Neuropeptidomics applied to studies of mammalian reproduction

Abstract
Neuropeptidomics, a mass spectrometry-based technique which aims to uncover the complete suite of neuropeptides present in a tissue, organ or cell from the brain or nervous system, has found application in studies examining physiological responses (e.g., food intake, appetite and reproduction). Neuropeptides (and peptide hormones) have long been known as regulators of mammalian physiological processes, particularly reproduction. These peptides are derived from precursor proteins and become active via proteolytic processes and post-translational modifications. A relatively large number of neuropeptides, mainly formed in the hypothalamus or the anterior pituitary of mammals, have been specifically associated with reproduction, including GnRH, NPY, PYY and kisspeptin. Here, we will present an overview of neuropeptides, their roles in reproduction and the application of neuropeptidomics in this field. We address the advantages of neuropeptidomics in reproductive studies including the high throughput identification, profiling and quantification of neuropeptides present in reproductive tissues and also discuss some of the challenges. The application of neuropeptidomics to the field of reproduction will provide the foundation for a greater understanding of how neuropeptides act to regulate reproductive function.

Keywords
Peptidomics • Neuropeptides • Reproduction • Mass spectrometry

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Received 01 July 2013
Accepted 07 October 2013

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1. Neuropeptides and processing

Neuropeptides and peptide hormones are important intercellular signalling molecules [1]. Due to their effects on neural (neuropeptides) and non-neural tissues (peptide hormones), they act to regulate the functions of the brain and the peripheral organ systems that confirm their important roles in the physiological functions of all species. The distinction between neuropeptide and peptide hormone has to do with their site of synthesis and action, wherein neuropeptides are secreted from neuronal cells and signal to neighbouring cells (primarily neurons). In contrast, peptide hormones are secreted from neuroendocrine cells and typically act on distant tissues after transport through the blood. There is some overlap though with a number of peptide hormones also having neuronal function. The term “neuropeptides” was first coined by David de Wied in 1971 owing to their discovery in the nervous systems [2]. Neuropeptides can be neurotransmitters or neuromodulators.

Neuropeptides, typically 3-50 amino acids in size, are derived from precursor proteins (or prohormones) that are converted to the final active peptides via a series of enzymatic processing steps followed by modifications such as amidation, acetylation, phosphorylation, sulfation and glycosylation. These post-translational modifications act to regulate the activity of these peptide hormones [3]. Many steps are involved in the processing of bioactive peptides (Figure 1). Neuropeptide precursors are synthesised on ribosomes at the endoplasmic reticulum and processed through the Golgi. The production (including from alternate splicing events) [4,5] and proteolytic processing [6,7] of neuropeptides is tissue-specific, and this can result in altered quantities and altered activity of the final peptide products. The most common processing involves the endoproteases including prohormone convertase 1 (PC1, also known as prohormone convertase 3) and prohormone convertase 2 (PC2),
neuropeptides or multiple copies of a single neuropeptide may be released. Furthermore, a single precursor molecule can be cleaved at different sites resulting in different sets of peptides being produced in different cell types leading to an increase in neuropeptide complexity [10,11].

Post-translational modification may serve two important roles: (1) to confer biological activity or (2) to enhance the stability of the final peptide product [1]. Of all the modifications, C-terminal amidation, N-terminal pyroglutamic acid and acetylation are frequently encountered. Proopiomelanocortin (POMC) is a typical example of tissue-specific and post-translational processing: adrenocorticotropic hormone (ACTH), a 39 amino acid unmodified peptide, is produced from POMC in the anterior pituitary, whereas α-melanocyte-stimulating hormone (α-MSH), a 13 amino acid which is both N-terminally acetylated and C-terminally amidated, is produced from the same precursor in the intermediate pituitary. Post-translational modification can also affect the affinity of peptides for their receptors, for example, the β-endorphin stimulation of opioid receptors is reduced by acetylation at the N-terminal end; and this modification produces analgesia [8].

While there are predicted to be only around 100 genes that encode neuropeptide precursors, the observed diversity of biologically active peptides (in excess of 250) is the result of alternate splicing, proteolytic processing and post-translational modification [9]. Many forms of neuropeptides can be produced from a single neuropeptide precursor; for example, a single neuropeptide, multiple distinct neuropeptides or multiple copies of a single neuropeptide may be released. Furthermore, a single precursor molecule can be cleaved at different sites resulting in different sets of peptides being produced in different cell types leading to an increase in neuropeptide complexity [10,11].

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Figure 1. GnRH processing scheme. The first stage of processing involves the removal of the N-terminal sequence by a signal peptidase forming the preprohormone. In the presence of prohormone convertases, the prohormone is classically cleaved at dibasic sites (e.g. Lys-Arg site in GnRH). The subsequent steps are the removal of C-terminal basic residues (Arg, Lys) as well as the C-terminal Gly by carboxypeptidases. An amidating enzyme, peptidyl amidating monoxygenase, converts the C-terminal Gly residue to a C-terminal amide to produce mature GnRH.

and to a lesser extent, prohormone/proprotein convertase 5A (also known as prohormone/proprotein convertase 6A) [8]. Metalloprotease and endothelin convertase families are considered as new endopeptidases [1]. The typical cleavage sites of the endopeptidases are at mono or dibasic residues; for example, Lys-Arg or Arg-Arg. The next stage of processing is the removal of C-terminal basic residues present in most processing intermediates. Carboxypeptidase E (CPE) is the exopeptidase primarily responsible for this C-terminal trimming, although carboxypeptidase D has also been shown to act in this role. However, non-classical cleavage (such as cleavage at aliphatic and aromatic residues) has also been observed; for examples, endothelin converting enzyme-2,carboxypeptidase A5 and carboxypeptidase A6 [8].

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2. The role of neuropeptides in reproduction

Mammalian reproductive physiology is highly regulated by the interactions between the hypothalamus, pituitary and gonads in what is commonly described as the hypothalamo-pituitary-gonadal axis [12]. Two of the most important reproductive hormones are luteinizing hormone (LH) and follicle-stimulating hormone (FSH) which are known as gonadotropins as they stimulate the gonads, that is the testes in males and the ovaries in females. Both LH and FSH are produced in the anterior pituitary where their secretion is stimulated by gonadotropin-releasing hormone (GnRH), which is produced in the hypothalamus. LH and FSH are required for the maturation and development of the gonads and the synthesis and secretion of the gonadal steroid hormones. During the ovulatory cycle, steroid hormones such as the estrogens, provide feedback to the hypothalamus and the pituitary to modulate GnRH secretion and the pituitary gonadotropin response to each GnRH surge [13].

A great number of neuropeptides, such as GnRH, neuropeptide Y (NPY), peptide YY (PYY) [14], kisspeptin, RFamide-related peptides (the RFRPs) [15,16], galanin, galanin-like peptide, oxytocin and α-MSH [17] have been implicated in regulation of reproductive functions in mammals.

GnRH is a ten amino acid peptide that is highly conserved across mammalian species. The release of GnRH is influenced by many factors such as olfactory signals, nutrition and stress [18]. Changes in GnRH have an impact on reproductive systems [19]. It is a critical requirement to have sufficient pulsatile GnRH so that the functions of reproduction are retained. For example, too low pulsatility of GnRH causes hypothalamic amenorrhea and infertility in women, whereas high pulsatile GnRH leads to polycystic ovarian syndrome [8]. GnRH is generally believed to be the only hypothalamic neuropeptide that controls the synthesis and release of pituitary gonadotropins. GnRH secretion in the hypothalamus is modulated by many factors, among them several neuropeptides, such as NPY, kisspeptin, gonadotropin inhibitory hormone (GnIH), and others.

NPY is composed of 36 amino acids and is abundantly present in the central nervous system (CNS) and the peripheral sympathetic nervous systems. In addition to NPY, the two orthologues NPY[2-36] and NPY[3-36] act to modulate feeding behaviour [20,21]. NPY and its orthologues are mainly synthesised in the arcuate nucleus of the hypothalamus. It is transported in vesicles along axonal projections to regions such as the lateral hypothalamic area. Importantly, the involvement of NPY in the regulation of reproductive systems has been shown [14]. NPY neurons are found in close proximity to GnRH neurons in the preoptic area. The correlation between pulsatile release of NPY and GnRH in the median eminence was reported by Woller et al. (1992) [22]. However, the effects of NPY on GnRH release depend on the environment of the endocrine system, for example, NPY may inhibit plasma LH and FSH in the absence of gonadal steroids [14]. Furthermore, PYY is produced by endocrine cells in the gastrointestinal tract and is structurally related to NPY. The peptide has recently been shown to inhibit food intake. PYY acts as a stimulator of LH and FSH secretion, as well as an inhibitor of GnRH secretion in vitro [23], but the effects of PYY on GnRH release in vivo are not yet known.

Kisspeptin, a 54 amino acid peptide (kisspeptin-54), was discovered by Lee et al. (1996) [24]. Kisspeptins are produced mainly by neuronal clusters at discrete hypothalamic nuclei [25]. They are known as key regulators of GnRH neurons. Kisspeptin-54 and its N-terminally truncated form (kisspeptin-10) were reported as LH stimulators. Kisspeptin-10 was demonstrated to stimulate GnRH release in rat hypothalamic explants and in vivo with intracerebral injection in sheep [17]. The influence of kisspeptins on GnRH neurons and LH is mediated via the GPR54 receptor. Genetic inactivation of GPR54 in humans and mice caused impaired puberty progression and sexual immaturity [23].

GnIH, a 12 amino acid peptide, and its orthologues are novel hypothalamic neuropeptides that were initially identified in...
avian species and have since been found in other vertebrates (from fish to humans). Mammalian GnIH orthologues (known as RFRPs) inhibit the release of gonadotropin across mammalian species [16]. It has been reported RFRP-3 acts as GnRH-stimulation inhibitor in mammals. Recently, RFRP-1 was also found to inhibit GnRH release in hamsters. Whether RFRP-1 and RFRP-3 co-inhibit the HPG axis in mammals or each peptide functions independently warrants further investigation [15,16].

Other neuropeptides, such as galanin, galanin-like peptide, oxytocin and α-MSH have been shown to regulate the release of GnRH or LH [17]. While galanin stimulates the secretion of GnRH, galanin antagonists and anti-serum obstruct the LH surge and ovulation [17]. Galanin acts on receptors on GnRH neurons that might be involved in the process of forming the GnRH pulse shape. Galanin-like peptide, a 60 amino acid peptide, is also capable of activating galanin receptors. Galanin-like peptide stimulates LH release and is also implicated in the secretion of GnRH [17]. Oxytocin is a nonapeptide that also induces GnRH release with its action on GnRH neurons, and is speculated to be regulated by estrogen [17]. Interestingly, α-MSH is a 13 amino acid derived from POMC that has bipolar effects on LH release. α-MSH has been demonstrated to have both stimulatory and inhibitory effects on LH release depending on when it is injected, before or after the LH surge, respectively. Again, the action of neuropeptides on GnRH may vary between species [18].

In summary, there are several hundred known neuropeptides present in the hypothalamus and presumably many more in other organs, however, their interactions and biological functions are not yet fully understood. The advance of technologies to identify, characterise and quantify neuropeptides is critical to unravelling the complex interplay between neuropeptides, receptors and their environment.

3. Traditional methods of neuropeptide analysis

The technique of radioimmunoassay (RIA), developed by Berson and Yalow in the 1960s [26], is a sensitive in vitro method for peptide quantification. RIA has been a popular tool to quantify neuropeptides and/or hormones in biological samples, such as NPY in rat brain [27], adrenals and blood [28]; leptin, corticosterone and insulin in rat blood [29]; orexin A and NPY in the plasma and hypothalamus of rats [30]; and galanin in rat brain [31]. Although RIA is a relatively sensitive technique capable of absolute quantification of peptide levels, due to its antibody-based detection method, it is not always specific for a single peptide isoform. RIAs either identify peptides based on their N- and/or C- terminal sequence or post-translational modified residues (e.g., phosphorylation, sulfation, acetylation, and glycosylation) [3]. However, unless specific antibodies are available for the different isoforms, RIAs do not distinguish between these modifications. Enzyme-linked immunosorbent assays (ELISA) which utilise antibodies along with a colourimetric reagent, have in part taken over from RIAs because of their ease of use, despite lower sensitivity in some cases [32,33]. Commercial ELISA kits have been widely used in quantitative neuropeptide studies, for example, NPY quantification [34]. These antibody-based techniques are simple, sensitive, specific, rapid and easily automated, however, only neuropeptide isofoms that interact with specific antibodies can be detected.

Traditionally, the primary structure of peptides has been obtained by Edman sequencing. The method was developed by Edman in 1950 and continues to play an important role in sequencing peptides or proteins [35]. Automated Edman degradation has been used for neuropeptide sequencing in many different species; for examples, NPY in porcine brain [36], GnRH in dogfish brain [37] and periviscerokinins in insect perisymphatic organs [38]. Heine et al. (1997) successfully sequenced peptides in human urine samples by using an Edman-based sequencing technique [39]. Edman degradation sequencing is very sensitive, reliable and its interpretation is straightforward, but it is restricted to pure peptides and cannot analyse peptides with N-terminal modifications, for example those modified by acetylation, formylation or pyroglutamination [35].

The other techniques which have been used in neuropeptide mapping are immunohistochemistry (IHC) and immunocytchemistry (ICC). IHC can be applied to characterise neuropeptides and their distribution, for example, in the pre-mammillary area of the rat hypothalamus [40]. Likewise, ICC can be used to improve our understanding of the distribution of the peptides and assist in identifying the function of the neurons synthesizing and secreting the neuropeptides [11].

4. Neuropeptidomics

The high throughput discovery of neuropeptides has been enabled by a relatively new technique coined peptidomics [41] and is defined as the study of endogenous peptides present within the cell, tissue or an organism. The expressions “peptidome” and “peptidomics” came into use in 2001 [42-45]. Typical proteomics studies involve the digestion of proteins using trypsin or other proteases to yield peptides suitable for analysis by liquid chromatography (LC) coupled to mass spectrometry (MS). Peptidomics studies focus on the analysis of the native peptide forms, i.e. without ex vivo digestion. The technique has found application in the discovery, study and quantification of neuropeptides that were traditionally reliant on using the techniques in the previous section. Peptidomics offers advantages over these techniques due to its speed, sensitivity and capability in the simultaneous characterisation and quantification of neuropeptides. Hundreds to thousands of neuropeptides can be separated and identified in a single LC-MS/MS experiment [46]. For example, a single antibody-based assay may yield information of one neuropeptide (or related family of neuropeptides), whereas MS-based peptidomics can be utilised to characterise suites of neuropeptides simultaneously and furthermore, can be applied to the quantification of individual neuropeptide isofoms (including truncated or modified forms) [3]. Peptidomics is a promising tool to characterise and quantify known neuropeptide molecules, but additionally to discover novel neuropeptides.
5. Neuropeptidomics techniques and applications

The techniques used for neuropeptidome studies primarily involve MS, often coupled to peptide separation strategies such as high performance liquid chromatography (HPLC) or capillary electrophoresis (CE). Two commonly employed workflows are outlined in Figure 3.

5.1. ESI versus MALDI

Mass spectrometry approaches have been successfully applied to the characterisation of neuropeptides from tissue extracts. Two commonly used types of mass spectrometers in peptidomic research involve the use of electrospray ionisation (ESI) or matrix-assisted laser desorption / ionisation (MALDI) [47]. They differ in the way the protein/peptides are ionised, and each of them has certain advantages. While MALDI is able to deal with complex mixtures and is tolerant to the presence of salts, ESI can be easily interfaced with separation strategies such as LC and CE [1]. Due to its decreased susceptibility to impurities, MALDI is a direct tool for peptidomic analysis of biological samples. The singly charged ions produced in MALDI make it less complicated in terms of data interpretation compared to ESI in which multiple charge states are generated for larger peptides [47]. Typically MALDI ionisation is coupled with mass analysis using a time-of-flight (TOF) mass analyser, whereas ESI ionisation may be found as the ionisation source associated with TOF, quadrupole, ion trap or hybrid mass analysers [48].

MALDI-TOF MS has been used as a direct or LC-combined technique to profile the peptidome of cell organelles, whole cells, tissues and organs of several species [47]. The tissues are either first placed in extraction solution or loaded directly on the MALDI plate (in a technique known as MALDI imaging) (Figure 3). For direct tissue profiling by MALDI, Dircksen et al.

![Figure 3. Commonly employed workflows in neuropeptidomic studies. In MALDI imaging, tissue sections are placed directly on a sample plate, coated with a matrix solution and then peptides are analysed after ablation of the tissue surface by a laser. This generates MS spectra that allow the location of a peptide to be determined. In a typical ESI-LC-MS/MS experiment, tissue is dissected and homogenised before extraction. The endogenous peptides are then separated by HPLC and analysed by tandem mass spectrometry generating a spectral collection that can be searched against databases to identify the peptide components.](image-url)
(2011) [49] and Hauser et al. (2011) [50] showed the feasibility of MALDI-TOF MS through the identification of neuropeptides in the water flea Daphnia pulex and the parasitic wasp Nasonia vitripennis respectively. The peptide extract can also be separated and fractionated using LC before loading on MALDI target plate. A diverse range of applications of MALDI-TOF MS in endogenous peptide analysis has been reported; for example, the discovery of active peptides in vesicles of the exocrine, a trial gland of Aplysia californica [51], and its application in the study of invertebrate [52,53] and vertebrate neurons [54]. Molluscan neurons were the first single cell studied by MALDI MS. Studies examining learning, memory and behaviour have been conducted on several marine and freshwater invertebrates (e.g. the molluscs A. californica and Lymnaea stagnalis) because of their simple nervous system and the relatively large size of their neurons. Furthermore, the MALDI imaging technique has evolved as a powerful tool to map neuropeptide distribution in complex organs [55]. MALDI imaging is comparable to immunohistochemistry in that the information derived from both techniques involves the localisation of specific peptides. The technique has been applied in neuropeptide profiling in rat spinal cord [56], and in the localisation of multiple neuropeptides in the brain and neuroendocrine organs of the crab Cancer borealis [57]. Advantages of MALDI imaging over traditional methods include its relatively simple application and ability to produce valuable information pertaining to the spacial localisation of multiple target molecules simultaneously.

In ESI, the ions are produced as an aerosol from the liquid phase and transferred directly to the gas phase following desolvation [58]. As such, ESI can be directly coupled with LC or CE (online) (more detail in the next section). This enables ESI MS instruments to detect and fragment very low amounts of peptides/neuropeptides. Thus, ESI MS is a perfect tool for peptide profiling and amino acid sequencing [47]. The peptidome of the Drosophila CNS, the rat brain including the motor cortex, the striatum and the thalamus, and human urine have all been analysed by ESI approaches [59]. In addition, the application of ESI has improved the coverage of the rat brain peptidome. Skold et al. (2002) detected 1500 peptides and protein fragments in the rat brain using nano-ESI-LC-MS/MS [60], however, many of these peptides were revealed to be the result of post-mortem degradation and not true neuropeptides. The implementation of sample preparation techniques, including the use of microwave irradiation, has reduced post-mortem protein degradation (see section 6) allowing the detection and identification of endogenous neuropeptides. In 2006, Dowell et al. reported the identification of 56 putative neuropeptides in the hypothalamus and striatum of rats using microwave irradiation to arrest post-mortem degradation [51]. Using ESI-LC-MS/MS and thermal denaturation, Colgrave et al. (2011) were able to identify more than 100 neuropeptides in the bovine hypothalamus including GnRH, α- and β-MSH, the enkephalins and neuropeptide Glu-lle [46]. Romanova et al. (2012) used capillary LC-ESI-ion trap MS/MS to compare the peptidome profile between saline- and amphetamine-treated rats [62].

5.2 Separation strategies

The diversity of neuropeptides present in tissue extracts varies greatly between different tissues or cells, ranging from hundreds to potentially thousands of neuropeptides in the brain, cerebrospinal fluid or blood. Furthermore, the complexity is confounded by the dynamic range within biological samples with endogenous peptide concentrations in the low pg/mL levels and highly abundant proteins in mg/mL concentrations [41]. For example, the high concentration of albumin in human plasma interferes with peptide analysis of low concentration biomarkers [63-65]. Thus, a separation, and sometimes depletion, is needed prior to peptide identification by MS.

HPLC is one of the most useful techniques for separating peptides from complex mixtures [1]. In contrast to proteomics, 2D-PAGE has limited application in peptidome analysis because the peptides are normally lower than 10 kDa which cannot be retained and visualised on a gel [41]. Hence, HPLC is more applicable, sensitive, robust and easily automated for peptide separation. In order to avoid the incidence of co-elution of peptides in complex mixtures and to improve sensitivity, micro or nanoscale LC (micro- or nano-LC) has been applied. The very low flow rates delivered by micro- or nano-LC results in an increased acquisition time allowing a greater number of peptides to be analysed or enables a longer time per analysis improving the spectral quality for low abundance peptides [41]. For example, using nano-LC-ESI-QTOF-MS analysis, a total of 550 endogenous neuropeptides were identified in hypothalamic rat brain tissues [66]. In addition, numerous separation modes such as size exclusion, reversed-phase (RP) and ion exchange can be used in LC, however RP is more often employed [1]. The complexity of the biological samples can be reduced by combining different separation modes (e.g. two-dimensional (2D) chromatography) before MS analysis. The application of 2D LC using strong cation exchange (SCX) chromatography to separate peptides in the first dimension and reversed-phase in the second dimension has resolved highly complex samples. Holm et al. (2005) developed a 2D LC with SCX-RP approach to characterise peptides in rat brain and differentiate peptide expression in less and more distressed rats [67]. Ion exchange chromatography combined with RP is a popular choice for proteomic studies, but the combination of two stages of RP chromatography is still used with great success, especially for peptidomic studies. Dowell et al. (2006) discussed some limitations of SCX in 2D LC including low compatibility, separation selectivity and efficiency due to high salt concentrations and lack of charge of some neuropeptides [61]. His group employed 2D RP-RP LC to analyse neuropeptides in the rat hypothalamus and striatum punches. The neuropeptide extracts were fractionated by high pH off-line RP in the first dimension and low pH in the second dimension, resulting in an increased number of neuropeptide identifications (48%).

Besides LC, CE has been considered for peptide separation. CE combined with MS (CE-MS) has become a popular tool in peptidomics because of its high efficiency, fast analysis time and low sample consumption [68]. To date, CE is frequently
interfaced with ESI for peptide analysis. CE-MS has a wide range of applications. Javerfalk-Hoyes et al. (1999) confirmed the application of CE-MS/MS in identification of peptides and neurotransmitters in discrete marmoset brain regions (the striatum, thalamus, hypothalamus and frontal cortex) [68]. An extensive review of CE-MS has been published by Herrero et al. (2008) [69].

5.3. Neuropeptidomic discovery

Peptidomics is the most advanced tool for neuropeptide discovery. Due to the complexity of endogenous peptides and their specific biological activities [3], different techniques have been applied in order to identify and localize neuropeptides and their post-translational modifications present in different species, from invertebrates to vertebrates. The first peptidomics studies were performed in the CNS of locust and cockroach by using ESI-TOF and MALDI-TOF MS in which 12 and 44 peptides were detected respectively [70]. The peptidomes of the CNS of these insects were profiled and their physiological processes were revealed to include solitary and gregarious phases in locusts. Over the years, improvements in technology have led to increased depth of coverage and numbers of peptides identified. A recent review summarises different peptidomics techniques and bioinformatics approaches for endogenous peptide analyses [1].

The hypothalamus and pituitary are well-studied organs because of their important roles in regulating physiological processes such as reproduction, lactation, fluid balance, and metabolism. By using nano-ESI-LC-MS, up to five hundred neuropeptides were detected in the rat hypothalamus/pituitary [69,71,72]. However, few peptidomics studies to date have specifically focused on reproduction. Table 1 summarises peptidomic studies across the animal kingdoms with a specific focus on reproduction and includes the number of neuropeptides and/or endogenous peptides detected in different species and the techniques employed. The peptides reported in these studies were derived from a new processing mechanism, post-translationally modified or encoded from novel neuropeptide genes (Table 1). Zatylny-Gaudin et al. (2010) confirmed the novel neuropeptides detected in Sepia officinalis (GNLFRFamide called sepFRF1) as the first RFamide family that is not derived from the FMRFamide-related peptide (FaRP) precursor [73]. Some of the novel peptides such as galanin/galanin-like peptides were first identified in invertebrates (e.g. Ciona intestinalis) [74]. In addition, the gene encoding arginine-vasopressin-like peptide (APVL) has been found in Tribolium and several ant species despite its absence in many holometabolous insects [75,76]. Colgrave et al. (2011) detected a number of neuropeptides in bovine hypothalamus which had not been found in any species. They were present as post-translationally modified and un-modified forms [46]. These studies highlight the role of peptidomics in the high throughput identification and characterization of multiple neuropeptide forms as well as shedding light on their processing pathways.

5.4. Quantification of neuropeptides

The important contribution of peptidomics is not only in the identification of peptides present in tissues or brain regions, but also the determination of the biological activities of the peptides. One of the potential ways to obtain this information is via quantitative peptidomics. The level of neuropeptides detected in animal tissues at different physiological states could indicate the involvement of these peptides in physiological functions [8]. For example, the amount of hypothalamic peptide expression may

Table 1. Peptidomic studies across the animal kingdom specifically focusing on reproduction.

<table>
<thead>
<tr>
<th>Species</th>
<th>Organs</th>
<th>No. of peptides</th>
<th>Techniques</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nasonia vitripennis (parasitic wasp)</td>
<td>Ventral nerve cord, brain (subesophageal ganglion and corpora cardiaca)</td>
<td>24</td>
<td>MALDI-TOF-MS</td>
<td>[50]</td>
</tr>
<tr>
<td>Sepia officinalis (cuttlefish)</td>
<td>CNS (optic lobes, subesophageal mass, supraesophageal mass, nerves endings)</td>
<td>4 RFaRPs (1 novel)</td>
<td>Micro-LC-ESI-MS/MS</td>
<td>[73]</td>
</tr>
<tr>
<td>Daphnia pulex (water flea)</td>
<td>Brain-optic ganglia</td>
<td>40</td>
<td>MALDI-TOF-MS</td>
<td>[49]</td>
</tr>
<tr>
<td>Ciona intestinalis (sea squirt)</td>
<td>Neural tissues</td>
<td>33 (26 novel)</td>
<td>MALDI-TOF and ESI-QTOF MS</td>
<td>[74]</td>
</tr>
<tr>
<td>Tribolium castaneum (red flour beetle)</td>
<td>CNS and peptide release sites (retrocerebral complex, thoracic perisym pathetic organs, abdominal perisym pathetic organs, inka cells)</td>
<td>49</td>
<td>MALDI-TOF-MS and ESI-QTOF-MS</td>
<td>[75]</td>
</tr>
<tr>
<td>Locusta migratoria ( migratory locust)</td>
<td>Corpora allata</td>
<td>17 (9 novel)</td>
<td>MALDI-TOF-MS, ESI-QTOF-MS and nano-LC-QTOF-MS</td>
<td>[47,107]</td>
</tr>
<tr>
<td>Schistocerca gregaria (desert locust)</td>
<td></td>
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<tr>
<td>Rattus norvegicus (rat)</td>
<td>Pituitary</td>
<td>142 (22 novel)</td>
<td>Nano-LC-MS/MS, LC ETD combined LC CID</td>
<td>[102]</td>
</tr>
<tr>
<td>Bos taurus (cow)</td>
<td>Hypothalamus</td>
<td>140 (6 novel)</td>
<td>ESI-LC-MS/MS</td>
<td>[46]</td>
</tr>
</tbody>
</table>
increase or decrease in response to food intake in animals; that means the peptide could be a regulator/modulator of food intake or energy balance.

Isotope labelling and label-free quantification approaches using MS may be employed for peptide quantification. These quantitative techniques measure MS signal intensities of either labelled peptides or targeted analytes [81]. In isotopic labelling studies, a stable isotopic tag, either heavy or light, can be incorporated into the extracted peptides of the two samples (e.g. control vs. experimental animals). The relative levels of the peptides can be calculated by the difference in the MS peaks (intensity or area) of the two groups. To obtain reproducible and reliable results, the reagent should react with a common group, e.g. peptide side-chain, and show efficient labelling. The stability of the reagent is also taken into account. Hou et al. (2012) labelled neuropeptides in the mixture of five neuropeptide standards with H4/D4-succinic anhydride with the purpose to compare the quantification ability of three different MS systems [77]. It was shown that capillary LC-ESI-ion trap has higher detection limits than ultra-performance LC-ESI-QTOF-MS and MALDI-TOF MS. Moreover, acetic anhydride was used as a stable isotope in one of the early studies on the effect of water deprivation on the pituitary peptide levels in mice [78].

Relative quantification has commonly been applied to examine the regulation of murine pituitary and brain upon food/water deprivation or drug administration [8]. In combination with ESI-QTOF-MS, Brockmann et al. (2009) employed differential isotope labelling to quantify neuropeptides in the bee brain [71]. Similarly, several studies by Che et al. (2002, 2005) have applied isotopic tags for quantitative neuropeptidomics in Cpeafacr mice [78,79]. The stable isotopic tags that have been developed for proteomics studies, such as Isotopic Code Affinity Tags (ICAT) or Isobaric Tagging for Relative and Absolute Quantification (iTRAQ), have limited application in neuropeptidomics. For an example, the ICAT reagent cannot be used in the quantification of endogenous peptides because Cys residues are absent in most neuropeptides. In an analogous fashion, iTRAQ relies upon labelling the primary amines (N-terminal amine or Lys side-chain). Not all mature neuropeptides contain lysine and in many instances the N-terminal amine is absent due to modification (acetylation, pyroglutamination). Che et al. (2005) have compared the use of non-standard isotopic tags (including succinic anhydride and 4-trimethylammoniumbutyryl reagent) for peptide analysis in the rat pituitary [79]. Both the techniques were demonstrated to be effective tools in the relative quantification of neuropeptides [72].

In addition to labelling techniques, the MS-based technique of multiple reaction monitoring (MRM, also known as selective reaction monitoring, SRM) has recently been implemented for peptide analysis. MRM is not a new method as it has been largely used in drug metabolite quantification for more than 30 years [80]. In the MRM process, a triple quadrupole instrument (QqQ) is set up to maximally detect target compounds. As long as the mass and structure of the peptide is known, it is possible to predict the precursor m/z and a fragment m/z of the analyte of interest [81]. The MS analysis time is focused only on target analytes, wherein the precursor (or parent) ion and product (or fragment) ions are selected for ion transmission in quadrupoles 1 and 3 respectively. The resultant signal is proportional to the amount of analyte [80]. These measurements may be multiplexed allowing up to 1000 peptides to be analysed in a single run [82]. Therefore, MRM is one of the most sensitive and specific methods for quantification of known analytes [83]. MRM has been extensively used in quantitative studies of small molecules in the pharmaceutical industry such as therapeutic agents (e.g. warfarin, triazolam, and antibiotics) [84-86] and drugs of abuse (e.g. heroin, cocaine and cannabinoids) [87,88].

MRM approaches have been recently extended to peptidomics; for example, MRM studies have been conducted to quantify peptides in plasma and tissue biomarkers. Xi et al. (2012) developed an MRM method to detect NPY family neuropeptides present in human plasma [89]. Ling et al. (2010) showed the feasibility of MRM in identification of disease-related peptide biomarkers in human urine samples [90]. The technique has been applied to quantify the level of native peptides in the serum of hepatocellular cancer patients, chronic liver disease patients, and disease free controls [91].

5.5. Bioinformatics

The automated identification of neuropeptides based on their MS and MS/MS data are facilitated by the use of software programs and algorithms such as Mascot [92], SEQUEST [93], XITandem [94], Peaks Studio [95], Phenyx [96] and ProteinPilot [97]. These programs were originally developed for protein identification and as such employ protein databases and typically rely on the identification of at least two peptides per protein. The application of database searching tools for the identification of neuropeptides, which commonly exist as a single peptide product excised from a larger precursor protein sequence, is not trivial. The primary obstacle related to efficient neuropeptide identification employing standard protein databases is the selection of the appropriate database and search parameters. For example, in a proteomic workflow, the theoretical peptides are restricted to those produced by endoprotease cleavage, for example C-terminal cleavage at Arg and Lys residues in the case of trypsin. In peptidomic analyses, the difficulty is the selection of an appropriate enzyme. In reality, neuropeptide precursors are processed by a suite of proteases. Typically peptidomic database searches employ a no enzyme search, but this dramatically increases the search space and hence time required for each search. Several neuropeptide prohormone databases have been constructed, e.g. Swepep [98], to facilitate neuropeptide identification. Binary logistic and expert system tools such as the NeuroPred discovery tool are used to predict annotated prohormones [99,100]. Consequently, the predicted peptide library can be used in MS analyses.

Neuropeptide identification can be obtained not only by protein database searches, but also by de novo peptide sequencing and/or peptide sequence tag search strategies [1]. In the database search strategy, peptide spectra are compared...
with theoretical peptides derived from the protein database. In contrast to protein database search strategies, de novo sequencing only chooses the best possible sequence which has the highest score from all linear amino acid combinations. Due to the absence of prior database information, this approach is only suitable for high quality spectra and is time consuming and requires manual verification. Partial sequences (sequence tags) which are found by an integrated or separate de novo sequencing program represent a semi-automated strategy. Obtained sequence tags can be used in BLAST searches against the genome or prohormone sequence of closely-related species. Moreover, the combination of the three different methods gives the best chance of identification.

In summary, bioinformatics is a very important tool to predict or discover putative hormones in newly sequenced genomes and also has the ability to identify signalling peptides for prohormone confirmation. By integrating genome sequence data with the knowledge of peptide processing and distribution, bioinformatics is a complementary field that supports peptidomics [1].

6. Neuropeptidomics challenges

Peptidomics was evolved from proteomics; however, at its current stage of development, a number of challenges remain. While proteolytic enzymes are commonly used in proteomics in order to produce peptide fragments that are amenable to detection and identification by MS, enzymes are not required in peptidomics studies aimed at examining the endogenous peptide profiles. Although the exclusion of enzyme digestion in peptidomics results in less sample preparation steps in peptidomics, there are inherent challenges in the analysis of large peptides (30-50 amino acids in length) and in the confident identification of single peptide products as detailed in the previous section.

In proteomics studies, a protein is identified by one or more peptides derived from enzymatic cleavage. In the study of neuropeptides, each peptide has a specific biological function. Therefore, the aim of peptidomics is to identify the complete suite of neuropeptides present in a given sample [9]. Furthermore, neuropeptides are generally do not possess basic C-terminal residues. C-terminal Lys and Arg are normally a consequence of proteolytic cleavage by trypsin in a typical proteomics experiment. Due to the absence of the positively charged residues in neuropeptides, they are less amenable to ionisation and hence detection.

One major difficulty in neuropeptidomics is post-mortem degradation. It has been shown that proteins and peptides in brain tissues are substantially degraded by active peptidases and proteases released from cells and organelles within minutes of animal sacrifice [3]. Moreover, highly abundant proteins also interfere with the analysis of neuropeptides as they are degraded to yield peptides in the same mass range. Because of the limited dynamic range of most mass spectrometry techniques, it is very difficult to detect the low levels of endogenous neuropeptides in the extract [3]. Richter et al. (1999) reported that more than 95% of peptides detected in human blood serum were from protein degradation due to proteolysis [101]. Similar findings have been reported in mouse brain tissues [3]. In addition, the post-mortem process could change the amount or type of post-translational modifications observed in bioactive peptides. For example, 85% of an endogenous phosphopeptide, corticotrophin-like immediate lobe peptide (CLIP), was dephosphorylated within 3 minutes post-mortem [102].

Several techniques have been employed to minimize protein degradation. Focused microwave irradiation was used to inactive proteases in the brain of rats and mice. The temperature of the brain was increased to 90°C within 1.4s resulting in enzyme denaturation and effectively arresting protein and peptide degradation. After microwave irradiation, neither neuropeptides nor proteins were degraded and post-translational modified peptides remained intact [103]. This method has been shown to be more effective on fresh or thawed tissues than frozen tissues. In another method, the tissues were heated to 80°C by the microwave directly after the brain is removed. There was no evidence of degraded neuropeptides, however, degradation fragments of haemoglobin were produced [79]. It has been shown that the use of protease inhibitors does not inhibit proteolysis completely [104]. In recent years, thermal stabilisation has proven to be an effective tool in preventing protein degradation. The thermal stabilisation combines heat and pressure under vacuum and can be applied to frozen tissues [104]. This technique is able to retain post-translational modified peptides and is a very useful way to study the peptidome [105,106].

7. Future directions

Over the past decade, a significant number of neuropeptides related to mammalian reproduction have been identified using peptidomics approaches. While this relatively new technique has been extensively employed in studies of neuropeptides regulating stress and nutrition, its application in the study of reproductive function has yet to be fully exploited. Recent years have seen an increasing number of studies aimed at the high throughput discovery or peptidomic profiling of important reproductive tissues, yet very few quantitative studies have been reported. The application of peptidomics to the study of reproductive function will enable a better understanding of the central regulation of reproduction. In depth knowledge of peptide processing, tissue specific peptide expression and modification, as well as structure-function relationships will shed light on how a variety of sensory, metabolic and endocrine signals are integrated at the molecular level to allow the hypothalamus to control mammalian reproduction.

Acknowledgements

The authors wish to thank Dr Tamara Leahy and Dr Gene Wijffels for providing helpful feedback and advice on the manuscript.
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Neuropeptidomics applied to studies of mammalian reproduction


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