragmentation events. This approach enhances the accurate quantification of low-abundance peptides by integrating their signal across the entire chromatographic elution profile. As a result, peptidomics LFQ approaches commonly employ precursor ion-based integration strategies to capture the full elution profile of endogenous peptides.<sup>164,165</sup>

DDA and DIA are routinely used in untargeted discovery peptidomics as both acquisition modes aim to maximize the

number of peptides that can be identified and quantified. Typically, discovery-based experiments aim to screen peptides across a broad dynamic range while measuring their relative abundance across multiple samples.<sup>166</sup> By profiling the differential abundance of peptides between a control and experimental group, insights into how peptide regulation is altered on a global, comprehensive scale can be achieved.<sup>142,167</sup> This hypothesis-generating approach is, therefore, suitable not only for biomarker discovery and validation but also for naturally allowing investigators to quantify unique peptide differences for downstream targeted analysis of their involvement in signaling pathways. Further, tuning acquisition parameters for low-abundance peptide ions has been demonstrated to boost their identification and quantity confidence.<sup>156,168</sup>

Although DDA and DIA are instrumental in discovery-based peptidomics for probing the global peptide landscape, targeted acquisition methods like MRM and PRM provide the precision needed for quantifying specific peptides with greater sensitivity and reproducibility. MRM leverages the mass filtering capabilities of a triple quadrupole mass spectrometer where specific precursor ions are selected and fragmented with transitions (precursor-to-fragment ion pairs) monitored for quantification.<sup>169,170</sup> This approach is highly selective but requires detailed prior knowledge of target peptides, often requiring significant assay development. PRM, derived from MRM, uses high-resolution hybrid quadrupole-orbitraps or time-of-flight instrumentations to collect full MS/MS spectra for the targeted precursor ions, providing enhanced specificity by monitoring all fragment ions simultaneously.<sup>171</sup>

While relative quantification methods are commonly used to compare peptide abundances across samples and depend on the fold changes in peptide expression, there is a need to provide precise absolute concentration measurements of peptides. Absolute quantification, commonly referred to as AQUA, relies on spiking a known concentration of synthetic stable isotope-labeled peptide internal standards (SIL-IS) into samples.<sup>172</sup> When combined with SRM or PRM, AQUA enables precise quantification of target peptides in complex matrices by detecting both the native peptide and SIL-IS. This technique is commonly applied for bottom-up proteomics, where quantitative measurements occur at the peptide level from proteolytic peptide fragments.<sup>173</sup> This approach eliminates variability associated with inter-sample comparisons, making it particularly valuable for applications requiring high accuracy, such as biomarker validation and allergen quantification. Ultimately, while targeted methods bring substantial rigor to quantitation, they demand a high level of specificity in peptidomics due to the diversity of native peptides being studied.

## Post-translational modifications

Post-translational modifications (PTMs) are covalent processes where various modifying groups are added to or replace the side chain of one or more amino acids within a peptide sequence. Common PTMs for endogenous peptides include



but are not limited to glycosylation, phosphorylation, citrullination, C-terminal amidation, N-terminal acetylation, and pyroglutamation on glutamic acid and glutamine.<sup>174</sup> PTMs function as critical mediators for cellular signaling by influencing peptides' and proteins' function, activity, stability, and interaction with other biomolecules. Often, endogenous peptides only become bioactive once modified, which allows them to dynamically regulate cellular processes, including hormonal signaling, immune response, and neuromodulation.<sup>11,175–178</sup> As such, PTM processing of endogenous peptides opens avenues for targeted therapeutic intervention.<sup>16,179</sup>

Mass spectrometry-based characterization of modified peptides accounts for diagnostic features imparted by PTMs, including mass shifts, fragmentation patterns, and chromatographic retention.<sup>180–182</sup> For untargeted profiling, most software for peptide identification offer variable PTM and fixed PTM searching abilities, allowing researchers to identify the differentially modified peptides from the sample.<sup>183–185</sup> Generally, PTMs affect the peptides' overall chemical and physical characteristics, which can be exploited for their specific enrichment and purification.<sup>186</sup> Glycosylation, for example, involves the addition of carbohydrates onto certain amino acid residues, which increases peptide hydrophilicity.<sup>187</sup> Hydrophilic interaction liquid chromatography (HILIC) can relatively enrich the hydrophilic glycopeptides from the unmodified hydrophobic peptides. After enrichment, the interfering spectral background signal is lower, resulting in cleaner spectra and a higher signal-to-noise ratio for the glycopeptides.<sup>188</sup> Cui *et al.* have similarly employed electrostatic repulsion hydrophilic interaction chromatography (ERLIC) to achieve simultaneous enrichment of both glycosylated and phosphorylated peptides from the MM.1S cell lysate to elucidate PTM crosstalk.<sup>189</sup> Citrullination, the deimination of arginine, imparts a very small shift (+0.984 Da) which can be readily masked by deamidation (+0.894 Da) or <sup>13</sup>C isotopic peaks (+1.0033 Da).<sup>190</sup> To enhance their detection, Shi *et al.* developed a biotin thiol tag-assisted MS method to enrich and derivatize the citrullinated peptides, discovering 691 citrullination sites from 432 proteins.<sup>191</sup>

Different fragmentation techniques can also characterize modified peptides and determine PTM site localization. Collision-induced dissociation (CID) and high-energy collisional dissociation (HCD) induce fragmentation by colliding peptide ions with inert gas molecules (*e.g.*, nitrogen or helium) to predominantly cleave amide bonds along the peptide backbone (C–N), generating sequence-informative b- and y-ions that are ideally suited for *de novo* sequencing and identification.<sup>192,193</sup> Electron transfer dissociation (ETD) involves transferring an electron to multiply charged peptide ions, resulting in sequence-independent radical-driven fragmentation along the NH–C<sub>α</sub> to generate c- and z-ions.<sup>194,195</sup> This type of fragmentation can better retain intact labile moieties such as glycans and phosphates than HCD.<sup>196,197</sup> Both dissociation methods are complementary and can generate a wealth of sequence-specific information in the resulting tandem MS spectra.

For specific PTMs of interest, instead of an untargeted approach, a targeted approach combining both dissociation methods can be used for characterization and quantification.

Among these, electron transfer/high-energy collision dissociation (ETHCD) is a hybrid technique that produces a mixture of c/z and b/y ions for detailed annotation of peptides and their PTMs, especially for glycopeptides.<sup>198,199</sup> For instance, Yu *et al.* have used a known list of oxonium ions to selectively trigger ETHCD for glycopeptides and achieved the first global profile of O-glycopeptides in both mouse and human pancreatic islets, discovering that signaling peptides such as insulin and BigLEN are glycosylated.<sup>200</sup> Riley *et al.* subsequently determined that HCD is the preferred method for N-glycopeptide analysis, whereas ETHCD fragmentation is indispensable for site-specific O-glycopeptide analysis.<sup>201</sup> Using these techniques, glycosylated neuropeptides and peptide hormones have been discovered in the nervous system of mammals and crustaceans.<sup>202,203</sup> Recently, D-amino acid-containing peptides (DAACPs) have been discovered as unusual neuropeptide PTMs across different phyla, including chordates, arthropods, and mollusks.<sup>204</sup> Unlike other PTMs, peptide L/D isomerization does not induce a mass shift readily discernable by traditional neuropeptidomic approaches, thus requiring extensive sample processing for their characterization and functional assays to test their physiological and behavioral implications.<sup>205–208</sup> However, ion mobility-mass spectrometry (IM-MS) uniquely resolves isomeric species through gas-phase separation based on peptide ions' mass, charge, and collisional cross-section values.<sup>209,210</sup> In particular, site-specific identification of DAACPs can be achieved first by online reversed phase chromatography followed by ion mobility analysis of epimeric fragment ions.<sup>211</sup> This pioneering strategy has since been adopted to screen for DAACPs in the central nervous system of both vertebrates and invertebrates.<sup>212–216</sup>

In quantitative bottom-up proteomics, it is common to aggregate peptide fragments into a single quantitative value for each protein-coding gene. On the other hand, in neuropeptidomics, quantitation techniques must be adapted to account for the diverse and often modified nature of endogenous peptides, which differ from the predictable tryptic peptides typically analyzed in bottom-up proteomics. Unlike tryptic peptides, which result from enzymatic digestion and are typically unmodified and within a narrow mass range, endogenous peptides can have a broader range of PTMs, non-tryptic cleavages, and variable lengths. The *in vivo* abundance of endogenous peptides and their modified counterparts can vary significantly, with many existing at very low levels, thus requiring careful optimization of instrument settings to minimize modified peptide signal suppression.<sup>159</sup> Specifically, SRM and PRM in peptidomics require tailored transitions that account for these unique features, often necessitating extensive optimization of precursor and fragment ion selections for each peptide target.<sup>217,218</sup>

## Mass spectrometry imaging

Mass spectrometry imaging (MSI) leverages the specificity and sensitivity of mass spectrometric techniques to map and image the spatial distributions of diverse biomolecules directly.<sup>219</sup>

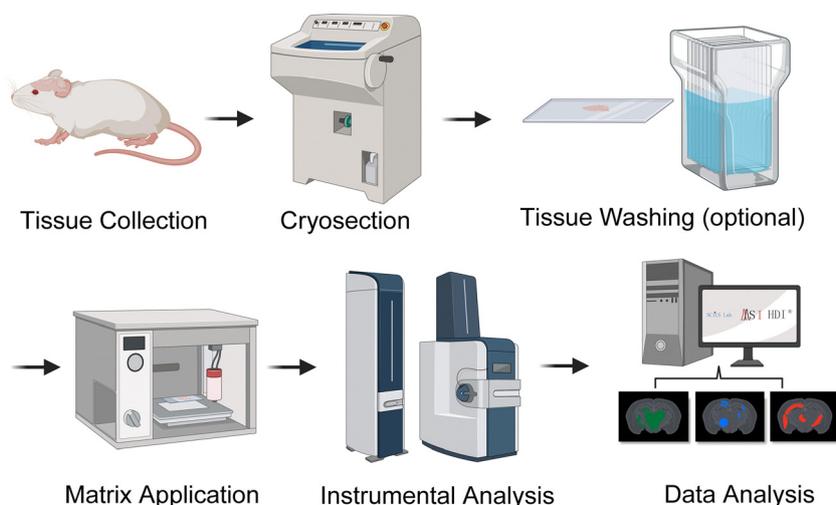


Unlike other imaging techniques, such as immunohistochemistry (IHC), fluorescent tags, or radio-immunoassays, MSI requires no prior knowledge of the targeted class of molecules, and thousands of molecules can be imaged in a single MSI experiment, covering a mass range from 1  $m/z$  to as high as 120 000  $m/z$ .<sup>220</sup> Sample preparation is relatively simple, making them amenable to thin tissue sections. MSI of proteins was first introduced by Caprioli *et al.* in 1997 by utilizing matrix-assisted laser desorption/ionization (MALDI) technique, and it allows spatial mapping of all classes of molecules, including lipids, metabolites, peptides, and intact proteins or protein complexes.<sup>221</sup> MALDI is another soft ionization routinely used for MSI experiments as it is much more tolerant of impurities while enabling fast data acquisition.<sup>222</sup> Fig. 4 shows an example of a typical MALDI-MSI workflow. Aside from MALDI, other ionization techniques for MSI have been employed, such as desorption/ionization (DESI), atmospheric pressure MALDI, surface-assisted laser desorption/ionization, and secondary ion MS (SIMS).<sup>223–226</sup>

Before imaging, tissue sections must be properly prepared for the analyte of interest. Upon dissection, tissues need to be flash frozen or embedded into imaging matrices to preserve the tissue orientation and structure and prevent molecule degradation. Common embedding matrices include gelatin, carboxymethyl cellulose (CMC), paraffin, and optimal cutting temperature (OCT) compounds.<sup>227–229</sup> Although paraffin and OCT compounds are common embedding reagents, both matrices are known to suppress analyte signals and need to be properly cleaned before MSI.<sup>229,230</sup> Although tissue washing can remove interfering signals, it may also lead to diffusion, negatively affecting the analytes' native localization. In these instances, fresh frozen tissue or gelatin and CMC embedding are recommended sample preparation methods for peptide imaging.<sup>231</sup> Further, imaging endogenous and tryptic-digested

peptides is particularly challenging due to their relatively low concentration compared to interfering lipid species. Lipids can be washed off tissues before imaging using organic solvents to remove these abundant molecular species to prevent their masking effect.<sup>232,233</sup> Because neuropeptides are typically present in low abundance, several strategies have been developed, including removing formaldehyde-induced peptidyl crosslinks and employing alternative aqueous tissue washes.<sup>234,235</sup> These have increased neuropeptide coverage while increasing their signal intensities by 5-fold.<sup>235,236</sup>

During imaging, mass spectra are generated for each defined pixel of the tissue slice, revealing the molecular composition and their relative abundance. The colored images from the experiment can reflect the abundance of specific ions across the whole tissue section, akin to an ion heat map. Similar to LC-MS/MS quantification techniques, both relative and absolute quantification can be achieved through MS imaging. In relative quantification, ion signals across tissues can be normalized using various imaging processing software methods, such as HDI, MSiReader, and SciLS lab software.<sup>237,238</sup> The normalized intensity of the ions can then be used to compare tissue slices from different experimental conditions. Internal standards can also be used for relative and absolute quantification in MSI.<sup>239,240</sup> Isotopically labeled standards are frequently used in MSI to correct for variations in ionization efficiency and matrix effects, enhancing the precision of quantitative analyses in complex samples. For instance, Bergman *et al.* integrated specific concentrations of multiple deuterated metabolites into the solvent stream for DESI imaging.<sup>241</sup> This approach enhances the accuracy and reliability of quantification methods in mass spectrometry imaging experiments. In addition to internal standards, calibration curves are commonly employed in MSI for accurate quantification. These curves, as demonstrated by Jadoul *et al.*, involve adding known concentrations of



**Fig. 4** General overview of mass spectrometry imaging (MSI) workflows. Thin tissue sections are mounted on a plate and tissue washing can remove interfering ion signals. The tissue slices are then coated with or without a matrix, depending on the ionization technique employed. The produced ions are detected by a mass spectrometer, generating mass spectra at each spatial coordinate. MSI constructs mass spectrometric images corresponding to specific molecular weight values. This allows for the simultaneous imaging of many different molecules from a single tissue section.



analytes to tissue homogenate, establishing a relationship between signal intensity and concentration.<sup>242</sup> Further, comprehensive peptide identification and structural probing are crucial but difficult to achieve simultaneously. Using nanosecond photochemical reaction (nsPCR)-based click chemistry with 2-nitrobenzaldehyde (NBA), researchers developed a method to enhance neuropeptide identification and structural analysis by enabling on-demand removal of surrounding matrices, demonstrated with MALDI-MSI.<sup>243</sup> This strategy achieves high labeling efficiencies (>90%) and facilitates neuropeptide visualization in complex tissues, with broad applicability for proteins up to 155 kDa across diverse samples and amine-containing metabolites.<sup>244</sup> The 3D spatial peptidomics information provided by MSI can be integrated with micro-computed tomography for increased multiplexed mapping of neuropeptides in the brain microanatomy.<sup>245</sup>

## Neuropeptidomic applications and challenges

Many MS-based approaches have been employed to profile and quantify the neuropeptidome of various animals. Nontargeted LFQ strategies are used extensively to study the endogenous peptidome change for several animal model systems across vertebrates and invertebrates, including mice, rats, crustaceans, nematodes, and insects.<sup>5,246,247</sup> DeLaney *et al.* used a multifaceted approach to quantitatively investigate the neuropeptidome content in fed and unfed *Cancer borealis* tissue extracts by LFQ and spatially map neuropeptides of intact neural tissue.<sup>157</sup> Wu *et al.* similarly conducted LFQ analysis across several regions of the brain on a rodent model of intense stress to discover novel neuropeptides involved in predator-stress-related behavior with implications for post-traumatic stress disorder.<sup>248</sup> In total, 328 peptides were quantified from several key neuropeptide families, including ProSAAS, tachykinin, and opioid peptides. Probing the neuropeptidomes of early-branching lineages of *Cnidaria*, *Porifera*, and *Ctenophora* can offer insights into the evolutionary origins of peptidergic nervous systems.<sup>249,250</sup> These findings suggest that animal peptidergic neurons first evolved from sensory-secretory cells, with neural function highly dependent on the complexity of neuropeptide-receptor interactions.<sup>251</sup> Further, DIA and LFQ methods have emerged as powerful techniques to account for low-abundance peptides that may be unaccounted for in DDA analyses while reducing spectral complexity due to labeling and maintaining high degrees of reproducibility and coverage.<sup>252</sup> For example, DeLaney *et al.* have used DIA-MS LFQ analysis for neuropeptide profiling for *C. borealis* hemolymph, successfully quantifying 217 neuropeptides compared to previous reports, 8 of which were documented to be significantly changed upon feeding for the first time.<sup>253,254</sup>

As discussed above, peptidomics can be carried out using various quantification strategies. With untargeted LFQ, even scarce samples can be relatively quantified without the risk of sample loss, and the number of comparisons is not limited by

the channels provided by the labeling strategies. For example, Anapindi *et al.* conducted discovery-based LFQ experiments to quantify over 1500 neuropeptides from a total of 200 LC-MS/MS runs, assessing their relationship with migraine and opioid-induced hyperalgesia (OIH) and discovering that pituitary adenylate cyclase-activating polypeptide mechanistically links chronic migraine with OIH.<sup>247</sup> Because neuropeptides are commonly amidated, the authors accounted for this PTM by characterizing the peptide false discovery rate by adjusting false amidation assignments and normalizing the run-to-run variability from such extensive instrument time.<sup>255,256</sup> Labeling methods can be used to minimize these batch effects and simultaneously screen multiple endogenous peptides. Sauer and Li leveraged the DiLeu isobaric tags to investigate how neuropeptide expression in neural tissues of *Callinectes sapidus* is affected by copper toxicity across different time points (1, 2, and 4 hours of copper exposure).<sup>257</sup> Several neuropeptides from RFamide, allatostatin, orcokinin, and pigment-dispersing hormone families were significantly changed due to copper exposure. With DiLeu isobaric labeling, 4 conditions were analyzed in a single instrument run, enabling the time-course study to be done in an efficient and high-throughput way. Similarly, a 10-plex version of this tag was integrated with label-free techniques to assess the mouse hypothalamic neuropeptidome and proteome of germ-free and conventionally raised mice.<sup>258</sup> Commercially available tagging reagents such as TMT and iTRAQ are commonly used for proteomics and tryptic-digested peptides; however, they have not been extensively used for endogenous peptidomics, although similar benefits provided by the isobaric labeling should be retained.<sup>111,259</sup>

Isotopic labeling strategies, including dimethyl labeling, SILAC, and <sup>18</sup>O labeling, are also widely used to quantify endogenous peptides.<sup>94,260,261</sup> Succinic anhydride and 4-trimethylammoniumbutyryl (TMAB) have been shown to label the mouse pituitary peptidome whereby deuterium incorporation generated channels with mass differences between the heavy and light labeled forms of 4 Da and 9 Da, respectively.<sup>262</sup> Accordingly, TMAB reagents resulted in better separation and resolution than succinylated peptides and detected a broader range of peptides as the increased mass difference at the MS<sup>1</sup>-level minimized isotopic envelope overlap. Reductive dimethylation on the N-terminus and lysine residues with isotope-labeled formaldehyde is another strategy used for neuropeptide quantitation.<sup>263,264</sup> These have been used to probe neuropeptide expression and localization across various environmental stressors in marine animals, including oxygen, salinity, and temperature fluctuations.<sup>265</sup>

Untargeted, profiling-based MS quantitation approaches provide a global understanding of the peptidome as these techniques require no prior knowledge of cellular content. Targeted quantitation techniques, such as SRM/MRM and PRM, require knowledge of specific peptides of interest to compare their expression level between control and experimental groups. These methods are desirable when analyzing bioactive peptide families. However, targeted approaches must account for dynamic peptide regulation, including proteolytic



processing and PTMs. Informed from a previous discovery-based LFQ analysis to profile diet-induced neuropeptide changes, an extended SRM assay of 15 endogenous peptides from the *Rattus norvegicus* hypothalamus has been developed, discovering a significant upregulation of VGF-derived peptides as a result of a high caloric diet.<sup>266,267</sup> Additionally, circulating peptide hormones are often present in low concentrations and require the high specificity of immunoassays, as matrix effects frequently hinder their detection from the complex composition of circulating body fluids.<sup>268</sup> Neuropeptide Y (NPY), for example, is the most abundant neuropeptide in the brain and is prone to peptidolysis where NPY fragments are known to mediate distinct effects due to varying receptor affinities.<sup>269</sup> The low concentration of NPY peptides, typically at picomolar concentrations, makes them difficult to quantify, even with techniques such as MRM in human plasma.<sup>270</sup> However, combining the high affinity of monoclonal antibodies, using isotope-labeled analogs, and controlling for NPY chemical degradation makes it possible to assay the NPY family at subpicomolar concentrations.<sup>271</sup> Similar targeted SRM-MS immunoassays have been developed for insulin-like growth factor 1 and parathyroid hormones.<sup>272–274</sup>

Recently, PRM techniques have garnered attention in neuropeptidomics for both relative and absolute quantification, as their mass accuracy and sensitivity rival SRM. Instead of measuring one transition at a time, all fragment ions of a selected precursor are measured in parallel.<sup>275</sup> The renin-angiotensin system (RAS), a regulatory pathway controlling arterial blood pressure, produces angiotensin peptides by enzymatic cleavages from the prohormone angiotensinogen (AGT) with some peptides are short as five.<sup>276</sup> Previous SRM-MS assays to study angiotensin peptides have yielded roughly 10 amol levels on the lower limit of detection for both relative and absolute quantitation.<sup>277–279</sup> Angiotensin peptides in the brain, however, may be present at even lower concentrations and become trace-limited in regulatory regions with predicted concentrations from 400 zmol to 3 amol.<sup>280,281</sup> To address these very low concentrations, PRM has been used to identify angiotensin peptides at very low limits of detection from 300 zmol to 5 amol using synthetic standards with roughly 10 amol sensitivity for quantification.<sup>282</sup> PRM has also been used for targeted profiling experiments. For instance, Cockx *et al.* recently developed a discovery-driven targeted peptidomics strategy utilizing PRM to profile and quantify the neuropeptides of interest.<sup>283</sup> With DDA LC-MS/MS LFQ analysis, they first generated a spectral library using 427 synthetic known and theoretical peptide sequences, resulting in a cumulative library of 510 peptide ions. By using RT scheduling for these low-abundance samples along with the sensitivity and specificity of PRM, they quantified 172 neuropeptides while functionally validating flp-7 and flp-11 as novel nictation regulating peptides in *C. elegans*. It is also possible to target the neuropeptide biosynthetic pathway directly to elucidate neuropeptide processing. For instance, affinity chromatography can be used to purify neuropeptides that are substrates of

carboxypeptidase E (CPE), an enzyme commonly found in the final processing site for neuropeptide biosynthesis.<sup>284</sup> This allows the analysis of peptides in genetically defined cell types with insights into peptide isoforms resulting from proteolytic cleavages and other PTMs with respect to CPE processing.<sup>285</sup>

## Conclusion

Neuropeptide analysis has advanced significantly, driven by improvements in MS instrumentation, quantitative strategies, and data analysis workflows. The inherent challenges of neuropeptide analysis including low *in vivo* abundance, structural diversity, and complex functions have driven the development of both label-free (LFQ) and label-based strategies. While isotopic labeling methods such as dimethyl labeling and DiLeu tags improve quantitation and multiplexing, their additional processing steps can lead to sample loss, making DIA-based LFQ methods suitable for neuropeptidomic studies. Despite rapid progress, neuropeptidomics still lags behind proteomics regarding workflow standardization, enrichment techniques, and computational analysis. A major limitation is the high structural variability of neuropeptides, which necessitates the development of specialized MS methodologies tailored to specific neuropeptide families and their PTMs. Nonetheless, advances in hybrid MS fragmentation techniques and IM-MS have further improved neuropeptide and PTM analysis, particularly for glycopeptides and DAACPs. Additionally, enhanced spatial resolution in MSI is needed to better map neuropeptide distributions at the cellular level. IM-MS can be combined with MSI to probe neuropeptide spatial localization with enhanced sensitivity and specificity. Innovations in sample preparation and targeted techniques such as MRM and PRM have enabled the absolute quantification of neuropeptides, even in small-volume samples with significantly low limits of detection and quantification. These advancements are crucial for studying neuropeptide-mediated signaling in physiological and pathological contexts. Furthermore, integrating single-cell mass spectrometry with spatial peptidomics to resolve neuropeptide heterogeneity at the cellular level presents a promising approach.<sup>286</sup> These innovations will further refine neuropeptide quantification, allow for more precise functional characterization, and ultimately enhance our understanding of neuropeptide dynamics in health and disease. By leveraging these advances, MS-based neuropeptidomics will continue to be instrumental in unraveling the complex neurochemical landscape of the brain.

## Author contributions

A. E. Ibarra: conceptualization, writing – original draft, review and editing, visualization. W. Wu: writing – original draft, review and editing, visualization. H. Zhang: writing – original draft, review and editing. L. Li: conceptualization, supervision, writing – review and editing.



## Data availability

No primary research results, software or code have been included and no new data were generated or analysed as part of this review.

## Conflicts of interest

There are no conflicts of interest to declare.

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