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REVIEW ARTICLE

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Recent advances in mass spectrometry analysis of neuropeptides

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Abstract

Due to their involvement in numerous biochemical pathways, neuropeptides have been the focus of many recent research studies. Unfortunately, classic analytical methods, such as western blots and enzyme-linked immunosorbent assays, are extremely limited in terms of global investigations, leading researchers to search for more advanced techniques capable of probing the entire neuropeptidome of an organism. With recent technological advances, mass spectrometry (MS) has provided methodology to gain global knowledge of a neuropeptidome on a spatial, temporal, and quantitative level. This review will cover key considerations for the analysis of neuropeptides by MS, including sample preparation strategies, instrumental advances for identification, structural characterization, and imaging; insightful functional studies; and newly developed absolute and relative quantitation strategies. While many discoveries have been made with MS, the methodology is still in its infancy. Many of the current challenges and areas that need development will also be highlighted in this review.

KEYWORDS

imaging, mass spectrometry, microdialysis, neuropeptides, posttranslational modifications, quantitation

Abbreviations: ACN, acetonitrile; AMT, accurate mass time; Aβ, amyloid-β protein; BK, bradykinin; CCAP, crustacean cardioactive peptide; CCS, collision cross sections; CE, capillary electrophoresis; CHCA, α-cyano-4-hydroxy-cinnamic acid; CID, collision-induced dissociation; CNS, central nervous system; cryo-IM-MS, cryogenic IM-MS; DAACPs, D-amino acid containing peptides; DDA, data-dependent acquisition; DESI, desorption electrospray ionization; DH, diapause hormone; DHB, 2, 5-dihydroxybenzoic acid; DIA, data-independent acquisition; ECD, electron-capture dissociation; EDTA, ethylenediaminetetraacetic acid; ESI, electrospray ionization; ETD, electron-transfer dissociation; EThcD, electron-transfer/higher-energy collision dissociation; ExD, electron activated dissociation; FA, formic acid; FDRs, false discovery rates; FFPE, formaldehyde-fixed paraffin embedding; FTICR-MS, Fourier-transform ion cyclotron resonance MS; HCD, high-energy collision dissociation; HCl, hydrochloric acid; HOAc, acetic acid; HRAM, high-resolution accurate mass; ICAT, isotopic-coded affinity tag; IM-MS, ion mobility MS; IQ, informed quantitation; LC, liquid chromatography; LESA, liquid extraction surface analysis; LFQ, label-free quantitation; LSI, laserspray ionization; M, molarity; MALDI, matrix-assisted laser desorption/ionization; MD, molecular dynamics; mDa, milliDalton; MeCAT, metal-coded affinity tag; MeOH, methanol; MS, mass spectrometry; MS1, single stage MS; MSH, melanocyte-stimulating hormone; MS/MS, tandem MS; MTBE, methyl-tert-butyl ether; MWCO, molecular weight cut-off; *m/z*, mass-to-charge ratio; NIMS, nanostructure initiator MS; NMR, nuclear magnetic resonance; OCT, optimal cutting temperature; PACAP, pituitary adenylate cyclase activating polypeptide; PEI, polyethylenimine; pHPMA, poly[*N*-(2-hydroxypropyl) methacrylamide]; PRM, parallel reaction monitoring; PTMs, posttranslational modifications; RIAs, radioimmunoassays; SIMS, secondary ion MS; SP, substance P; TFA, trifluoroacetic acid; TOF, time of flight; TOF/TOF, tandem TOF.

1 | INTRODUCTION

The nervous system is one of the most highly regulated parts of the human body, and signaling molecules are well known for their roles in behavior, controlling bodily homeostasis, and processing incoming information (Herlenius & Lagercrantz, 2004; Hokfelt et al., 2000; Li & Sweedler, 2008; Xie et al., 2011). Any perturbation of this system can have detrimental effects on an organism, leading to temporary or long-term biochemical changes. Neuropeptides, one of the largest classes of neuronal signaling molecules, are well known for playing prominent roles in the nervous system (Herlenius & Lagercrantz, 2004; Hokfelt et al., 2000; Li & Sweedler, 2008; Xie et al., 2011). However, the comprehensive analyses of the neuropeptidome, the entire range of neuropeptides able to be expressed, remain to be challenging due to global diversity of their size, sequence, and function.

The diversity of neuropeptides can be first observed at the biological synthesis level. This review only focuses on the typical neuropeptide biosynthesis pathway and not on alternative ways of endogenous peptide production. A typical neuropeptide biosynthesis starts with the translation of a prepropeptide RNA chains. A prepropeptide may contain several neuropeptide copies, which are revealed after multiple processing steps. Initially, a propeptide is produced from the prepropeptide via proteolytic cleavages, splicing events, or introduction of posttranslational modifications (PTMs) (Li & Sweedler, 2008). The result is a propeptide which is packaged into vesicles where they are stored before release. A strong stimulation, such as high frequency firing, elicits site-specific enzymes to produce the final, biologically active peptides that are released from the neuron. Mature neuropeptides released in the extracellular space 'travel' through the body to reach (distant) organs/tissues/cells which contain receptors where they bind. The latter are sometimes referred to as neuropeptide targets. The final neuropeptides generally range in length from 3 to 70 amino acids long (Buchberger et al., 2015). The signaling targets can be within the same neuron produced, within the same organ, or in an entirely different tissue. In addition, neuropeptide anabolism, catabolism, and thus function may even vary depending on the destination of the signaling target (von Bohlen & Halbach, 2005). To further increase the chemical diversity, neuropeptides can have isoforms that may only vary by one residue but have widely different functions within the body. All these factors lead to a high, natural complexity that is difficult to characterize even with complete genetic coverage.

The development of sophisticated analytical tools or simplified networks are required for deep neuropeptidomic analysis. To decrease the complexities of neuropeptide analysis, many researchers have adopted different, similar animal models, such as crustaceans or mice, to characterize neuropeptidomic changes (Che et al., 2005; Chen et al., 2014; OuYang, Liang, et al., 2015; Yin et al., 2011; Zhang et al., 2015). Due to homology between neuropeptides from different species, many of the results and insights obtained from these simpler systems can be readily transferred to more complex organisms, such as humans (Bruzzone et al., 2006; Schmerberg & Li, 2013; Yew et al., 2005; Yu et al., 2014). As the full complement of neuropeptides has yet to be fully discovered, even with the aid of these model organisms, it is important to develop and implement more advanced technology.

To fully characterize neuropeptides, we require methodology that is selective, sensitive, and swift, all while being cost-effective and capable of providing dynamic temporal and spatial information. In the past, researchers have focused on the use of antibody-based, electrochemical, bioluminescent, or other biological assays to characterize neuropeptides (Li & Sweedler, 2008). For example, radioimmunoassays (RIAs) were very popular at one time due to being highly sensitive and selective (Li & Sweedler, 2008), even to familial isoforms (Jarecki et al., 2013), but their high cost and inability to simultaneously study multiple analytes, spatially and quantitatively, limits their global use. Unlike these classical methods, mass spectrometry (MS) has begun to meet all the necessary requirements for scientists to fully study neuropeptides. In general, MS measures the mass-to-charge ratio (m/z) of an analyte of interest. These instruments are capable of analyzing neuropeptides down to low attomole ranges while providing mass accuracy down to a few ppm and resolution to differentiate between not only different neuropeptides but also familial isoforms (Andren et al., 1994; Dowell et al., 2006; Hui et al., 2012). While the development of high-resolution, accurate mass (HRAM) instrumentation allows for identification at the single stage MS (MS1) level, masses can also be selected for tandem MS (MS/MS). Peptide precursor ions are fragmented, producing characteristic fragments. As such, both known and novel analytes can be characterized and/or confidently identified. In conjunction with online or offline separations, MS is claimed to be capable of analyzing “entire proteomes” in a short amount of time (Hebert et al., 2014), making it an excellent tool to study the full complement of neuropeptides in a system (Castro et al., 2014; Hui et al., 2013; Predel et al., 2010, 2018; Xie et al., 2011). Furthermore, the development of MS imaging (MSI) has allowed to obtain highly accurate spatial information of several hundred analytes in one experiment. In addition, several strategies have been also developed (label-free and label-based) to quantitatively study neuropeptide changes, such as due to a biochemical or environmental stressor

(Buchberger et al., 2015; Southey et al., 2014; Yin et al., 2011). It should be noted that proper handling and separation of the samples are key to acquiring quality data, especially in the case of specialized MS techniques such as in vivo sampling methods and MSI (Buchberger et al., 2015; Gemperline, Chen, et al., 2014; Li et al., 2009; OuYang, Liang, et al., 2015). Overall, MS provides an attractive ability to examine the full complement of neuropeptides qualitatively and quantitatively.

While it seems that MS provides all the necessary qualities to study neuropeptides, many of the techniques used are still far from perfect. Figure 1 provides a pictorial representation of the possible workflows taken when studying neuropeptides with a mouse used as a model organism. This review will focus on the technological advancements and discoveries made, along with the challenging areas that still need development.

2 | SAMPLE PREPARATION

Sample handling is the first step where researchers need to be cautious to be accurate and consistent. Neuropeptides are often present at low abundance in a background containing all sorts of contaminants (e.g., salts, lipids). They are prone to proteolytic degradation, so sample handling is crucial. Yet it is often the least optimized step compared to downstream well-established instrumental MS methodologies (Buchberger et al., 2015; De Haes et al., 2015; Romanova & Sweedler, 2015; Yu et al., 2014). While salts and lipids compete with neuropeptides for ionization and suppress peptide signals, proteolytic degradation or other protein-modifying enzymes can rapidly change composition of the neuropeptidome, leading to inconsistent and sometimes confounding results.

2.1 | Prevention of neuropeptide degradation

Neuropeptides are subject to rapid proteolysis at room temperature. To preserve neuropeptide integrity, flash-freezing of the tissue is convenient to use and widely applied (Han et al., 2015; Sterkel et al., 2011). Other options do exist, including boiling (Altelaar et al., 2009; Sturm et al., 2010; Zhang, Wang, et al., 2018) and microwave irradiation (Wardman et al., 2010). Heat denaturation, which was introduced to eliminate postmortem degradation, can be adapted for a wide range of tissues (Svensson et al., 2009) and has been proven effective. Colgrave et al. (2011) have shown that hypothalamic tissue treated with a stabilization device yielded twice the number of mature neuropeptides than those detected in the untreated samples. The Stabilizer T1 (Denator), heat stabilization system has been shown to successfully increase neuropeptide identifications compared to other tissue preservation methods. It is worth noting that a high number of identifications may not indicate successful prevention of neuropeptide degradation, but rather abundant peptide signal may be due to high levels of postmortem degradation (Fridjonsdottir et al., 2018; Yang et al., 2017). Protease inhibitors also serve a similar purpose; for example, Onorato et al. (2019) recently showed that recovery of neuropeptide $(Pyr)^1$ apelin-13 from blood samples was only observed when samples were treated with a stabilization cocktail consisting of HALT® protease inhibitor (Thermo Fisher Scientific), 0.25 mM phenylmethanesulfonyl fluoride and 25% guanidine HCl (v/v). Protease inhibitors are also added to biological liquids, such as crustacean hemolymph (Chen, Ma, et al., 2009).

2.2 | Extraction strategies

Several workflows exist depending on the type of information sought from the sample (Buchberger et al., 2015; Dallas et al., 2015; Yu et al., 2014). Tissue homogenization and peptide extraction are procedures that affects identification rate in neuropeptidomics. Homogenization typically employs manual tissue grinding (i.e., using a pestle on snap-frozen tissue), sonication, or cell disrupter devices. Homogenization and extraction are performed in the presence of solvents or buffers which can dissolve peptides and simultaneously deactivate proteases in the sample. One of the most generally utilized buffers in such application is acidified methanol (Adamson et al., 2016; Budamgunta et al., 2018; Chen, Jiang, et al., 2010; Hui et al., 2013; Lavore et al., 2018; Sterkel et al., 2011; Van Bael, Watteyne, et al., 2018; Ye et al., 2015) which contains 90% methanol (MeOH), 9% glacial acetic acid, and 1% water. It is reported to be able to extract neuropeptides from single neurons (Zhang, Khattar, et al., 2018). This buffer system is further optimized by Zhang et al. for a "mixing on column" protocol, an approach that includes four steps with varying aqueous and methanol compositions. This hybrid protocol was able to capture hydrophobic peptides as well as hydrophilic peptides simultaneously and create up to fivefold more neuropeptide identifications (Petruzziello et al., 2012; Yu, Khani, et al., 2015; Zhang, Petruzziello, et al., 2012). Chen, Ma, et al. (2009) also demonstrated that the use of acidified methanol with a protease inhibitor additive is efficient for trace-level neuropeptide analysis in hemolymph samples. However, C-terminal methylation, an enzyme-assisted extraction artifact, might happen to some neuropeptides (Stemmler et al., 2013). Although use of acidified methanol is prevalent, a 0.25% acetic acid solution (DeAtley

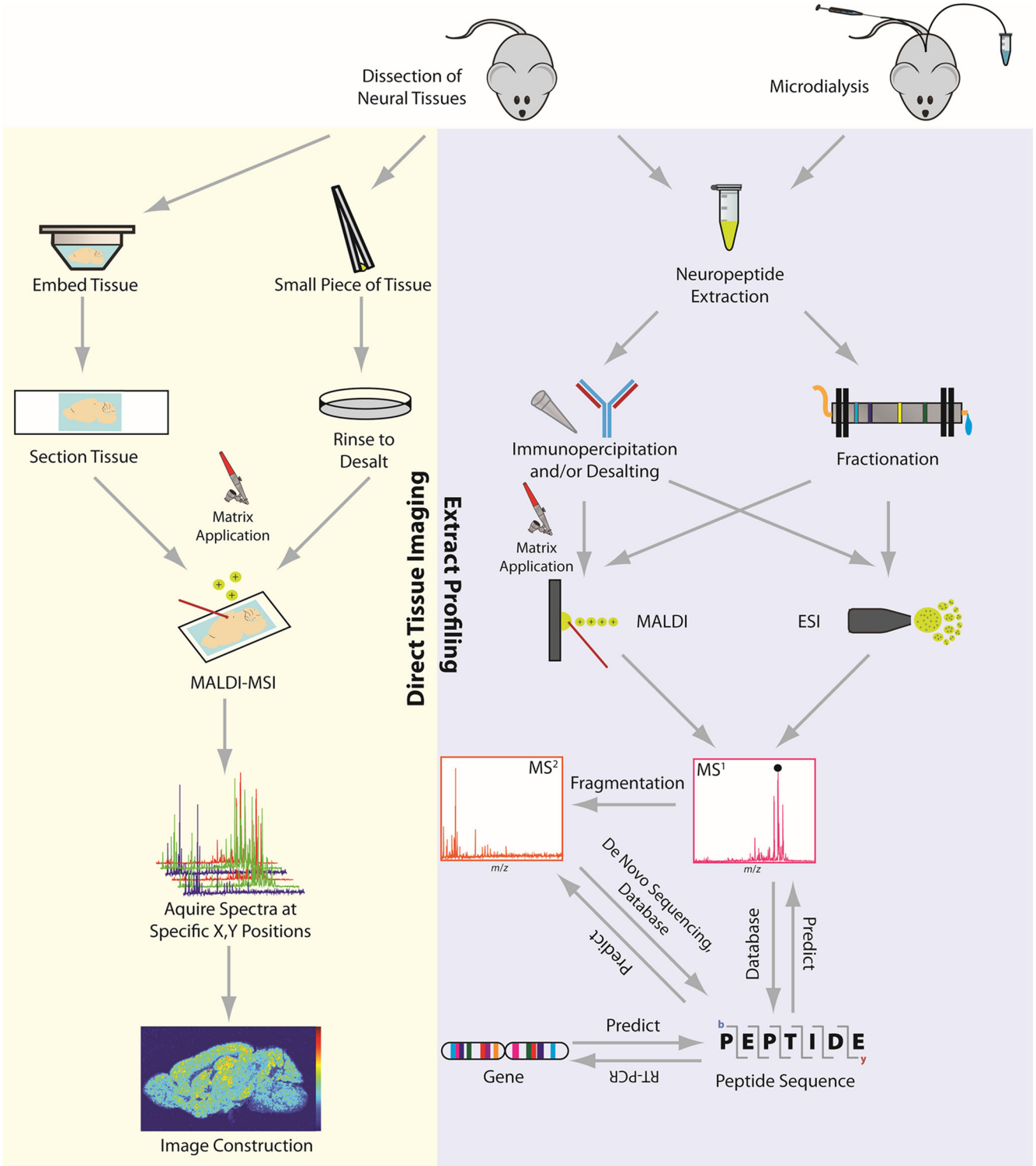

FIGURE 1 General workflow and strategies for investigating neuropeptides by mass spectrometry. Two major routes: extract profiling and tissue imaging [Color figure can be viewed at wileyonlinelibrary.com]

et al., 2018; Fridjonsdottir et al., 2018) has been shown to produce higher quality neuropeptide signal than acidified methanol (Dowell et al., 2006). Therefore, examples of effective strategies are to perform either multiple peptide extractions on the same tissue homogenate (Petruzziello et al., 2013), collect, and combine the supernatant fraction from each extraction (Yang et al., 2017, 2018). Alternatively, a peptide extraction can be followed by the addition of salt to the peptide extract supernatant to further precipitate remaining proteins (Gomez-Ramos et al., 2018).

A wide variety of organic buffers have been used in the recent years to extract neuropeptides from several biological matrices using acids such as formic acid, trifluoroacetic acid, and ethylenediaminetetraacetic acid (summarized in Table 1). For tissues that are difficult to homogenize, like bone tissue, more corrosive extraction buffers such as 1.2 molarity (M) hydrochloric acid (HCl), and 20% acetonitrile (ACN) are necessary (Gatenholm et al., 2019). Additionally, delipidation strategies using *n*-hexane (Van Bael, Edwards, et al., 2018) or methyl-*tert*-butyl ether in MeOH (Li, Zhou, et al., 2020) can also be applied during neuropeptide extraction. In lieu of organic solvent extraction buffers, molecular weight cut-off (MWCO) filters have also been used for neuropeptide purification and isolation of a particular size of neuropeptides. For example, neuropeptides from sea cucumber radial nerves can be extracted using either artificial sea water (Chieu, Suwansa-Ard, et al., 2019) or simple urea-based cell lysis buffers (Chen et al., 2019) followed by MWCO filters. However, extra care must be taken using these methods to avoid peptide degradation by catabolic enzymes. For biological samples containing abundant high molecular weight proteins, such as hemolymph (Fredrick & Ravichandran, 2012), a combination of extraction using acidified methanol and ultracentrifugation through MWCO filters are necessary for neuropeptide analysis (Liu, Buchberger, et al., 2019).

2.3 | Enrichment and sample clean-up

Generally, crude neuropeptide extract still contain soluble contaminants, such as salt, which can degrade mass spectral quality and result in decreased peptide signal in MS measurements (Constantopoulos et al., 1999), and desalting neuropeptide extract is especially important for biological samples that are suspended in proteomics/peptidomics

TABLE 1 Examples of various organic buffers applied for neuropeptide extraction from different biological material

Biological material	Extraction buffer	Reference(s)
Various	90% MeOH, 9% HOAc, 1% water	Adamson et al. (2016); Budamgunta et al. (2018); Chen, Jiang, et al. (2010); Hui et al. (2013); Lavore et al. (2018); Sterkel et al. (2011); Van Bael, Watteyne, et al. (2018); Ye et al. (2015)
Whole sea anemone	90% MeOH, 9% water, 1% FA	Hayakawa et al. (2019)
Rat spinal cord tissues	80% MeOH, 10% water, 10% FA	Tillmaand et al. (2020)
Mice cecum	37.5% MeOH, 12.5% chloroform, 50% water	Keller et al. (2020)
Starfish	70% MeOH, 5% HOAc	Kim et al. (2016)
Bed bug	50% MeOH, 1% FA	Predel et al. (2018)
Beetle		Ragionieri and Predel (2020)
Stick insect tissues	50% MeOH, 1% TFA	Liessem et al. (2018)
Various	0.25% HOAc	DeAtley et al. (2018); Dowell et al. (2006); Fridjonsdottir et al. (2018)
Dog saliva	80% ACN	Wang et al. (2019)
Human plasma		Kirwan et al. (2018)
Oyster ganglia	90% ACN, 0.1% TFA	Schwartz et al. (2019)
Monkey plasma and cerebrospinal fluid	ACN	Lee et al. (2018)
Citrus psyllid colonies	10% trichloroacetic acid, 2% 2-mercaptoethanol in acetone	Fleites et al. (2020)
Sea urchin	60% acetone, 40% water, 1% HCl	Monroe et al. (2018)
Aplysia abdominal ganglia	80% acetone, 10% water, 10% FA	Anapindi et al. (2018)
Rat spinal cord tissues	Dry ammonium sulfate with 0.01 M EDTA	Do, Ellis, et al. (2018)
Bone	1.2 M HCl in 20% ACN	Gatenholm et al. (2019)

Abbreviations: EDTA, ethylenediaminetetraacetic acid; FA, formic acid; TFA, trifluoroacetic acid.

buffers. Examples of popular commercial methods for desalting neuropeptide extract typically involves solid phase extraction utilizing reversed phase resin (i.e., C4, C8, or C18), such as Millipore ZipTip pipette tips (Sigma-Aldrich) and Pierce Desalting Columns or Tips (Thermo Fisher Scientific), or a hydrophilic polymer sorbent, such as Oasis hydrophilic-lipophilic-balanced cartridges (Waters). These types of tools are critical for peptidomics workflows because they not only desalt, but also concentrate neuropeptide samples. Additionally, pooling several tissues, organs, and neurons into one sample is often necessary when concentrated neuropeptidomic content is desired for comprehensive neuropeptide identifications. Other methods of concentrating neuropeptides are by utilizing monoclonal antibodies immobilized on magnetic beads (Vocat et al., 2020), automated solid-phase extraction (Bardsen et al., 2019), and large volume sample stacking using capillary electrophoresis (CE) (DeLaney & Li, 2019a). A recently developed technique to quickly concentrate and desalt neuropeptides involve dispensing a droplet of tissue extract onto a sample target consisting of a hydrophobic circle surrounded by a hydrophilic ring, which allows separation between salts and neuropeptides to occur directly on the MS sampling plate (Wang et al., 2017; Yoon et al., 2018).

2.4 | MS imaging

Unlike tissue homogenization, direct analysis of intact tissue is a simpler way that enables comparing localization from individual samples or animals which is usually important to determine its biological relevance. For example, intact somata were analyzed after aspiration by a pipette and transfer onto an MS sampling plate for neuropeptide profiling (Diesner et al., 2018; Neupert, Fusca, et al., 2018). Liquid extraction surface analysis (LESA) is a direct tissue sampling technique that has recently been commercialized by HTX Technologies as the SepQuant droplet probe and has been used successfully for neuropeptide analysis (Kertesz et al., 2015). Pioneered by Caprioli et al. (1997), MSI has also emerged as an attractive technology for localizing neuropeptides. Neuropeptide MSI experiments require sectioning tissue into 10–20 μm thick slices. Tissues have to be embedded into scaffold materials, such as gelatin (Chen, Cape, et al., 2010; OuYang, Chen, et al., 2015; Ye et al., 2015), sucrose (Verhaert et al., 2010), gelatin containing sodium salts of carboxymethyl cellulose (Resetar Maslov et al., 2019), at the time of snap-freezing to facilitate sectioning and preserving tissue integrity. Once sectioned, the tissue can be directly mounted onto a glass slide or sample plate for matrix application with an airbrush or automatic matrix sprayer (Andersson et al., 2008; Ye et al., 2012). Spectral quality can be improved by washing the tissue sections with organic solvents (e.g., ethanol, methanol, acetone, water, or different mixtures of these solvents) before matrix application to remove salts and lipids which negatively influence the matrix crystallization process and signal quality (Buchberger, Vu, et al., 2020; Kaletaş et al., 2009; Meriaux et al., 2011; Seeley et al., 2008). The most common matrices for neuropeptide imaging include α-cyano-4-hydroxy-cinnamic acid (CHCA) (Chen, Ma, et al., 2009; Pratavieira et al., 2014) and 2,5-dihydroxybenzoic acid (DHB) (Chen, Cape, et al., 2010; Ye et al., 2015; Zimmerman et al., 2009). More details on MSI are described in a separate section below.

2.5 | Microdialysis

Though tissue homogenization and direct tissue analysis are complementary in gaining insight into sample composition and localization, they all require sacrificing animals. This makes it impossible to track real-time change in vivo and brings in unwanted variations among animals if following time course changes is the real objective. As an emerging as well as underdeveloped technique, microdialysis offers the capability to monitor spatio-temporal dynamics of neuropeptides over a certain time period upon external stimulus via a probe implanted into the tissues of interest that allows continuous sampling from the extracellular space (Kushikata & Hirota, 2011). When sampling from extracellular space, the concentration gradient drives the analytes to diffuse across the dialysis membrane, which has a certain MWCO filter depending on the substances of interest (OuYang, Liang, et al., 2015). Due to the small probe size, animals endure minimal physical damage and associated neurological disturbance. Long-term sampling can be accomplished while animals are still alive and freely moving. It has found its applications in a wide variety of tissues and organs, including skin (Baumann et al., 2019), hypothalamus (Guzman-Ruiz et al., 2015; Kurian et al., 2015), hippocampus (Takeda et al., 2011), spinal cord (Wu, Zhang, et al., 2015), and kidney (Wesson et al., 2015) in vertebrates as well as neuronal organs in crustaceans (Behrens et al., 2008; Jiang et al., 2016; Liang et al., 2015; Schmerberg et al., 2015).

Despite its attractiveness, challenges still exist for microdialysis sample preparation. High temporal resolution (shorter intervals for collection of individual samples) is desired for microdialysis measurements, but this must be considered with MS sensitivity factor by selecting an appropriate sampling volume. Balancing low neuropeptide concentration in vivo (1–100 pM), small sample volumes generated by microdialysis (1–10 μl) (Zhou et al., 2015) and low recovery rate (20%–30%) (Schmerberg & Li, 2013) makes the choice of instrument even more important. It has been demonstrated that adding organic solvents, especially ACN,

to dialysate is able to prevent adsorptive loss of low-abundance neuropeptides by hydrophobic interactions with membrane surfaces (Maes et al., 2014; Zhou et al., 2015). By treating the dialysis membrane and fused silica tubing with polyethylenimine, recovery was improved by 1.2- to 80-fold (Zhou et al., 2015). This only benefited the detection of peptides that carried a net positive charge, though, probably due to reduced electrostatic interaction between peptides and the microdialysis probe. An array of affinity-enhanced microdialysis approaches have been tested by Schmerberg and Li (2013), and they observed antibody-coated magnetic nanoparticles to provide the greatest enhancement in neuropeptide recovery. Other efforts to increase peptide recovery include a study by Van Wanseele et al. (2017), who tested several liquid chromatography (LC) columns and mobile phases to find the combination for optimal recovery of neuropeptides (Cortecs®C18+ column with a mobile phase containing methanol as organic modifier and acetic acid as additive) from microdialysate of a solution containing peptide standards. Another advancement in microdialysis probe sampling include nonspecific perturbing of the tissue of interest to elicit a biochemical response. Al-Hasani et al. (2018) developed a microdialysis probe containing optical fibers for the purpose of stimulating neuronal peptide release which is subsequently collected in the probe perfusate. The peptide profile resulting from nonspecific techniques such as this can be used to generate additional research questions that can be answered by more specific techniques, such as expression knock-out experiments. Although microdialysis is useful for performing in vivo experiments, the recovery rate of neuropeptides is relatively low.

Overall, each sample handling step strives to increase neuropeptide signal by decreasing interfering signal while minimizing sources of neuropeptide loss. However, the variety of chemicals and solvents used by different research groups (even for similar tissue types) illustrates the need for continued evaluation and comparison between these different extraction and sampling systems. Ideally, there would be a workflow that is unanimously agreed upon to produce optimal neuropeptide signal, but it is our opinion that there would likely exist multiple workflows tailored for individual subclasses of neuropeptides and specific underlying questions to address.

3 | DISCOVERY/SEQUENCE IDENTIFICATION

3.1 | MS in general peptide structural elucidation strategies

Before the introduction of MS, neuropeptides were identified during searches for endogenous molecules that produced a physiological effect, and Edman degradation was used as a standard method to determine the primary sequences (Yu, Liang, et al., 2015). This strategy requires a substantial amount of sample, especially from tissue types with scarce neuropeptide content, and a priori knowledge of the analyte of interest since it is a "function first" approach. With its high-throughput capability, MS, especially when coupled with electrospray ionization (ESI) sources, allows thousands of peptides to be measured simultaneously. One of the pioneers in the field, Dominic Desiderio, demonstrated the utility of MS for endogenous (neuro)peptide structural analysis early on (Desiderio & Yamada, 1982; Desiderio et al., 1993; Kusmierz & Desiderio, 1992; Mahajan & Desiderio, 1978; Yamada & Desiderio, 1982). By alternating between MS and MS/MS, records of both intact mass and fragment information (to determine the sequence) can be obtained. Matching these two pieces of information to the respective genome reveals exact neuropeptide sequences, their origins, as well as functions. However, not all organisms have their genome fully characterized, which sometimes makes genomic-based database searching unfeasible. This is overcome by de novo peptide sequencing, a technique that can provide neuropeptide sequences solely based on tandem MS data, without the need for a complete genome. MS has greatly shifted discovery of neuropeptides from the identification of a single peptide to the characterization of multiple peptides representing entire peptidomes.

Various fragmentation techniques have been developed, see Table 2. Collision-induced dissociation (CID), the conventional vibrational activation, has been widely used (Ye et al., 2013; Zhou et al., 2013). However, CID has been criticized for preferentially cleaving the weakest bonds, no matter of location in the peptide backbone or side chains, such as with PTMs. Once a bond is cleaved, the internal energy is released and the product will not be further activated, which sometimes leaves spectra with few dominating peaks to interpret (Medzihradszky & Chalkley, 2015; Seidler et al., 2010). Furthermore, the loss of PTMs can be detrimental to some studies. To generate a better-quality spectrum, an alternative fragmentation approach is the beam-type CID or high-energy collision dissociation (HCD). It accelerates all ions across the chamber instead of the ion trap, permitting multiple collisions, and therefore fragments might break up further to create products equally distributed along the backbone (Jedrychowski et al., 2011; Medzihradszky & Chalkley, 2015). Fragmentation by CID in a triple quadrupole and HCD in an Orbitrap mass analyzer for the structural characterization of neuropeptide receptor antagonists were compared (Silva et al., 2018). Similar qualitative and structural information was seen between

the two mass analyzers, though higher confidence structural assignments were seen from the HCD-obtained data (Silva et al., 2018). Another comparison was performed by Tu et al. between HCD in an orbitrap, HCD in an ion trap, and CID in an ion trap using an Orbitrap Fusion Lumos where they achieved the highest number of identifications using HCD in the orbitrap, then using HCD in the ion trap, and the lowest amount from CID in the ion trap (Tu et al., 2016). Despite being less sensitive than CID due to the higher ion volume requirement to generate a spectrum, HCD has become more and more popular due to its better data quality and ability to record all products across a wide mass range (Silva et al., 2018).

Two MS/MS methods complementary to collision-based activation that have been developed are electron-capture dissociation (ECD) (Zubarev et al., 2000) and electron-transfer dissociation (ETD) (Syka et al., 2004), where a radical ion is formed and undergoes fragmentation to yield almost exclusively peptide backbone fragmentation, thus preserving labile PTMs. Following their introduction, both electron-based methods, particularly ETD, have been gaining popularity among researchers studying PTMs in proteomics (Sobott et al., 2009). While still relatively new, ECD and ETD have the potential to be a critical component of neuropeptide sequencing. Unlike the digested protein fragments observed in bottom-up proteomics with predictable C-termini and similar lengths, neuropeptides tend to have varying sizes from a few to several dozens of residues. For example, some FMRFamides in invertebrates have only four amino acids whereas CCK-58, as indicated by its name, has 58. Furthermore, endogenous proteolytic processing leads to the production of peptides containing multiple internal basic residues (histidine, lysine, and arginine) which hold higher charges states in the gas phase, for which CID and HCD show limited performance. Fortunately, that is where ETD outperforms the former two (Hui et al., 2011). Combining CID or HCD with ETD provided complementary spectra for Sasaki et al. (2013) in their study on endogenous peptides from a human endocrine cell line, and ETD helped identify a previously unknown large peptide, VGF[554–577]-NH_2. Rathore et al. (2015) developed a strategy to perform two dissociation techniques, CID and ETD, in one analysis without a decrease in duty cycle. Facilitated by the temporal separation gained through ion mobility MS (IM-MS) (see *Ion mobility MS* section), a single packet of precursor ions can give rise to b- and y-type ions containing spectra and c- and z-type ions containing spectra. A hybrid strategy was further developed by Hui et al. and Jia et al. where a bottom-up approach using CID and HCD fragmentation was coupled with a top-down strategy employing ETD fragmentation to

TABLE 2 Four common ion activation and dissociation techniques—CID, HCD, ETD, ECD, and EThcD—for fragmenting peptides

Method	Mass analyzers	Fragment ions	Advantages	Disadvantages
CID	Quadrupole ion trap, triple quadrupole	b-type, y-type	Fast, sensitive, more efficient for low charge ions, induces glycan fragmentation	Low mass resolution, loss of labile modifications
HCD	Orbitrap	b-type, y-type	Fast, high mass resolution, induces glycan fragmentation	Less sensitive and slower than CID, loss of labile modifications
ETD	Ion trap, ion trap-orbitrap hybrid	c-type, z-type	Retains neutral and labile modifications, faster than ECD, suitable for higher charge state ions (>2+)	Slow, inefficient fragmentation for low charge state precursors, less accessible instrumentation
ECD	FTICR or selected time-of-flight	c-type, z-type	Retains neutral and labile modifications	Less accessible instrumentation
EThcD	Orbitrap	b-type, y-type, c-type, z-type	Rich sequence-specific fragment ion information, more suitable for characterization of neuropeptides with labile PTMs	Longer duty cycle, requires the production of higher charge state of precursor ions

Abbreviations: CID, collision-induced dissociation; ECD, electron-capture dissociation; ETD, electron-transfer dissociation; EThcD, electron-transfer/higher-energy collision dissociation; HCD, high-energy collision dissociation.

reveal more structural details of large neuropeptides (Hui et al., 2011; Jia et al., 2012). This represents a new route to discovery and characterization of large neuropeptides since neither of these fragmentation techniques could manage to provide a complete picture of a large neuropeptide alone. Rather than using CID, HCD, or electron activated dissociation (ExD), Vrkoslav et al. (2018) have shown that in-source decay fragmentation can be used to produce fragment ions for peptide structure characterization in single-stage matrix-assisted laser desorption/ionization (MALDI) instruments lacking precursor ion-selection capabilities. To improve the coverage and quality of neuropeptide sequencing by in-source decay, Neupert reports a method for N-terminal derivatization using 4-sulfophenyl isothiocyanate (Neupert, 2018). This radical based dissociation technique enables the fragmentation of intact peptide ions, where traditional dissociation techniques are inefficient.

Chemically derivatized peptides can carry some distinct fragmentation patterns and/or improve fragmentation, and some of them can be utilized for sequencing. Dimethyl labeling is one of the well-established methods that has been employed in neuropeptide identification studies (Fu & Li, 2005; Hsu et al., 2005; Ma et al., 2009), as it features enhanced a1-ion signal for N-terminal determination and simplified MS/MS interpretation. Dimethyl labeling is also effective for analyzing dipeptides and tripeptides (Tang et al., 2014). Short neuropeptides (2–3 residues) are difficult to characterize. They have low molecular weights, complicating the desalting process, and can be hydrophilic, decreasing compatibility with conventional C18 columns. However, these short peptides are still bioactive and potentially important; their MS analysis benefits from derivatization with Marfey's reagent (Bobba et al., 2012). Acetylation is another example of methods that target primary amines (Yew et al., 2009). A nanosecond timescale photochemical click-chemistry based enhancement for neuropeptide detection was developed by Li et al. (2019) to remove matrix components to decrease matrix effects and spectral complexity. A few other chemical derivatization schemes have been developed in recent years but have yet applied to neuropeptide studies. Kim, Shin, et al. (2011) reported an oxazolone chemistry for incorporation of Br signature to the C-terminus, which populates MS/MS spectra with a series of y-ions bearing a Br signature for easier interpretation. Isothiocyanate analogues with basic moieties have been demonstrated to derivatize peptides and significantly improve the MS sensitivity, while promoting Edman-type cleavage and maintaining other sequence fragments for easy sequencing (Wang et al., 2009). Cationization by alkali metals have also been shown to improve de novo sequence coverage of small peptides (<15–20 residues) (Logerot & Enjalbal, 2020). The peptide derivatization strategy reported by Frey et al. appends tertiary or quaternary amines to the peptide's carboxyl groups present at the C-terminus and in aspartic and glutamic acid side chains. As the amine appended, the charge state of that peptide increases, improving its ETD fragmentation efficiency (Frey et al., 2013). Charge state manipulation and distribution of neuropeptides were further studied by Nielsen and Abaye where it was found that the use of electrolyte additives or supercharging reagents was sufficient to alter the observed charge states and total ion signal (Nielsen & Abaye, 2013). Bongaerts et al. (2020) recently studied the use of several supercharging agents on neuropeptide ionization and concluded the effects to be highly dependent on the peptide. While supercharging agents can alter charge state distributions to something more desirable, care must be taken to choose the appropriate one for each analyte.

3.2 | Data independent analysis

While improvements in fragmentation techniques have paved the way for the increased identification and characterization of neuropeptides, traditional discovery/shotgun proteomics strategy using data-dependent acquisition (DDA) is still limited by the number of MS/MS spectra abled to be collected. This is problematic for the analysis of more complex samples because only a small fraction of analytes can be selected and fragmented. As the most abundant precursor ions are selected for fragmentation, DDA biases detection to higher abundance or more readily ionizable species. Data-independent acquisition (DIA) can address some shortcomings of DDA, expanding proteome and peptidome coverage through its increased MS acquisition abilities (Chapman et al., 2014). DIA methods involve the isolation and fragmentation of multiple precursor ions within a window simultaneously, with windows spanning the whole m/z range of interest, followed by the use of software to deconvolute the more complicated MS/MS spectra containing fragments from several precursors. This approach generates fragment ions of all precursors in a sample instead of solely the highest abundance ones. The information gathered from every sample component can thus be accessed later as well with the evolution of better software, increasing the capabilities for untargeted analysis. While the additional information enables a wider coverage and increases reproducibility of analysis, the subsequent data deconvolution becomes exponentially more complex. An up-to-date and comprehensive review was written by Zhang, Ge, et al. (2020) addressing several different DIA schemes, as well as software tools for analysis and library

TABLE 3 Various software tools designed specifically for advancing neuropeptide research

Type of tool	Name	Brief description	Link to resource
Prediction	ENPG	Neuropeptide prediction	https://sourceforge.net/projects/enpg/
	NeuroPID	Neuropeptide precursor and neuromodulator prediction	http://neuropid.cs.huji.ac.il/
	NeuroPIpred	Insect neuropeptide prediction	https://webs.iiitd.edu.in/raghava/neuropipred/
	NeuroPP	Neuropeptide precursor prediction	NA
	NeuroPred	Neuropeptide prediction	http://neuroproteomics.scs.illinois.edu/neuropred.htm
	NeuroPred-FRL	Neuropeptide prediction	http://kurata14.bio.kyutech.ac.jp/NeuroPred-FRL/
	SignalP	Signal peptide prediction	http://www.cbs.dtu.dk/services/SignalP/
Database	BLAST	Sequence alignment search tool	http://www.ncbi.nlm.nih.gov/BLAST/
	DINeR	Insect neuropeptide database	http://www.neurostresspep.eu/diner/
	NeuroPep	Database of neuropeptides, their genes, precursors	http://isyslab.info/NeuroPep/
	SwePep	Endogenous peptide database	NA
MS Data Search	IggyPep	Hybrid de novo and genome wide-database search	NA
	NeuroPedia	Searchable neuropeptide database and spectral library	http://proteomics.ucsd.edu/Software/NeuroPedia.html
	PRESnovo	Motif prescreening before de novo sequencing	https://www.lilabs.org/resources

building so this will not be addressed again in this review.

While DIA is increasingly being incorporated into proteomics analysis workflows, it is slow to be applied to neuropeptidomics, a field that would benefit greatly from a decrease in high-abundance bias. This is made evident by the work by Kwok et al. (2020), where they developed a sensitive method for the detection of 42 bioactive peptides and hormones using DIA. A side-by-side comparison performed by Delaney and Li (2019b) demonstrated the utility and benefits of incorporating DIA over DDA into the neuropeptidomics workflow. An impressive improvement was seen in the number of neuropeptide identifications, sequence coverage, and technical and biological reproducibility, further demonstrating the utility of applying a DIA workflow to neuropeptidomics analysis. While it has been demonstrated that a DIA approach can provide benefits over DDA analysis, Saidi et al. also saw an advantage to using parallel reaction monitoring (PRM) to perform targeted peptide quantitation of neuropeptides (Saidi et al., 2019). The authors compared the use of DIA with PRM analysis and observed an increase in variability and decrease in performance associated with DIA, indicating DIA has larger advantages in an untargeted capacity, rather than in targeted analyses. These few explorations into DIA for neuropeptide analyses demonstrate promise for utilizing the advantages of DIA for analysis, though it seems to be slow to be incorporated into the neuropeptidomics workflow, potentially due to a lack of tailored software tools and spectral libraries.

3.3 | Peptide bioinformatics: Database search software/de novo sequencing advances

3.3.1 | Peptide sequence prediction and databases

Traditional proteomics workflows compare MS-generated fragmentation data to genome-generated databases to determine which proteins are found in a sample. Unfortunately, this workflow does not transfer directly to neuropeptides; a comprehensive specific endogenous (neuro)peptide database does not exist. Several independent initiatives have been initiated in the past. If a species does not have its genome fully sequenced, there is not an easily obtained database to compare against. Furthermore, the fact that neuropeptides go through a series of modifications involving several endopeptidases before final maturation/neuronal release introduces some degree of unpredictability of their final active sequences, meaning that these genomic-generated databases may not be accurate. Therefore, not much can be learned about neuropeptides without robust

bioinformatics tools even with a complete genome database. To predict neuropeptide sequences in silico from a genome and construct a reliable database, multiple algorithms have been developed and tested, which has been well-reviewed in several publications (Boonen et al., 2008; Hayakawa et al., 2019; Yu et al., 2014). We have compiled a list of tools and resources, including sequence prediction tools, database compilations, and tools to search MS spectra, specifically developed to benefit the MS identification of neuropeptides in Table 3. Generally, when studying a new organism, the genome of which is available, the online BLAST program allows extraction of all potential neuropeptide prohormones (NPPs) with known NPPs from related species (Christie, 2015; Conzelmann et al., 2013). The deduced NPPs are processed to remove signal peptides using the online program SignalP 5.0 (Almagro Armenteros et al., 2019; Christie, 2015; Petersen et al., 2011), after which they are ready to be submitted to neuropeptide prediction tools such as NeuroPred (Han et al., 2015; Hummon et al., 2003; Tegge et al., 2008), ENPG (Hayakawa et al., 2019), NeuroPred-FRL (Hasan et al., 2021), and specific for insect neuropeptide prediction, NeuroPIpred (Agrawal et al., 2019). Another resource for insect research is DINeR, a database for neuropeptide sequences and functionality (Yeoh et al., 2017). In another homology-based search, Ofer and Linial (2014) reported a machine learning scheme, Neuropeptide Precursor Identifier (NeuroPID), that can be trained on hundreds of identified NPPs and used to predict metazoan NPPs. NeuroPP, another tool for neuropeptide precursor prediction has also been developed for improved screening (Kang et al., 2019). Burbach presented an inventory of known neuropeptides, classified in families according to shared structural properties (http://www.neuropeptides.nl) (Burbach, 2010), which is included in another database additionally compiled of genes and precursors called NeuroPep (Wang, Wang, et al., 2015). SwePep, while not currently active, was also an endogenous peptide specific database that improved MS analysis (Falth et al., 2006). However useful, these databases are not searchable directly with MS/MS data. NeuroPedia, a specialized neuropeptide database and spectral library that is directly searchable using MS/MS data was constructed, improving identification efficiency, sensitivity, and reliability (Kim, Bark, et al., 2011). Instead of using homology-based or de novo sequencing database filtration-based searches, Menschaert et al. developed a genome-wide database searching method combined with de novo sequencing, IggyPep. Compared to using limited-sized database searches, a 30% increase was seen in identification rate when searching the sea urchin neuropeptidome (Menschaert et al., 2010). This approach was later adapted to include enhanced homology-based gene discovery to discover new prohormones and neuropeptides, previously unidentified by the original IggyPep method (Monroe et al., 2018). Also using genomic information, Jarecki et al. discovered novel neuropeptides through searching *Ascaris suum* libraries of expressed sequence tags and preliminary genome survey sequences (Jarecki et al., 2011). The field of neuropeptidomics faces challenges as many of the model organisms for analysis do not have a fully sequenced genome. To address these informatics challenges, as well as others with endogenous peptide specific concerns in mind, like technical difficulties arising from a lack of enzymatic digestion, a streamlined analytic framework was developed for large-scale peptidomics (Jarecki et al., 2011). By incorporating database mining and predicting fragmentation patterns, many neuropeptides could be identified and 21 putative novel neuropeptides were discovered (Jarecki et al., 2011). Also with the goal of improving endogenous neuropeptide analysis, Secher et al. developed a full workflow, from sample extraction to bioinformatic analysis, for increased identification and insight into function through a prioritization scheme for biologically relevant peptides (Secher et al., 2016).

While not developed specifically for neuropeptide analysis, PEP Search (http://www.mycompoundid.org/mycompoundid_IsoMS/searchSmallPeptide.jsp) (Tang et al., 2014) can be used for the identification of small neuropeptides, such as dipeptides and tripeptides. Besides specialized endogenous (neuro)peptide search engines, common proteomics database search programs can be used to identify neuropeptides, though the translation may not be that straightforward. To provide a reference for people who want to use a common database search program, Akhtar et al. (2012) elaborated on the strengths and weaknesses of several of these programs (OMSSA, X!Tandem, and Crux) to identify neuropeptides.

3.3.2 | De novo Sequencing

If genomic information is too scarce to create a thorough NP database, de novo sequencing can be used to derive amino acid sequences of peptides solely based on MS/MS fragmentation spectra. Since the late 1990s, a handful of de novo sequencing tools have been developed (e.g., PEAKS, PepNovo). A more comprehensive review of de novo sequencing tools can be found in other reviews (Allmer, 2011; Ma & Johnson, 2012). As high resolving power and accuracy are extremely important when deriving a peptide sequence, modern mass spectrometers will continue to make de novo sequencing easier with instrumental advances, which in turn requires new de novo sequencing software tools to be developed accordingly to work with certain type of instruments.

For example, pNovo was designed for use with HCD fragmentation (Chi et al., 2010). UniNovo was introduced two years later, claiming to be able to work well for spectra from various types of fragmentation methods (CID, ETD, HCD, and CID/ETD) (Jeong et al., 2013). Later, Ma et al. presented a novel de novo sequencing program, Novor, offering improvements in both the speed and accuracy for peptide de novo sequencing analyses (Ma, 2015), compare to PEAKS (Mazurais et al., 2015). Most recently, DeepNovo was introduced by Tran et al. (2017), an innovative deep learning-based approach for de novo sequencing, outperforming PEAKS, PepNovo, and Novor. This method was later adapted to create DeepNovo-DIA for analyzing DIA data (Tran et al., 2019). While not created for endogenous peptide analysis, the field of neuropeptidomics benefits from incorporation of these tools into the neuropeptide analysis workflow.

Neuropeptide identification has been facilitated by these various advances and can be further improved through preliminary processing before database searching. PRESnovo was developed to take advantage of the common conserved sequence motifs found in many neuropeptides as a prescreening method to improve the subsequent de novo sequencing (DeLaney et al., 2020). By searching through a predefined motif database, probable motifs can be assigned to each precursor from a MS/MS spectrum, which increases correct identifications seen through PEAKS, compared to without PRESnovo prescreening (DeLaney et al., 2020). Preprocessing was also shown to be beneficial for the detection of neuropeptides, using a MATLAB-based workflow and statistical analysis (Salisbury et al., 2013).

After receiving the results from a database search, the confidences of identifications must be evaluated, commonly using statistical false discovery rates and dummy databases (Jeong et al., 2012). This is important for measuring the integrity and confidence in identification assignments. Using a mixed species database, the assignment fidelity and false positive percentages were compared after the acquisition of single species neuropeptidomic data using Orbitrap, ion trap, and quadrupole time-of-flight (TOF) instruments (Anapindi et al., 2018). While all platforms saw a decrease in identifications during the use of the mixed database, the Orbitrap data was least negatively affected (Anapindi et al., 2018). Overall, the quantity, quality, and reliability of neuropeptide identifications depends on the careful consideration of neuropeptide sequence prediction, database selection method, as well as the search method and fidelity evaluation parameters. While there are various tools available for identification (and possibly support quantitative analysis), these software are not created for the characterization of endogenous peptides specifically; instead, modern day software requires researchers to state that no enzyme digestion is performed. The field of neuropeptidomics could benefit from development of effective bioinformatic tools able to perform identification without specification of an enzyme or able to interpret results at the endogenous peptide level, rather than having to compromise and use the "digested peptides" function at the software-designated protein level.

4 | STRUCTURAL ANALYSIS

4.1 | Posttranslational modifications

As described above, neuropeptide synthesis begins with a large precursor protein that undergoes cleavage by proprotein convertases. These processed peptides are subject to various PTMs, all of which can affect neuropeptide binding affinity, lifetime, and function (Hokfelt et al., 2000). PTMs along with proteolytic processing leads to the generation of distinct structures of bioactive peptides. Such PTMs, such as phosphorylation, sulfation, and glycosylation, may be introduced before or after proteolytic processing. While studies to determine the presence of PTMs are important, it is also of interest to understand the mechanisms for modification of neuropeptides (Hook et al., 2018). Location of a PTM, whether on the precursor peptide or on the bioactive peptide, may also be of importance. Multiple prolactin variants were recently identified and their regulation patterns were found to differ (Qian et al., 2018). Glycosylation of the mature natriuretic peptide hormone family alters processing, whereas the O-glycosylation of the propeptide decreases cleavage frequency and leads to fewer bioactive peptides in circulation (Hansen et al., 2019). In addition to the effects from propeptide modifications, altered receptor activation and increased stability of the bioactive peptides were also observed when glycosylation was located on the receptor binding region of the mature peptide (Madsen et al., 2020). Whereas formerly, bioactive neuropeptide PTMs were thought to be conserved to terminal amino acids (for protective effects against degradation) as well as the precursor proteins (for cleavage purposes), though PTMs at other positions along the neuropeptide backbone are likewise observed (Baggerman et al., 2004; Busby et al., 1987; Hummon et al., 2003).

The most common PTMs on neuropeptide termini include pyroglutamate modification of the N-terminus (Gade & Marco, 2015; Lee et al., 2010; Monroe et al., 2018; Salisbury et al., 2013), which is thought to protect the peptide from enzymatic degradation (Hayakawa et al., 2019), and C-terminal amidation, which is required for the biological activity of many neuropeptides (Anapindi et al., 2018;

Salisbury et al., 2013; Secher et al., 2016). To evaluate the importance of neuropeptide amidation, Van Bael et al. designed a gene knockout experiment targeting three putative neuropeptide amidation enzymes in *Caenorhabditis elegans*, an organism able to survive without neuropeptide biosynthesis enzymes. Their findings indicated the dependence on C-terminal amidation for reproduction, drastically interfering with the quantity and success of egg-laying, further highlighting the importance of such PTMs (Van Bael, Watteyne, et al., 2018).

Another common peptide hormone PTM is acetylation. Biological roles of acetylation include to increase peptide stability, by protecting the peptide from enzymatic degradation, and to regulate receptor affinity (Van Dijck et al., 2011; Zhang, Petruzziello, et al., 2012). During the characterization of pro-opiomelanocrtin related hormones, Yasuda et al. (2011) identified novel triacetylation of α-melanocyte-stimulating hormone (α-MSH). Acetylation has also been found to exist as a tissue specific modification of mouse hemokinin-1, detected only in the brain and not in peripheral tissue, indicating a brain specific functional role for this PTM (Deliconstantinos et al., 2017).

Cysteine disulfide crosslinking of peptides is an important PTM observed in neuropeptides (Jia et al., 2012). It provides structural rigidity and contributes to a peptide's three-dimensional structure, essential for receptor recognition and peptide function. Challenges in MS analysis of disulfide crosslinked molecules include its low abundance and low fragmentation efficiency, owing to the stability of the disulfide bond. Yu, Khani, et al. (2015) developed a targeted ETD-based method and data mining scheme to improve the recognition and localization of endogenous disulfide bonds in rat neuropeptides, enabling future studies to target this PTM in a more high throughput manner. To improve disulfide bond characterization, Bhattacharyya et al. (2013) developed *DisConnect*, an open source software, to determine disulfide connectivity of peptide hormones, peptide toxins, and proteins, and to characterize disulfide foldamers. In-source reduction methods have also been shown to successfully map disulfide bond linkages in peptides (Cramer et al., 2017; Stocks & Melanson, 2018, 2019; Ye et al., 2015). A vendor neutral software tool, DiSulFinder, was designed to identify peptide backbone fragments with both intact or cleaved sulfur-sulfur or sulfur-carbon bonds (Liang et al., 2018). Liang et al. were able to quickly provide identifications for disulfide linkage determination in the interchain disulfide-linked crustacean cardioactive peptide and insulin fragment peptide (Liang et al., 2018).

Glycation, a PTM associated with age, altering protein structure and function, has also been shown to modify neuropeptides. The different types and binding sites of glycation for the neuropeptide substance P (SP) were investigated by Lopez-Clavijo et al. Using a multimodal MS approach, the authors were able to confidently assign binding sites and identify intermediate products to understand glycation and its different types, paving way for studies of glycation on other neuropeptides (Lopez-Clavijo et al., 2012).

Acidic modifications such as phosphorylation of serine, threonine, or tyrosine and sulfation of tyrosine can benefit from the use of negative ion mode MS analysis and are commonly analyzed through such methods (DeLaney, Phetsanthad, et al., 2020). With only a mass difference of 0.0095 Da between the phosphorylation and sulfation modifications, and both capable of modifying tyrosine residues, HRAM instruments must be used to resolve these small differences (for more isobaric PTMs see the *Isobaric PTMs* section). Using a high-resolution Fourier-transform ion cyclotron resonance MS (FTICR-MS), tyrosine sulfation was identified and localized during the top-down analysis of a sex ganglia-specific peptide in *Hirudo medicinalis*. Sulfation was confirmed through high mass accuracy measurements as well as characteristic isotopic abundance shifts consistent with sulfur isotopes (Hsu et al., 2017). In another study, during a multi-MS platform neuropeptidomic characterization of the rat habenular nuclei, novel sulfation sites were discovered on secretogranin I prohormone and confirmed through an additional targeted MS analysis (Yang et al., 2018). In summary, although it is difficult to differentiate between the two PTMs, it can be achieved using the proper MS tools. In addition, enzymatic tools may help conclusively establish sulfation (de Vries et al., 2005). Neuropeptidomics can benefit from the increased characterization of these two PTMs, as there are many sulfated neuropeptides with unknown function (Seibert & Sakmar, 2008).

Furthermore, phosphorylation is known to induce dynamic modifications of neuropeptides and is of great interest for characterization as potential biomarkers because of its common occurrence (Yasuda et al., 2011). Over 50 novel neuropeptide phosphorylation sites were discovered by Secher et al. by a newly developed bioinformatics tool. Functional studies show that phosphorylation of α-MSH reduces its binding to melanocortin receptors. Serine phosphorylation of neuropeptides were of much higher abundance compared to intracellular proteins in the rat brain (Secher et al., 2016). While insect phosphorylated neuropeptides are rare, Sturm and Predel were able to identify phosphorylation of CAPA pyrokinin in *Lamproblatte albipalpus* that is interestingly taxon specific. Phosphorylation has not been observed in the closely related species *Periplaneta americana* even

though both cockroach share identical neuropeptides sequences. This suggests some specific development within the peptidergic system of *Lamproblatta albipalpus* requiring phosphorylation for function (Sturm & Predel, 2014). As phosphorylation is known to differentially modify neuropeptides in diverse ways due to the dynamic nature of neuropeptides, Lietz et al. did a study to determine a global status of the phosphorylated neuropeptidome of bovine dense core secretory vesicles through characterizing phosphorylation stoichiometry and site motifs of phosphopeptides. Among a wide range of phosphosites detected, SxE was found to be the most prevalent motif (Lietz et al., 2018). They also found differential regulation of neuropeptides, as expected, on many neuropeptides with both known and unknown function, confirming that there is ample room for future studies into the roles of neuropeptide phosphorylation.

4.2 | Glycosylation

Glycosylation is among the most ubiquitous and complex PTMs in biology, with a diverse range of structural possibilities leading to a variety of functional effects. These include an improved metabolic stability to increase peptide hormone circulatory half-life (Flintegaard et al., 2010). There are several types of glycosylation, primarily N- and O-linked. Glycosylation micro- and macro-heterogeneity has been observed on hormones and peptide hormones as well, demonstrating the high degree of diversity of glycans able to modify neuropeptides (Bousfield et al., 2015). Glycosylation is also shown to affect neuropeptide receptors (Quistgaard et al., 2014). Cao et al. analyzed the biosynthesis pathway of calcitonin, a peptide hormone implicated in cancer, and discovered O-glycosylated calcitonin. They observed that both hormone forms responded similarly when the cells were challenged with biosynthetic enzyme inhibitors (Cao et al., 2017). This observation demonstrates the diverse range of glycosylation effects, as it has also been shown to alter response to enzymatic activity (Goettig, 2016).

The characterization of glycosylation is important because unlike other simpler PTMs with a static mass shift, glycan composition, as well as the connectivity and configuration of their glycosidic bond, can vary. To increase detection sensitivity and specificity as well as provide improved sequence coverage, Yu et al. used a targeted analytical method employing oxonium ion-triggered electron-transfer/higher-energy collision dissociation (EThcD) (Yu et al., 2017). Demonstrating its utility for neuropeptides modified by glycosylation, several glycosylated signaling peptides were analyzed and several glycoforms were identified. Additionally, novel glycosylated insulin-B chain, insulin-C peptide, and BigLEN, a potential body weight regulating neuropeptide, from mouse and human tissue were reported (Yu et al., 2017). They could distinguish two isobaric monosaccharides, GalNAc and GlcNAc (Yu et al., 2017) through their distinct diagnostic oxonium ion fragmentation profiles (Halim et al., 2014). This targeted method enables higher quality fragmentation spectra to be obtained, along with reducing instrument time required for glycopeptide analysis. Cao et al. (2020) also employed oxonium ion-triggered EThcD to characterize both N- and O-linked glycosylated neuropeptides in crustaceans (Figure 2). In a pursuit to improve the characterization of glycopeptides, Riley et al. systematically compared several fragmentation methods and dissociation energies. The authors found the optimal dissociation methods to differ between N- and O-linked glycans (Riley et al., 2020). While these results were obtained through enzymatically digested peptides, the differences in optimal fragmentation methods likely hold true for endogenous peptides. Thus, the characterization of each type of glycosylated neuropeptides, whether N- or O-linked, should include considerations for each fragmentation method before use.

Advances in glycoinformatics to aid in glycopeptide characterization include the compilation of several glycomics databases, such as GlyTouCan (Tiemeyer et al., 2017) and glypy (Klein & Zaia, 2019), for glycan identification (Campbell et al., 2014; Ranzinger et al., 2015). There has also been the development of many software programs such as MSFragger (Kong et al., 2017), GlycReSoft (Klein et al., 2018), and O-pair search with MetaMorpheus for O-glycopeptides (Lu et al., 2020). Byonic, a glycoproteomics search program recently added the capability for a glycan "wildcard search" to improve detection of glycans without a priori knowledge of their mass (Roushan et al., 2020). This is beneficial in the neuropeptidomic studies of organisms with incompletely sequenced genomes and lack of knowledge of potential glycans. More detailed information on the glycomics databases and bioinformatics tools available can be found in various platforms (Aoki-Kinoshita, 2017; Dallas et al., 2012; Tsai & Chen, 2017; Woodin et al., 2013). A table of useful information for recent MS-based strategies and software tools for glycopeptides is included within a recent review article (Cao et al., 2021).

In a large-scale effort to map O-linked glycosylation on peptide hormones, Madsen et al. found almost a third of the 279 identified peptide hormones to be O-glycosylated, serving as a basis for global O-glyconeuropeptide discovery (Madsen et al., 2020). While peptide hormone glycosylation seems common, it is still of low abundance and is still difficult to detect, let alone characterize and quantify. Thus, several strategies involving enrichment and derivatization schemes were developed. Interested readers are encouraged to examine the reported by Liu,

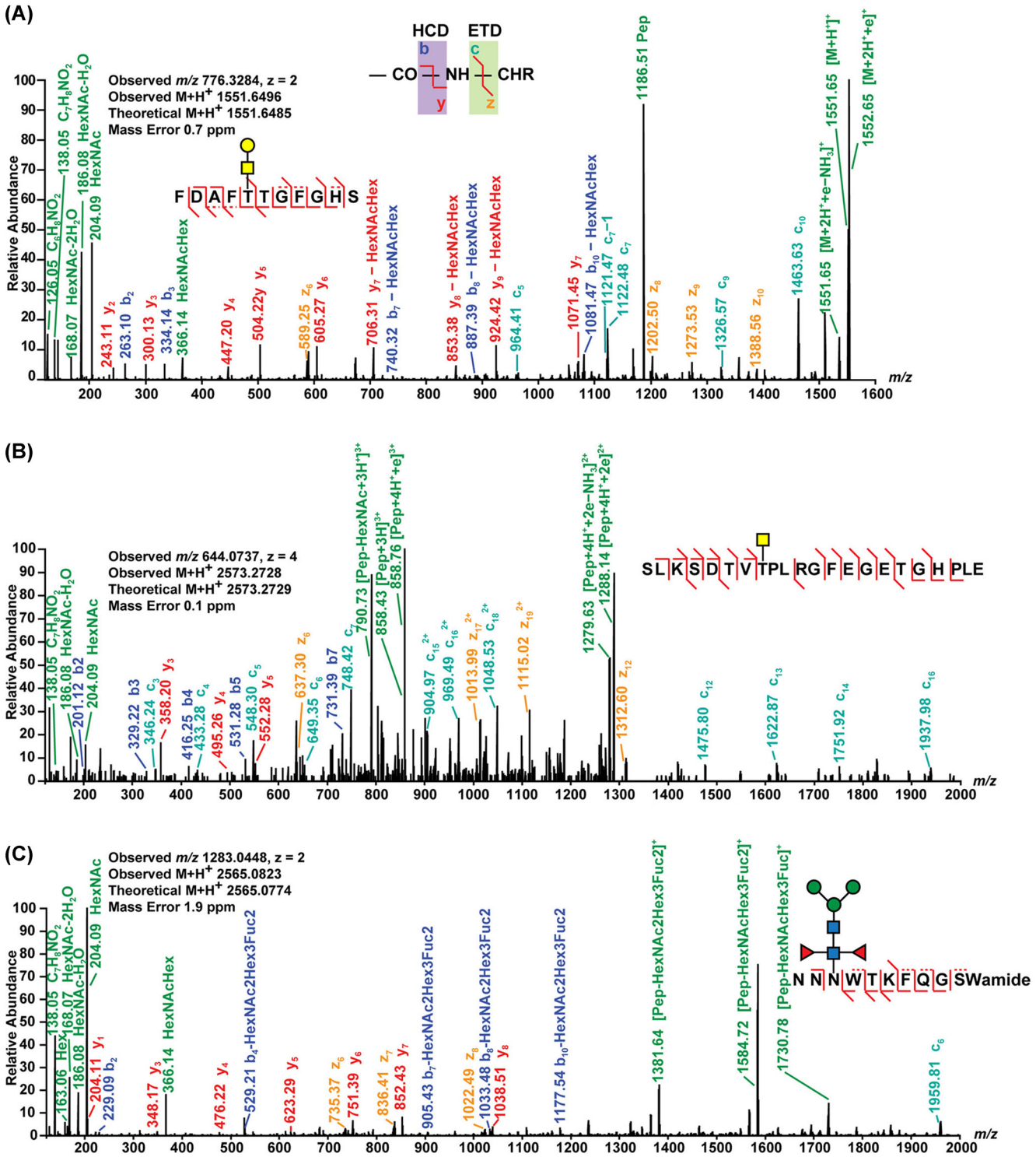

FIGURE 2 Using EThcD, both N- and O-linked glycosylated neuropeptides are identified. EThcD spectra of an (A) O-linked orcomyotropin neuropeptide discovered in rock crab *Cancer irroratus* nervous system, an (B) O-linked truncated crustacean hyperglycemic hormone precursor-related neuropeptide discovered in blue crab *Callinectes sapidus* nervous system, and an (C) N-linked B-type allatostatin (AST-B) neuropeptide discovered in *C. sapidus* nervous system. Reprinted with permission from (Cao et al., 2020). EThcD, electron-transfer/higher-energy collision dissociation [Color figure can be viewed at wileyonlinelibrary.com]

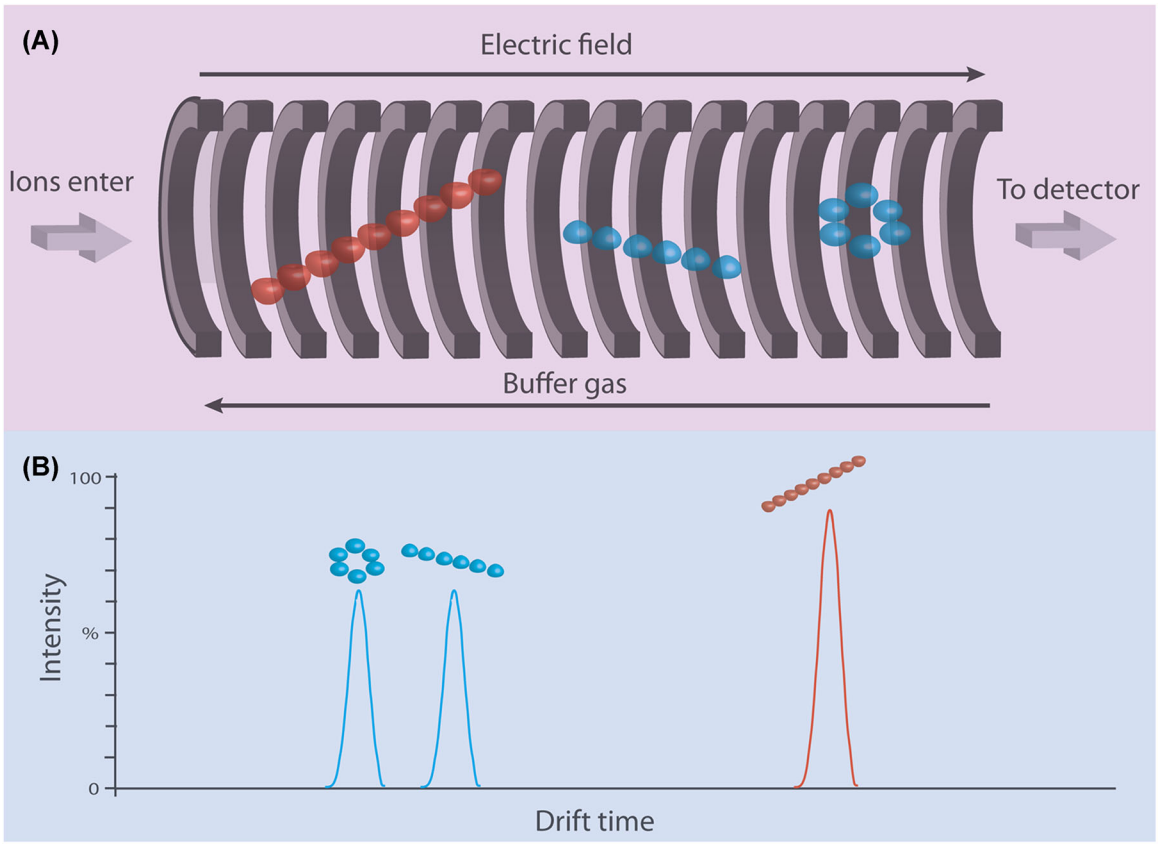

FIGURE 3 Schematic diagrams of analyte ions separation in a drift tube (small ion in blue, large ion in red). (A) Analytes are ionized and enter the drift tube. Small ions travel faster in the drift tube due to less collision with the buffer gas. In this example, the small ion has two conformations: compact ring conformation and unfolded linear conformation. Same ion with compact conformation will travel faster than unfolded linear species. (B) Drift time profile of analyte ions [Color figure can be viewed at wileyonlinelibrary.com]

Cao, et al. (2019) for more information on different strategies for the isolation and characterization of glycosylated neuropeptides. Additionally, a comprehensive review about glycopeptide quantitation was published very recently (Delafield & Li, 2020).

4.3 | Ion mobility MS

Isobaric species are challenging to study with MS due to their identical nominal masses, especially in a discovery-based mode. Nonetheless, differentiation between different isobaric peptides is important as different isobaric neuropeptides may have different properties and bioactivity. IM-MS is an analytical technique that separates gas-phase ions based on their differences in collisional cross section (mobility) through the buffer gas, which originate from differences in size and shape. The separation mechanism of ion mobility is demonstrated in Figure 3. IM-MS for structure elucidation of isobaric peptides when MS measurements is reviewed by Li et al. (2020). Lamont et al. (2017) utilized IM-MS and detected two coeluting isobaric peptides, which they identified as the opioid neuropeptides, leucine enkephalin, and N-acetylated alpha-melanocyte stimulating hormone. Aspartic acid isomerization to isoaspartic acid is suggested to play a role in apoptosis and protein stability, but the crucial differentiation via MS remains to be challenging. Sargaeve et al. (2011) demonstrated the ability to distinguish between these isomers using diagnostic fragment ions produced by ExD fragmentation methods.

Naturally occurring amino acids in peptides and proteins are typically of the L-isoform, with the D-isoform being rare. Even so, D-amino acid containing peptides (DAACPs) can be found in nature and are the focus of many studies as this "unnatural" stereoisomer can have implications for 3D conformation, bioactivity, and degradation. While many studies have been performed on DAACPs, little of this has been applied to the neuropeptidome. DAACPs can differentially regulate neuropeptide activity by altering affinity to its receptors. Using a combination of IM-MS, computer modeling, cell-based assays and results from prior functional studies (Bai et al., 2013), the Sweedler group discovered and evaluated several analogues of the D-amino acid containing

neuropeptides GFFD and GTFD in the sea slug, *Aplysia californica* (Do, Checco, et al., 2018). Careful modeling led to correctly predicting activities with a feeding circuit related receptor, showing the change from L-Ala to D-Ala to alter peptide activity (Do, Checco, et al., 2018). The Sweedler group has led many recent efforts in understanding bioactive DAACPs through studying *A. californica*. One of their workflows analyzes the relative abundances of key chirality-reporting fragment ions to distinguish between neuropeptide L- and D-epimers (Bai et al., 2011). Analyzing single neurons with MALDI tandem MS, identification of D-isoforms of endogenous peptides was demonstrated directly from cells and tissue (Bai et al., 2011). In addition, they evaluated several protocols for untargeted DAACP discovery, again using sea slug neurons (Livnat et al., 2016). Their validated approach involves screening for resistance to aminopeptidase M digestion, inducing a retention time shift between epimers, and comparing the endogenous peptide with synthetic standards leading to the discovery of two peptides with D-isomers. Only one of these peptides appeared to be bioactive (Livnat et al., 2016). For a neuropeptide natively present as both L- and D-residue containing forms, both were found to activate their newly identified receptor, with the D-epimer being the more stable (Checco et al., 2018). The same group also discovered ten new DAACPs in the central nervous system (CNS), two of which were found to be the first animal DAACPs with more than one D-amino acid residue (Mast et al., 2020). This demonstrates the dynamic nature of D-isomerization to alter neuropeptides, highlighting the importance of D-epimer localization.

Benefits of DAACPs include enhanced metabolic stability; they are protected from many endogenous enzymes that only recognize the L-amino acid variant. Demonstrating the utility of modified neuromodulators in their exploration for improved pharmacological peptides, Magafa et al. created a variety of neurotensin analogues. Using various combinations of D-amino acids and an unnatural amino acid, they discovered several modified neurotransmitters with improved enzymatic stability, establishing a basis for the rational design of novel pharmaceutical neuromodulators (Magafa et al., 2019). While there have been several method developments for endogenous DAACP detection and identification, specific D-residue peptide localization tends to be complex or expensive (Soyez et al., 2011). Jia et al. demonstrated the utility of a MS fragmentation-based IM-MS method to localize D-amino acid residues in bioactive peptides in a single MS analysis (Jia et al., 2014, 2016). As peptide epimers are chromatographically separated, each can be fragmented by CID before ion mobility separation to indicate the presence and location of a D-amino acid. The increasingly known variability and complexities of the effects of this PTM are why DAACPs will retain interest in the future.

4.4 | Conformational analysis by IM-MS

While neuropeptides are often considered as 2D entities, it is important to note that these analytes have 3D structures that can widely vary. When combined with molecular dynamics (MD) simulations, IM-MS is able to provide gas-phase peptide ion structural insights at the atomic level. With IM-MS, analyte structure is determined from experimental values measuring temperature-dependent rotationally averaged collision cross sections. It is hypothesized that these reflect the gas-phase ion conformations originating from

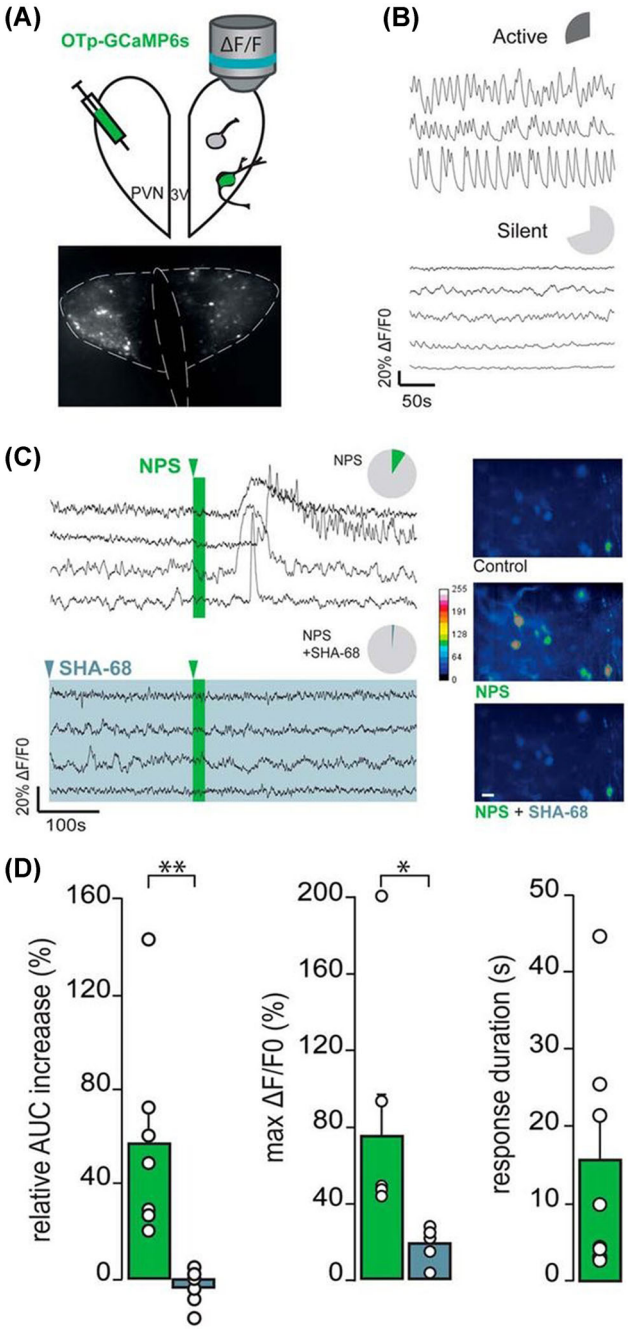

FIGURE 4 (See caption on next page)

solution-phase after desolvation (Jurneczko & Barran, 2011). Compared to other biophysical techniques, such as X ray crystallography or nuclear magnetic resonance (NMR) spectroscopy, IM-MS is sufficiently specific and sensitive to ascertain structural information using impure, trace amount of sample (Scarff et al., 2008). Moreover, whereas X ray crystallography and NMR provide an averaged structure, IM-MS obtains snapshots of short-lived intermediates and conformational transitional states and thus can be used to interrogate dynamic heterogeneity (Gidden & Bowers, 2002; Gidden et al., 2001). In fact, quite a few studies (Bereszczak et al., 2012; Jenner et al., 2011; Shi et al., 2012, 2014; Wyttenbach et al., 2009) report analyte ion gas phase structure and conformational dynamics, which provides important insights into what occurs in solution.

The conformation of neuropeptides is a very relevant aspect with respect to their biological function. Bradykinin (BK), a nine residue neuropeptide, has been a model peptide both for conformational dynamics studies (Papadopoulos et al., 2012; Pierson & Clemmer, 2015; Pierson et al., 2010, 2011, 2013; Voronina & Rizzo, 2015) and the development of systemic IM-MS strategies for structural studies in general. IM-MS assisted by MD revealed 10 independent populations of structures in solution and three gas-phase quasi-equilibrium conformations due to combinations of three cis and trans prolines (Pierson & Clemmer, 2015; Pierson et al., 2010, 2011, 2013). The Clemmer group investigated penultimate prolines in SP. In a detailed and step-by step manner to elucidate the spontaneous peptide cleavage pathway, they showed trans to cis configurational changes to be key in initiating nonenzymatic degradation (Conant et al., 2019). Inspired by the fact that penultimate proline residues are frequently found in neuropeptides, Glover et al. (2015) utilized IM-MS to probe the effect of penultimate proline on neuropeptide conformations. Besides protecting peptides from enzymatic degradation, penultimate Pro also plays a key role in increasing the conformational heterogeneity of neuropeptides, which may be important for receptor affinities and thus function.

IM-MS is able to distinguish between cis and trans isomers of Pro-containing peptides (Shi et al., 2016; Warnke et al., 2015). While different observed conformations are attributed to the isomerization of proline using specific criteria, they do not always indicate cis/trans conformers; IM-MS has limitations for structure elucidation. To this end, the non-proline containing neuropeptide Y wild type and naturally occurring proline containing mutant were investigated by Lietz et al. Though typical cis/trans isomerization hallmarks were present, the presence of these isomers were excluded (Lietz et al., 2016). IM-MS and MD analyses have their limits and require other methods for validation. While Konig et al. (2017) were originally unable to prove DAACP in cicada hypertrehalosemic neuropeptides using IM-MS, they later showed that other techniques, such as NMR, may be required to confirm the proposed 3D structure (Konig et al., 2019).

Some neuropeptides are active through self-oligomerization (Cowley et al., 1992; Smith & Griffin, 1978) and IM-MS has proven instrumental to study this process. For instance, important insights have been obtained on the amyloid fibril formation that is a central implication in neurodegeneration, including Alzheimer's or Parkinson's diseases (Bernstein et al., 2009; Bleiholder et al., 2011). Subsequently, IM-MS studies of various Leu-enkephalin mutants highlighted the importance of characterizing dimer and higher oligomers in determining possible protofibril structures that a peptide system can access (i.e., single β-sheet or doublesheet steric zipper) (Bleiholder et al., 2013; Do et al., 2014).

A number of studies (Heck, 2008; Kaddis & Loo, 2007; Kondrat et al., 2013; Konijnenberg et al., 2013; McAllister et al., 2015) demonstrated that certain peptide and protein ions in the gas phase retain a memory of their solution structures upon ionization (e.g., ESI). How exactly the structure in the gas-phase mimics the solution phase remains to be clarified. The Russell group (Fort et al., 2014; Servage et al., 2015; Silveira, Fort, et al., 2013; Silveira, Servage, et al., 2013) used cryogenic IM-MS (cryo-IM-MS) to

FIGURE 4 In hypothalamic slices, NPS stimulates silent OXT neurons via NPSR but does not stimulate active OXT neurons. (A) Schematic drawing of the PVN OXTpr-GCaMP6s virus infusion and subsequent [Ca^{2+}] imaging of OXT neurons. (B) Basal activity of two distinct subpopulations of OXT neurons (dark gray: active; light gray: silent) illustrated by typical ΔF/F0 traces. Pie charts represent the proportion of active (up) and silent (down) OXT neurons: ns = 11, n OXT neurons (nn) = 237. (C) Pie charts of proportion of responsive OXT neurons to NPS application alone (2 μm, 20 s; ns = 11, nn = 24 of 237; green) or in the presence of NPSR antagonist (SHA-68 100 μm, >15 min; ns = 6, nn = 3 of 135; light blue) and typical ΔF/F0 traces. Pseudo-color video extract of identified OXT neurons through GCaMP6s imaging [Ca^{2+}] in control conditions (gray), in presence of NPS (green) or NPS + SHA-68 (light blue) (stacks of 50 images/10 s of recording). Scale bar = 20 μm. (D) Relative AUC increase and maximal ΔF/F0 of OXT neurons in presence of NPS (ns = 11; green) or NPS + SHA-68 (ns = 6, light blue). Only response duration of OXT neurons in presence of NPS (ns = 11; green) are represented here. White circles represent average value per slice. *$p < .05$ (Student's t test). **$p < .01$ (Student's t test). Reprinted with permission from (Grund et al., 2017). NPS, neuropeptide S; NPSR, NPS receptor; ns, n slices; OXT, oxytocin [Color figure can be viewed at wileyonlinelibrary.com]

reveal that intramolecular interactions can stabilize the kinetically trapped SP dehydrated conformer in a time scale of several milliseconds. The use of cryo-IM-MS for the study of analyte structure and is reviewed by Servage et al. (2016).

Besides peptide inherent secondary structure, external environmental factors also affect peptide conformational preference in the gas phase. IM-MS studies have investigated temperature (Berezovskaya et al., 2013; Zilch et al., 2007), activation voltage (Pierson et al., 2010), solvent composition (Pierson et al., 2011), and metal binding (Chen et al., 2011).

5 | MS IMAGING

Until recently, the most popular way to obtain spatial information of molecules was immunohistochemistry and RIA. However, due to the selectivity of the antibodies used, immunoassays are unable to acquire information from more than one analyte. This is a disadvantage when working with limited amount of sample. Within the last years, MSI has emerged as an alternative method to circumvent this disadvantage. Through MSI, molecular ion images are generated of a surface (e.g., tissue or tissue slice). By rastering a laser along a predefined (x, y) grid, a mass spectrum is acquired at each grid square (i.e., pixel). Ion specific images are then generated by bioinformatic tools. As such, MSI has the capability to generate hundreds of images from a single experiment. The investigation of neuropeptides and their spatial distribution patterns has been accomplished by MSI throughout several organisms for several applications (Altelaar et al., 2005; Berisha et al., 2014; Buchberger, Vu, et al., 2020; Chen & Li, 2010; Chen, Cape, et al., 2010; Chen, Hui, et al., 2010; De Haes et al., 2015; Hanrieder et al., 2012; Herbert et al., 2010; Jia et al., 2012; Mark et al., 2012; Monroe et al., 2008; OuYang, Liang, et al., 2015; Pratavieira et al., 2014; Romanova et al., 2009; Shariatgorji et al., 2014; Ye et al., 2013, 2015). While the general workflow has become very well defined (Figure 1), several modifications have been explored and implemented to improve the quality and depth of MSI data. Several reviews discuss these in the context of neurobiology (Buchberger et al., 2018; Gemperline, Chen, et al., 2014; Hanrieder et al., 2015; OuYang, Liang, et al., 2015).

5.1 | Ionization, identification, and instrumentation

While several ionization techniques exist, only a small subset has been used for MSI in biological relevant experiments. MALDI MSI was first developed by the Caprioli group, who successfully imaged proteins and peptides in thin tissue slices of the rat pituitary and pancreas (Caprioli et al., 1997). MALDI still remains the most utilized ionization methods for MSI of biomolecules, including metabolites, lipids, and proteins (Eriksson et al., 2013). Alternative ionization methods employed include desorption electrospray ionization (DESI) (Wiseman et al., 2008), nanostructure initiator MS (NIMS) (Sturm, Greer, Chen, et al., 2013; Yanes et al., 2009), and secondary ion MS (SIMS) (Altelaar et al., 2005; Jiang et al., 2014; Lanni et al., 2014; Monroe et al., 2008; Ogrinc Potocnik et al., 2017), but MALDI MSI has been a predominant technique utilized in neuropeptide-related studies (Buchberger, Vu, et al., 2020; Chen, Cape, et al., 2010; Chen, Hui, et al., 2010; Herbert et al., 2010; Jia et al., 2012; Lanni et al., 2014; Pratavieira et al., 2014; Verhaert et al., 2007, 2010; Ye et al., 2013, 2015). Crustacean neuronal tissues have been studied under various MSI conditions to understand the functional roles of neuropeptides through mapping their localization (Buchberger, Vu, et al., 2020; Chen & Li, 2010; Chen, Cape, et al., 2010; Chen, Hui, et al., 2010; Jia et al., 2012; OuYang, Liang, et al., 2015; Ye et al., 2013). The alternative ionization methods suggest various advantages over MALDI, such as being matrix-free, preventing analyte diffusion (see sample preparation below). They also have distinct disadvantages. For example, while NIMS is excellent for metabolites, lipids, and so forth, it has been shown to not ionize neuropeptides efficiently when compared to MALDI (Sturm, Greer, Chen, et al., 2013). For additional information on MSI, we refer the reader to an in-depth review on developments in high resolution MALDI MS relevant for neurobiology (DeLaney, Phetsanthad, et al., 2020). For a summary of the advantages and disadvantages of different MSI ionization sources (i.e., MALDI, SIMS, NIMS, DESI, and LAESI, and LESA), we refer the reader to Table 1 in a recent review (Rocha et al., 2017).

With only minor amino acid differences between neuropeptides in the same family, methods for confident identification need to be in place. Classically, MS/MS of singly charged ions, which are primarily produced during MALDI ionization, is inefficient, leading to poor fragmentation and thus inconclusive identifications. Also, due to the varying distribution of analytes across a tissue, the motion of constantly rastering across the tissue makes it difficult to be able to fragment a mass that was originally detected in a previous raster step. Thus, accurate mass matching followed by subsequent tissue extract ESI MS/MS analysis (Ly et al., 2019) have been common ways to identify analytes. With the development of modern instrumentation, such as the HRAM Orbitrap, identification of analytes with similar masses has become more reliable (Verhaert et al., 2010). Yet, tandem MS is still difficult on singly charged ions. Significant effort has been put in developing hybrid methods of MS and MS/MS occurring in a single square form to facilitate the

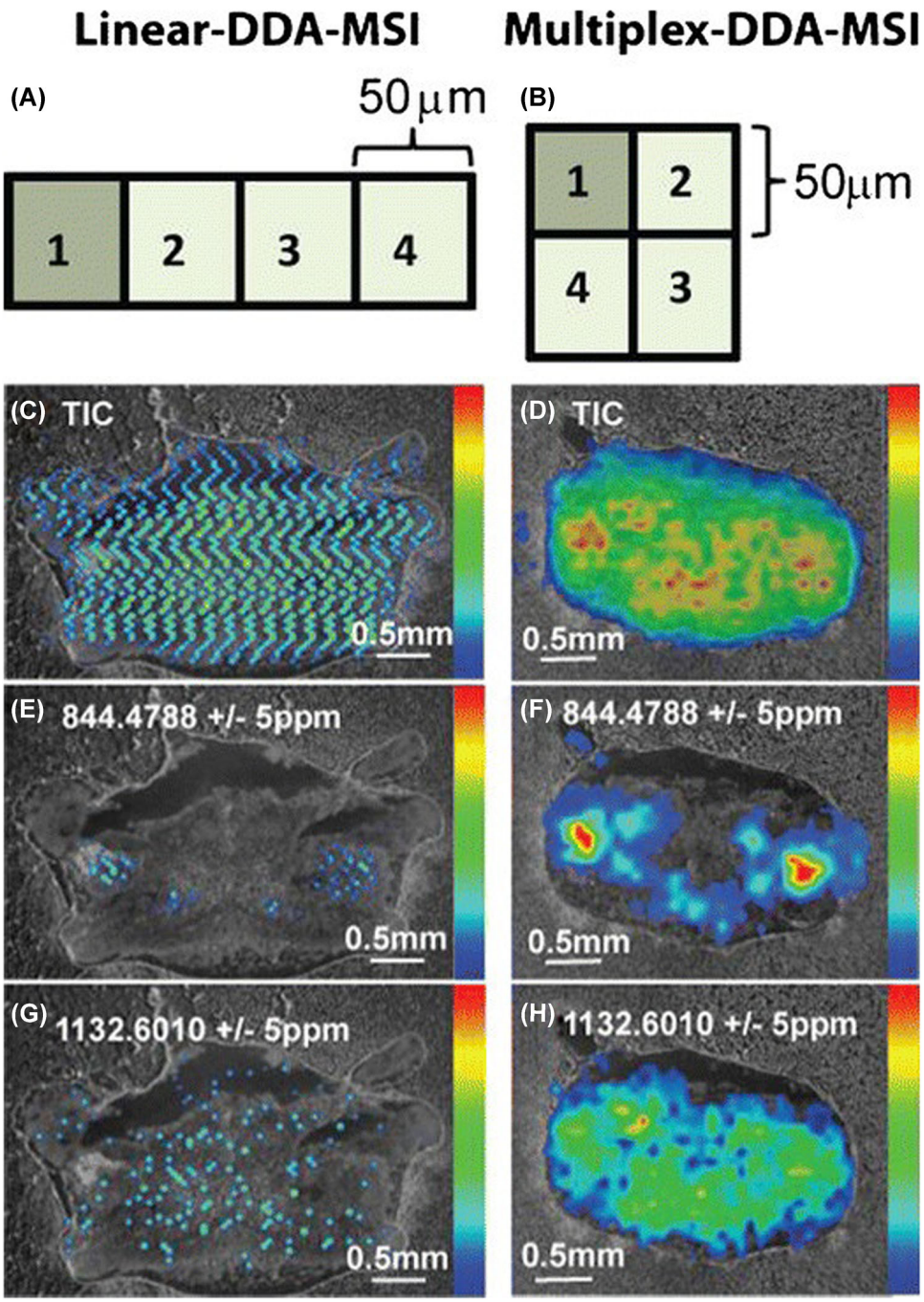

FIGURE 5 Comparison between linear and spiral DDA MS imaging. (A, B) Illustrate the step motion and size, respectively, while (C–H) demonstrate image quality obtained from both with high mass accuracy. Reprinted with permission from (OuYang, Chen, et al., 2015) [Color figure can be viewed at wileyonlinelibrary.com]

isolation of ions identified in first pass MSI spectra (OuYang, Chen, et al., 2015). These hybrid methods also increase the image quality, as shown in Figure 5 (OuYang, Chen, et al., 2015).

Singly charged ions produce the simplest spectra, which means that MALDI-TOF analyses allow for the widest mass range that can be analyzed. Tandem TOF (TOF/TOF) mass analyzers have a theoretically infinite mass range, with analytes larger than 50 kDa being imaged with high signal (Leinweber et al., 2009; van Remoortere et al., 2010). Unfortunately, most TOF/TOF mass spectrometers lack the mass accuracy and resolution that allow for differentiation between closely massed neuropeptides of interest (Verhaert et al., 2010). On the other hand, Orbitraps and FTICR instruments have a limited mass range, leading to several larger neuropeptides of interest not being imageable as singly

charged ions. Several methods to handle these larger mass analytes have been developed, such as in situ digestion and generating multiply charged ions by MALDI (Cillero-Pastor & Heeren, 2014; Dreisewerd, 2014; Groseclose et al., 2007). Dependent on the sample preparation conditions, multiply charged ions are usually produced by using laserspray ionization (LSI) at atmospheric, intermediate, or high vacuum (Chen et al., 2014; Hale et al., 2021; Inutan et al., 2011; Trimpin et al., 2011). Trimpin and coworkers have analyzed a 12+ charge state cytochrome c by atmospheric pressure (AP)-MALDI on a Thermo Fisher Q Exactive mass spectrometer (Trimpin et al., 2010).

Data processing represents challenges as well, particularly in high-throughput data collection. Pipelines for automated identification of unique peptides were developed (e.g., MSI-Query) (Bruand et al., 2011). Software packages developed to view MS images are vendor-specific (e.g., Thermo Fisher ImageQuest and TissueView, Waters HDImaging, Bruker SCiLS lab, etc.) or more generic/open source (e.g., MSiReader, Cardinal, msIQuant) (Bemis et al., 2015; Källback et al., 2016; Robichaud et al., 2013). Many packages are utilized to identify masses unique to the tissue (which may be known or unknown), such as the program written to perform accurate mass matching with an intensity threshold (Buchberger, Sauer, et al., 2020). A particular program has been published for normalization and quantitation-based studies (Kallback et al., 2012). This software enables the quantitation of SP in mouse brain structures, which correlated well with previous studies (Kallback et al., 2012).

One area that has gained a lot of attention is spatial resolution, defined as how small the pixels can get in MSI. Higher spatial resolution allows MSI of smaller biological tissues down to even single cells (Boggio et al., 2011; Xie & Fidler, 1998; Zimmerman et al., 2011). Two factors play major roles in the maximum resolution achievable in MALDI MSI: matrix crystal size and focusing of the laser. The crystal size is dependent on the matrix and application method used (see Section 5.2). It is especially instrumental advancements which have provided most improvements in this context. Commercial instruments allow for small pixels with oversampling, but this can lead to poor signal in tissues with already low analyte concentrations. However, with home-built instruments, some groups have achieved 5-μm resolution without oversampling (Guenther et al., 2011; Kompauer et al., 2017; Mark et al., 2012; Rompp & Spengler, 2013), which allows imaging discrete cellular structures (Boggio et al., 2011; Dueñas et al., 2017; Xie & Fidler, 1998; Zimmerman et al., 2011). It should be noted that this typically lowers the throughput of the instrument due to the longer acquisition time, but some companies have developed scanning laser beams to lessen this time (Ogrinc Potocnik et al., 2015). Alternatively, Zimmerman et al. (2009) have achieved this by placing individually stretched cells on an ITO-coated slide and analyzing them with a Bruker Ultraflex II MALDI-TOF/TOF (laser beam diameter is between 5 and 30 μm), which allowed them to acquire MS and MS/MS images of neuropeptides throughout the cell body (>0.5 mm diameter). The Bruker rapifleX MALDI TOF/TOF mass spectrometer has become a popular choice for peptidomics (Vu et al., 2020) due to its high spatial resolution (<20 μm) and the fast laser repetition rate, and its ability to scan the full area of a pixel while the sample stage moves continuously, allowing rapid acquisition of MSI data. Continued instrumental development allows researchers to do more single cell studies, and quality reviews discuss the next challenges that need to be met to advance the field (Berman et al., 2010; Boggio et al., 2011; Xie & Fidler, 1998; Zimmerman et al., 2011).

5.2 | Sample considerations

Proper sample handling is crucial for maintaining the spatial distribution and abundance of biomolecules in a sample, allowing for maximum spatial resolution, sensitivity, and reproducibility of an MSI experiment (Goodwin, 2012). Studies of postmortem changes in peptide and protein abundance in brain tissue demonstrate the necessity for sample collection protocols that limit sample degradation (Goodwin et al., 2008; Skold et al., 2007). To preserve sample integrity, samples are typically flash frozen either using liquid nitrogen or dry ice. Alternatively heat stabilization, often using, for example, a Stabilizor T1, (Goodwin et al., 2010; Sturm, Greer, Woodards, et al., 2013). Fixation methods, such as formaldehyde-fixed paraffin embedding (FFPE), are commonly used to preserve samples in biomedical research, but this procedure is reported not be compatible with MSI FFPE is known to result in crosslinking between peptides and proteins, which is predicted to have a negative impact on MSI. Optimized protocols for MSI on FFPE material involve deparaffination, antigen retrieval, and trypsin digestion before analysis (Casadonte & Caprioli, 2011; De Sio et al., 2015). MSI of proteins and neuropeptides has been performed in rat brain FFPE samples, after deparaffination and tryptic digestion, and in *Penaeus monodon* shrimp (Chansela et al., 2012; Lemaire et al., 2007; Stauber et al., 2008). A protocol for performing MSI of neuropeptides from FFPE tissue without antigen retrieval and enzymatic digestion has also recently been developed (Paine et al., 2018). Alternatively, alcohol fixation methods have been used to fix samples without any of the complications of FFPE (Chaurand et al., 2008). The PAXgene system is an alcohol fixation system commercially available that can be

used to fix samples before MSI, although use of this system is not as widespread as FFPE (Ergin et al., 2010). Interestingly, DHB matrix can also be used as a one-step tissue preservation and peptide extraction solvent (Alim et al., 2019; Romanova et al., 2008). Multiple reviews discuss sample preparation in more depth (Buchberger et al., 2018; DeLaney, Phetsanthad, et al. 2020; Gemperline, Chen, et al., 2014; Goodwin, 2012; OuYang, Liang, et al., 2015).

Traditionally, before MSI analysis, the typical (frozen) tissue samples are sectioned into 10–20 μm thick slices, which is roughly the thickness of a single mammalian cell (Crossman et al., 2006). Before sectioning, samples are usually embedded in a support substance to aid in sectioning. These can be water and gelatin that do not interfere with the MSI analyses of neuropeptides. Other polymer-based support substances such as optimal cutting temperature (OCT) medium is known to contaminate the sample and suppress ion formation (Buchberger et al., 2018; OuYang, Liang, et al., 2015). A novel embedding material, poly[*N*-(2-hydroxypropyl)methacrylamide], was tested on mouse lung and bumblebee samples and it was found to be suitable for MALDI MSI with low background signal and ion suppression effects (Strohalm et al., 2011). Also egg yolk seems to be an appropriate embedding material for MSI of neuropeptides in rat pituitary, preventing OCT contamination (Sosnowski et al., 2015). The sectioned samples are transferred to a simple glass or a metal coated glass slide, depending on instrumentation, using either a thaw-mount method or with double-sided tape (Goodwin et al., 2012). Certain intact tissues are thin enough to bypass the sectioning step and can be directly analyzed by MALDI MS. Examples of this are crustacean pericardial organs and cardiac ganglion (Buchberger, Vu, et al., 2020; DeLaney & Li, 2020; Zhang, DeLaney, et al., 2018), and the insect corpus cardiacum (Verhaert et al., 2007; Verhaert et al., 2010). It is important to note that fragile samples, such as pericardial organs, benefit from immediate MS analysis after dissection to prevent tissue degradation.

There are multiple treatment steps that can be taken before matrix application. For protein analysis, washing tissue sections with organic solvents such as xylene, chloroform, or alcohols has been shown to increase detection through removal of contaminating compounds, such as lipids and salts (Lemaire et al., 2006; Seeley et al., 2008). However, washing steps risk to cause delocalization or loss of low molecular weight or hydrophilic neuropeptides if not optimized (OuYang, Liang, et al., 2015; Yu et al., 2014). Nonetheless, proper optimization of tissue washes has been shown to be effective at enhancing neuropeptide signal (Vu et al., 2020). Reduction of salt adducts has also been shown using a condensation/matrix recrystallization procedure after matrix deposition (Monroe et al., 2007). An aqueous MSI tissue wash containing sodium phosphate salts resulted in detection of a complementary cohort of neuropeptides compared to control, unwashed tissue (Vu et al., 2021). MSI of neuropeptides in *Aplysia* nervous tissue utilized a tissue stretching method to fragment the tissue into small pieces, which minimizes analyte diffusion and salt adduct formation (Zimmerman et al., 2009). Another option is the application of trypsin to the sample to digest larger proteins or certain large neuropeptides to the mass range ideal for higher resolution instrumentation (Cillero-Pastor & Heeren, 2014; Groseclose et al., 2007). Optimization of digestion times, proteases, and matrix application has been performed in brain tissue to improve the repeatability of trypsin digestion (Diehl et al., 2015; Heijs et al., 2015). Other protocols look to improve trypsin digestion reliability and reproducibility utilizing graphene oxide-immobilized enzyme reaction (Jiao et al., 2013) or microwave irradiation and hydrogel discs (Taverna et al., 2015).

The choice of matrix and matrix application method is critical for ionization of the target analytes while limiting diffusion in MALDI MS analysis (Kaletaş et al., 2009). Common matrices include CHCA and DHB for the analysis of peptides and sinapinic acid and DHB for the analysis of larger proteins. Matrix concentration, solvent composition, and deposition temperature are factors that impact matrix crystal size and therefore spatial resolution. Hulme et al. observed that a higher concentration of matrix and higher deposition temperature (i.e., drier deposition) resulted in high spatial resolution (15–25 μm), but using a lower concentration and temperature resulted in more neuropeptide identifications but at lower spatial resolution (50 μm) (Hulme et al., 2020). Many new matrices are being investigated for application in MSI experiments of all analyte types, from metabolites up to proteins (Buchberger et al., 2018; Dreisewerd, 2014). The derivatization of chemical compounds with amines, including catecholamine neurotransmitters and neuropeptides, by reacting primary amines with pyrylium salts have been proposed as a novel matrix for MSI of primary amine compounds, which are usually challenging to detect (Shariatgorji et al., 2015). Additionally, graphene has been used as matrix on brain tissue to detect lipids and small peptides (Friesen et al., 2015). The choice of matrix is important for studies using LSI to produce multiply-charged ions. For example, when using 2-nitrophloglucinol as a matrix, multiply-charged ions are produced at both vacuum and atmospheric conditions, but some matrices, like CHCA, only produce singly charged ions at all conditions (Chen et al., 2018; Inutan et al., 2011, 2012). To apply matrix for MSI experiments, several different options are available, including robotic spotters, airbrush, and automated spraying devices. Robotic spotters, such as

acoustic droplet ejectors (Aerni et al., 2006) and inject printers (Baluya et al., 2007; Franck et al., 2009) apply small amounts of matrix in spots of approximately 150-200 um diameter in an ordered array across the tissue. Automated spraying systems, such as the TM-Sprayer or M5 Sprayer (HTX Imaging), pneumatic spraying devices, and the ImagePrep (Bruker Daltonics) vibrational sprayer, allow more control compared to manual airbrush. Use of these automated methods has been shown to produce higher quality data compared to other methods (Gemperline, Rawson, et al., 2014). Our group has reviewed recent advances (from years 2017 to 2020) in MSI washes, matrices, and other sample preparation considerations for neuropeptide analysis (Buchberger et al., 2018; Vu et al., 2020).

5.3 | Special applications of MSI

While the application of MSI to directly answer biological questions has boomed, MSI has also been utilized for new applications. For example, the 3D analysis of structures in a heterogeneous tissue (Chen, Hui, et al., 2009; Dueñas et al., 2017; Jones et al., 2012; Trede et al., 2012). For example, consecutive sections have been analyzed in 3D to demonstrate the spatial variability of several crustacean neuropeptides (Chen, Hui, et al., 2009). MSI has been used in 3D cell culture studies (Fernandes, 2004; Li & Hummon, 2011), for example, to understand the depth of drug penetration or the production of different metabolite due to changing environments (e.g., normoxia vs. hypoxia). Another analytical technique that has been combined with MSI is microfluidics, to study neuropeptide secretion from a cell (Jo et al., 2007; Zhong et al., 2012). Finally, unlike spot analysis, analyte traces analyzed by MSI has become a way to add temporal information to this spatial technique (DeLaney & Li, 2019a; Wang et al., 2011; Zhang, Jia, et al., 2012; Zhang, Ye, et al., 2012; Zhang et al., 2013). Initially, this was used to analyze CE or LC traces of tissue extracts, but MSI of traces has evolved to direct analysis of microdialysates (OuYang, Liang, et al., 2015). This combination has also proven to be remarkably accurate for quantitative analysis, allowing both relative and absolute concentrations of neuropeptides to be obtained (Zhang, Ye, et al., 2012; Zhang et al., 2013).

6 | ADVANCES IN QUANTITATION

To analyze a system for biological relevance, quantitative tools are necessary. Most techniques are compatible with both ESI and MALDI sources, but special considerations should be made for either ionization method. ESI is well known for consistency, but often ESI is done in conjunction with LC separation. Any run-to-run variation will need to be corrected for by bioinformatic tools. MALDI, on the other hand, is notorious for inconsistent ionization (such as due to variability in matrix crystallization). This makes MALDI, without further methodological developments, inherently semiquantitative. With that in mind, we will focus below on the development of relative (i.e., comparative) versus absolute (i.e., actual) quantitation with both label versus label-free methodology for the study of neuropeptides (Figure 6). Many recent quality reviews exist, and only the major contributions will be highlighted below (Buchberger et al., 2015; Fricker, 2018; Fricker et al., 2006; Li & Sweedler, 2008; Romanova et al., 2013; Yin et al., 2011).

6.1 | Labeling-based methods

The incorporation of standard isotopes has revolutionized MS for quantitative analysis. The more variations of isotopes we have, the more samples we will be able to compare, which will thus increase analysis throughput. There are several different ways to incorporate isotopes into an analyte of interest, including in vivo metabolic labeling. This is done usually by culturing cells with a heavy isotope, for example, in the form of an amino acid, allowing it to be incorporated during the synthesis of other cells (Ong et al., 2002; Potts et al., 2016). Heavy amino acids have also been added into the diets of animals (Kruger et al., 2008; Zanivan et al., 2011) and plants (Lewandowska et al., 2013). A simplified protocol has been created to quantify fruit fly neuropeptides by growing differential isotopically labeled yeast that can be fed to different groups of flies (Kunz et al., 2018). Isotopic neuropeptides can also be administered intranasally or intravenously into animals (Lee et al., 2018). While decreasing variability in the population analyzed, full incorporation of the isotopes can take a long time depending on cell turn over, especially in animals, leading to a high cost. The number of samples that can be compared is limited by the number of isotopes of an element. This methodology has been mainly used in protein quantification (Geiger et al., 2010; Kruger et al., 2008; Lewandowska et al., 2013; Ong et al., 2002; Potts et al., 2016; Zanivan et al., 2011), although it could be adapted for neurochemical cell culture studies.

Another variation of this MS labeling method is in vitro chemical tags. Generally, the analytes of interest are derivatized with a chemical tag that includes stable isotopes, which produce well-defined mass differences between the samples at either the MS1 or MS2 level. For neuropeptidomic studies, MS1-based quantitation is becoming more and more common. In particular, duplex dimethyl labeling

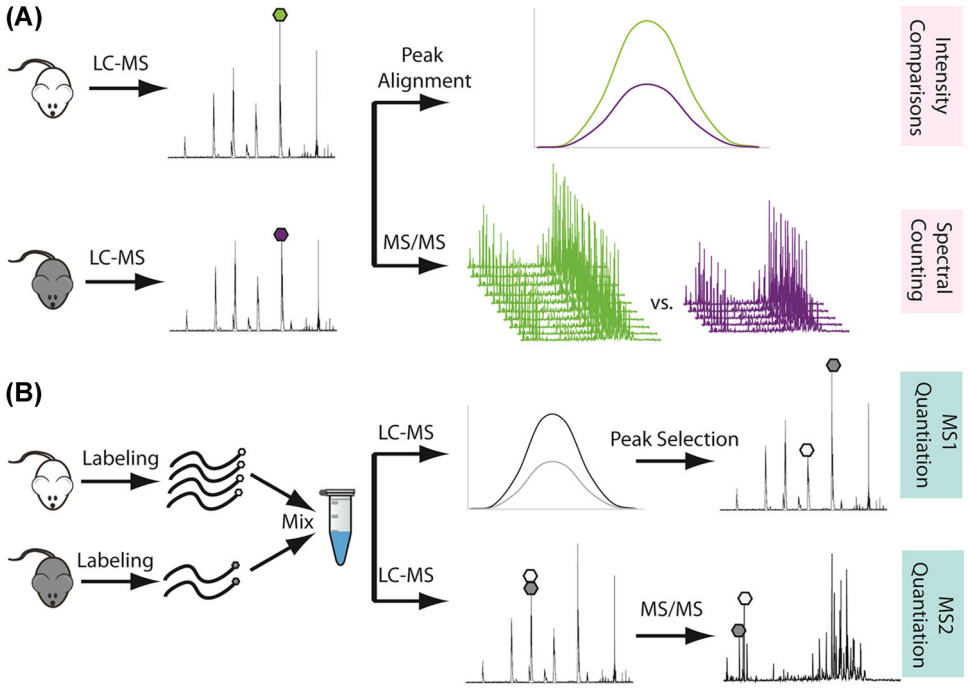

FIGURE 6 Two major types of quantitation in MS. (A) Label-free quantitation strategies, include intensity comparisons and spectral counting. (B) Label-based quantitation. These techniques can be done at both MS1 and MS2 levels [Color figure can be viewed at wileyonlinelibrary.com]

has been used thanks to its simplistic and quick labeling on all primary amines (e.g., N-terminus and ε-lysine). For example, the Li lab has utilized this method for studying the dynamic changes in neuropeptides due to environmental stress (Buchberger, DeLaney, et al., 2020; Buchberger, Sauer, et al., 2020; Chen, Hui, et al., 2010; Chen et al., 2014; Liu, Buchberger, et al., 2019; Zhang et al., 2015). Wilson et al. achieved in vivo quantitation of Leu-enkephalin and Met-enkephalin after on-column dimethyl labeling of microdialysis perfusate from rat brain (Wilson et al., 2018). Dimethyl labeling was expanded from 2 to 5 plex (Boersema et al., 2008; Buchberger, Sauer, et al., 2020; Hsu et al., 2006; Tashima & Fricker, 2018). Isotopic dimethyl *N*,*N*-leucine (iDiLeu) and mass defect-based *N*,*N*-dimethyl leucine (mdDiLeu) also contains five spaced channels, which allows for relative or absolute quantitation (Greer et al., 2015; Zhong et al., 2019). By labeling four channels with neuropeptide standards to construct a calibration curve, the fifth channel can be used to calculate the absolute concentration of an unknown sample. Care should be taken that, when all five channels are in use and ESI is chosen as the ionization technique, isotopic impurities and charge state differences may lead to overlapping peaks and thus inaccurate quantitation (Greer et al., 2015). In terms of MS1-based quantitation, other options exist to label the N-terminus, such as succinic anhydride (Bark et al., 2009; Fricker, 2006; Hou et al., 2012; Rubakhin & Sweedler, 2008), acetic anhydride (Che & Fricker, 2002), and 4-trimethylammoniumbutyryl (Che et al., 2005). Amino acid specific labels, including isotopic-coded affinity tag, metal-coded affinity tag, and tyrosine-specific cysteine labeling (Ahrends et al., 2007; Choi et al., 2010), are also accessible to study neuropeptides. All these labeling schemes come with similar considerations to dimethyl labeling, iDiLeu, and mdDiLeu. While a balance between multiplexing and spectral complexity is a major concern, the development of tags with smaller spaces alleviates some of this burden. Unfortunately, these small spacing usually requires high-resolution instrumentation, which may not be readily available for many labs. For example, neutron-encoded (Hebert, Merrill, Stefely, et al., 2013), mdDiLeu (Hao et al., 2017), and dimethyl pyrimidinyl ornithine (Frost et al., 2017) tags all take advantage of isotopic mass defect, but high multiplexing requires resolution only achievable by top tier instrumentation.

The use of chemical tags that quantitate at the MS/MS level can also decrease the MS1 spectral complexity occurring above. Instead of producing mass shifts at the MS1 level, all the differentially labeled peptides occur at the same mass in the initial MS1 scan. If the associated peak is selected for fragmentation, characteristic reporter ions are created, usually in the low mass range where no interference occurs. Unlike at MS1 level, where theoretically every analyte can be quantified, one is limited by the peptides which get selected for fragmentation. Thus the duty cycle of an instrument can play a major role in the depth of quantitation achieved. Many commercial tags,

such as isobaric tags for relative and absolute quantitation (iTRAQ) and tandem mass tags (TMT), have been used (McAlister et al., 2012; Rubakhin & Sweedler, 2008). iTRAQ has even been used for single cell analysis, allowing the relative quantities of peptides to be obtained by MALDI MS (Rubakhin & Sweedler, 2008). Unfortunately, the cost of these commercially available labels limits their use. *N*,*N*-Dimethyl leucine (DiLeu) is one, low cost example for MS2 level quantitation, and it has been expanded from 4- to 21-plex quantitation (Frost et al., 2020; Frost, Greer, & Li, 2015; Frost, Greer, Xiang, et al., 2015; Liu et al., 2020; Xiang et al., 2010). Recently, DiLeu and iDiLeu have been combined to form a strategy called hybrid offset-triggered multiplex absolute quantification (HOTMAQ) which enables the formation of an internal standard curve at the MS1 level, peptide sequencing and identification at the MS2 level, and peptide quantification at the MS3 level (Zhong et al., 2019). With the multitude of channels all these tags contribute, both absolute and relative quantitation is possible. Most of these tags have not been applied to neuropeptide quantitation, but they would provide a practical way to compare several samples in one instrumental run.

6.2 | Label-free methodology (LFQ)

LFQ techniques are more frequently used in the study of neuropeptides. Unlike labeling strategies, label-free methods allow one to compare an infinite number of samples. The simplest label-free method is based on signal intensity, meaning that the signal intensity in the spectra, or more accurately the area under the curve in the LC chromatogram, correlates with the analyte concentration. Relative quantities are easily found by just comparing samples at either the MS1 or MS2 (i.e., MS/MS) level, although peak alignment and other postprocessing aspects need to be considered due to run-to-run variability (Jiang et al., 2012; Johansson et al., 2006; Ranc et al., 2012). For example, Ranc et al. (2012) utilized the extracted ion chromatograms to obtain relative quantities of different endogenous peptides in the tree shrew visual system. It should be noted that at the MS/MS level, multiple reaction monitoring, or monitoring of only specific, characteristic fragments, and PRM, or monitoring of all fragments, can lead to lowering the limit of detection by 100-fold (Bobba et al., 2012; DeAtley et al., 2018; Pailleux & Beaudry, 2014; Saidi et al., 2019; Song & Liu, 2008; Wang et al., 2014; Yang et al., 2017).

To acquire the absolute concentration of neuropeptides, (a) a calibration curve is required (Chung-Davidson et al., 2020; Schmerberg et al., 2015; Song & Liu, 2008; Wang et al., 2014); (b) a synthetic, isotopic internal standard, also known as an AQUA peptide is added (Bozzacco et al., 2011; Ozalp et al., 2018; Salem et al., 2018); or (c) a peptide standard similar to the peptide of interest (Dong et al., 2018) to be used as a proxy. Several software packages assist in processing these large datasets, including commercial software packages (SIEVE, PEAKS, Proteome Discoverer, etc.) or open access platform (e.g., Skyline). Several groups have developed their own pipelines, such as using accurate mass time (Wu, Monroe, et al., 2015), informed quantitation (Wu, Monroe, et al., 2015), and DeCyder MS (Johansson et al., 2006; Kaplan et al., 2007).

Many of the above informatics tools also assist in another LFQ method: spectral counting. Unlike peak area/signal intensity measurements, spectral counting is dependent upon the number of times an analyte is selected for fragmentation. Similar to MS/MS label-based quantitation, only high concentration molecules are analyzed due to the limited duty cycle of an instrument. Also, care should be taken on instrumental parameters, such as scan and exclusion parameters to increase sampling depth (Zhou et al., 2012). Relative comparisons are easily done by comparing the number of MS/MS spectra collected between analytes, although validation of the smaller differences will be particularly important. Furthermore, only estimates can be made based upon total protein concentration (Neilson et al., 2011). When comparing SIEVE peak area analysis and spectral counting on peptides in the rat suprachiasmatic nucleus, it was revealed that spectral counting provided a richer characterization of differences in differential peptide abundance when rats were analyzed at different circadian rhythm points (Southey et al., 2014). Furthermore, when compared to SILAC and spectral counting for quantifying proteins, spectral counting was found to be able to quantitate ~50% more proteins before a limit being set (Collier et al., 2010). This shows the power of spectral counting and LFQ for neuropeptidomic analysis, and it is likely that these approaches will be increasingly used.

6.3 | DIA quantitation

DIA strategies can be applied to improve quantitative studies, however the use of label-based quantitation adds additional complexity to the MS or MS/MS spectra collected. This makes spectral deconvolution and accurate quantitation difficult to achieve. Therefore, LFQ is commonly used in conjunction with DIA. In fact, a recent comparison study between LFQ DIA and isobaric tag labeling with DDA demonstrated similar performance between workflows (Muntel et al., 2019). Performing 10 LFQ DIA analyses demonstrated better quantitative accuracy while a single multiplex TMT labeled DDA analysis resulted in an increase in identified

proteins and quantitative precision (Muntel et al., 2019). This demonstrates the capability of LFQ DIA for quantitation; although, to fully leverage the benefits of DIA, specific DIA labeling strategies need to be developed. mdDiLeu (Zhong et al., 2020), NeuCode SILAC (Hebert, Merrill, Bailey, et al., 2013; Hebert, Merrill, Stefely, et al., 2013), and MdFDIA (Di et al., 2017), are labeling strategies that have been successful for DIA quantitation. The latter two rely on metabolic labeling and are thus restricted to cell culture applications, and none of these techniques have been applied to neuropeptidomics. Developments in the DIA quantitative analysis to more broadly study the brain proteome are summarized by Li, Gonzalez-Lozano, et al. (2020).

Parker et al. (2015) applied a LFQ DIA strategy in a targeted phosphoproteomics analysis to understand signaling of the peptide hormone insulin. The increased throughput and reproducibility, enabled by DIA analysis, led to the quantitation of 86 protein targets affected by insulin (Parker et al., 2015). In a new Skyline software application (MacLean et al., 2010), Schmerberg et al. (2015) performed quantitation of LFQ DIA MS/MS data in a pseudo-multiple reaction monitoring analysis of crustacean neuropeptides. They were able to identify and quantify several neuropeptides from microdialysate and their changes across the feeding study illustrating the sensitivity of the method (Schmerberg et al., 2015). Saidi et al. (2019) evaluated the utility of label-free and isotopic dilution DIA methods for targeted quantitation of neuropeptides and found an increase in variance when compared to PRM methods. This could be attributed to the increase in cycle time for the DIA method, which decreases the points per chromatographic peak acquired. DeLaney and Li optimized the DIA duty cycle for crustacean neuropeptides by considering various isolation windows and *m/z* ranges. They also evaluated the quantitative accuracy and observed experimental errors between 18.0% and 32.8% (DeLaney & Li, 2019b). Potential improvements to this method could include the use of label-based quantitation. While the capabilities and applications for DIA has expanded over the years, it will benefit from additional improvements and new labeling strategies for accurate and reproducible quantitation.

6.4 | Special considerations: MSI

High throughput data collection is key in developing new analytical techniques. Thus, the application of quantitative methods to imaging was a natural transition to acquire both spatial and quantitative information in a single instrumental run. Some applications have been discussed briefly above (see Section 5.3) (Zhang, Ye, et al., 2012; Zhang et al., 2013; Zhong et al., 2012), but the streamlining of methods has obtained a lot of attention recently for drugs and metabolites (Pirman, 2015; Sun & Walch, 2013). While this has not been fully developed for neuropeptidomics, it could be easily implemented in the future. It should be noted that these techniques still require further development to become more common practice in the scientific community (Cillero-Pastor & Heeren, 2014).

As stated above, there are label-free and label-based techniques for acquiring quantitative information from samples. LFQ is the most commonly used in MSI, including the use of a calibration curve or an internal standard (Clemis et al., 2012; Goodwin et al., 2012; Groseclose & Castellino, 2013; Hamm et al., 2012; Lanekoff & Laskin, 2017; Nakanishi et al., 2014; Rodrigues et al., 2014; Shariatgorji et al., 2014). In these cases, usually the standard (s) are either spotted onto the tissue or added to the MALDI matrix solution before its application. Alternatively, the use of multiple isotopically labeled standards can be sprayed onto the tissue section for use as internal standards (Dewez et al., 2021). Koeniger et al. (2011) have taken a more unique approach by taking nearby, separate sections for MSI and LC-MS quantitation, since serial sections have similar analyte concentrations. This approach requires homogenous tissues. MSI, label-based quantitation applications are still novel. The only published example utilizes a duplex-isotopic immunohistochemical staining azo dye which, after laser energy absorption, produces signature reporter ions separated by 5 Da (Wang, DeGnore, et al., 2015). While all these methods seem promising, without the ability to process this data quickly, the throughput of quantitative MSI is limited. Some groups have produced software for on-tissue calibration curve quantitation, both open source (e.g., MSiReader) or commercial (e.g., SCiLS or Quantinetix) (Kallback et al., 2012; Robichaud et al., 2013). More effort needs to be applied to developing additional bioinformatics tools in this area.

The generation of a calibration curve using a peptide standard to absolutely quantify neuropeptides is considered the gold standard. This can be performed on a variety of instruments and does not require many biological replicates to produce confident results. However, peptide standard synthesis is expensive. Additionally, if the neuropeptides selected for quantification exclude other comodulating neuropeptides involved in a particular biochemical pathway, incorrect conclusions might be drawn. As only a small number of discovered neuropeptides have been functionally evaluated, there is high risk of not selecting all the neuropeptides involved in the pathway. In this case, it is better to perform global profiling analyses to detecting as many neuropeptides as possible simultaneously. Therefore, the next best alternative for quantitation is to either incorporate isotopes into animals before sample collection or to chemically

derivatize the animal samples after collection. For both methods, the optimal means would involve detection of the same neuropeptides in all conditions, while also not detecting any non-modified neuropeptides. Additionally, in chemical derivatization, a 100% labeling efficiency would be achieved. Overall, global profiling of changes in neuropeptide expression can serve as a foundation to understand neuropeptide function and dysregulation.

7 | FUNCTIONAL STUDIES

Neuropeptides impact a large and diverse array of physiological processes (Insel, 2010; Mills et al., 2020; Neumann & Landgraf, 2012; Steinhoff et al., 2014; Wang et al., 2021; Xu et al., 2020). Functional elucidation is not trivial notably due to neuropeptide co-transmission capabilities (Nusbaum et al., 2017) and pleiotropic nature (Souza-Moreira et al., 2011). Changes in neuropeptide abundance and localization can act as a foundation for functional studies since dysregulation of these characteristics indicate an abnormal or disease state (DeLaney, Buchberger, & Li, 2018) (see elsewhere in this review). Thus, MS-based quantification of neuropeptides can be exploited to understand neuropeptide expression level changes under physiological and pathological conditions. For example, Ye et al. (2017) profiled neuropeptide expression changes due to differential food intake and functionally validated the role of significantly changed neuropeptides by injecting them into rats. This section focuses on physiology- and microdialysis-based functional studies where neuropeptides are the target analyte. Yet, it is also worth noting that neuropeptide receptor dynamics also play a critical role in neuropeptide function (DeLaney et al., 2018).

7.1 | Physiology-based functional studies

Besides MS, other techniques are often used to understand neuropeptide function, and these characterizations are critical for development of therapeutics. For example, pituitary adenylate cyclase activating polypeptide, known to improve cornea health, is shown by Kovacs et al. (2020) to be resistant to degradation in solution, demonstrating its potential for use in eye drops. A common technique to investigate neuropeptide function is overexpression of the peptide in an animal model. Transgenic mice overexpressing thyrotropin-releasing hormone exhibit higher blood pressure and heartbeat rate (Landa et al., 2020). Since the development of transgenic animal models is difficult and costly, so alternative methods are often preferred.

Neuropeptide function are often characterized by examining physiological effects in vitro or ex vivo. Such studies allow the researcher to control experimental parameters better than in in vivo experiments. For example, somatostatin/allatostatin-C ArSS2 standards have a relaxation effect on dissected starfish tube foot, apical muscle, and cardiac stomach muscle contractions (Zhang, Yanez Guerra, et al., 2020). Muscle contractions are typically recorded using a timer or by connecting the tissue to a force-displacement transducer or similar instrument recording contraction force. Manual counting is advantageous when studying small animals as employed in a recent study on the effect of adipokinetic hormone Carmo-HrTH-II neuropeptide on heartbeat rate (Katali et al., 2020). Since certain invertebrates, including decapod crustaceans, have neurogenic hearts, neuropeptide modulation of cardiac function has become a field of interest, broadly reviewed by Calabrese et al. (2016). Marciniak et al. (2020) showed that FMRF6 causes a decrease in beetle heartbeat rate and an increase in hindgut contractions. Dickinson et al. perfused shrimp pyrokinin PevPK2 neuropeptide onto a lobster heart to observe an increase in heartbeat rate and amplitude and decrease in heart contraction duration. Altering the peptide sequence resulted in a loss of activity (Dickinson et al., 2015). The Dickinson group used semi-intact heart preparations to evaluate the neuropeptide modulation of heart (Wiwatpanit et al., 2012) and also published a review on crustacean neuropeptide modulation of pattern generating systems (Dickinson et al., 2016). Cardiac assays have also been performed on mammals. Studneva et al. (2019) administered forms of galanin to myocardial injury-induced rats and recorded blood pressure and heart rate in vivo.

Additional methods to investigate neuropeptide function measure biochemical effects. Wei et al. (2020) cultured crab hepatopancreas tissue and applied crustacean cardioactive peptide (CCAP), measuring an increase in nitric oxide and resulting improved bacterial clearance in the medium. To investigate the impact of neuropeptides on reproduction, Chieu, Turner, et al. (2019) incubated dissected sea cucumber ovarian tubules in solutions containing gonad-stimulating peptide and observed oocyte maturation. Hao et al. (2019) injected newly synthesized diapause hormone (DH)-like peptides into locusts and found some peptides to induce diapause in eggs. Atkins et al. (2018) applied neuropeptides, including arginine vasopressin to excite rat optic nerves ex vivo to evaluate their involvement in the regulation of circadian rhythm.

One strategy is to minimize sample handling, as seen in the use of microfluidic platform to culture neurons in a capillary and directly analyze secreted neuropeptides by MS (Lee et al., 2016). While in vitro and ex vivo experiments have their clear benefits, there is a push towards in vivo

approaches, particularly when translation to therapeutics is aimed for.

7.2 | Microdialysis-based functional studies

Microdialysis probes in or adjacent to the location injected with peptides can be used to collect local perfusates. Guvenc-Bayram et al. (2020) observed an increase in prostaglandin in mice hypothalamic injected with nefastin 1, indicating that this peptide activates the arachidonic acid-cyclooxygenase and -lipoxygenase signaling pathway. Our lab has recently investigated neuropeptides implicated in circadian rhythm using microdialysis (Liang et al., 2015), after the development of a protocol for the in vitro microdialysis of a neuropeptide standard as well as the in vivo microdialysis sampling of neuropeptides from a live crab (Behrens & Li, 2010). Using microdialysis coupled with MS, Mabrouk and Kennedy (2012).

Bulbul et al. administered neuropeptide-S into Parkinson's disease-induced rats and observed increased dopamine levels (collected via microdialysis) 7 days after the administration. This suggests that the peptide has protective effects in the brain (Bulbul et al., 2019). Grund et al. (2017) examined potential anxiety disorder therapeutics and saw that neuropeptide S stimulates oxytocin release (Figure 4). Cui and Smith (2019) studied the neuronal regulation of obesity and demonstrated an increase in agouti-related peptide release when G_s-linked G protein-coupled receptors were activated. Willie et al. (2012) combined intracerebral microdialysis and electroencephalography/electromyography with motor activity monitoring to study the effect of orexin neuropeptides in brain injury. In addition to roles in biochemical signaling, certain neuropeptides have measurable behavioral effects. Lee et al. delivered oxytocin neuropeptide into mice and observed a decrease in the rate by which mice self-administered the drug methylphenidate (CNS stimulant) along with differential regulation of dopamine levels (collected via microdialysis from mice that were randomly implanted in the right or left brain side) between different brain regions (Lee et al., 2019). A logical next step is to expand the number of simultaneously measurable characteristics, particularly during in vivo experiments, and to increase the sensitivity for neuropeptide detection, such as by improving sample preparation methods (see Section 2).

8 | CONCLUSIONS

In the last decade, significant advances in MS instrumentation and associated technologies have accelerated the progress of neuropeptide research, enabling high throughput neuropeptidome characterization. As the biological importance of neuropeptides is increasingly realized, we predict that more people will be attracted to study them and conduct more in-depth investigations. However, compared to well-established proteomic workflows and tools, there are still many technological gaps to be filled, and implementation of advancements in proteomics tools should be more readily applied to neuropeptidomics. Techniques capable of reliably enriching scarcely distributed neuropeptides and removing interfering substances are in high demand. As neuropeptides vary in length and structure, there is a need for customized MS approaches to be developed based on each particular family, class, and even isoform of neuropeptides being targeted to enable obtaining comprehensive MS/MS spectra. For MSI, areas of interest are developing more robust sample preparation techniques, improving spatial resolution, increasing throughput, and development of quantitation methods. Algorithms that are capable of integrating prohormone cleavage preferences would be beneficial in performing mature neuropeptide prediction from genomes as they are increasingly being sequenced. Further advances in bioinformatics must keep up such that all MS data will be interpreted in a convenient fashion while providing rich chemical information. Although empirical determination of individual neuropeptide functions is highly valuable, the time it takes to do so can be considered a bottleneck step in the overall pipeline from discovery to therapeutics. To improve and facilitate interpretation of neuropeptidomics data, methods capable of elucidating neuropeptide comodulation must be developed. Finally, further development of sensitive, reliable quantitation approaches that can handle limited sample amount will be key to allow cross comparisons of neuropeptides in a high throughput manner. While the field of neuropeptide analysis by MS has seen great advances over the years, the incorporation of more advanced techniques and tools in the future will greatly benefit our understanding of neuropeptides and neurochemical signaling.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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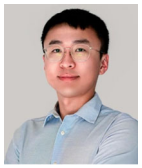

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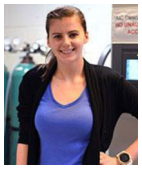

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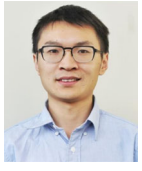

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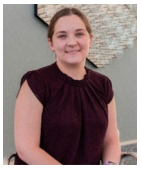

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