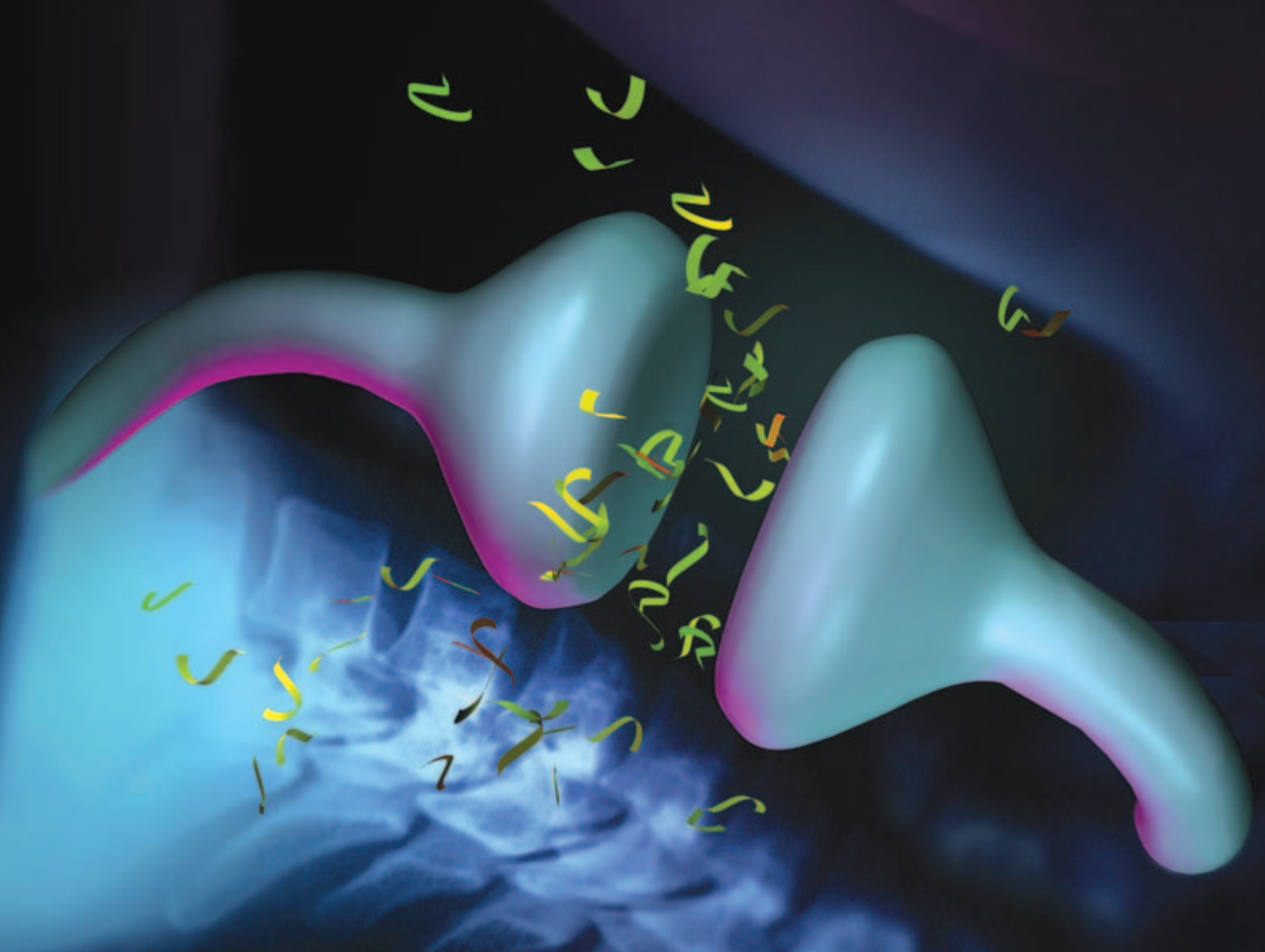

Neuropeptidomics: MS Applied to the Discovery of Novel Peptides from the Brain

BY COMPARING THE PROTEINS AND PEPTIDES IN DISEASED AND NORMAL TISSUES, RESEARCHERS CAN IDENTIFY DIFFERENTIAL EXPRESSION PATTERNS THAT MAY LEAD TO BIOMARKERS.

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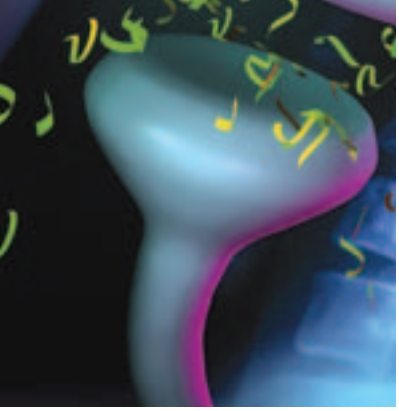
The characterization of genomes of an ever-growing number of species, including the human genome, has provided new technologies and theories necessary to tackle the evaluation of very complex biological systems (*1–3*). In the field of proteomics, valuable information about the function of the molecular products encoded in the genome is obtained, and modern proteomics approaches have significantly improved the possibilities for high-throughput identification of proteins. However, core proteomics tools, including 2D gel electrophoresis (2DGE) in combination with MS, are limited to the analysis of proteins >10 kDa. Other technologies are therefore necessary to identify small proteins and peptides in the samples (Figure 1).

The expressions "peptidome" and "peptidomics" came into use in 2001 and are analogous to the terms "proteome" and "proteomics" (*4–7*). Peptidomics involves the comprehensive analysis of the peptide content of a certain cell, organ, body fluid, or organism. It complements molecular biology approaches in its ability to characterize the processing of translation products, including changes in expression or posttranslational modifications (PTMs) of peptides and small proteins. By comparing the proteins and peptides in samples of diseased tissue with those in normal tissue, researchers can identify differential expression patterns that may lead to the identification of novel biomarkers. In this article, we discuss neuropeptidomics approaches in brain lysates and tissue sections to study changes in peptide expression in different animal disease models and to identify novel, potentially biologically active neuropeptides.



Neuropeptides—the basic facts

Proteins and peptides play a pivotal role in human life because they are involved in almost all physiological processes. In the early 1970s, de Wied and colleagues used the term "neuropeptide" for the first time to describe an endogenous substance synthesized in nerve cells and involved in nervous-system functions (*8*). The definition has evolved from this origin-oriented one to a functional one (*9*). Neuropeptides are present throughout the central nervous system as well as in peripheral organs such as the pancreas, the adrenal glands, and the cells of the immune system (*10*, *11*).

By definition, neuropeptides are smaller than regular proteins, usually contain only 2–50 amino acid residues, and have less complex 3D structures. Posttranslational processing of polyprotein precursors into active neuropeptides occurs in the cell body by a common mechanism. The processing enzymes include endopeptidases that cleave the propeptide at specific sites, usually at pairs of the basic amino acids lysine and/or arginine, which are subsequently removed from the C-terminus by carboxypeptidase enzymes.

Several peptides can be produced from a single polypeptide. In addition, neurons with the same gene encoding a protein may release different neuropeptides because of differences in the way each neuron processes the polypeptide. In brief, peptides are produced by a complex network of a relatively small number of proteases and peptidases functioning at different sites, such as intracellularly, upon secretion, and in the extracellular environment. However, proteases and peptidases also regulate the degradation and inactivation of peptides (*12*).

The receptors for many of the neuropeptides found in nervous tissue have been cloned. With very few exceptions (*13*, *14*), they are coupled to G proteins and have the characteristic seven membrane spanning α helices. Neuroactive peptides are removed more slowly than small-molecule transmitters from the synaptic cleft, where the mechanisms of peptide removal are diffusion, proteolysis by extracellular peptidases, and receptor–peptide internalization. The slow removal of neuropeptides contributes to the lengthy duration of their effects.

Historically, the isolation of a novel neuropeptide was achieved by extraction from hundreds of kilograms of tissue from selected brain areas or other organs. The discovery by Mutt and Tatemoto that C-terminal amidation was a special feature of neuropeptides led to faster and more sensitive extraction procedures (*15*). More recently, molecular biology procedures have been used to discover additional neuropeptides. For example, calcitonin gene-related peptide was shown to exist as a result of alternative mRNA splicing of the calcitonin gene (*16*). The subtractive hybridization approach was used to discover the hypocretins and orexins, which are peptides that regulate feeding and wakefulness behavior (*17*). Orphan G-protein-coupled receptors (GPCRs) have been used as targets to identify and isolate their endogenous ligands, for example, nociceptin, the natural ligand for the opioid-like receptor (*18*, *19*).

Neuropeptidomics

Proteomics has recently been combined with peptidomics, a technology aimed at thorough visualization and analysis of small endogenous polypeptides in the molecular-mass range 1–20 kDa (*4–6*, *20*). Peptidomics is a relatively new direction in proteomics research that covers the gap between proteomics and metabolomics and overlaps with both areas. However, early contributions to this field in terms of novel peptide identifications and characterizations were made from experiments on invertebrates, such as locust, fruit fly, and flesh fly, even before the term peptidomics was invented (*21*, *22*). Peptidomics methodologies are generally based on separating complex endogenous peptide mixtures by LC—usually nanoscale capillary reversed-phase LC (nanoLC; *23*, *24*), multistep chromatographic approaches (*25*, *26*), or gel- or liquid-based isoelectric focusing (*27*)—combined with MS to identify peptides.

Another approach for the discovery of peptides from brain samples is the partial purification of homogenate from the biochemically defective $Cpe^{fat/fat}$ mouse. These mice have a point mutation within the coding region of the carboxypeptidase E (CPE) gene that causes the CPE enzyme to be inactive and rapidly degraded (*28*). CPE is an important enzyme in the biosynthesis of many neuropeptides; thus, the mice accumulate peptide-processing intermediates that contain C-terminal basic residues (*28*). Because of the extended C-terminus, these peptide precursors may subsequently be isolated from tissue extracts by affinity chromatography (*29*).

Hence, neuropeptidomics is the technological approach for detailed analyses of endogenous peptides from the brain (*5*, *23*, *30–34*). The levels of peptides in the brain reflect certain information about physiological status; this information can be revealed when MS is used to generate broad profiles of the dynamic neuropeptide pattern. The neuropeptidomics approach is an excellent example of how to further reveal the way neuropeptides are diversified from a single gene, which releases a variety of regulated, biologically active peptides.

The neuropeptidomic detection system has to accomplish several tasks simultaneously—detect and quantify peptide patterns in the sample, compare and select peptides that differ in abundance more than normal biological variation, and identify and further characterize the selected peptides. This qualitative and quantitative procedure has been termed "differential peptide display" (*4*, *23*, *35*). The MS approach, with either ESI (*36*) or MALDI (*37*), permits sensitive, simultaneous detection of peptide changes in complex mixtures of hundreds of different peptides. The resolution and specificity of a neuropeptide analysis are further enhanced by coupling MS to LC or other high-resolution separation techniques.

Importance of sample preparation

Postmortem enzyme activity plays an important role in the integrity of the peptide and protein content in the brain as well as in detecting PTMs of proteins and peptides. We have found that substantial protein and peptide degradation occurs in brain tissue within minutes postmortem (*23*) and that instant denaturation of the brain allows neuropeptides to remain intact and minimizes

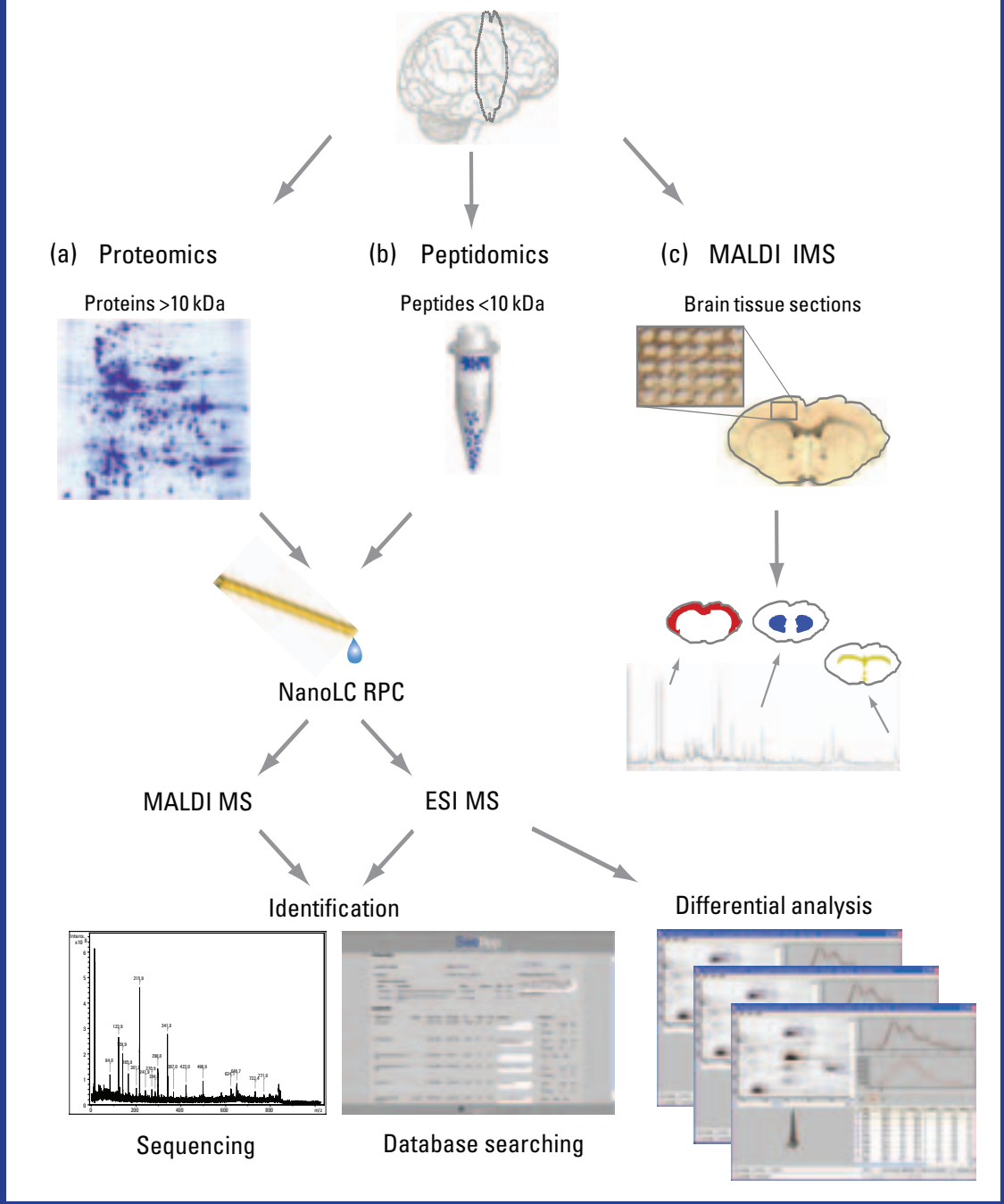
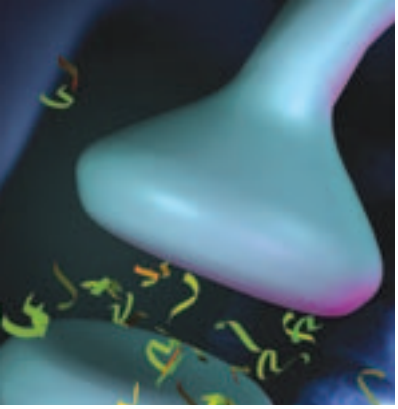


FIGURE 1. Workflows.

(a) Proteomics usually involves separation by 2DGE, detection of proteomic changes by difference GE (DIGE), and identification of tryptic peptides by MS. (b) In the peptidomics approach, partial purification of homogenized tissue is followed by nanoLC and ESI MS or MALDI MS. Acquired mass spectra are semiquantitatively compared among samples. The SwePep database significantly speeds up the MS identification of complex tissue samples by comparing acquired data to the peptide masses stored in the database. This intermediate identification step is fast and singles out possible peptide matches, which can later be confirmed by MS/MS. (c) Imaging MALDI MS allows simultaneous mapping of peptides and small proteins in thin tissue sections with a lateral resolution down to ~30–50 µm. Molecular images from a raster over the sample surface are created. The laser position is fixed, and the sample plate is repositioned for consecutive spots. In brain tissue sections, each spot produces a mass spectrum of typically 200–500 protein and peptide peaks present within the area. MALDI TOF/TOFMS can identify detected peptides and small proteins on the tissue section (up to ~2000–3000 Da). Larger proteins must be tryptically digested directly on the tissue or purified using conventional proteomics.



degradation of proteins by proteolysis (*33*). To inactivate the enzymes, we used focused microwave irradiation to sacrifice rats and mice in our studies. Such irradiation raises the brain temperature to 90 °C within 1.4 s, thereby rapidly denaturing the enzymes and preventing postmortem degradation of proteins and peptides (*38*). In our earlier experiments that used traditional sacrificing methods, fragments from hemoglobin were a major peptide source and were detected at high levels in the brain (*23*). Interestingly, none of these hemoglobin-derived peptide fragments were detected in microwave-irradiated brain tissue. It is clear from these and other studies that the microwave irradiation procedure has several advantages (*23*, *33*, *39*, *40*): the relatively low abundance neuropeptides remain intact, the proteolytic degradation of proteins is minimized, and the PTMs of the neuropeptides are conserved.

A conventional method to block postmortem degradation of proteins and peptides is paraformaldehyde perfusion of the brain. This procedure is commonly used in immunohistochemical methods to detect neurotransmitters and neuromodulators and in individual neurites to produce excellent spatial information (*41*). However, this procedure reduces the intensity of the peptide peaks in MS, probably because many peptides become cross-linked into the polymer matrix by fixation (*42*).

Another method to prevent postmortem degradation is to use a microwave to heat the brain instantly after it is removed (*30*). Although there is a delay of several seconds before the brain is heated to 80 °C, this does not seem to contribute to neuropeptide degradation. Nevertheless, it should be noted that several hemoglobin degradation fragments were detected, although it is not clear whether these fragments were generated during the peptide-extraction procedure or the in vitro microwave heating or were, in fact, degradation products due to the postmortem delay before the sample was frozen (*30*).

Our laboratory has used an alternative, novel sample-inactivation method for instant fixation of brain samples, brain tissue sections, and other tissues. Direct and rapid fixation of snap-frozen (within 10 s) brain tissue with a tissue-fixation instrument (Denator Biotechnology) showed a peptide display pattern similar to the in vivo, focused-microwave-irradiated brains. This device permits controlled and uniform heat transfer, thereby rendering the sample proteins denatured and inactivated with their primary structures conserved. This method also conserved the phosphorylations of neuropeptides and proteins.

When we performed western blots with phosphospecific antibodies, the phosphorylation levels of the protein MAPK were found to be similar in the instantly heat-fixed, snap-frozen brain tissue and in the corresponding tissue of in vivo focused-microwave-irradiated brains. Phosphorylated proteins are known to be subject to variable rates of dephosphorylation during the postmortem interval (*43*). Accordingly, the phosphorylation levels of MAPK were reduced by 85% in striatal brain tissue subjected to a 3-min postmortem delay. When the phosphorylated corticotropin-like intermediate lobe peptide (CLIP) was measured, we detected a similar 85% reduction of phosphorylation in 3-min postmortem-delay samples (unpublished data).

Discovery of novel endogenous peptides

We have developed a comprehensive peptidomics approach to analyze complex tissue extracts from the brains of rats and mice. Using nanoflow capillary LC/ESI MS with automatic-switching tandem MS, we detected and identified known neuropeptides, novel endogenous peptides, and endogenous peptides with PTMs that had not previously been described for those particular peptides (*33*). Our targets were neuropeptides from the hypothalamus, a phylogenetically ancient region of the mammalian brain that plays an important role in mediating physiological processes such as reproduction, lactation, fluid balance, and metabolism. Because neuropeptides are involved in these processes, the hypothalamus is an excellent source for studies of functionally relevant neuropeptides and their PTMs.

After homogenization and partial separation of the brain tissue on a 10-kDa centrifugal filter, we separated the peptide extracts by reversed-phase LC and analyzed them by ESI MS. We detected and sequenced the peptides with quadrupole TOF, linear ion trap, and FT ion cyclotron resonance mass spectrometers. Mass spectra were collected in the m/z range 300–1200 Da. The data were converted and imported into DeCyder MS (GE Healthcare) for 2D and 3D visualization and for qualitative and semiquantitative interpretation. In a typical experiment, we could detect >550 peptide ions that produced distinct MS peaks during the 60-min gradient (*23*, *33*). Sequence information for the peptides was obtained in the data-dependent acquisition mode. On the basis of intensity, the mass spectrometer automatically performs collision-induced dissociation tandem MS on selected precursor peptide ions during the nanoLC separations.

As a complement to ESI MS, capillary LC and MALDI TOF/TOF/TOF combinations were successfully used to identify and quantify neuropeptides (*44*). In this procedure, the peptide samples were separated on a reversed-phase column and directly spotted onto MALDI target plates or onto prespotted targets with an on-line fraction collector. When conventional MALDI targets were used, the matrix solution was mixed with the peptide sample before deposition with a microdosage pump. Typically, the flow rate was ~1 μL/min and the deposition rate was ~2 spots/min. The MALDI targets were then analyzed in automated-acquisition mode—first, MS was performed; then, MS/MS analysis of data-dependent, selected precursor ions was done. A considerable advantage when MALDI MS is used is the off-line coupling of the LC separation. This allows replicate analyses and in-depth selection of specific precursor ions, thereby increasing the efficiency of identifying low-abundance peptides.

The MS/MS data were analyzed and interpreted with the search engines Mascot, X!TANDEM, and TurboSequest with criteria such as "no enzymatic cleavage" and various common peptide modifications (e.g., N-terminal acetylation and C-terminal amidation). Unsolved spectra often needed software-assisted de novo sequencing or manual sequencing. The proposed peptide sequences were then compared with the nonredundant database of the National Center for Biotechnology Information to

establish the peptide identities with the Basic Local Alignment Search Tool (www.ncbi.nlm.nih.gov/BLAST) with the instruction to "search for short nearly exact matches".

Table 1 provided in Supporting Information summarizes the data on endogenous peptides and small proteins identified from rat- and mouse-brain tissues in our laboratory, with the exception of ~40 novel endogenous peptides that will be published separately. The total number of uniquely identified peptides is 74, 48 from mouse tissue and 51 from rat tissue. The peptide sequences are conserved between the two species with a few exceptions, for example, met-enk-RSL/RGL, neuropeptide-EI, bigLEN, and littleSAAS, which differ by 1–4 amino acids.

Recently, other researchers have confirmed several of the novel endogenous peptides that we have published (*30*, *33*, *45*). Most of the peptides listed in Table 1 are cleaved enzymatically at characteristic processing sites for prohormone convertases. Moreover, the majority of the peptides originate from known neuropeptide precursors, annotated in UniProt. Many of the classical neuropeptides, such as neurotensin, substance P, neurokinin A, CLIP, and β-endorphin, were identified both in rat- and mouse-brain tissues (Supporting Information).

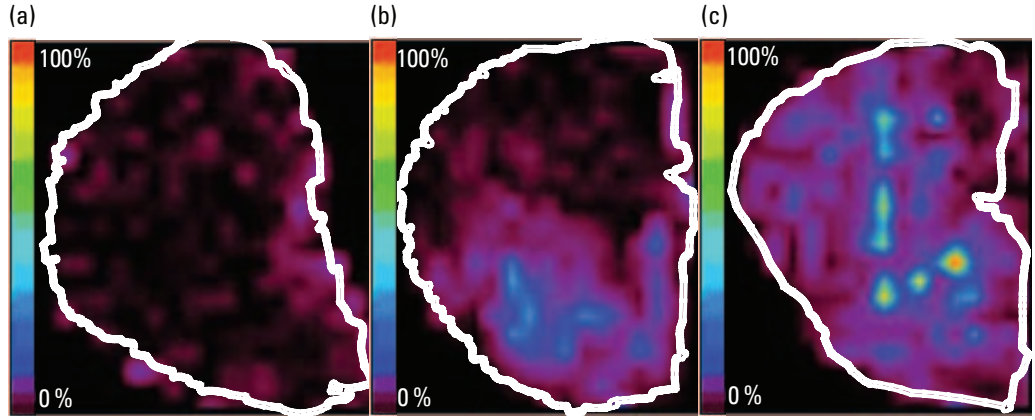


FIGURE 2. Imaging MALDI MS of one hemisphere of coronal mouse-brain tissue sections shows the ion-intensity distribution of a 2770-Da peptide from (a) control (saline-treated), (b) MPTP-lesioned, and (c) MPTP/L-DOPA treated animals, respectively.

Other laboratories have subsequently used similar approaches to detect and identify novel peptides from the brain. For example, in mouse-brain pituitary, several peptides or protein fragments were detected, such as a modification of the POMC-derived J-peptide, an N-terminal fragment of VAP-33 protein, and a novel modified form of β-tubulin (*30*). Furthermore, novel peptides from the protein precursors proenkephalin A, prodynorphin, chromatogranin B, chromatogranin C, protachykinin 1, phosphatidylethanolamine binding protein, neurogranin, synapsin 1, synapsin IIb, ProSAAS, neuropeptide Y receptor type 4, neurosecretory protein VGF precursor, and homeobox protein engrailed-1 were identified in rat striatum that was subjected to the in vivo focused-microwave treatment (*44*, *45*).

Endogenous peptides from brain extracellular fluid of live rats were obtained by in vivo microdialysis and were analyzed by nanoLC coupled to MS/MS. These peptides were possible candidates for neurotransmitters, neuromodulators, and markers of synaptic activity or brain-tissue damage. An interesting approach to examining the neuroactivity of the novel proenkephalin fragments was to infuse the peptide into the brain while monitoring amino acid neurotransmitters by microdialysis sampling combined with CE (*46*). Three of the six tested proenkephalin peptides induced significant increases in various neuroactive amino acids. This study demonstrates that this combination of methods can identify novel neurotransmitter candidates and screen for potential neuroactivity.

SwePep database

Accurately predicting all of the neuropeptides produced by a particular gene is difficult, because of the large variation in PTMs and the complex processing pattern that a peptide precursor undergoes while it is being proteolytically cleaved to a biologically active neuropeptide (*47*). Most software for peptide sequencing is designed for identification of enzymatically cleaved peptides and proteins. Using this software to identify endogenous peptides is troublesome, slow, and gives poor results. The SwePep database (www.swepep.org; *47*) is constructed to speed up the process of identifying endogenous peptides in complex tissue samples analyzed by MS.

The database contains 2438 unique neuropeptides and 25,047 small (<10-kDa) proteins. The neuropeptides are derived from 1643 precursor proteins from 394 different species. All peptides have searchable descriptors, such as mass (monoisotopic and average), p*I*, tissue origin, subcellular location, modifications, precursor information, and organism affiliation. The data in SwePep are being collected from data produced in-house (*33*), UniProt (www.ebi.uniprot.org/uniprot-srv/index.do), and the literature. New peptides and protein fragments from MS data are divided into classes—biologically active peptides, potential biologically active peptides, and uncharacterized peptides. This organization makes the data in SwePep consistent and provides the ability to store information about peptides that may have a biological function that may be unproven.

In the identification process, the experimental peptide masses are compared with the peptide masses stored in the database, both with and without PTMs. This search takes <1 min and suggests ≤2 peptides for a specific experimental mass. A peptide-mass match from SwePep is a suggestion for an identification and needs to be confirmed by the analysis of the corresponding tandem mass spectrum. For the time being, the validation is done manually; however, tandem mass spectra are saved in the data-

ENDOGENOUS PEPTIDES FROM BRAIN EXTRACELLULAR FLUID WERE CANDIDATES FOR NEUROTRANSMITTERS, NEUROMODULATORS, AND MARKERS OF SYNAPTIC ACTIVITY OR BRAIN-TISSUE DAMAGE.

base, and in the near future it will be possible to perform the validation process automatically.

We recently used SwePep to investigate the identities of neuropeptides collected from hypothalamic tissue samples from a rat. The MS data were automatically analyzed by DeCyder MS, and ~400 specific peptide masses were assigned. To quickly identify known peptides, we compared the generated mass list with the neuropeptide masses stored in SwePep. The absolute difference between the theoretical and experimental masses was set to be ≤0.2 Da for a match to be valid. SwePep suggested 54 neuropeptide candidates, and out of these, 31 were verified by tandem MS. This fast identification of previously stored peptides permits us to put more effort into the task of identifying novel peptides.

A model for predicting the processing of mammalian neuropeptide precursors was also recently presented to facilitate the MS characterization of neuropeptides from genetic information (*48*). This model limits the number of putative peptides expected from a novel prohormone and reduces the time needed to analyze MS data.

Neuropeptides in disease models

MS-based peptidomics technology in combination with sophisticated bioinformatics tools has great potential for the discovery of biologically relevant peptides as biomarkers for diseases (*49*–*52*). A biomarker is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention" (*53*). To this end, peptidomic approaches have been applied to profile complex samples, including bodily fluids, cell cultures, and animal models (*20*, *54*, *55*).

A number of recent neuropeptidomic analyses focused on biomarker discovery in cerebrospinal fluid (CSF). CSF is regarded as the sample matrix closest to the brain where the pathology of the central nervous system occurs (*56*). CSF peptides and proteins may be derived directly from neuronal cells in the brain, or they may be actively transported across the blood–brain barrier and alter the composition of CSF. Profiling peptides and small proteins reveals several thousand individual peptide signals per sample (*57*, *58*). These signals have been screened for differential peptide display in various neurodegenerative disorders (*59*, *60*). For example, biomarker candidates were selected from >6000 different peptide signals in CSF of Alzheimer's patients; these were compared with peptides from cognitively unimpaired subjects and from patients suffering from other primary dementia disorders (*60*). Twelve candidates were identified as fragments of the possibly neuroprotective neuroendocrine protein VGF and one as C3f, a descendant of complement factor C3.

We have investigated neuropeptide expression in experimental models of Parkinson's disease (the MPTP mouse model and 6-OHDA rat model; *61*). Parkinson's disease is a common neurodegenerative disorder that is characterized by the progressive loss of dopamine-producing neurons in the brain and affects movements, muscle control, and balance. The neurotoxins MPTP and 6-OHDA cause cell death in the nigrostriatal dopamine pathway. Using the peptidomics approach mentioned earlier, we found a number of differentially expressed peptides and small proteins, for example, PEP-19 (*55*, *62*, *63*).

PEP-19 is a 6.7-kDa polypeptide that belongs to a family of proteins involved in calcium transduction through their ability to interact with calmodulin. Calcium ions play fundamental roles in cell signaling and controlling processes such as neurotransmitter release, muscle contraction, transcriptional regulation, and cell death. Failure of calcium buffering or intraneuronal calcium homeostasis contributes to calcium-mediated cytotoxic events in the pathogenesis of neurodegenerative diseases (*64*).

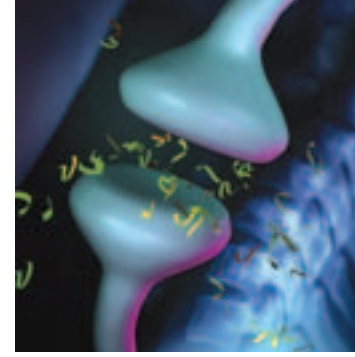
We used imaging MALDI MS (IMS; *65*) to further characterize the striatal loss of PEP-19 in MPTP-treated animals. In this technique, the proteins and peptides in thin sections are directly imaged (*66*). Typically, a frozen tissue section is mounted on a target plate and is covered with an energy-absorbing matrix, for example, sinapinic acid. The mass spectrometer measures the specific molecular weight of molecules that are desorbed from the surface of the tissue. Arrays of mass spectra are assembled into ion images, thus visualizing the spatial distribution of selected molecules.

In our case, thin tissue sections from the mouse brain were mounted on MALDI target plates, coated with matrix solution, and analyzed by MALDI TOFMS. In addition to previously identified, highly abundant small proteins and peptides in the mass range 5–25 kDa (e.g., thymosin-β4, thymosin-β10, ubiquitin, cytochrome oxidase, myelin basic protein, FKBP-12, and calmodulin), the image analysis showed a significant decrease in the expression of PEP-19 in the striatum of MPTP-treated animals compared with a control. Another peptide found to be regulated in the mouse model of Parkinson's disease is a so-far-unidentified peptide at mass 2770 Da. This peptide is almost absent in all the saline-treated control animals but is up-regulated in animals with MPTP-induced lesions and animals treated with MPTP/L-DOPA (Figure 2; unpublished results).

Future perspectives

A substantial number of neuropeptides may still be unidentified. In addition, the human genome contains ~550 GPCR genes, 25% of which have no known ligands and many of which may be neuropeptides (*10*). Improved approaches and technologies are therefore necessary to unravel unidentified neuropeptides from complex biological matrices, such as the brain.

Handling of tissue samples is important when neuropeptides are analyzed by MS. The peptidomics approach is sensitive and capable of detecting a large number of biologically active peptides at low levels in one analysis if the tissue was instantly denatured (*33*). The background of highly abundant protein fragments, caused by postmortem and sampling degradation, will



suppress signals from the neuropeptides. Obviously, a low level of degradation peptides will always exist in the sample as part of the protein-to-peptide homeostasis. The peptidome and the degradome of a sample are directly linked to the proteome. The distribution of these is regulated by protein synthesis, protease activity, and protease-inhibitor activity. A disease state may change this distribution and may thus be used to characterize it.

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Marcus Svensson, Karl Sköld, Anna Nilsson, and Maria Fälth are graduate students; Katarina Nydahl is a researcher; and Per E. Andrén is a professor of neurobiology and director of the Medical Mass Spectrometry Laboratory at Uppsala University (Sweden). Svensson and Sköld are working in the area of neuroproteomics, MS, and endogenous peptides. Nilsson's main focus is neuropeptidomics of models of Parkinson's disease with nanoLC MS and imaging MALDI MS. Fälth is working with bioinformatics solutions for identifications of endogenous neuropeptides. Nydahl's current position is study director at AstraZeneca R&D (Sweden). Andrén's interests include using electrospray and MALDI MS to study neuropeptides in live animal or viable tissue systems. Current work involves the application of imaging MALDI MS and nanoLC MS of the brain, particularly in Parkinson's disease applications. Per Svenningsson is a physician and an associate professor in pharmacology at the Karolinska Institutet (Sweden). His research interest is serotonin–dopamine interactions and signal transduction in animal models of Parkinson's disease, depression, and psychostimulant abuse. Address correspondence about this article to Andrén at the Laboratory for Biological and Medical Mass Spectrometry, Uppsala University, Box 583, SE-75123 Uppsala, Sweden (per.andren@bmms.uu.se).

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