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# Neuropeptidomics Mass Spectrometry Reveals Signaling Networks Generated By Distinct Protease Pathways in Human Systems: Commentary Review

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## Abstract

Neuropeptides regulate intercellular signaling among cells of the central brain and peripheral nervous systems, and the endocrine system. Diverse neuropeptides of distinct primary sequences of various lengths, often with post-translational modifications, coordinate and integrate regulation of physiological functions. Mass spectrometry-based analyses of the diverse neuropeptide structures in neuropeptidomics research is necessary to define the full complement of neuropeptide signaling molecules. Human neuropeptidomics has notable importance in defining normal and dysfunctional neuropeptide signaling in human health and disease. Neuropeptidomics has great potential for expansion in translational research opportunities for defining neuropeptide mechanisms of human diseases, providing novel neuropeptide drug targets for drug discovery, and monitoring neuropeptides as biomarkers of drug responses. In consideration of the high impact of human neuropeptidomics for health, an observed gap in this discipline is the few published articles in human neuropeptidomics compared to, for example, human proteomics and related mass spectrometry disciplines. Focus on human neuropeptidomics will advance new knowledge of the complex neuropeptide signaling networks participating in the fine control of neuroendocrine systems. This commentary review article discusses several human neuropeptidomics accomplishments that illustrate the rapidly expanding diversity of neuropeptides generated by protease processing of pro-neuropeptide precursors occurring within the secretory vesicle proteome. Of particular interest is the finding that human-specific cathepsin V participates in producing enkephalin and likely other neuropeptides, indicating unique proteolytic mechanisms for generating human neuropeptides. The field of human neuropeptidomics has great promise to solve new mechanisms in disease conditions, leading to new drug targets and therapeutic agents for human diseases.

## Graphical abstract

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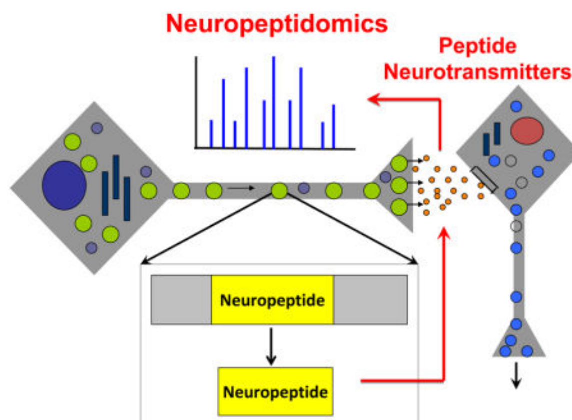


## Introduction

Neuropeptides mediate intercellular signaling in the nervous and endocrine systems in the integrated and coordinated control of physiological functions (figure 1) [1–5]. The tremendous diversity of the huge spectrum of neuropeptides highlights their significance in multiple regulatory functions. In the nervous system, neuropeptides function and peptide neurotransmitters for chemical communication among neural circuits in the brain and in the peripheral sympathetic and parasympathetic nervous systems. Neuropeptides link communication signals between nervous and endocrine systems. And in endocrine functions, peptide hormones regulate physiological homeostasis and responses to environmental stresses that involve all organ systems.

### Neuropeptide and classical small molecule neurotransmitters in the nervous system

Neuropeptides function as peptide neurotransmitters along with the classical small molecule neurotransmitters, the two main categories of neurotransmitters in the nervous system. Neuropeptides are represented by diverse peptide sequences typically consisting of about 3–40 amino acid residues, and many contain post-translational modifications. It is estimated that hundreds to thousands of different neuropeptides are utilized in numerous organisms, with many yet to be discovered. The neuropeptides regulate pain, appetite, cognition, and migraine via the endorphin, NPY, galanin, and CGRP peptides, respectively, as examples. In contrast to neuropeptides, the classical neurotransmitters consist of small molecules that are largely generated by modifications of single amino acids, such as norepinephrine synthesized from tyrosine and serotonin synthesized from tryptophan. Classical transmitters such as acetylcholine are synthesized by enzymatic reactions, in this case, from choline and acetyl-CoA by choline acetyl transferase. The 'neuropeptide' and 'classical' neurotransmitters together mediate intercellular signaling in the nervous system among neurons, as well as glia cells [1–5].

The distinct primary sequences of neuropeptides defines their selective and potent biological actions, mediated in large part by G-protein coupled receptors. A given neuropeptide may function in both the nervous and endocrine systems (Table 1). For example, adrenocorticotropin hormone (ACTH) is a neuromodulator in brain, and also regulates



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peripheral glucocorticoid metabolism controlled by pituitary and adrenal gland. Enkephalin neuropeptides function as neurotransmitters in the brain in the regulation of behavior and pain, and are also involved in peripheral actions of intestinal motility, immune cell functions, and related [6, 7]. The neuropeptides β-endorphin, neuropeptide Y (NPY), galanin, corticotropin-releasing factor, vasopressin, insulin, and numerous others control prominent physiological functions of pain, feeding behavior and blood pressure regulation, cognition, stress, water balance, and glucose metabolism, respectively. The plethora of physiological systems regulated by neuropeptides, thus, illustrates that neuropeptides participate in the control of all organ systems. Furthermore, neuropeptides function across different organisms including invertebrate, mammals, and human.

## Advantages of neuropeptidomics analyses of neuropeptide structures by mass spectrometry

Clearly, the diversity of neuropeptide functions indicates the essential requirement to understand their peptide sequence structures, including post-translational modificatoins, that define molecular mechanisms of their biological actions. The development of mass spectrometry analyses of peptides has provided tremendous new knowledge of the peptide structures of neuropeptides. Even the early discovery of the TRH (thyrotropin releasing hormone) peptide hormone by Roger Guillemin and Andrew Schally, Nobel prize recipients in 1977, utilized mass spectrometry to elucidate the amino acid sequence of thryotropin releasing hormone [8, 9], among the first identified hypothalamic releasing hormones. At that time, discovery of the radioimmunoassay (RIA) method by Rosaly Yalow in 1960 [10] allowed sensitive measurements of insulin and neuropeptides through the subsequent decades, and was recognized with award of the Nobel prize to Rosalyn Yalow in 1977. Subsequent advances in high resolution mass spectrometry analyses of neuropeptides allows unbiased, objective definition of peptide amino acid sequence structures that are not possible with antibody-based neuropeptide detection methods including ELISA and RIA immunoassays.

The defined mass of each neuropeptide molecule predicts its amino acid sequence structure with high accuracy. Moreover, the combination of liquid chromatography coupled with mass spectrometry (LC-MS/MS) enables analyses of hundreds, and nearly thousands, of neuropeptide structures in single experiments. With the rapid acquisition of large LC-MS/MS datasets of neuropeptides, development of neuropeptide-focused bioinformatics strategies are required for appropriate identification. Together, the high throughput LC-MS/MS analyses of peptides of the nervous and endocrine systems is termed ‘neuropeptidomics’. The term ‘neuropeptidomics’ refers to global identification and quantitation of peptide profiles in neuroendocrine systems by LC-MS/MS technologies.

Neuropeptidomics now allows systems analyses of intercellular signaling networks in the regulation of nervous and endocrine control, especially in human functions of physiological homeostasis, human disease conditions, and responses to changes environmental conditions including drug therapeutics. Investigation of neuropeptidomics signatures in human disease allows opportunities for advancing mechanistic knowledge of disease progression, biomarkers for disease states, and new peptide targets for drug discovery and development.

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The profound importance of neuropeptide regulation of human conditions provides the rationale for rapid advances in neuropeptidomics investigation in human health and disease.

### Human neuropeptidomics: current status in the field

While the significance of neuropeptidomics regulation of human physiology is undisputed, a search of the literature indicates the current paucity of neuropeptidomics research compared to proteomics mass spectrometry to gain protein structural information. PubMed searches of ‘peptidomics’ indicates 366 published articles, and search of ‘neuropeptidomics’ yields 32 articles; but search of ‘human neuropeptidomics’ indicates only 9 articles published (Table 2). In contrast, the proteomics field shows substantially greater research activity. ‘Proteomics’ studies number 55,388 articles to date (PubMed), and ‘human proteomics’ is described so far in 30,141 articles (Table 2). Search of ‘peptide mass spectrometry’ covers both proteomics and peptidomics since proteomics is conducted by analyses of tryptic peptide digests, and is illustrated by 50,069 articles published to date. The few articles published on ‘human neuropeptidomics’ clearly demonstrates the high need for expansion of the neuropeptidomics field to gain new understanding of neuropeptide regulation of human biological systems.

For this reason, this review focuses on ‘human neuropeptidomics’ to illustrate strategies for expansion and acceleration of human neuropeptidomics research. A spectrum of related studies is reviewed here covering human neuropeptide diversity, human protease pathways for neuropeptide biosynthesis, secretory vesicle proteomics systems that are utilized for neuropeptide production, and networks of secreted neuropeptide profiles that undergo coordinate regulation in physiological responses. These studies raise important questions to be investigated in future studies. Development of NeuroPedia, a neuropeptide database and spectral library, facilitates bioinformatics analyses of neuropeptidomics data [11]. Human neuropeptidomics research has great promise to solve new mechanisms in human neurobiology that can lead to new drug targets and therapeutic treatments for human diseases. It is, therefore, predicted that human neuropeptidomics is becoming an expanding field of broad interest to biomedical sciences.

## Diversity of Human Neuropeptide Profiles Revealed by Neuropeptidomics: Explosion of Intercellular Signaling Peptides to be Discovered

The diversity of human neuropeptides is tremendous and will expand tremendously with neuropeptidomics analyses of the peptide sequence structures identified in the central and peripheral nervous system, and the endocrine systems. Neuropeptidomics identification of known and current neuropeptides will first require knowledge of the pro-neuropeptide precursor proteins that undergo proteolytic processing to generate neuropeptides. Neuropeptidomics experiments will identify numerous peptides, and the question will arise as to which are neuropeptides or peptides of other biological processes. This question can be answered to a large extent by examination of the pro-neuropeptide sequences.

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## Pro-neuropeptide precursors of active neuropeptides

Active neuropeptides are generated from inactive pro-neuropeptide precursor proteins, also known as prohormones for those in the endocrine systems. A pro-neuropeptide precursor may contain one copy of the active neuropeptide as represented by by pro-neuropeptides for NPY, galanin, and VIP (vasoactive intestinal polypeptide) (supplemental figure S1) [5]. Or, a pro-neuropeptide may contain several related copies of the neuropeptide; for example, proenkephalin (PE) contains four copies of (Met)enkephalin, one copy of the related (Leu)enkephalin, and one copy each of the neuropeptides ME-Arg-Gly-Leu and ME-Arg –Phe (supplemental figure S1). Further, a pro-neuropeptide may contain several different neuropeptides with each having distinct biological actions; for example, the proopiomelanocortin precursor contains β-endorphin, ACTH (adrenocorticotropin hormone), and α-MSH (melanocyte stimulating hormone). Of particular interest is the tissue-specific expression of the POMC-derived neuropeptides.

While the primary sequences of pro-neuropeptides differ, as the distinct sequences of neuropeptides are the basis for their specific biological actions, they share the similar feature neuropeptides often flanked by dibasic residues at their $NH_2$- and COOH-termini within the pro-neuropeptide (supplemental figure S1). The dibasic residues Lys-Arg (KR) most often flank the neuropeptides; and the dibasic sites Lys-Lys, Arg-Arg, and Arg-Lys also occur. These paired basic residues have been found to represent sites of proteolytic processing to liberate neuropeptides from their precursor proteins. Processing may also occur at monobasic Arg sites as well as at monobasic residue sites (as in POMC). Processing at nonbasic residues may occur but is not as well-defined as processing at dibasic residues of pro-neuropeptides.

## Intervening peptide sequences within pro-neuropeptides

It is of interest that analyses of pro-neuropeptide sequences indicates that major portions of the pro-neuropeptides have unknown function. Known active neuropeptides have been the subject of peptide neurotransmission through synthesis, secretion, and activation of specific receptors However, there are many intervening peptide sequences, present between known active neuropeptides, but little attention has been given to investigating such intervening sequences. Neuropeptidomics is an ideal strategy to answer the question of what peptide products are generated from pro-neuropeptides? Neuropeptidomics may reveal new and existing neuropeptides.

## Neuropeptidomics reveals that pro-neuroeptides are converted to intact intervening sequences and known active neuropeptides

Neuropeptidomics analyses by nano-liquid chromatography tandem mass spectrometry (LC-MS/MS) of human secretory vesicles (isolated from human pheochromocytoma) is capable of revealing a repertoire of processed peptides derived from a single pro-neuropeptide [12]. Furthermore, LC-MS/MS in one experiment provides data for the spectrum of peptide products derived from multiple pro-neuropeptides. These experiments were conducted with a low molecular weight (MW) pool (obtained by a 10 kDa Millipore filtration membrane).

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Neuropeptidomics illustrated the multiple peptide products in human pheochromocytoma secretory vesicles derived from proenkephalin (PE), pro-NPY, pro-SAAS, and the chromogranins A, B, and C (CgA, CgB, CgC, respectively) [12]. Features of peptides derived from PE and CgA are highlighted.

Neuropeptidomics revealed numerous extended forms of (Met)enkephalin that included "intervening" sequences of non-enkephalin domains of proenkephalin (figure 2). Furthermore, intervening peptide sequences that do not include enkephalin were identified. Some intervening peptide domains were not detected (residues 145–161). Significantly, the presence of such intervening peptide sequences has not been observed in prior studies.

These neuropeptidomics findings identified numerous CgA-derived peptide domains of catestain, vasostatin, parastatin, and related neuropeptides derived from CgA (supplemental figure S2). Several intervening peptides derived from CgA were identified. The low MW pool did not include all intervening peptides, which may indicate that their presence with large intermediate polypeptides greater than 10 kDa that are derived from the CgA precursor of ~68 kDa.

The presence of intact intervening peptide sequences and known active neuropeptides derived from pro-neuropeptide precursors indicates that an explosion of new neuropeptides will be revealed by neuropeptidomics. The question of possible biological functions of the intervening peptides will be of interest to address in future research. Neuropeptidomics is key for unbiased analyses of all peptides derived from precursor proteins, in contrast to traditional focused immunoassays that each measure only one neuropeptide. Findings in the field have, thus far, only observed the 'tip of the iceberg' of the full spectrum of neuropeptides. It will be exciting to gain understanding of the full spectrum of neuropeptides derived from pro-neuropeptide precursors, and define their biological functions.

## Protease cleavage sites of pro-neuropeptides observed by neuropeptidomics data: dibasic residue processing and novel cleavage sites of pro-neuropeptide processing

Neuropeptidomics of human secretory vesicles (human adrenal medullary pheochromocytoma) has defined proteolytic peptide products of several pro-neuropeptides including proenkephalin, pro-NPY (neuropeptide Y), pro-SAAS (Ser-Ala-Ala-Ser related peptides), chromogranin A, chromogranin B, and secretogranin II (SCG2) that has also been known as chromogranin C [12]. Evaluation of the adjacent peptide sequences at the N- and C-termini of identified peptides revealed that these peptides were derived from processing at classical dibasic residue sites of pro-neuropeptides, and also at novel cleavage sites.

Classical dibasic residue cleavage site motifs flanking the N- and C-termini of endogenous peptides were prevalent, shown by LOGO maps, which illustrated processing at KR, KK, RK, and RR sites (figure 3a). These data are consistent with pro-neuropeptide processing by proteases known to possess cleavage specificities for dibasic residue sties. These proteases consist of the subtilisin-like proprotein convertases and cysteine cathepsin protease pathways (described in section on human protease pathways).

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Novel cleavage sites of pro-neuropeptides were also identified by neuropeptidomics data and illustrated by LOGO maps (figure 3b). There analyses found an abundance of acidic amino acids (E, glutamate) at the P1 position of putative cleavage sites (cleavage site is P1-↓P1’). These cleavage sites occur at the junctions of known active neuropeptides and intervening sequences of pro-neuropeptides. Thus far, proteases cleaving at glutamate residues for neuropeptide production have not yet been identified.

Based on searches of the literature to date, the study by Gupta et al., 2010 [12] is among the first to report on human pro-neuropeptide cleavage sites via neuropeptidomics. Future human neuropeptidomics research will benefit from comprehensive of endogenous peptides among human neuroendocrine tissues that will likely define new and existing neuropeptides that are generated by novel and classical proteolytic processing sites of human pro-neuropeptides.

## Bioinformatics for Neuropeptidomics by NeuroPedia: Neuropeptide Database and Spectral Library

Bioinformatics addressing the particular features of neuropeptideomics data is necessary to facilitate high quality data analyses. Global analyses of neuropeptidomics expression data is necessary for understanding the role and regulation of neuropeptide forms in health, disease, and drug treatments. However, the unique properties of neuropeptides, with respect to very short and very long non-tryptic peptide sequences, presents difficulties for identification from tandem mass spectrometry data (MS/MS) with popular database search tools (such as SEQUEST or Mascot) [13, 14]. Short neuropeptides can lead to inaccurate search results because the database search tools usually assign lower scores to short peptides. Alternatively, long or non-tryptic neuropeptides are difficult to identify because most database search tools are trained for tryptic peptides cleaved at K/R and because peptide fragmentation processes for long neuropeptides is usually inefficient. Further, searching larger databases takes more time because of the number of comparisons and reduces the number identifications with the caveat of greater choices for false positives [15]. Thus, while some neuropeptides can be identified with current bioinformatics approaches, complete neuropeptidomics requires novel computational tools for identifying both short and long neuropeptides by MS/MS [11].

For these reasons, NeuroPedia is being developed as a specialized neuropeptide database and spectral library that is directly searchable using mass spectrometry data [11]. NeuroPedia improves sensitivity by targeted searching of a small neuropeptide sequence database, and provides enhanced identification efficiency, sensitivity, and reliability. NeuroPedia spectral libraries are compatible with the publicly available spectral library search tool M-SPLIT [16] and can integrate with other spectral library formats. NeuroPedia provides annotated spectrum images for every library spectrum and separates spectral libraries by species, enzyme digestion, and MS instrument.

The NeuroPedia spectral library contains a total of 3401 identified spectra in MGF files. The NeuroPedia sequence database contains 847 neuropeptides from human, chimpanzee, mouse, rat, cow, sea hare, thesus macaque, and leech. Using InsPecT or any other database

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search tool, new MS/MS data can be searched against this sequence database. NeuroPedia offers the advantages of being rapid and precise identification of small or nontryptic neuropeptides.

Comparison of NeuroPedia with the online neuropeptide repository (at www.neuropeptides.nl) shows that this resource is not designed to allow identification from MS/MS data [17]. This repository provides non-searchable neuropeptide sequences, gene names, precursor names and expected human brain expression Users must search their data using other peptide database search tools and caompre the results against the neuropeptide list. This process is less sensitive and utilizes time-consuming manual matching of searches to information in current resources.

The www.neuropeptides.nl are complemented by recent development of NeuroPep [18] which is a comprehensive resource of neuropeptides, which holds 5949 non-redundant neuropeptide entries from 493 vertebrate and invertebrate organisms. Each peptide entry of the database contains organisms, tissues, families, names, modifications, 3D structures (if known), and literature references. It is noted that NeuroPep and www.neuropeptides.nl provide information about neuropeptides, but they do not cover computational strategies for neuropeptide identification as addressed by NeuroPedia.

Recently, accurate assignment of significance to neuropeptide identifications was developed using Monte Carlo k-permuted decoy databases [19]. The straightforward Monte Carlo permutation testing can be combeind with existing peptide identification software for accuarte neuropeptide detection.

NeuroPedia uniquely utilizes neuropeptide spectral libraries to enhance neuropeptide identification by MS/MS methods. Expansion of NeuroPedia involving neuropeptidomics investigators will significantly advance human neuropeptidomics knowledge, as well as neuropeptidomics in numerous organisms. Because the breadth of the diversity of neuropeptides has yet to be fully understood, continued development of NeuroPedia will be instrumental in defining the full spectrum of neuropeptide structures that regulate biological systems in humans and all organisms. Further, arrangement of NeuroPedia for integration with other ongoing neuropeptide and peptide bioinformatics systems for MS/MS identification will benefit the field for comprehensive analyses of human neuropeptidomics.

## Human-Specific Protease Pathways for Neuropeptide Biosynthesis

Proteases for processing pro-neuropeptides are required for the production of biologically active neuropeptides from inactive precursor proteins. Two distinct protease pathways for subtilisin-like proprotein convertases and cysteine cathepsin proteases have been demonstrated to participate in neuropeptide biosynthesis (figure 4). Notably, emerging evidence illustrates the human-specific cathepsin V of the cysteine protease pathway as a human-specific protease for production of neuropeptides in human biological systems [20]. This exciting finding implicates human-selective approaches for regulating neuropeptide production in physiological systems.

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## Distinct protease pathways for neuropeptide biosynthesis

The two protease pathways for processing at dibasic residues were identified through different approaches of (1) gene homology cloning of mammalian protease genes based on homology to the yeast kex 2 gene that generates α-mating factor peptide hormone, and (2) activity-based mass spectrometry identification of proenkephalin and pro-neuropeptide processing proteases in mammalian systems. These approaches identified distinct protease pathways for pro-neuropeptide processing consisting of (1) the subtilisin-like proprotein convertases (PC1/3 and PC2) protease pathway [5, 21] and the (2) cysteine cathepsin L and cathepsin V protease pathway (figure 6) [5, 20, 22]. Protease of both pathways cleave primarily at dibasic residue cleavage sites within pro-neuropeptides.

PC1/3 and PC2 family members participate in neuroendocrine production of neuropeptides, demonstrated through gene knockout and gene expression studies, and by neuropeptidomics characterization in mice with knockout of the PC1/3 or PC2 genes [5, 21–24]. Because of the limited scope of this review article, readers are referred to the extensive literature on PC1/3 and PC2 [5, 21–17] that indicate the current knowledge of PC1/3 and PC2 in neuropeptide production.

## Human-specific cathepsin V and human cathepsin L of the cysteine protease pathway for neuropeptide production

The neuropeptide cysteine protease pathway was elucidated by identify major pro-neuropeptide cleaving activity, using recombinant proenkephalin (PE) as substrate, in secretory vesicles where pro-neuropeptide processing occurs [5]. Chemical probe labeling of the PE-cleaving activity with the activity-based probe DCG04 for cysteine proteases allowed identification of the enzyme mass spectrometry [28], identifying cathepsin L as the processing protease. Cathepsin L gene knockout in mice confirmed its major role in the production of multiple neuropeptides including ACTH, α-MSH, β-endorphin, CCK (cholescystokinin), NPY, and others [5, 22]. Furthermore, the colocalization of cathepsin L with neuropeptides in secretory vesicles indicates the secretory vesicle as a new organelle location for cathepsin L for producing bioactive peptides.

Notably, the human genome possesses highly homologous human cathepsin L and human cathepsin V proteases [20, 29, 30]. No orthologues of human cathepsin V is present in mouse and other mammalian species [29, 30], indicating that cathepsin V is a human-specific protease gene. Mouse cathepsin L possesses high homology with human cathepsin V (74.6% homology in protein sequence), which is greater than the excellent homology of human cathepsin L and mouse cathepsin L (71.5% homology in protein sequence) (supplemental figure S3a). These homologies predict that human-specific cathepsin V can participate in neuropeptide production.

Indeed, human cathepsin V participates in generating enkephalin neuropeptide from its proenkephalin (PE) precursor [20]. Gene silencing of cathepsin V (by siRNA) substantially reduced production of enkephalin by more than 80% (supplemental figure S3b). Further, cathepsin V cleaves PE at dibasic residue sites to generate PE-derived intermediates and (Met)enkephalin. Cathepsin V is present in secretory vesicles with enkephalin, and cathepsin

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V is present human brain regions that produce enkephalin neuropeptides. These data demonstrate the significant role of human-specific cathepsin V in enkephalin neuropeptide production. It will be important to conduct human neuropeptidomics studies to gain understanding of human neuropeptides that are produced by human cathepsins V and cathepsin L. Human-focused studies will further define the roles of the cysteine protease pathway of cathepsins V and L with the subtilisin-like protease pathway of PC1/3 and PC2 convertases.

## Human Secretory Vesicle Proteome for Neuropeptide Biosynthesis, Storage, and Secretion

Neuropeptide production occurs largely in secretory vesicles which then store and secrete neuropeptides for intercellular signaling. The pro-neuropeptides and the processing proteases function within the environment of the secretory vesicle proteome. In neurons, pro-neuropeptide processing occurs within secretory vesicles during their transport along the axon to nerve terminals for neuropeptide release as neurotransmitters. Proteomic studies of human neuropeptide-containing secretory vesicles can identify the functional protein categories utilized for neuropeptide production and secretion.

The protein architecture of human dense core secretory vesicles (DCSV) that produce neuropeptides was subjected to quantitative proteomics and systems biology analyses of human DCSV purified from human pheochromocytoma [31]. Over 600 human DCSV proteins were identified with quantitation of over 300 proteins, revealing that most proteins participate in producing neurotransmitters and neurohumoral factors, consisting of pro-neuropeptides, proteases, and neurotransmitter/neurohumoral factor proteins. Proteins that regulate the internal secretory vesicle conditions included ATPases, chaperones, and those that regulate reduction-oxidation conditions. Protein functions in biochemical, secretory, and morphological functions of secretory vesicles are also present.

Organization of human secretory vesicle proteomics data in a systems biology format provided knowledge of soluble and membrane proteins of the organelle (figure 5). Protein interaction networks were analyzed by Cytoscape, a platform for complex network analyses and visualization [31, 32]. Construction of potential DCSV protein interaction networks was achieved by query of proteins reported to display protein-protein interactions in the Michigan Molecular Interaction (MiMI) database [31]. The human DCSV network map represents a model of the protein architecture of this organelle (figure 5). These protein systems support the function of secretory vesicles for production and secretion of neuropeptides.

## Modeling Neuropeptidomics in Human-Induced Pluripotent Stem Cells Differentiated into Neurons

Neuronal models of human neuropeptide producing cells will enhance investigation of the human proteases responsible for producing profiles of neuropeptides in neuropeptidomics studies. Investigation of protease pathways utilized in the human nervous system of the brain and peripheral sympathetic and parasympathetic systems is necessary to gain

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knowledge of human neuropeptidomics biosynthesis. Human patient-derived human-induced pluripotent cells (hiPSC) differentiated into neurons, as well as glia, provide an ideal strategy to define the human neurobiology of neuropeptide systems.

The first question to address about hiPSC neurons is their ability to synthesize and secrete neuropeptides. Recent studies show that hiPSC neurons produce enkephalin, dynorphin, and other neuropeptides that are secreted in an activity-dependent manner [33]. Moreover, the classical catecholamine neurotransmitters are also produced and secreted, indicating that hiPSC neurons model human neurotransmitter production and secretion. These seminal findings show that hiPSC neurons derived from patients provide human models of the human neurobiology of neuropeptides in health and disease.

Model hiPSC neurons from patients with neurological, neurodegeneration, mental disorders and many compromised conditions of brain dysfunctions [33–35] provide windows into human neuropeptide functions. Neuropeptidomics to define profiles of neuropeptides in disease compared to normal conditions may reveal new mechanisms of human disease conditions. These so-called 'disease-in-a-dish' studies are revolutionizing mechanistic studies of human disease conditions and will underscore the significance of neuropeptidomics systems in human health and disease.

## Human Neuropeptidomics to Define Neuropeptide Networks in Health and Disease

Related neuropeptides function together in the regulation of particular physiological processes. For example, regulation of blood pressure is achieved by the vasoactive neuropeptides including angiotensin, bradykinin, vasopressin and others. The control of pain relief is regulated by the endogenous opioid neuropeptides composed of the related enkephalin, β-endorphin, dynorphin, and nociceptin neuropeptides [6, 7]. Neuropeptidomics allows evaluation of the dynamic changes in neuropeptide profiles that regulate physiological functions. Furthermore, neuropeptidomics provides knowledge of how they are regulated in response to therapeutic drug agents used to treat human disease conditions.

For example, the angiotensin converting enzyme (ACE) inhibitor, captopril, is utilized as an anti-hypertensive drug. ACE converts angiotensin I to angiotensin II which is a vasoconstrictor and elevates blood pressure. Captopril inhibits ACE to result in reduced blood pressure. While captopril blocks the formation of angiotensin II, the drug regulates multiple vasoactive neuropeptides as demonstrated by neuropeptidomics studies [36]. Quantitative neuropeptidomics with MRM demonstrated that captopril not only decreases plasma angiotensin II but also regulates bradykinin and kallidin in a temporal manner (figure 6, for rats administered captopril). Future extension of these strategies to human plasma neuropeptidomics will be valuable to define how changes in networks of neuropeptides participate in the regulation of physiological processes.

Future human neuropeptidomics research will be significant for gaining new mechanistic understanding of human disease, providing new neuropeptide system components as drug targets for discovery of new therapeutic agents, and monitoring disease and therapeutic

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responses by neuropeptide biomarkers defined via neuropeptidomics investigation. Human neuropeptidomics research is predicted to undergo remarkable expansion and contribute to improving human health.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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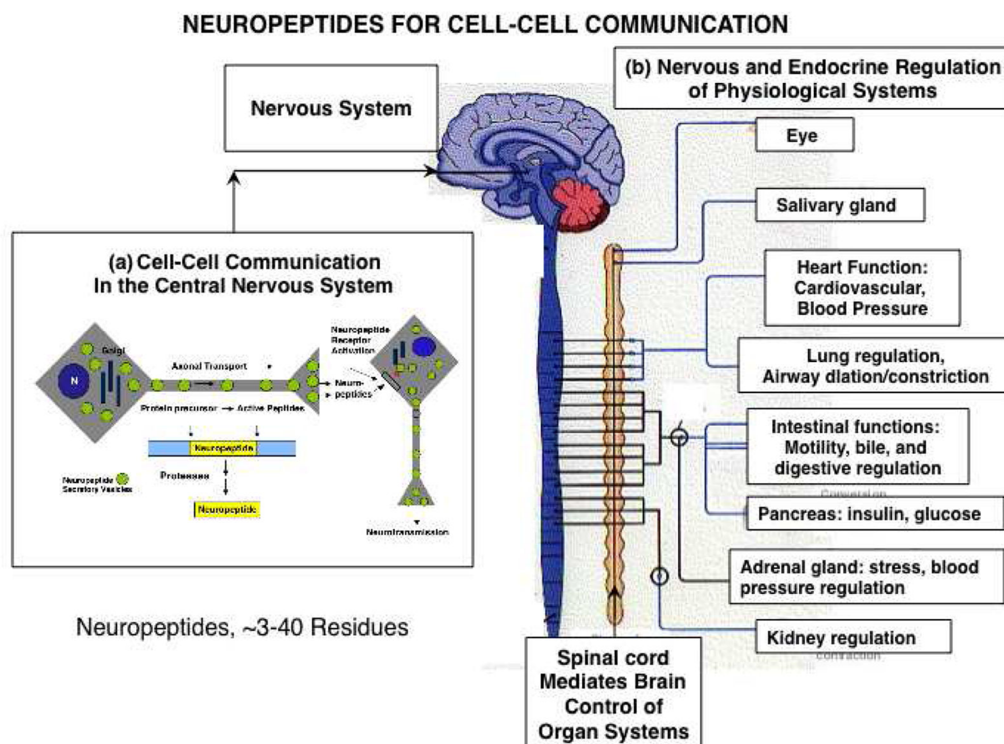


**Figure 1. Neuropeptides for neuronal and endocrine cell-cell communication**

(a) Neuropeptides in the central nervous system of brain. Brain neuropeptides function as peptide neurotransmitters to mediate chemical cell-cell communications among neurons. Neuropeptides are synthesized within secretory vesicles that are transported from the neuronal cell body via the axon to nerve terminals. The pro-neuropeptide (or prohormone) is packaged with the newly formed secretory vesicle in the cell body, and proteolytic processing of the precursor protein occurs during axonal transport and maturation of the secretory vesicle. Mature processed neuropeptides are contained within secretory vesicles at the synapse where activity-dependent, regulated secretion of neuropeptides occurs to mediate neurotransmission via neuropeptide activation of peptidergic receptors.

(b) Neuropeptides in the peripheral nervous system and endocrine systems for regulation of physiological organ functions. The peripheral nervous system regulates all organ systems, linking the central nervous system of the brain with peripheral neuronal control of physiological functions. In the body, neuropeptides also function as hormones that mediate endocrine cell-cell communication.

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Neuropeptidomics of Proenkephalin-Derived Peptides

**Figure 2. Human proenkephalin-derived neuropeptides assessed by neuropeptidomics**
Neuropeptidomic studies investigated endogenous peptides derived from human proenkephalin in secretory vesicles of human pheochromocytoma [12]. Neuropeptides derived from human proenkephalin (PE) are illustrated with respect to their location within PE. Peptides were identified by ion-trap and QTOF MS/MS, combined with InsPecT (Ins) and Spectrum Mill (SM) bioinformatics analyses of MS/MS data at 1% FDR (with the exception of Leu-enkephalin that was indicated at 5% FDR). Peptides identified under each of these conditions were mapped to PE, illustrated by colored lines: QTOF MS/MS data analyzed by InsPect (Ins, orange) or Spectrum Mill (SM, yellow), and ion-trap (Trap) analyzed by insPect (Ins, green) or SM (olive). Within PE, the active enkephalin neuropeptides sequences are shown in yellow. Dibasic cleavage sites are highlighted by boxes; in addition, monobasic residues within PE are shown. (Hyphens at the end of some lines indicate peptides that were split between two lines.)

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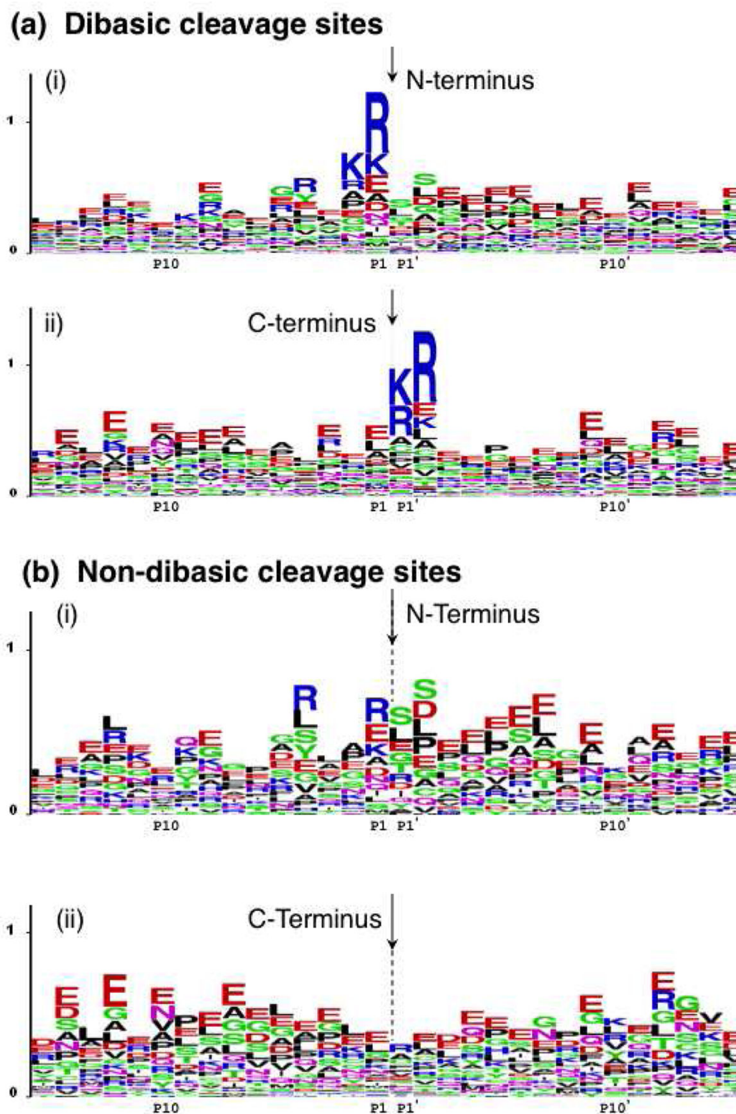


**Figure 3. Neuropeptidomics reveals processing of pro-neuropeptides at dibasic residue sites at and non-dibasic residues**

(a) Dibasic residues cleavage sites. Dibasic residue sites are major proteolytic cleavage sites of pro-neuropeptides in human secretory vesicles. Sequence LOGO maps of the N- and C-termini of neuropeptides in human secretory vesicles were generated by evaluation of adjacent residues to the identified peptides with their pro-neuropeptide precursors, as we reported [12].

*(i) N-terminal cleavage sites of identified neuropeptides*. The N-termini of peptides are indicated by the arrow, with illustration of the flanking amino acid sequences present within the peptides' pro-neuropeptides. The x-axis shows residues at the P1-P15 positions relative to the cleavage sties at P1-↓P1′ residues. The relative frequency of amino acids at each position is illustrated (y-axis).

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*(ii) C-terminal cleavage sites of identified neuropeptides*. The C-termini of peptides are indicated by the arrow, with residues of respective pro-neuropeptides flanking the C-termini at P1′-P15′ positions.

(b) Novel non-dibasic residue cleavage sites. Non-dibasic residue cleavage sites of pro-neuropeptides are illustrated after removal of the dibasic sites observed in the data.

*(i) N-terminal cleavage sites*. P1-P15 residues flanking the N-termini of identified peptides within their pro-neuropeptides are illustrated.

*(ii) C-terminal cleavage sites*. P1′-P15′ residues flanking the C-termini of identified peptides within their pro-neuropeptide precursors are illustrated.

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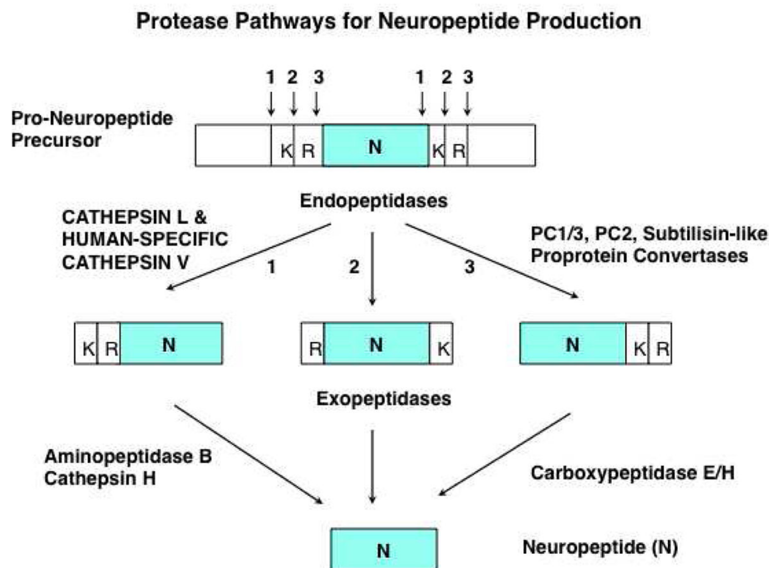


**Figure 4. Human cysteine and serine protease pathways for neuropeptide production**

Distinct cysteine protease and subtilisin-like protease pathways participate in proneuropeptide processing [5]. The cysteine proteases cathepsin L and human-specific cathepsin V in secretory vesicles functions as a processing enzyme for the production of neuropeptides. Human cathepsin L and cathepsin V cleave at dibasic residue sites within the pro-neuropeptide. Subsequence to cathepsins L and V, an aminopeptidase is needed to remove N-terminal basic residues, and carboxypeptidase E (CPE) removes C-terminal basic residues to generate active neuropeptides. The subtilisin-like protease pathway involves the proprotein convertases PC1/3 and PC2. The PC enzymes preferentially cleave at the C-terminal side of dibasic processing sites, which results in peptide intermediates with basic residue extensions at their C-termini that are removed by carboxypeptidase E.

In mammalian species other than human, cathepsin L with the PC1/3 and PC2 proteases participate in neuropeptide production.

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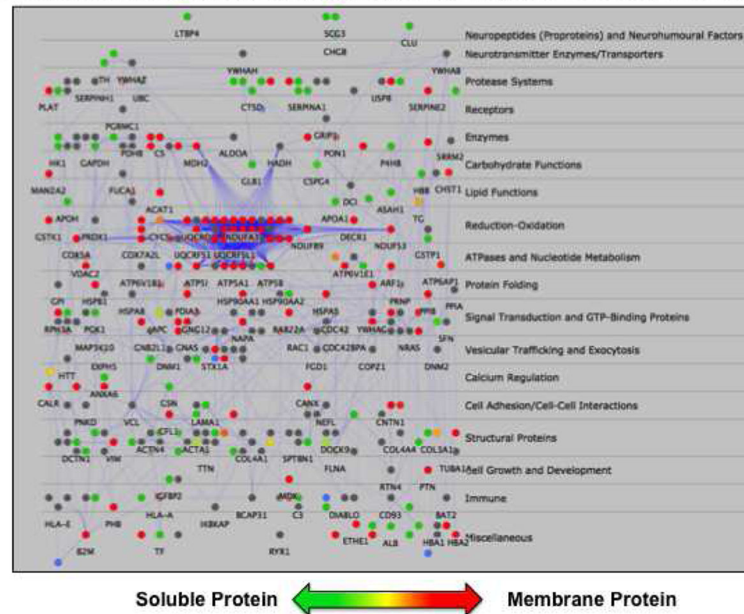


**Figure 5. Proteomics and systems biology of human neuropeptide secretory vesicles**

Proteomics data of human neuropeptide-containing secretory vesicles (dense core secretory vesicle, DCSV, type) was subjected to Cytoscape systems biology analyses for predicting protein interaction networks [31, 32]. The functional protein categories are shown on the right hand side. Based on quantitative NASF data of the MS/MS identified proteins, proteins are indicated as soluble (green circles) or membrane (red circles) proteins, or present in both soluble and membrane at similar levels (yellow circles). Proteins shown as gray circles were identified, but not quantitated since they did not meet the criteria for quantitation of lat least 3 identifications out of 4 nano-LC-MS/MS runs.

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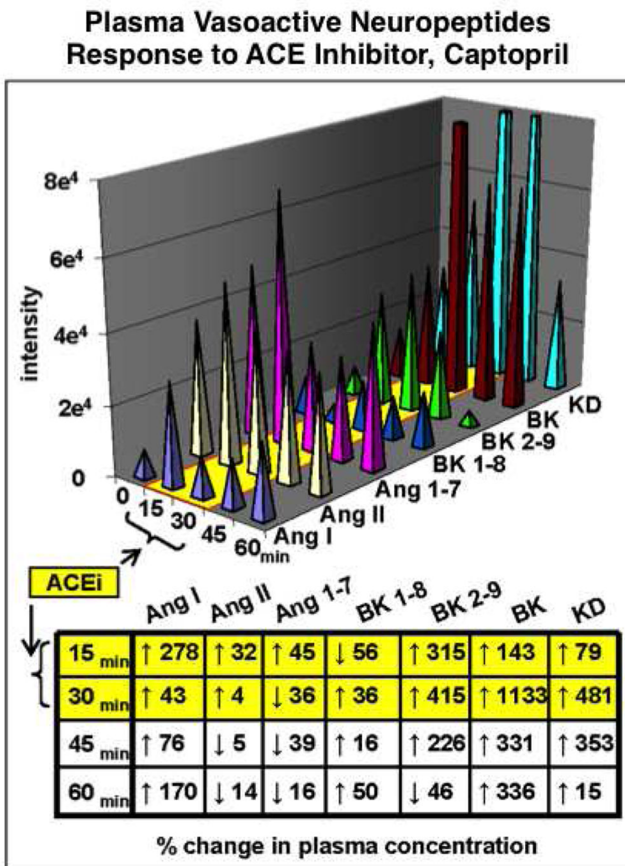


| | Ang I | Ang II | Ang 1-7 | BK 1-8 | BK 2-9 | BK | KD |
|---|---|---|---|---|---|---|---|
| 15 min | ↑ 278 | ↑ 32 | ↑ 45 | ↓ 56 | ↑ 315 | ↑ 143 | ↑ 79 |
| 30 min | ↑ 43 | ↑ 4 | ↓ 36 | ↑ 36 | ↑ 415 | ↑ 1133 | ↑ 481 |
| 45 min | ↑ 76 | ↓ 5 | ↓ 39 | ↑ 16 | ↑ 226 | ↑ 331 | ↑ 353 |
| 60 min | ↑ 170 | ↓ 14 | ↓ 16 | ↑ 50 | ↓ 46 | ↑ 336 | ↑ 15 |

**Figure 6. Vasoactive neuropeptides regulated by ACE inhibitor drug therapeutics**

The effects of an ACE (angiotensin converting enzyme) inhibitor, captopril, on levels of plasma vasoactive peptides was analyzed in time-course studies by nano-LC-MS/MS with quantitation using stable isotope-labeled internal peptide standards [36]. ACE inhibitors are utilized as anti-hypertensive drugs. Chromatographic separation of target peptides and multiple reaction monitoring (MRM) provided quantitation of angiotensin I (Ang I), Ang II, Ang1-7, bradykinin 1-8 (BK 1-8), BK-2-9, and kallidin (KD). Results show significant reduction by the ACE inhibitor of the angiotensin peptides, with an interesting concomitant increase in plasma bradykinins and kallidin (potent vasodilators). The percent change in plasma concentration at different times after drug administration is shown in the table below the bar graph. Results illustrate the utility of simultaneous profiling of multiple peptides using mass spectrometry analysis to monitor drug-induced changes in vasoactive neuropeptides.

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**Table 1**

Neuropeptides in the Nervous and Endocrine Systems

| Neuropeptides | Physiological Functions |
|---|---|
| Enkephalins | Analgesia, pain relief |
| β-Endorphin | Analgesia, pain relief |
| Dynorphin | Analgesia, pain relief |
| CRH | Stress, glucocorticoid production |
| ACTH | Steroid production |
| α-MSH | Skin pigmentation, appetite |
| Insulin | Glucose metabolism |
| Glucagon | Glucose metabolism |
| Galanin | Cognition |
| NPY | Obesity, blood pressure |
| Somatostatin | Growth regulation |
| Vasopressin | Water balance |
| Calcitonin | Calcium regulation, migraine |
| Cholecystokinin | Learning, memory, appetite |

Neuropeptides function as peptide neurotransmitters and peptide hormones.

Examples of several neuropeptides and their biological functions are shown in this table.

ACTH, adrencorticotropin hormone; α-MSH, α-melanocyte stimulating hormone; NPY, Neuropeptide Y; CRH, corticotropin releasing hormone.

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## Table 2

Neuropeptidomics Compared to Proteomics Publications

| Topic | Number of Citations (PubMed) | Dates of Publications |
|---|---|---|
| Neuropeptidomics: | | |
| Human Neuropeptidomics | 9 | 2006 – 2015 |
| Neuropeptidomics | 32 | 2004 – 2015 |
| Peptidomics | 366 | 2001 – 2015 |
| Proteomics: | | |
| Human Proteomics | 30,141 | 1998 – 2015 |
| Proteomics | 55,388 | 1998 – 2015 |

Search of PubMed was conducted using the key words listed above. The number of publications shown for each search (as of July, 2015) is shown. It is realized that use of related search phrases or terms will also reveal published articles on these topics; however, the recent terminology of "neuropeptidomics" and "peptidomics" in the literature is shown to occur since approximately the year 2001. A tremendous difference is observed in the number of articles published for 'human neuropeptidomics' compared to 'human proteomics'. Among the 'neuropeptidomics' and 'peptidomics' topics, 'human neuropeptidomics' is a small portion of such published studies to date.