Extending the scope of neuropeptidomics in the mammalian brain

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Abstract
Neuropeptides are signaling molecules of intermediate size that are involved in neurotransmission and endocrine regulation. Complete monitoring of neuropeptides using neuropeptidomics approaches remains an important goal for describing targeted physiological regulation pathways. Considerable effort has been expended, particularly in terms of technique and methodology development, to extend the scope of neuropeptidomics. The capability of peptide characterization has been gradually improved, thus responding to increasing demands for broad detection and determination of various peptides. In this review, we discuss some achievements for the improvement of peptide identification coverage and their application for brain diseases and studying consequences of drug applications.

1. Introduction
The role of neuropeptides continues to receive great attention in the fields of basic neuroscience as well as clinical and pharmaceutical research. As molecules of a size falling between proteins and classical neurotransmitters, neuropeptides carry out specific functions in diverse physiological processes and pathways [1]. For example, neuropeptides can act on G-protein coupled receptors, generating intracellular cascades and subsequently leading to response of cells, microcircuits, organs and ultimately behaviors. They are indispensable for species from worms to humans, for example in the regulation of sleep, food-intake, sexual function and reward processing. Importantly, neuropeptides seem to never work alone, but one generally finds that a set of neuropeptides, rather than a single neuropeptide, is involved in a given brain disorder or physiological pathway [2,3]. Without doubt, a complete characterization of the neuropeptideome will be of great interest for studying brain functions, discovering biomarkers or characterizing drug effects.

Mass spectrometry (MS)-based neuropeptidomics, as a sub-branch of proteomics, has now emerged as the method of choice for high throughput sequencing of endogenous neuropeptides in biological samples [4–6]. Compared to proteomics that focuses on characterization of digested peptides, neuropeptidomics shares many technical implementation aspects but also clearly requires some different methodologies due to the biological and chemical properties of endogenous peptides. As particular neuropeptides can have distinct bioactive effects, even if they are derived from the same precursor, the characterization of every member of an entire neuropeptidome becomes highly desirable in many cases for example in the discovery of biomarkers. In addition, many neuropeptides have long sequences containing often more than thirty amino acids, and exhibit low informative tandem MS spectra as...
well as high hydrophobicity. These features inevitably present substantial challenges upon many procedures such as peptide extraction, separation, identification and quantitation [7], which in turn has stimulated technical and methodological development efforts for more powerful neuroproteomics approaches [8,9].

In the last decade, extensive efforts have been expended to improve the peptide identification coverage by developing novel methods and have yielded exciting advances [10–17]. Current integrated peptidomics approaches allow the identification of hundreds of prohormone-derived peptides even from tiny amounts of body fluid or tissue samples [15,18,19]. The species investigated have also been extended from the common laboratory animals such as mice and rats to uncommon species including tree shrew [14], bee [3] and crab [20]. Very recently, the application of quantitative neuroproteomics has received increased interest for characterizing neuro peptide changes in studies of central nervous system diseases or drugs. This review reports advances in neuroproteomics, with a particular focus on the improvement of neuropeptide identification and its biological applications.

2. Increased peptide identification coverage in neuroproteomics

2.1. Sample treatment

Sample treatment is an initial but crucial step that remarkably affects peptide identification coverage. Neuropeptides, as well as various proteins, are subject to rapid proteolysis under room temperature. This degeneration results in the decrease or even disappearance of neuropeptides from tissue samples. Moreover, the degeneration of proteins produces a large number of peptide fragments. The presence of these fragments complicates the LC–MS/MS analysis because fragments compete with native neuropeptides during sample extraction, peptide ionization and fragmentation. Previous studies have shown that preparation of brain samples from live animals after euthanasia without deactivation of enzymes results in the production of a large amount of neuropeptide fragments. Different techniques, such as snap-freezing, microwave heating and laser heating, have been developed to reduce peptide degeneration [21,22]. While snap-freezing is convenient to use, the stability of the treated tissue is generally poor, and often insufficient for quantitative analysis. In contrast, heating using microwave or laser allows the production of high quality stabilized samples because enzymes are permanently deactivated, protecting endogenous peptides from proteolysis. Particularly, the laser heating system is easy to conduct and maintains the morphology of the tissue much better than microwave heating. Intact morphology is useful for precise extraction of brain regions of interest, particularly when relatively small nuclei are targeted. Peptide extraction and subsequent treatment is another procedure that affects identification rate in neuroproteomics. Brain samples contain a vast number of peptides, which have extremely different abundances, lengths, hydrophobicities and chemical properties. The use of different solvents and/or procedures has pronounced effects on peptide profiles detectable in brain tissue samples. The extraction or preservation of diverse neuropeptides in a single solution, for example the commonly used aqueous buffers [2,4], results in a substantial loss of peptides [23]. Similar phenomena were found during direct mixing of different solutions in a multi-stage extraction procedure [7]. Alternatively, a mixing on column strategy, which serially loads organic and aqueous extracts, allows simultaneous retention of a large number of hydrophilic and hydrophobic peptides on the column, consequently increasing the peptide identification rate [7].

2.2. Targeted and directed LC–MS

High accuracy MS has become a method of choice in neuroproteomics, mainly due to the presence of many long peptides in the central nervous system. The use of high accuracy measurements of both precursor and productions can provide an informative and promising approach for peptide sequencing, thus facilitating the improvement in the assignment of fragment ion series [15,24,25]. However, the use of high resolution MS is only part of the solution, because the overall peptide identification coverage critically depends on the capability of a method for the fragmentation of low abundant peptides.

In a typical LC–MS/MS analysis, many hundreds or even thousands of peptide features can be detected in MS1, leading crowded peptide peaks in a limited elution time (Fig. 1A). Tens or even hundreds of peptide features are thus present within several seconds (Fig. 1B), which far exceed the fragmentation capability of a mass spectrometer, in particular for those operated with FT-MS/MS mode. In a common data dependent LC–MS/MS analysis, peptides of highest intensities are submitted to fragmentation within an acceptable scan period. This makes the sequencing of low abundant peptides in a single or repeated data-dependent LC–MS/MS analysis difficult due to the limited scan speed of current LC–MS/MS systems, even if precautions like dynamic exclusion are used [26]. To deal with these issues, directed or targeted LC–MS/MS have been developed to guide the features into fragmentation, by which high quality tandem MS spectra could be acquired for a vast number of peptides or desired peptides.

A targeted LC–MS/MS analysis strategy allows the MS/MS analysis on precursor ions of interest during analysis of complex biological samples (Fig. 2). As only specified ions are selected for fragmentation, MS/MS parameters, for example resolution and maximum accumulation time in FT-MS systems, can be optimized and thus increase the capability of generation of high quality tandem MS spectra. Illustrating this advantage, targeted LC–MS/MS analysis has been applied in the tree shrew neuropeptidome analysis to screen neuropeptides that are identical to their counterparts in one or more related mammalian species [14]. Inclusion mass lists of previously reported peptides identified from other species including mice, rats, and humans were thus submitted to LC–FT-MS/MS analysis. The application of targeted LC–MS/MS analysis allowed the identification of several classical neuropeptides, despite the fact that they exist in the tree shrew brain at low concentrations.

Compared to targeted LC–MS/MS analysis, directed peptide sequencing strategy facilitates a comprehensive MS/MS analysis on non-redundant precursor ions and thus increases
Fig. 1 – Representative LC–MS chromatogram of a brain extract. (A) A large number of peptide features can be detected within a 80-min gradient. (B) A representative MS² spectrum of peptide features collected within a 10 s interval.

Fig. 2 – Schematic illustration of targeted and directed LC–MS/MS analysis for a sample containing features with similar retention times on an LC column. Rt: retention time, CSD: charge state dependent.

the depth of analysis [26,27]. A directed LC–MS/MS analysis is conducted using an inclusion mass list of precursor ions which can be prepared based on analysis of the full scan LC–MS data that are acquired from the same sample in advance using the identical LC elution profile. The precursor ions are selected according to retention time and intensities of peptides in LC–MS analysis. For example, a proteomics study has demonstrated that when inclusion lists are divided into segments of 3–5 min, the number of possible target masses can increase to as many as 3000 in a 1-h LC
gradient, leading to a significant increase in the identification coverage [26].

Charge state directed (CSD) LC-MS/MS analysis is a variant of commonly used directed LC-MS/MS analysis strategy, which is performed by scanning peptides according to their charge states rather than using dedicated inclusion mass lists. Although a directed LC-MS/MS analysis can significantly increase the capability of detection of low abundant peptides, some peptides are still unable be sequenced if too many other peptides co-elute at the same retention time. This is particularly true when using the low scan speed instrument such as FT-MS spectrometers, which has lower sensitivity and slower scan speed than low resolution MS/MS conducted in other detectors such as ion traps [28]. By comparison, CSD LC-MS/MS analysis demonstrates its advantage if just the peptides of a specified charge state(s) are fragmented in a LC run. As the targeted precursor ions are much reduced, optimized MS/MS parameters can be applied for acquisition of high quality tandem MS spectra for low abundant peptides. After the LC-MS/MS analyses of peptides of all desired charge states, the data from a given sample are pooled for database search. Although repeated LC-MS/MS analyses are required, CSD-LC-MS/MS analysis reduces the time needed for generation of inclusion lists in a commonly used targeted LC-MS/MS analysis. The application of such approach allows 206 peptides on average to be identified from mouse prohormones in a single brain sample that was extracted using 15 μl solutions per 1 mg of tissue. The cumulative number of peptides identified from three brain samples reached a value of 272 [18].

2.3. Complementary fragmentation approaches

Collision induced dissociation (CID) has become the method of choice for peptide identification in neuropeptidomics. Yet, identifications of the intact forms of long peptides are still difficult even with high resolution MS. Now this issue has been alleviated due to the implementation of alternative fragmentation techniques such as high energy collision dissociation (HCD) and electron transfer dissociation (ETD). Compared to CID, HCD offers more informative tandem MS spectra, thus increasing the peptide identification. ETD is a highly charge state-dependent fragmentation technique and is more suited for precursor charge states higher than 2, which is useful for the identification of large neuropeptides. Because of the complementarities, the combined use of ETD and other fragmentation techniques, particularly ETD and HCD, has remarkably improved the identification capability of peptides in the top-down sequencing strategy [29].

2.4. Neuropeptidomics of uncommon mammalian species

Although massive progress in neuropeptidomics has been achieved on common laboratory mammalian species, the analysis of complete neuropeptidome of uncommon species remains challenging because of the lack of proteome for direct database search. When the genome available for a particular species, the prohormone sequences can be predicted from the genome and consequently a database can be created for sequencing peptides from MS data, as has been done for example in Apis mellifera [30] and Zebra finch [31]. For some species without a fully sequenced genome, the identification of peptides mostly depends on de-novo sequencing, which is normally done at low throughput and is more suited for short peptides or peptides of highly informative MS spectra. To deal with issue, databases can be developed based on the collection of prohormones from related species. Such a database can be used for direct peptide sequencing or homology search, which has been successfully conducted for example in tree shrews. Tree shrews are similar in size to rats but phylogenetically very close to primates [32,33]. To date, neither a complete genome nor proteome is available for this species. Due to the highly conserved nature of neuropeptides [34], the majority of neuropeptides in tree shrews have identical or similar sequences to those in related mammalian species. Based on this principle, FT-MS/MS data from tree shrew brain were search against a predicted peptide database, which consists of predicted peptides from prohormones collected from related species in the Euarchoptera clade. Using this approach, 92 peptides that have identical sequences to those from one or more other species can be directly sequenced using database search. Meanwhile, 15 peptides with substituted amino acid residues were identified using homology search based on de novo sequencing results.

3. Neuropeptidomics for the investigation of CNS diseases and drug responses

Neuropeptides have been commonly quantified using immunoassay or radioimmunoassay [35,36] in the discovery of markers of brain diseases or in the interpretation of pharmaceutical mechanisms. However, this procedure is time-consuming, limited to known peptides, and it is often impossible to distinguish between similar peptides. Moreover, the throughput of immunoassays is extremely low, and limited to one or several peptides in practice. In contrast, quantitative neuropeptidomics can simultaneously provide a complete image of many peptides in the brain region of interest and thus has been increasingly used. Mass spectrometric approaches offer several advantages including higher sensitivity and throughput compared to traditional methods [37-42].

The performance of quantitative neuropeptidomics is highly approach-dependent. Currently, selected reaction monitoring (SRM), chemical labeling, label-free monitoring and are the three most commonly used approaches for quantify peptides [43-45]. SRM-based quantitation was initially developed for the analysis of small molecules, but now it has been adapted for the quantitation of a large number of peptides with monitoring of multiple transitions for each feature. SRM-based analysis is highly sensitive and is thus expected to be increasingly used in neuropeptidomics, although it requires extra informatics work to determine the transitions of tens to thousands of peptides and, correction of the retention time to make the quantitation reliable across different samples or different columns. Chemical labeling is a widely used method for relative quantitation. While the labeling performance depends on the agent used and chemical properties of peptides, the use of chemical tags, such as
trimethylammoniumbutyryl (TMAB) [46] and N,N-dimethyl leucines [47], considerably enhances the precision and even sensitivity of quantitation. Compared to chemical labeling methods, label-free quantitation is substantially less precise but offers broader scope for monitoring various peptides, in particular those exhibiting relatively large changes between investigated conditions. Nevertheless, because of its convenience and low-cost, label-free quantitation is increasingly used for monitoring peptide changes during drug application or disease diagnostics.

The application of quantitative neuropeptidomics has generated exciting achievements in the research of various brain diseases. For example, a neuropeptidomics approach has demonstrated its power in detecting, identifying, and simultaneously quantifying the levels of a considerable number of endogenous peptides, including known and novel ones in an animal model of Parkinson disease [2], revealing alterations of secretogranin-1, somatostatin, prodynorphin, and cholecystokinin peptides in the striatum of the animals affected by Dopamine depletion in an experimental model of the disease. Neuropeptidomics studies using labeling techniques have also been performed on animal models of obesity, and have shown that a number of hypothalamic peptides including neuropeptide Y, enkephalins, Melanin-concentrating hormone (MCH), and α-melanocyte-stimulating hormone (α-MSH) are involved in the regulation of body-weight and food-intake [46,48].

The application of neuropeptidomics in the field of CNS drug research has promoted the discovery of diverse neurochemicals that are involved in neuronal circuits. For example, general anesthesia during a neurosurgical procedure was shown to strongly impact the neuropeptide brain system, potentially reinforcing effects of general anesthesia. Along these lines, Fouillen and colleagues have performed a neuropeptidomics study in tree shrew hypothalamus during volatile isoflurane/nitrous oxide anesthesia administrated accompanying a neurosurgical procedure. An up-regulation of 12 hypothalamic peptides was detect, 6 from opioid family and 6 from other families [49], revealing novel neuropeptides whose levels are affected during the surgical procedure under general anesthesia.

Peptidomics analyses in rats have demonstrated the involvement of peptides in the process of neural adaptation after repeatedly exposure of amphetamine, a psychological stimulant [50]. The peptidome analysis was performed for different brain areas like dorsal striatum (dSTR), nucleus accumbens (NAcc) and prefrontal cortex (PFC) of rats that received amphetamine over 19 days. Among the 150–300 peaks detected using MALDI-MS, peptides like neuregulin, stathmin or thymosin beta-4 were found to be significantly altered by the drug treatment. These marker peptides are derived from proteins that have been recognized for their role in the neuroadaptation and drug-induced behavioral sensitization. These results show that after chronic treatment with amphetamine there are chemical changes in neuronal organization and pathways not only related to dopamine in the mesolimbic system. In general, the behavioral sensitization is associated with complex chemical adaptations in the brain reward circuit in pathways that regulate energy/metabolism, neurotransmission and neuroprotection.

Neuropeptidomics has also produced novel insights related to neurochemical alterations following chronic nicotine treatment [51]. The chronic use of nicotine, the main psychoactive ingredient of tobacco, alters diverse physiological processes and consequently generates physical dependence. The treatment with chronic nicotine for three months led to moderate changes in the levels of endogenous peptides, including not only five enkephalin opioid peptides up-regulated, but also 9 non-opioid peptides, in the dorsal striatum. The regulation of chronic nicotine treatment on the multiple peptidergic systems implies the involvement of addictive, metabolism and endocrine pathways in the preoccupation/craving phase of drug dependence. This finding linked for the first time other classes of neuropeptides with the chronic nicotine treatment and may reflect a neurochemical adaptation or compensation system for deficit of other neurotransmitter like dopamine, acetylcholine or serotonin.

4. Conclusion and future perspectives

After more than one decade development, MS-based neuropeptidomics has greatly extended its scope for monitoring endogenous peptides present in diverse species. Continuous efforts continue to be expended for the identification and quantitation of peptides, in particular those of long sequence and/or low abundance. Following the advances of neuropeptidomics, the qualitative and quantitative approaches will be transferred to various biological fields. Its application is expected to yield insights in the understanding of neurochemical communication of neurons in various behaviors such as food-intake, sleep, sexual function, learning and memory. As neuropeptides are crucial molecules involved in various physiological processes, they at the same time represent important candidates of biomarkers for diseases and potential drug targets for therapeutic interventions. Taken together, many exciting achievements can be anticipated in the near future in neuropeptidomics applications in the central nervous system.

Transparency document

The Transparency document associated with this article can be found in the online version.

REFERENCES


