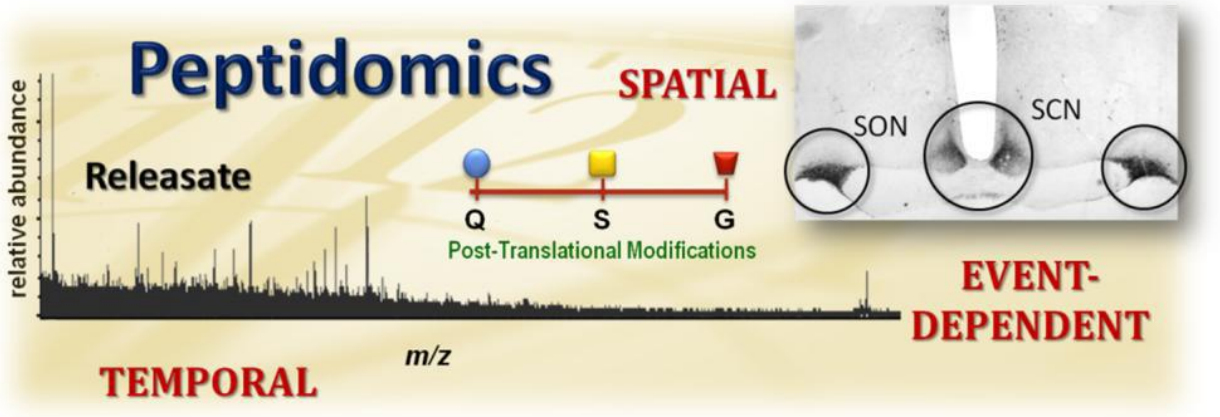
Direct Cellular Peptidomics of Hypothalamic Neurons



Research highlights

► Technological advances enable suites of peptides from hypothalamic neurons to be identified. ► Analytical chemistry protocols permit direct, high-resolution, high through-put peptide profiles. ► Secreted peptides can be measured in spatial, temporal, andstimulation-dependent contexts. ► A peptide must be both released and bioactive to be a *bona fide* neuropeptide. ► Peptidomic discovery reveals unanticipated complexity andpreviously unknown key peptides.

Frontiers in Neuroendocrinology 32 (2011) 377–386

Contents lists available at ScienceDirect

Frontiers in Neuroendocrinology

journal homepage: www.elsevier.com/locate/yfrne

Review

Direct cellular peptidomics of hypothalamic neurons

Jennifer W. Mitchell [a], Norman Atkins Jr. [b,1], Jonathan V. Sweedler [b,c,d], Martha U. Gillette [a,b,d,e,*]

[a] Dept. of Cell & Developmental Biology, University of Illinois at Urbana-Champaign, Urbana, IL, USA
[b] Neuroscience Program, University of Illinois at Urbana-Champaign, Urbana, IL, USA
[c] Dept. of Chemistry, University of Illinois at Urbana-Champaign, Urbana, IL, USA
[d] Dept. of Molecular & Integrative Physiology, University of Illinois at Urbana-Champaign, Urbana, IL, USA
[e] College of Medicine, University of Illinois at Urbana-Champaign, Urbana, IL, USA

ARTICLE INFO

Article history:
Available online 18 February 2011

Keywords:
Arginine vasopressin (AVP)
Gastrin-releasing peptide (GRP)
Hypothalamus
Little SAAS
Mass spectrometry (MS)
Neuropeptide
Peptidomics
Suprachiasmatic nucleus (SCN)
Supraoptic nucleus (SON)
Vasoactive intestinal peptide (VIP)

ABSTRACT

The chemical complexity of cell-to-cell communication has emerged as a fundamental challenge to understanding brain systems. This is certainly true for the hypothalamus, where neuropeptide signals are heterogeneous, localized and dynamic. Thus far, most hypothalamic peptidomic studies have centered on the entire structure; however, recent advances in collection strategies and analytical technologies have enabled direct, high-resolution peptidomic profiles focused on two regions of interest, the suprachiasmatic and supraoptic nuclei, including their sub-regions and individual cells. Suites of peptides now can be identified and probed for function. High spatial and analytical sensitivities reveal that discrete hypothalamic nuclei have distinct peptidomic signatures. Peptidomic discovery not only reveals unanticipated complexity, but also peptides previously unknown that act as key circuit components. Analysis of tissue releasates identifies peptides secreted into the extracellular environment and available for transmitting intercellular signals. Direct sampling techniques define peptide-releasate profiles in spatial, temporal and event-dependent patterns. These approaches are providing remarkable new insights into the complexity of neuropeptidergic cell-to-cell signaling central to neuroendocrine physiology.

1. Introduction

The hypothalamus comprises discrete brain nuclei that regulate and coordinate homeostatic processes, which range from osmotic balance, hunger/satiety and reproductive state to circadian rhythms and sleep. Hypothalamic nuclei, such as the suprachiasmatic nucleus (SCN) and the supraoptic nucleus (SON), are richly peptidergic [1,14]. The physiological functions of these nuclei—circadian regulation by the SCN and water balance, reproduction and affiliative behavior by the SON—depend upon intrinsic cell-to-cell neuropeptide signaling as well as at afferent and efferent innervations.

Several features of neuropeptides make them difficult to study. Peptide gene transcripts encode large prepropeptides, which undergo significant cell type-dependent processing and cleavage. Thus, prepropeptide mRNA expression does not allow one to predict which functional peptides will be produced from the gene. Neuropeptides are difficult to characterize biochemically, are physiologically active at a range of concentrations, some very low, and exhibit broad bioactivity across heterogeneous brain regions, especially throughout the neuroendocrine systems. Unlike classical neurotransmitters, where only one type is expressed per neuron and expression can characterize a brain region, multiple neuropeptides can be expressed in an individual neuron [44], processing products may be targeted differentially to distal cell regions [63] and a brain nucleus often expresses diverse neuropeptides in adjacent neurons [8]. Neuropeptides are released differentially with co-localized classical neurotransmitters, which significantly elevates the complexity and subtlety of neural signaling [39]. For these reasons, increasing our understanding of the full complement of neuropeptide signals is of significant interest to the field of neuroscience systems biology.

Processing the prepropeptide into functional neuropeptides occurs in multiple steps (Fig. 1). The prepropeptide transcriptional product contains both a signal sequence of amino acids and the sequences that will become functional neuropeptides. The signal sequence targets the molecule to the endoplasmic reticulum, where the NH_2-terminal signal peptide is cleaved. The resulting proneuropeptide transits to the Golgi system where it enters the secretory pathway via dense core vesicles (Fig. 1A). Because dense core vesicles lack synaptic docking proteins, which restrict release of clear vesicles containing classical neurotransmitters to synaptic release sites, neuropeptide release can occur at multiple sites in the cell.

* Corresponding author. Address: Dept. of Cell & Developmental Biology, University of Illinois at Urbana-Champaign, 610 S. Goodwin Ave., Urbana, IL 61801, USA. Fax: +1 217 244 1648.
E-mail address: mgillett@illinois.edu (M.U. Gillette).
[1] Present address: Center for Sleep & Circadian Biology, Northwestern University, Evanston, IL, USA.

0091-3022/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.yfrne.2011.02.005

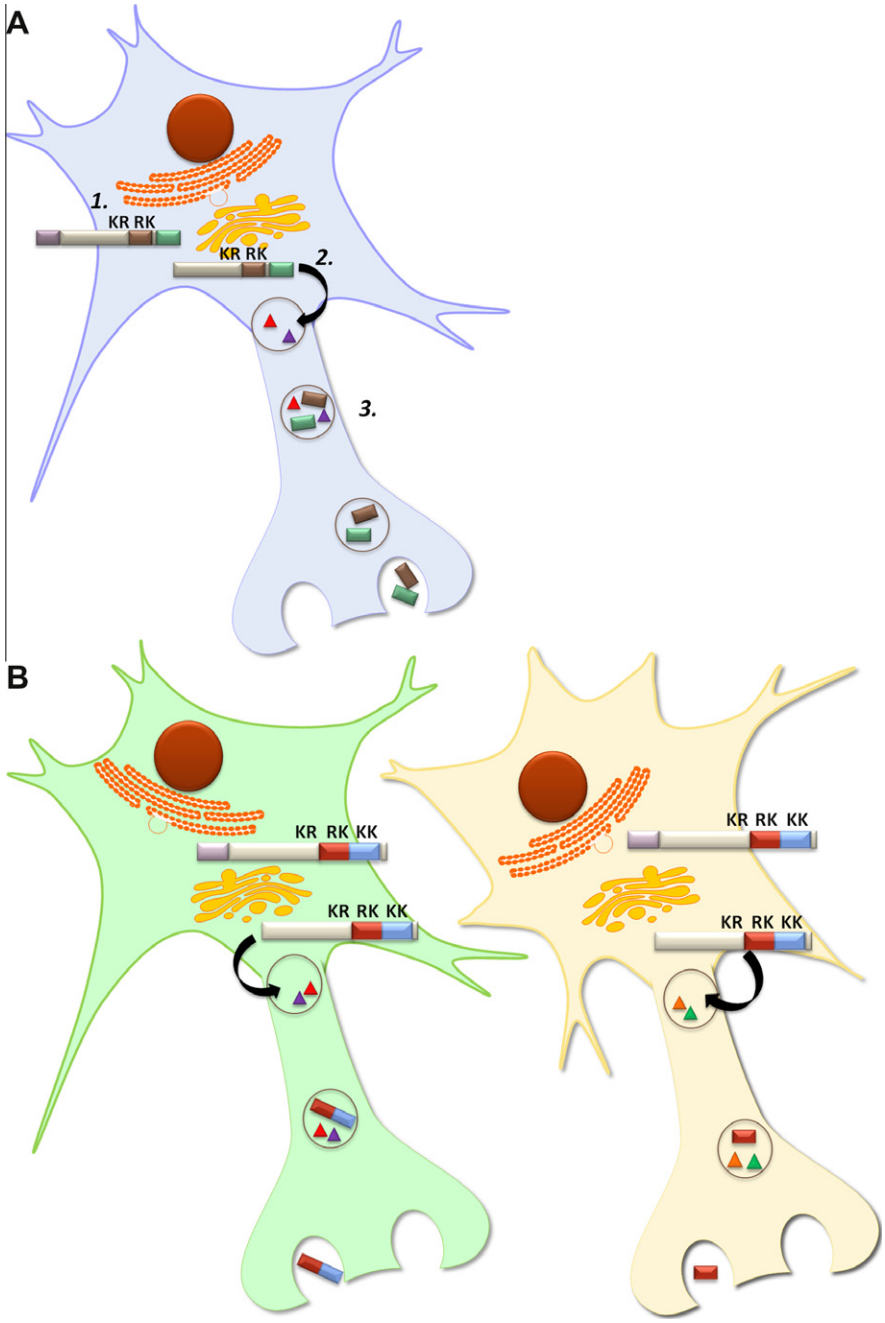


Fig. 1. Prepropeptide precursors undergo sequential steps of processing during which the peptide is generated and targeted. (A) Peptides are produced from larger precursors in multiple steps. 1. Proteolytic processing begins in the endoplasmic reticulum (ER) when the NH_2-terminal signal peptide is cleaved by signal peptidase. 2. The proneuropeptide is routed to the Golgi apparatus and packaged into dense-core secretory vesicles together with processing proteases, termed convertases (colored triangles). 3. As the secretory vesicle matures, proteolytic processing occurs, usually at dibasic cleavage sites (KK, KR, RK). The mature secretory vesicle contains fully processed, biologically active peptide ready for stimulus-initiated secretion by exocytosis. (B) Proneuropeptides may undergo tissue-specific processing by different convertases to generate distinct peptides. The processes illustrated here have been extensively studied [41,51,52,67].

Sequential processing steps convert the proneuropeptide into a collection of neuropeptides within a single vesicle. These proneuropeptide cleavages occur under the control of an array of endogenous intracellular propeptide convertases, which may differ between vesicles so that neuropeptide contents also differ (Fig. 1B).

A single proneuropeptide often produces multiple neuropeptides. Moreover, proneuropeptides may not be processed the same way in different cell types or even within the same cell type under different conditions. Cleavages can occur in a tissue-specific and even region-specific manner. Although proneuropeptides are predominantly cleaved at dibasic residues (lysine–arginine), only a small percentage of dibasic sites actually are cleaved [5]. This is noteworthy because post-translational cleavage and post-translational modifications (PTMs) can affect binding affinities, as well as regulate the bioactivity and stability of the neuropeptide. Antibody-based methods of detecting neuropeptides often cannot discriminate these subtle differences between peptides; therefore, immunohistochemical approaches are inadequate for comprehensively evaluating PTMs and alternate forms of known neuropeptides. They also require *a priori* information regarding potential peptide expression and, thus, are incapable of exhaustively examining the full peptide complement of peptidergic inputs, intrinsic

components and outputs that form brain networks. An emerging discipline that successfully addresses many of these limitations is *peptidomics*, a rapidly developing suite of technologies used in peptide investigation.

2. The evolution of peptidomics, from indirect to direct discovery

The potential for understanding functional differences in neuropeptides necessitates that their exact final form be determined. Cleavage prediction programs such as NeuroPred [61] are useful, especially in determining site-specific cleavage vs. extracellular degradation, but are limited by the incomplete database. Even if sequence information exists on the gene products expressed, only a list of theoretical [and/or] potential peptide products can be generated—not an accurate list of final peptide products. The final complement of peptides depends on the processing enzymes present. This emphasizes the need for direct measurement of peptides based upon their physical properties.

Each contemporary method of peptide analysis has relative merits and limitations (Fig. 2), as outlined below. While tedious large-scale biochemical isolations were used for the initial peptide discoveries, the current wealth of understanding about peptide chemistry, localization, physiology, functional context and pharmacology has been generated by, or has its origins in, information obtained by two indirect approaches, *in situ hybridization* and *immunohistochemistry*. Key points for consideration relevant to these two approaches are listed here.

(1) *In situ* hybridization and immunohistochemistry require *a priori* knowledge of a potential neuropeptide in order to generate sequence-specific anti-sense probes or antibodies. It follows that there may be neuropeptides not yet known that are important to brain function.

(2) These methods of detection are *indirect*, based on probes designed to be complementary to specific sequences of the prepropeptide mRNA or the peptide itself. They assume that the sequence is unique to the molecule of interest and binds with high affinity. A range of rigorous controls are required to demonstrate the specificity of the probe.

(3) The probe reports only that the complementary sequence is present, but does not report the actual size or full identity of the peptide. Nor does it reveal whether there are other peptides/proteins bearing a similar recognizable sequence. Antibody-binding may be altered or impeded by post-translational modifications (PTMs) or conformational changes in the peptide.

(4) *In situ* hybridization and immunohistochemistry are relatively low-throughput, with probes to only a limited number of peptides processed concurrently to determine relative localization.

(5) To be detected, bound probe must be amplified, which may introduce non-specific signals. A range of rigorous controls is required to demonstrate the lack of non-specific signals due to amplification.

(6) *In situ* hybridization and immunohistochemistry provide essential information regarding localization within the complexity of brain structure. Such approaches are the basis of functional neuroanatomy and neuropharmacology, with far-reaching fundamental and clinical consequences. There presently are no other methods that offer this type of spatial information.

Peptidomics is a newer, direct, discovery-based approach that uses mass spectrometry (MS) to provide an unbiased analysis of the peptides present at sufficient concentrations to be detected. Its performance depends on two key factors, the sampling methods and mass spectrometric instruments used (Fig. 2), with these per-

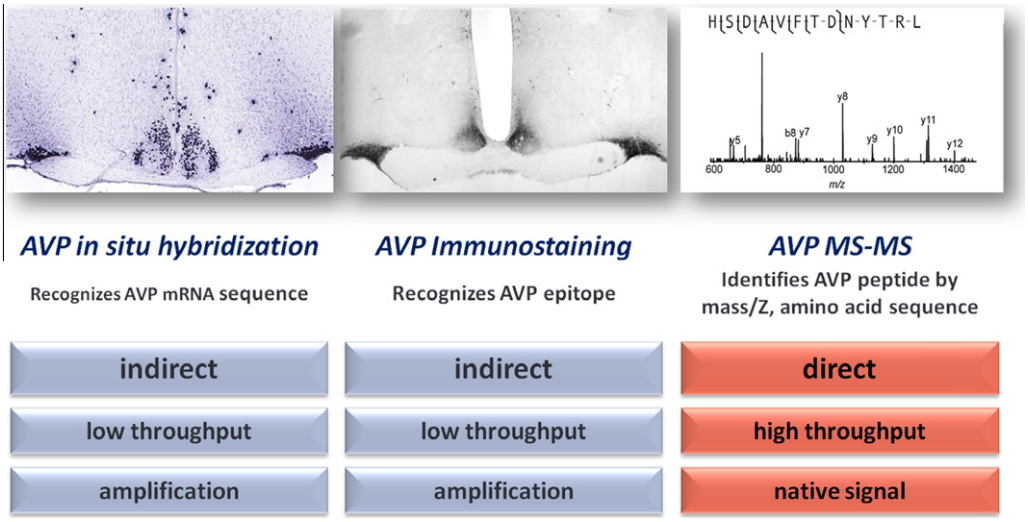


Fig. 2. Comparison of methods for arginine vasopressin (AVP) analysis and discovery: indirect vs. direct. Neuropeptides expressed in hypothalamic nuclei and neurons can be identified and analyzed by multiple methods. Each is based on different probes/reporters and assumptions. *In situ* hybridization (mouse [4]) and immunohistochemistry (rat) require *a priori* knowledge of the AVP mRNA and neuropeptide sequences, respectively, to generate complementary probes and antibodies. If the sequence is not unique, non-specific binding may occur. Mass spectrometry (MS) allows identification based on mass, charge and amino-acid sequence without prior information about neuropeptide sequence or content; however, spatial information is limited. Compared with indirect methods, direct MS-based analysis is high throughput: many peptides can be identified from one tissue isolation and their identities do not need to be known beforehand. Indirect methods of analysis are based on probes to specific sequences of the mRNA for the prepropeptide or peptide itself. They assume that the sequence is unique to the molecule of interest and that the probe binds with high affinity. A range of rigorous controls is required to demonstrate the specificity of the probe. The bound probe must be amplified to be detected, which may introduce non-specific signals. The probe reports only that the complementary sequence is present, but does not report the actual size or full identity of the peptide. Antibody-binding may be altered or impeded by post-translational modification or conformational changes in the peptide. In comparison, MS methodologies report the absolute physical identities of discrete components of the non-amplified native signal. For protein identification by MS, MS–MS is key. While MS provides an accurate measurement of the mass-to-charge of a peptide, MS–MS involves accurately measuring the mass of the peptide, fragmenting it, and then using the masses of the fragment ions to "sequence" the original peptide. The nomenclature of the fragment ions is specific to MS–MS and detailed in http://www.asms.org/whatisms/index.html and http://www.magnet.fsu.edu/education/tutorials/tools/.

formance details described in greater detail in several recent reviews [24,33,45,65]. These two aspects are critical for they determine the quality and completeness of the peptide profiles detected. Key features of MS-based peptidomics include the following.

(1) MS allows the confident identification of sample components without requiring prior information and, thus, has the potential to identify previously unknown and novel peptides.
(2) In contrast to anti-sense RNA probe- and antibody-based methods, peptidomics analysis is *direct*. It is capable of detecting post-translationally modified peptides, as long as the modifications are preserved during the extraction and measurement processes. This makes direct analysis by MS critical to identification of the full functional neuropeptidome.
(3) MS methodologies report the non-amplified native signal. In contrast to bottom-up proteomics where the proteins are digested via enzymes added to the samples [12], neuropeptidomics does not use digestive enzymes to generate fragments. The benefit is that the native forms of peptides are identified directly, and the native forms include the PTMs [24,33,59]. Elegant approaches now allow quantitative comparisons of the peptidome between two treatments, such as the work of Fricker measuring the role of various processing enzymes in hypothalamic peptide processing [50,70]. Obviously, for meaningful results, as many peptides as possible need to be identified from the tissue of interest [33].
(4) Compared with indirect methods, direct MS-based analysis is high throughput: many peptides can be identified from one tissue isolation. This includes identifying peptides derived from the same proneuropeptide, as the different final peptide products may have distinct physiological functions [9,67]. Because peptidomic analysis does not involve signal amplification, detectability is an issue. Detection requires sufficient starting material and sensitive mass spectrometric measurement approaches [7,10,46,71]. Some limitations are technological/instrumental in nature and these can directly affect the ability to characterize specific peptides; for example, the optimum method used to ionize the peptide can be both sample-type and peptide-sequence specific [7,46,71].
(5) A number of chemical and enzymatic changes in peptides occur, so that care must be taken to prevent changes in the sample during isolation and fractionation. Protein degradation can significantly hinder peptidomic analysis in two ways. First, highly abundant proteins can be degraded into peptides that interfere with the characterization of the less abundant peptides. Second, the peptides themselves can be degraded. The first solution to this issue is to make sure the sampling and extraction approaches minimize degradation. This can be done by heating the sample via microwave irradiation [9,28,29] or by rapid tissue isolation and boiling [27]. Other solutions include choosing tissues that are relatively high in peptides and low in degradative enzymes, such as the pituitary [20,25]. One can work with living brain slices. Although they may undergo protein/peptide degradation immediately after isolation, brain slices recover so that the peptides can be measured [37]. Another is preferentially enriching peptides either through the use chromatographic fractionation [18,32] or solid phase extraction (SPE) [37] (see below). This can help relieve the limitation of a low sample volume that may preclude direct sampling techniques.
(6) High-confidence identification based on physical properties discerned via MS-based peptidomics provides essential characterization of peptides within the complexity of the brain structure. There are few other methods that offer this type of fine-grained analytical information. The most common method of identification is the process of fragmenting the peptide in the mass spectrometer and examining the pieces to determine the original structure (so called tandem MS or MS–MS) [7,46,71].
(7) A disadvantage of the MS methods is the expense of the required specialized equipment; depending on constraint, a cost-benefit analysis may demonstrate a lower cost when normalized to the number of individual peptides analyzed. Similarly, optimum use of MS requires special approaches and training, although in some ways, this is no different than the training required for other complex tasks, such as cell physiology or brain slice preparation.

Multiple types of mass spectrometric methods are utilized for peptidomic and proteomic studies. The specific instrument used determines the quality of the information obtained. For evaluations of the available approaches and challenges of MS-based measurement in the analysis of brain peptides, see [2,45]. The MS approaches employed for direct peptidomics of hypothalamic nuclei and neurons are outlined in Fig. 3. At the most fundamental level, a mass spectrometer consists of (1) an ionization approach that vaporizes/ionizes the analyte (the peptide, in this case) without modifying it, (2) a mass analyzer that separates/encodes the ions based on their mass-to-charge ratio (m/z), and (3) a detector that registers the number of ions at each m/z value. The result is a mass spectrum, with each peak representing an analyte at a specific m/z and the peak height representing the relative amount of the analyte. Peptides that are normally considered similar (i.e., AVP and oxytocin) have unique amino-acid sequences, and thus have unique m/z values and are distinguishable.

Prior to ionization, the sample analyte can be concentrated, purified, or fractionated. This isolates peptides of interest, removes extraneous material, as well as increases the likelihood that the peptides reach a detectable threshold for analysis. Electrospray ionization (ESI) and matrix-assisted laser desorption/ ionization (MALDI) are the most common ionization approaches for peptides. Each has a distinct set of advantages/disadvantages for peptide measurements, such as types of samples (solid or liquid), sample volumes, ease of interfacing to specific fractionation approaches, and so oftentimes having both available leads to the least restrictions on neuropeptide research.

Mass analyzers with appropriate sensitivity, resolution and mass accuracy for peptidomic studies include a veritable alphabet soup of types—the ion trap (IT), time-of-flight (TOF), and Fourier transform ion cyclotron resonance (FTICR). Each mass analyzer has distinct operating principles and so has its own advantages and disadvantages. Rather than outline these in detail, interested readers are referred to http://www.asms.org/whatisms/index.html and http://www.magnet.fsu.edu/education/tutorials/tools/. If one has only a specific instrument available, then experiments must be designed accordingly with these strengths and weaknesses of each in mind. Differences in amino acid composition endow different peptides with unique mass and charge characteristics. Consequently, by combining these complementary approaches, we can enhance the completeness of the proteomic profile.

The evolution of peptidomics research has been aided by recent technical advances that have increased the overall sensitivity of the analysis. Peptidomics has moved beyond the generation of lengthy lists of compounds towards obtaining spatial, temporal, and functional information for peptides within discrete brain regions and cells. These studies have included comparative measurements in normal and pathophysiological states [6,21], dialysis sampling of the extracellular environment [11,30,35,40], and MS-based imaging [15–17,55,62].

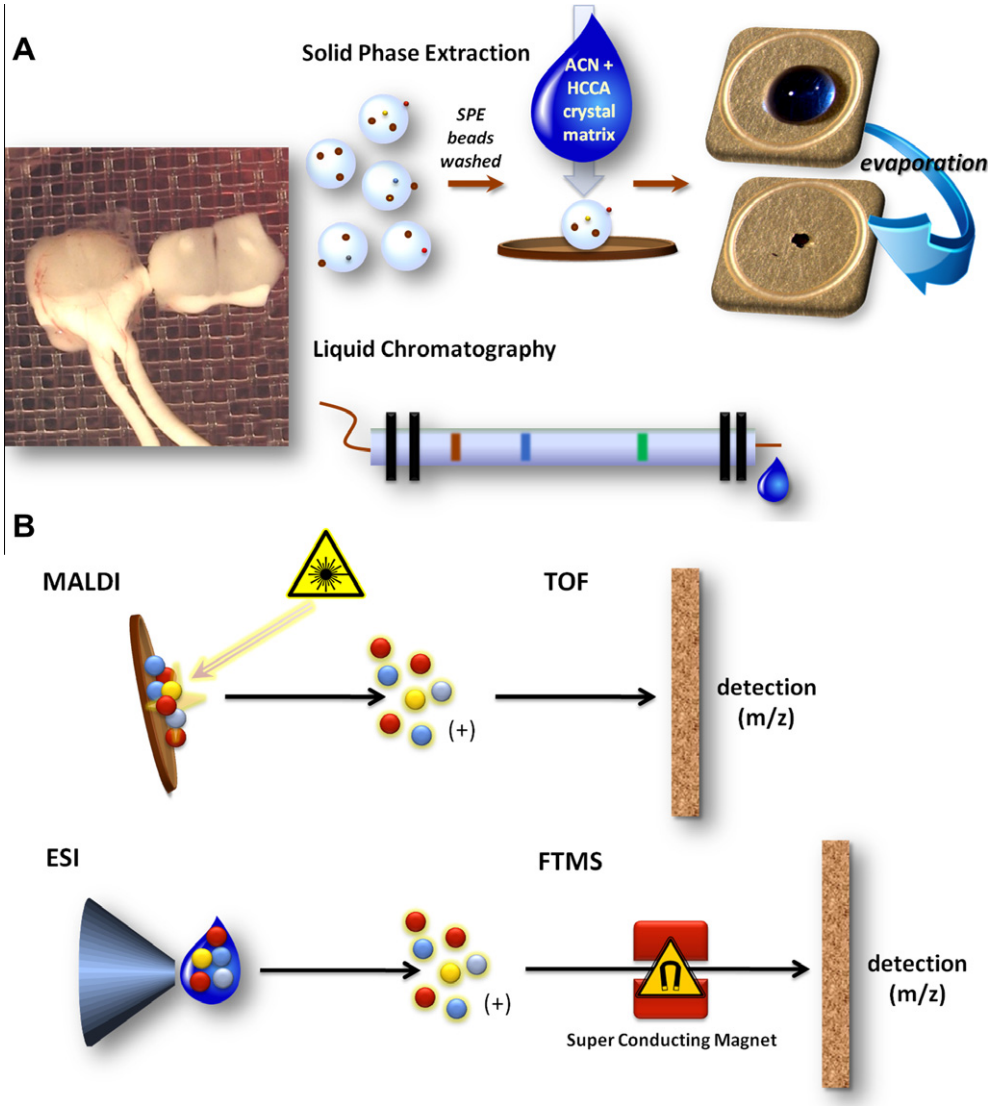


Fig. 3. Strategy for neuropeptide MS analysis from hypothalamic nuclei. (A) *Sample preparation.* Peptide analytes are collected from either horizontal or coronal hypothalamic slices, in this illustration, containing the SCN. In the top frame, analytes from samples (represented as colored circles) attach based on charge to solid phase extraction (SPE) beads. After collection, the beads are rinsed of salts and transferred to a MALDI target surface for elution of bound analytes with acetonitrile (ACN) and addition of α-cyano-4-hydroxycinnamic acid (HCCA) MALDI matrix solution. As the acetonitrile evaporates, analytes are concentrated with MALDI matrix onto discrete hydrophobic regions within the HCCA crystals on the pre-treated target plate. In the lower frame, analytes collected from tissue are enriched for peptides by removing large structural proteins and fractionation via liquid chromatography prior to MS analysis. (B) *MS analysis.* After the sample is enriched, it can be volatized and ionized by either matrix-assisted laser desorption/ionization (MALDI) or electrospray ionization (ESI). Following ionization, the analytes are subjected to a mass analyzer and detector for spectrophotometric analysis where their mass/charge ratio (m/z) can be determined. Spectrometric analyses include time-of-flight (TOF) MS or Fourier transform (FT) MS (also known as FTICR MS).

3. Identifying peptides from a single hypothalamic nucleus

In the past, MS-based studies have examined large morphological structures, such as the entire hypothalamus [22,27]. Only recently, with refinements in sample preparation coupled with higher sensitivity mass spectrometric analyses [22,27,53,64,66], have investigations of smaller brain nuclei been possible [13,43]. Utilizing the hypothalamic brain slice, Lee et al. [43] and Bora et al. [13] analyzed the SCN and SON, respectively. A brain slice preparation has the advantage of facilitating focus on small, anatomically defined brain structures that have established physiological functions.

Because of the higher mass accuracy of the measurements, FTICR MS and FTMS both were used in a comprehensive proteomic analysis of the SCN. This enabled us to achieve a high confidence in the peptide assignments and allow a greater range of PTMs to be confirmed [43]. Of the 102 SCN peptides identified, 33 had not previously been identified and 12 harbored PTMs. Many of the identified peptides were derived from known precursors expressed in the SCN. Vasoactive intestinal peptide (VIP), gastrin-releasing peptide (GRP), and somatostatin had been identified immunologically in neurons of the SCN's ventrolateral/central regions [1,48,49]. It is noteworthy that shorter peptides derived from the VIP and GRP proneuropeptides, and a peptide fragment of somatostatin, also were observed peptidomically [43]. The shortened forms of GRP and VIP had been identified previously in SwePep (http://www.swepep.org/) [31], a compendium of peptide information, whereas the short form of somatostatin had not been reported. Several expected full-length peptides may not have been identified due to short peptide life-times, rapid degradation, detection limits of the approaches used, or because the predicted full-length form may not be the bioactive species. Although it cannot be ruled out

completely that the shortened forms are due to peptide degradation, these specific peptides do conform to predicted cleavage sites identified by NeuroPred (http://neuroproteomics.scs.illinois.edu/neuropred.html) [61]. Prior studies examining localization of peptides used immunohistochemistry based on antibodies that would not distinguish full-length and shorter peptide forms. The unusual shortened forms of these well-known peptides would be interesting targets for functional studies.

In the SON, LC in conjunction with multiple MS platforms, including MALDI time-of-flight (TOF) MS, ESI ion trap MS and ESI FTMS, were utilized to enhance peptide identification [13]. Each platform excels at analyzing specific types of samples and analytes. As stated above, combining these complementary techniques facilitates detection of larger numbers of peptides than can be detected using only one method. Overall, the analysis yielded 85 peptides, including 20 unique peptides from known proneuropeptides. This is a significant increase from the 41 peptides identified from a larger sample containing not only the hypothalamus, but also the hippocampus and striatum [20], and from the 56 peptides from the hypothalamus and striatum [27]. The peptide identifications in the SON investigation are in agreement with these previous studies, and expand on them significantly.

In summary, these studies in the SCN and SON achieved high resolution, high quality identification of peptides in two discrete regions of the hypothalamus. With the discovery of numerous new hypothalamic peptides, these results are likely to prove a valuable asset for neuroendocrine research, as well as an essential peptide database that can be used to establish positive matches for MS analysis of peptides present in concentrations too low for tandem MS (MS–MS) analysis (see Section 6).

4. Identifying peptides from single hypothalamic cells

A yet more focused approach involves peptidomic investigations of single cells. MS at the single-cell level permits identification of peptides from the same precursor, as well as determination of peptides colocalized within the same cell [54,56]. Recent technical advances enabled MALDI MS peptide analysis of not only a distinct hypothalamic nucleus, as discussed above, but also of single cells within that nucleus [13,47].

The SON contains populations of well defined neurosecretory cells dedicated to producing neuropeptides/hormones that participate in a range of peripheral regulatory mechanisms. These cells, the magnocellular neurons (MCNs) of the SON, are part of the hypothalamic neuroendocrine system. At 30 μm in diameter, they are large relative to other mammalian neurons and are specialized for producing large amounts of peptide under acute stimulus conditions. Peptides can be released somatically within the SON, axonally at the neurohypophysis, and dendritically within the hypothalamus [52,57]. Whereas their axons access the portal vasculature to release peptides directly into the blood stream, somatodendritic peptide release participates in local regulation of neuron excitability [38,42]. Individual MCNs from the SON were manually isolated from a hypothalamic slice stabilized with glycerol [13]. Despite a marked decrease in the amount of peptides detected without enzyme-assisted dissociation, AVP, neurophysin II, and a cocaine- and amphetamine-regulated transcript (CART)-related peptide were identified by MALDI MS using mass-matching to an SON peptide database (see Section 6).

In contrast to acute isolation of cells, culturing neurons has the advantage of permitting regeneration of long neurites, which are lost during acute cellular separation. Providing conditions that favor the development of dendrites and axons increases the potential peptides detectable by MS analysis. By sampling through the application and crystallization of small-volumes of the MALDI matrix, 2,5-dihydroxybenzoic acid (DHB), directly onto cultured cells, peptide profiles were obtained from spatially distinct, identifiable neurons within a co-culture containing SON neurons [47]. MS analysis detected 10 previously characterized peptides and 17 peptides that could not be assigned based on known databases [47]. Despite the reduction in peptide quantity and possibility that the *in vitro* environment may introduce artifacts, the single-cell approach provides new opportunities to probe the roles of peptides in cellular function, to elucidate cell-specific proneuropeptide processing, and to discover potential cell-to-cell signals in ways not possible *in vivo*.

5. Identifying peptides in specific hypothalamic releasates

The techniques discussed thus far involve peptide identification within a cell or tissue. In the quest to discover bioactive neuropeptides, it is critical to know which peptides are released. Tissue samples contain not only the final peptides present, but also partially processed forms that are *en route* to becoming bioactive. These partially processed peptides may even bind to the appropriate receptor, but they may never be released and so are not bioactive neuropeptides.

How are peptides released from specific hypothalamic sites identified? Novel sampling approaches have been developed that allow the selective characterization of peptides secreted at discrete locations within the hypothalamus. We modified an existing collection strategy [36] for use in the mammalian brain slice [37]. Micrometer-sized beads of solid phase extraction (SPE) material are applied directly to the tissue where they act as local sampling probes that bind nearby peptides secreted into the extracellular milieu. The SPE beads are made of a polymer resin that non-selectively binds peptides and proteins through hydrophobic interactions. In previous work [36], analyses of samples collected by these beads were able to discriminate profiles of secreted peptides at the level of individual neurites of single *Aplysia* bag cells in culture. A long-term goal would be collection and analysis at individual cells in tissue, discriminating neuronal vs. glial release, or even co-release profiles at neurites *in situ* within the brain.

This sampling strategy and analytic procedure, outlined in Fig. 3, involves the use of SPE beads either placed directly on or near the brain slice [36]. A related approach is to use SPE material within a pipette (C_{18} Zip Tips®, Millipore), which is then positioned on the surface of the slice and media containing the brain-slice releasate is drawn up so that it is pulled across the matrix (the 'pipette-pull' approach). Following collection, adherent peptides are eluted and analyzed by MALDI-TOF MS [36]. This enables tentative identification of peptide species based on molecular mass and charge. This analysis then is coupled with tandem MS to generate the unique molecular sequence. The utility of these SPE-based collection approaches was demonstrated by the initial characterization of circadian peptides secreted from the rat SCN [37]. The SCN was predicted to be richly peptidergic because it is innervated by diverse peptidergic afferents and its intrinsic neurons are peptidergic [48,49,69]. A combination of neurophysiology and SPE sampling was used to measure the most complete SCN peptide release profiles to date, including secreted analytes that may correspond to novel intercellular circadian factors [37].

5.1. Peptides are released in distinct profiles by specific stimulation events

In order to distinguish activity-dependent release from unrelated compounds present in the media, SPE bead samples are taken from the same location before and after stimulation. After treatment, the beads are removed for measurement and replaced with another set of beads. This sequential collection generates separate

samples collected before, during and after stimulation. Following sample collection, the beads are rinsed to remove salts and transferred to a MALDI target plate for sample elution with an organic solvent. Following addition of MALDI matrix to the sample spot, MALDI-TOF MS is performed on pre-stimulation and stimulation samples. These mass spectra are then compared for stimulation-dependent peaks of interest. Analytes of interest are re-measured for accurate mass with internal calibrants and, when possible, sequence identification is obtained by MS–MS.

Peptide spectra for SCN releasates from three separate parameters were determined by comparing mass spectra from bead samples collected before and during tissue stimulation [37]. First, a microdrop of elevated K^+ (55 mM KCl) was applied to the surface of the suprachiasmatic brain slice to elicit generalized release of peptides. KCl stimulation occurred at circadian time (CT) 6, which has been shown to result in the peak secretion of AVP [29,34]. Secondly, glutamate (5 min, 2 μl, 10 mM), which signals circadian clock resetting [26], was applied similarly to stimulate specific release of peptides. Glutamate stimulation was at CT 19, at which clock state glutamate mimics the circadian phase-advancing effects of environmental light [26,68]. Thirdly, using horizontal slices containing the SCN with optic nerves attached, direct retinohypothalamic tract (RHT) stimulation was utilized for localized release of peptides within the SCN [37].

Pre-stimulation mass spectra exhibited few released analytes. The dominant peaks (1078 and 1288 m/z) in the lower mass range corresponded to compounds resulting from the α-cyano-4-hydroxycinnamic acid (HCCA) MALDI matrix. These same matrix analytes were observed in MS analyses of control beads that were exposed to brain tissue and, therefore, were subtracted from the SCN releasate. In contrast, stimulation bead samples from all three parameters listed above produced complex spectra of stimulation-dependent compounds [37].

Various stimulation parameters resulted in peaks that match the predicted mass for arginine vasopressin (AVP), the first peptide established as being released from the SCN in a circadian pattern [29,34]. Mass peaks corresponding to other known or suggested SCN-related peptides also were observed [1,3,23,28,34,60,69]. These include, among others, substance P, the somatostatins, neurotensin and galanin [37]. These assignments were confirmed when possible using a variety of MS–MS approaches. Notably, three pro-SAAS-derived peptides, little SAAS, big LEN, and PEN [19], which had no previous association with circadian rhythms, are clearly distinguished in profiles of the RHT-stimulated releasates [37].

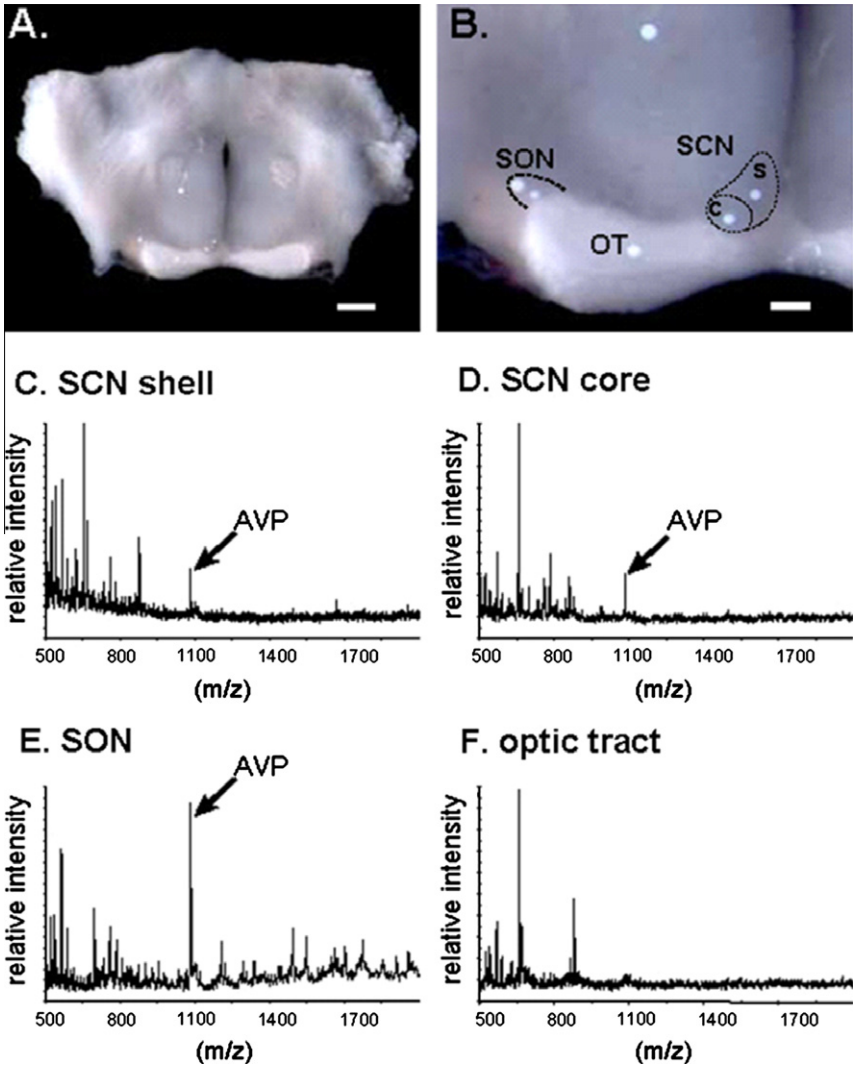


Fig. 4. Region-specific release can be resolved spatially with solid phase extraction (SPE)-bead probes. Peptide release can be measured with spatial precision, enabling releasate mass spectra to be obtained from spatially defined sub-regions of the hypothalamic brain slice. (A) Beads are positioned at discrete locations on the coronal brain slice (scale bar = 1.0 mm). (B) Discrete hypothalamic regions are outlined in this magnified image (scale bar = 0.2 mm) of SPE beads on SCN, SON, and optic tract regions. With KCl stimulation, AVP is detected in the releasate mass spectra obtained from (C) AVP-expressing SCN dorsomedial/shell, (D) ventrolateral/core SCN subregion, and (E) SON, but not (F), an adjacent AVP-negative optic tract (OT) region.

5.2. Peptide release is spatially and temporally defined

By placing SPE beads directly on the surface of the rat brain slice, spatially distinct releasate mass spectra can be obtained from specific sub-regions within brain nuclei, including the SCN and SON (Fig. 4). Known SCN neuropeptides are observed in releasates following stimulation with physiologically relevant paradigms, including several reported to alter clock timing. When bound peptides released from the SCN are eluted from the SPE beads and analyzed, multiple unknown compounds are observed. Mass spectra obtained from the stimulation/releasate profiles captured by beads are distinct between the SCN, SON, anterior hypothalamus (AHP) and optic tract (OT) (Fig. 4). AVP is detected in releasate only after stimulation and only from AVP-expressing SCN and SON; AVP is not detected in releasates from either the adjacent OT or the more distant AHP region [37].

This spatial analysis raises questions about roles of neurons within distinct areas of the SCN. SCN sub-regions have been identified based on immunohistochemistry, which reports localized expression of various peptides [6,8,48,49]. Most notable are the ventrolateral and central regions, which express VIP, little SAAS, and GRP, and the dorsomedial region, which is often defined by the expression of AVP. Peptides captured by SPE beads placed on the ventrolateral/central and dorsomedial SCN had almost identical mass spectra [37]. This supports recent immunocytochemical analyses that reported a sub-population of AVP cell bodies in medioventral SCN of rat [8]. Furthermore, AVP can be released from dendrites and soma in local circuits of these areas, whereas axonal release is likely extra-SCN in the subparaventricular zone to which these neurons project [48]. Extended sampling periods may resolve analytes that differ in amounts in different sub-regions.

Not surprisingly, the SCN releases peptides in a temporally defined manner. Release sampling has been used to assess temporal release patterns of peptides, but observations have been limited to known SCN peptides. Prior to applying peptidomic methods of analysis, AVP and VIP were the only peptides reported to be released in a circadian rhythmic pattern [29,34,60]. To evaluate the peptidome of released peptides, mass spectra were obtained over a 24-h collection period [37]. Mass matches of clearly discernable peaks were verified from the SCN peptidome (see Section 6). To discern release patterns, endogenous releasates were collected from coronal SCN slice preparations at 4-h sampling durations over six 4-h intervals of circadian time (CT): 0–4, 4–8, 8–12, 12–16, 16–20 and 20–24 h ($N = 2$ preparations for each CT interval) [37]. Samples were plated consecutively on the same MALDI target and analyzed consecutively on the same day without altering instrument settings to promote uniformity for relative comparisons of specific analyte peaks at different time intervals. AVP secretion over time was found to exhibit a circadian rhythm, with the largest mass peaks observed in samples obtained during mid-subjective day, near CT 8. This temporal profile corroborates the circadian pattern of AVP secretion from the rat SCN brain slice assayed by radioimmunoassay [29,34,60]. The circadian release patterns of AVP, angiotensin I, substance P, little SAAS and an unknown secreted compound with 1492.75 m/z were analyzed simultaneously; all but angiotensin I appear to undergo a circadian rhythm of release.

6. Use of a tissue-specific MS-peptide database to verify identity of putative mass matches in releasate

When measuring peptide release and when characterizing peptides in individual cells, adequate analyte is collected in order to determine m/z profiles of its components, but usually too little is collected to perform tandem MS sequencing. In order to verify the identity of putative mass matches in single cells or releasate

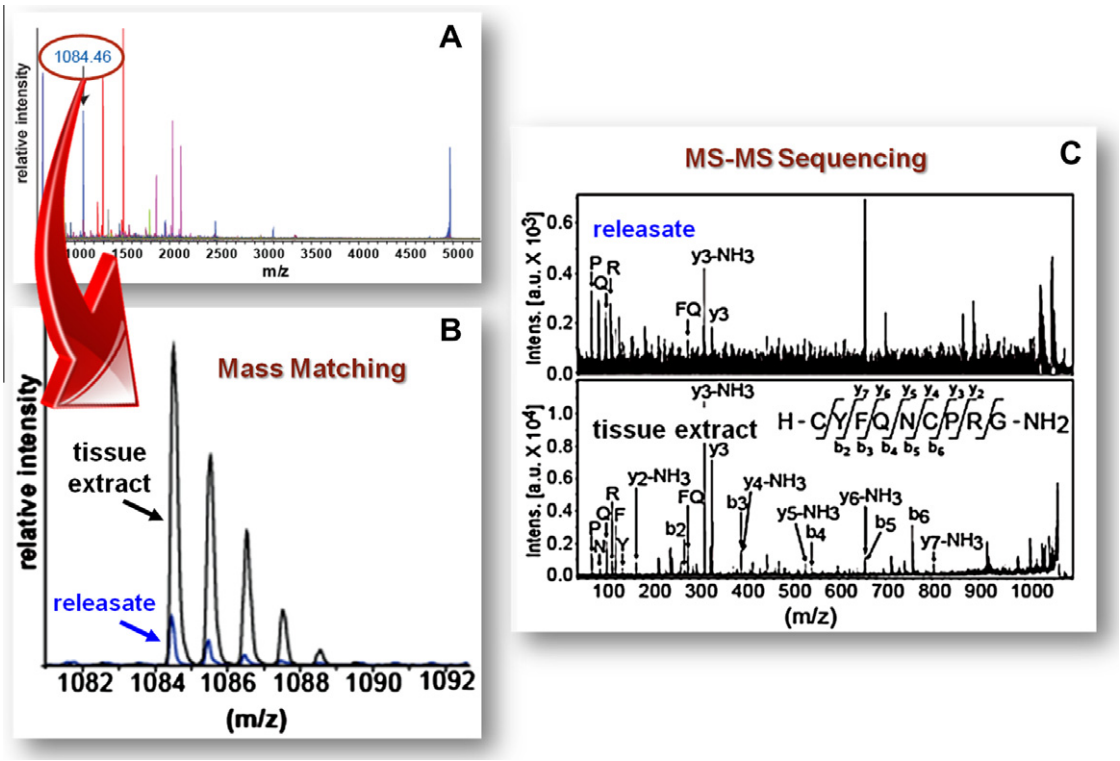


Fig. 5. A tissue-specific database can be used to verify the identity of lower abundance of peptides in releasate samples by mass-matching. (A) Peptides from SCN releasate can be analyzed by liquid chromatography (LC) coupled with mass spectrometry (LC–MS). In parallel, LC–MS is performed on the tissue extract from the defined brain region. (B) Secreted peptides generally are not collected in amounts sufficient for tandem MS (MS–MS) sequencing. Spectra from the releasate are compared with those obtained from tissue extract with the greater amounts of peptides. The LC–MS profiles of the tissue extract and the releasate are then *mass-matched*. (C) The MS spectrum obtained from releasate sample is compared to the MS–MS spectrum obtained from the matching extract analyte to establish the peptide sequence from the releasate [37].

samples, sequence information is acquired using MS–MS approaches from larger samples [37]. The exception was AVP, the only peptide collected in sufficient amounts for MS–MS sequencing directly from bead releasate samples. The most intense MS–MS AVP peaks matched the y_3 (and y_3-NH_3) fragment ions, as well as several ammonium ions. To obtain sufficient amounts of other peptides, larger samples are used. Hypothalamic tissue punches of SCN or SON are processed using peptide extraction protocols commonly employed for MS-based peptidomic studies. Following separation of these extracts by LC, hundreds of analytes with masses in the peptide range were detected and readily matched by mass to corresponding releasate analytes by MALDI-TOF MS–MS and, in some instances, separately with a linear trap quadropole-FT mass spectrometer for sequence confirmation (Fig. 5).

As one example of this strategy, SCN releasate data obtained both with pipette collections and with SPE beads positioned on the SCN identified an analyte with an observed monoisotopic mass that matched little SAAS [37], a processing product of the pro-SAAS prepropeptide [32]. This analyte is released endogenously in a circadian pattern, as well as in response to optic nerve stimulation, with an average m/z at 1783.97 and 1784.97, respectively. The FTMS spectrum was generated from the little SAAS releasate-matching analyte extracted from the SCN; this value represents an accurate match to within 0.5 ppm. The product ion spectrum generated from additional MS–MS sequencing matched nearly all of the y-ion series, confirming the sequence identity; thus, the SCN secretes the peptide little SAAS [37]. Following this strategy, the identities of many peptides observed in releasate samples were verified, although not all had been described previously.

7. Conclusions: the importance of ascertaining bioactivity

Peptides must have a function associated with cell-to-cell signaling to be judged to be neuropeptides. While a variety of approaches has been used to identify literally hundreds of brain peptides, this information alone is not enough to assign them as neuropeptides. Ascertaining bioactivity, therefore, becomes a central issue. Such bioactivity studies tend to be the bottleneck in the discovery process when compared to the higher throughput peptidomics approaches discussed thus far. While the necessary criterion for bioactivity is that the peptide induces a physiological response, this normally includes the identification of the receptor involved at sites of proposed action. However, recent work from Sasaki et al. [58] circumvents the need for identification of the receptors, identifying bioactive neuropeptides instead by their ability to raise intracellular calcium levels. This technique shows promise for cultured cells and brain slices, including the hypothalamus.

For intact hypothalamic nuclei, the use of the *in vitro* brain slice has proven to be an exceptional model for testing the bioactivity of identified peptides. In the case of little SAAS, which was identified in releasate from the SCN as secreted both endogenously and via RHT-stimulation [37], its function in clock resetting was tested directly [8,37]. Exogenous application of little SAAS onto the SCN brain slice causes resetting of the circadian clock in the early night [8,37]. Atkins et al. [8] demonstrated that little SAAS action is downstream of glutamate/NMDA receptor activation, but independent of VIP and GRP [8], the established mediators of the glutamatergic signal. Furthermore, a phase-delaying light pulse induces *c-FOS* expression in 50% of SCN neurons containing little SAAS. Thus, little SAAS acts in a novel, light-/glutamate-stimulated circuit to alter the time-state of the circadian clock. This bioactivity of stimulus-secreted little SAAS, which was discovered and identified by peptidomic MS analysis, establishes it as a functional neuropeptide in the SCN.

As described earlier, when preparing a tissue sample, the tissue must be handled to arrest enzymatic processing. Therefore, because both fully and partially processed products will be identified by MS, it is critical to determine which of these products are functional elements in cell-to-cell signaling. A partially processed protein may be recognized by a receptor and so provoke a response, but that form may never be released. Thus, to establish a true physiological role, MS analysis must identify the putative neuropeptides not only within a tissue or cell sample, but in releasate as well. Bioactive neuropeptide forms that mediate cell-to-cell signaling should be secreted into the extracellular space. Assessing release, especially with spatial and temporal resolution, is where the direct sampling by SPE beads has proven to be an extremely powerful strategy; it will continue to be so. This approach has the potential to discover the full complement of neuropeptides released locally, under diverse, functionally relevant stimulus conditions, and in response to natural stimuli *in vivo*. The resulting knowledge is necessary to fully understand hypothalamic signaling and modulation of neuroendocrine systems in health and disease. We fully expect that future studies that combine MS-based peptidomics, peptide release measurements and bioactivity tests will identify additional bioactive peptides within the hypothalamus and advance understanding of the many roles of neuropeptides in neuroendocrine physiology.

Acknowledgments

The project described was supported by award numbers P30 DA018310 from the National Institute on Drug Abuse (NIDA) to JVS, and HL086870 and HL092571 ARRA from the National Heart, Lung and Blood Institute (NHLBI) to MUG. We thank Maureen Holtz for preparing the manuscript. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

References

[1] E.E. Abrahamson, R.Y. Moore, Suprachiasmatic nucleus in the mouse: retinal innervation, intrinsic organization and efferent projections, Brain Res. 916 (2001) 172–191.
[2] R. Aebersold, M. Mann, Mass spectrometry-based proteomics, Nature 422 (2003) 198–207.
[3] A. Akabayashi, J.I. Koenig, Y. Watanabe, J.T. Alexander, S.F. Leibowitz, Galanin-containing neurons in the paraventricular nucleus: a neurochemical marker for fat ingestion and body weight gain, Proc. Natl. Acad. Sci. U S A 91 (1994) 10375–10379.
[4] Allen, Allen Mouse Brain Atlas [Internet]. Seattle (WA): Allen Institute for Brain Science. ©2009, (2009) <http://mouse.brain-map.org>.
[5] A. Amare, A.B. Hummon, B.R. Southey, T.A. Zimmerman, S.L. Rodriguez-Zas, J.V. Sweedler, Bridging neuropeptidomics and genomics with bioinformatics: prediction of mammalian neuropeptide prohormone processing, J. Proteome Res. 5 (2006) 1162–1167.
[6] M.C. Antle, R. Silver, Orchestrating time: arrangements of the brain circadian clock, Trends Neurosci. 28 (2005) 145–151.
[7] ASMS, American Society for Mass Spectrometry, ASMS, Santa Fe, NM, 2010 <http://www.asms.org/whatisms/index.html>.
[8] N. Atkins Jr., J.W. Mitchell, E.V. Romanova, D.J. Morgan, T.P. Cominski, J.L. Ecker, J.E. Pintar, J.V. Sweedler, M.U. Gillette, Circadian integration of glutamatergic signals by little SAAS in novel suprachiasmatic circuits, PLoS One 5 (2010) e12612.
[9] G. Baggerman, K. Boonen, P. Verleyen, A. De Loof, L. Schoofs, Peptidomic analysis of the larval Drosophila melanogaster central nervous system by two-dimensional capillary liquid chromatography quadrupole time-of-flight mass spectrometry, J. Mass Spectrom. 40 (2005) 250–260.
[10] M. Baker, Mass spectrometry for biologists, Nat. Methods 7 (2010) 157–161.
[11] H.M. Baseski, C.J. Watson, N.A. Cellar, J.G. Shackman, R.T. Kennedy, Capillary liquid chromatography with MS3 for the determination of enkephalins in microdialysis samples from the striatum of anesthetized and freely-moving rats, J. Mass Spectrom. 40 (2005) 146–153.
[12] B. Bogdanov, R.D. Smith, Proteomics by FTICR mass spectrometry: top down and bottom up, Mass Spectrom. Rev. 24 (2005) 168–200.
[13] A. Bora, S.P. Annangudi, L.J. Millet, S.S. Rubakhin, A.J. Forbes, N.L. Kelleher, M.U. Gillette, J.V. Sweedler, Neuropeptidomics of the supraoptic rat nucleus, J. Proteome Res. 7 (2008) 4992–5003.

[14] J. Bundzikova, Z. Pirnik, D. Zelena, J.D. Mikkelsen, A. Kiss, Response of substances co-expressed in hypothalamic magnocellular neurons to osmotic challenges in normal and Brattleboro rats, Cell. Mol. Neurobiol. 28 (2008) 1033–1047.
[15] R.M. Caprioli, T.B. Farmer, J. Gile, Molecular imaging of biological samples: localization of peptides and proteins using MALDI-TOF MS, Anal. Chem. 69 (1997) 4751–4760.
[16] P. Chaurand, S.A. Schwartz, R.M. Caprioli, Imaging mass spectrometry: a new tool to investigate the spatial organization of peptides and proteins in mammalian tissue sections, Curr. Opin. Chem. Biol. 6 (2002) 676–681.
[17] P. Chaurand, S.A. Schwartz, M.L. Reyzer, R.M. Caprioli, Imaging mass spectrometry: principles and potentials, Toxicol. Pathol. 33 (2005) 92–101.
[18] F.Y. Che, L. Yan, H. Li, N. Mzhavia, L.A. Devi, L.D. Fricker, Identification of peptides from brain and pituitary of Cpe(fat)/Cpe(fat) mice, Proc. Natl. Acad. Sci. U S A 98 (2001) 9971–9976.
[19] F.Y. Che, R. Biswas, L.D. Fricker, Relative quantitation of peptides in wild-type and Cpe(fat/fat) mouse pituitary using stable isotopic tags and mass spectrometry, J. Mass Spectrom. 40 (2005) 227–237.
[20] F.Y. Che, J. Lim, H. Pan, R. Biswas, L.D. Fricker, Quantitative neuropeptidomics of microwave-irradiated mouse brain and pituitary, Mol. Cell. Proteomics 4 (2005) 1391–1405.
[21] F.Y. Che, I. Vathy, L.D. Fricker, Quantitative peptidomics in mice: effect of cocaine treatment, J. Mol. Neurosci. 28 (2006) 265–275.
[22] F.Y. Che, X. Zhang, I. Berezniuk, M. Callaway, J. Lim, L.D. Fricker, Optimization of neuropeptide extraction from the mouse hypothalamus, J. Proteome Res. 6 (2007) 4667–4676.
[23] M.Y. Cheng, C.M. Bullock, C. Li, A.G. Lee, J.C. Bermak, J. Belluzzi, D.R. Weaver, F.M. Leslie, Q.Y. Zhou, Prokineticin 2 transmits the behavioural circadian rhythm of the suprachiasmatic nucleus, Nature 417 (2002) 405–410.
[24] E. Clynen, A. De Loof, L. Schoofs, The use of peptidomics in endocrine research, Gen. Comp. Endocrinol. 132 (2003) 1–9.
[25] D.M. Desiderio, Mass spectrometric analysis of neuropeptidergic systems in the human pituitary and cerebrospinal fluid, J. Chromatogr. B: Biomed. Sci. Appl. 731 (1999) 3–22.
[26] J.M. Ding, D. Chen, E.T. Weber, L.E. Faiman, M.A. Rea, M.U. Gillette, Resetting the biological clock: mediation of nocturnal circadian shifts by glutamate and NO, Science 266 (1994) 1713–1717.
[27] J.A. Dowell, W.V. Heyden, L. Li, Rat neuropeptidomics by LC-MS/MS and MALDI-FTMS: Enhanced dissection and extraction techniques coupled with 2D RP-RP HPLC, J. Proteome Res. 5 (2006) 3368–3375.
[28] D.J. Earnest, C.D. Sladek, Circadian rhythms of vasopressin release from individual rat suprachiasmatic explants in vitro, Brain Res. 382 (1986) 129–133.
[29] D.J. Earnest, C.D. Sladek, Circadian vasopressin release from perifused rat suprachiasmatic explants in vitro: effects of acute stimulation, Brain Res. 422 (1987) 398–402.
[30] M.R. Emmett, P.E. Andren, R.M. Caprioli, Specific molecular mass detection of endogenously released neuropeptides using in vivo microdialysis/mass spectrometry, J. Neurosci. Methods 62 (1995) 141–147.
[31] M. Falth, K. Skold, M. Norrman, M. Svensson, D. Fenyo, P.E. Andren, SwePep, a database designed for endogenous peptides and mass spectrometry, Mol. Cell. Proteomics 5 (2006) 998–1005.
[32] L.D. Fricker, A.A. McKinzie, J. Sun, E. Curran, Y. Qian, L. Yan, S.D. Patterson, P.L. Courchesne, B. Richards, N. Levin, N. Mzhavia, L.A. Devi, J. Douglass, Identification and characterization of proSAAS, a granin-like neuroendocrine peptide precursor that inhibits prohormone processing, J. Neurosci. 20 (2000) 639–648.
[33] L.D. Fricker, J. Lim, H. Pan, F.Y. Che, Peptidomics: identification and quantification of endogenous peptides in neuroendocrine tissues, Mass Spectrom. Rev. 25 (2006) 327–344.
[34] M.U. Gillette, S.M. Reppert, The hypothalamic suprachiasmatic nuclei: circadian patterns of vasopressin secretion and neuronal activity in vitro, Brain Res. Bull. 19 (1987) 135–139.
[35] W.E. Haskins, C.J. Watson, N.A. Cellar, D.H. Powell, R.T. Kennedy, Discovery and neurochemical screening of peptides in brain extracellular fluid by chemical analysis of in vivo microdialysis samples, Anal. Chem. 76 (2004) 5523–5533.
[36] N.G. Hatcher, T.A. Richmond, S.S. Rubakhin, J.V. Sweedler, Monitoring activity-dependent peptide release from the CNS using single-bead solid-phase extraction and MALDI TOF MS detection, Anal. Chem. 77 (2005) 1580–1587.
[37] N.G. Hatcher, N. Atkins Jr., S.P. Annangudi, A.J. Forbes, N.L. Kelleher, M.U. Gillette, J.V. Sweedler, Mass spectrometry-based discovery of circadian peptides, Proc. Natl. Acad. Sci. U S A 105 (2008) 12527–12532.
[38] M. Hirasawa, Y. Schwab, S. Natah, C.J. Hillard, K. Mackie, K.A. Sharkey, Q.J. Pittman, Dendritically released transmitters cooperate via autocrine and retrograde actions to inhibit afferent excitation in rat brain, J. Physiol. 559 (2004) 611–624.
[39] T. Hökfelt, O. Johansson, A. Ljungdahl, J.M. Lundberg, M. Schultzberg, Peptidergic neurones, Nature 284 (1980) 515–521.
[40] R.T. Kennedy, C.J. Watson, W.E. Haskins, D.H. Powell, R.E. Strecker, In vivo neurochemical monitoring by microdialysis and capillary separations, Curr. Opin. Chem. Biol. 6 (2002) 659–665.
[41] T. Kogel, H.H. Gerdes, Maturation of secretory granules, Results Probl. Cell. Differ. 50 (2010) 1–20.
[42] S.B. Kombian, D. Mouginot, Q.J. Pittman, Dendritically released peptides act as retrograde modulators of afferent excitation in the supraoptic nucleus in vitro, Neuron 19 (1997) 903–912.
[43] J.E. Lee, N. Atkins Jr., N.G. Hatcher, L. Zamdborg, M.U. Gillette, J.V. Sweedler, N.L. Kelleher, Endogenous peptide discovery of the rat circadian clock: a focused study of the suprachiasmatic nucleus by ultrahigh performance tandem mass spectrometry, Mol. Cell. Proteomics 9 (2010) 285–297.
[44] L. Li, E.V. Romanova, S.S. Rubakhin, V. Alexeeva, K.R. Weiss, F.S. Vilim, J.V. Sweedler, Peptide profiling of cells with multiple gene products: combining immunochemistry and MALDI mass spectrometry with on-plate microextraction, Anal. Chem. 72 (2000) 3867–3874.
[45] L. Li, J.V. Sweedler, Peptides in the brain: mass spectrometry-based measurement approaches and challenges, Annu. Rev. Anal. Chem. (Palo Alto Calif) 1 (2008) 451–483.
[46] Magnet Lab, National High Magnetic Field Laboratory, Tallahassee, Florida, 2010 <http://www.magnet.fsu.edu/education/tutorials/tools/>.
[47] L.J. Millet, A. Bora, J.V. Sweedler, M.U. Gillette, Direct cellular peptidomics of supraoptic magnocellular and hippocampal neurons in low-density co-cultures, ACS Chem. Neurosci. 1 (2010) 36–48.
[48] R.Y. Moore, J.C. Speh, R.K. Leak, Suprachiasmatic nucleus organization, Cell. Tissue Res. 309 (2002) 89–98.
[49] L.P. Morin, C.N. Allen, The circadian visual system, 2005, Brain Res. Brain Res. Rev. 51 (2006) 1–60.
[50] H. Pan, F.Y. Che, B. Peng, D.F. Steiner, J.E. Pintar, L.D. Fricker, The role of prohormone convertase-2 in hypothalamic neuropeptide processing: a quantitative neuropeptidomic study, J. Neurochem. 98 (2006) 1763–1777.
[51] Z.P. Pang, T.C. Sudhof, Cell biology of Ca2+triggered exocytosis, Curr. Opin. Cell. Biol. 22 (2010) 496–505.
[52] D.V. Pow, J.F. Morris, Dendrites of hypothalamic magnocellular neurons release neurohypophysial peptides by exocytosis, Neuroscience 32 (1989) 435–439.
[53] E.V. Romanova, S.S. Rubakhin, J.V. Sweedler, One-step sampling, extraction, and storage protocol for peptidomics using dihydroxybenzoic Acid, Anal. Chem. 80 (2008) 3379–3386.
[54] S.S. Rubakhin, R.W. Garden, R.R. Fuller, J.V. Sweedler, Measuring the peptides in individual organelles with mass spectrometry, Nat. Biotechnol. 18 (2000) 172–175.
[55] S.S. Rubakhin, W.T. Greenough, J.V. Sweedler, Spatial profiling with MALDI MS: distribution of neuropeptides within single neurons, Anal. Chem. 75 (2003) 5374–5380.
[56] S.S. Rubakhin, J.V. Sweedler, Characterizing peptides in individual mammalian cells using mass spectrometry, Nat. Protoc. 2 (2007) 1987–1997.
[57] N. Sabatier, C. Caquineau, G. Dayanithi, P. Bull, A.J. Douglas, X.M. Guan, M. Jiang, L. Van der Ploeg, G. Leng, Alpha-melanocyte-stimulating hormone stimulates oxytocin release from the dendrites of hypothalamic neurons while inhibiting oxytocin release from their terminals in the neurohypophysis, J. Neurosci. 23 (2003) 10351–10358.
[58] K. Sasaki, N. Takahashi, M. Satoh, M. Yamasaki, N. Minamino, A peptidomics strategy for discovering endogenous bioactive peptides, J. Proteome Res. 9 (2010) 5047–5052.
[59] M. Schrader, P. Schulz-Knappe, Peptidomics technologies for human body fluids, Trends Biotechnol. 19 (2001) S55–S60.
[60] K. Shinohara, S. Honma, Y. Katsuno, H. Abe, K. Honma, Circadian rhythms in the release of vasoactive intestinal polypeptide and arginine-vasopressin in organotypic slice culture of rat suprachiasmatic nucleus, Neurosci. Lett. 170 (1994) 183–186.
[61] B.R. Southey, A. Amare, T.A. Zimmerman, S.L. Rodriguez-Zas, J.V. Sweedler, NeuroPred: a tool to predict cleavage sites in neuropeptide precursors and provide the masses of the resulting peptides, Nucleic Acids Res. 34 (2006) W267–W272.
[62] M. Stoeckli, T.B. Farmer, R.M. Caprioli, Automated mass spectrometry imaging with a matrix-assisted laser desorption ionization time-of-flight instrument, J. Am. Soc. Mass Spectrom. 10 (1999) 67–71.
[63] F.L. Strand, Neuropeptides: Regulators of Physiological Processes, MIT Press, Cambridge, Mass., 1999.
[64] M. Svensson, K. Skold, P. Svenningsson, P.E. Andren, Peptidomics-based discovery of novel neuropeptides, J. Proteome Res. 2 (2003) 213–219.
[65] M. Svensson, K. Skold, A. Nilsson, M. Falth, P. Svenningsson, P.E. Andren, Neuropeptidomics: expanding proteomics downwards, Biochem. Soc. Trans. 35 (2007) 588–593.
[66] E. Theodorsson, C. Stenfors, A.A. Mathe, Microwave irradiation increases recovery of neuropeptides from brain tissues, Peptides 11 (1990) 1191–1197.
[67] G. Thomas, E. Herbert, D.E. Hruby, Expression and cell type-specific processing of human preproenkephalin with a vaccinia recombinant, Science 232 (1986) 1641–1643.
[68] S.A. Tischkau, J.W. Mitchell, S.H. Tyan, G.F. Buchanan, M.U. Gillette, Ca2+/cAMP response element-binding protein (CREB)-dependent activation of Per1 is required for light-induced signaling in the suprachiasmatic nucleus circadian clock, J. Biol. Chem. 278 (2003) 718–723.
[69] A.N. van den Pol, K.L. Tsujimoto, Neurotransmitters of the hypothalamic suprachiasmatic nucleus: immunocytochemical analysis of 25 neuronal antigens, Neuroscience 15 (1985) 1049–1086.
[70] J.H. Wardman, X. Zhang, S. Gagnon, L.M. Castro, X. Zhu, D.F. Steiner, R. Day, L.D. Fricker, Analysis of peptides in prohormone convertase 1/3 null mouse brain using quantitative peptidomics, J. Neurochem. 114 (2010) 215–225.
[71] V.H. Wysocki, K.A. Resing, Q. Zhang, G. Cheng, Mass spectrometry of peptides and proteins, Methods 35 (2005) 211–222.