Neuropeptidomics – Methods and Applications

KARL SKÖLD
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Abstract

The sequencing of genomes has caused a growing demand for functional analysis of gene products. This research field named proteomics is derived from the term proteome, which by analogy to genome is defined as all proteins expressed by a cell or a tissue. Proteomics is however methodologically restricted to the analysis of proteins with higher molecular weights. The development of a technology which includes peptides with low molecular weight and small proteins is needed, since peptides play a central role in many biological processes.

To study endogenous peptides and hormones, the peptidome, an improved method comprising rapid deactivation in combination with nano-flow liquid chromatography (LC) and mass spectrometry (MS) was developed. The method has been used to investigate endogenous peptides in brains of mouse and rat. Several novel peptides have been discovered together with known neuropeptides.

To elucidate the post mortem time influence on peptides and proteins, a time course study was performed using peptidomics and proteomics technologies. Already after three minutes a substantial amount of protein fragments emerged in the peptidomics study and some endogenous peptides were drastically reduced with increasing post mortem time. Of about 1500 proteins investigated, 53 were found to be significantly changed at 10 minutes post mortem as compared to control. Moreover, using western blot the level of MAPK phosphorylation was shown to decrease by 95% in the 10 minutes post mortem sample.

A database, SwePeP (a repository of endogenous peptides, hormones and small proteins), was constructed to facilitate identification using MS. The database also contains additional information concerning the peptides such as physical properties. A method for analysis of LC-MS data, including scanning for, and further profiling of, biologically significant peptides was developed. We show that peptides present in different amounts in groups of samples can be automatically detected.

The peptidome approach was used to investigate levels of peptides in two animal models of Parkinson’s disease. PEP-19, was found to be significantly decreased in the striatum of MPTP lesioned parkinsonian mice. The localization and expression was further investigated by imaging MALDI MS and by in situ hybridization. The brain peptidome of reserpine treated mice was investigated and displayed a number of significantly altered peptides. This thesis demonstrates that the peptidomics approach allows for the study of complex biochemical processes.

Keywords: mass spectrometry, proteomics, peptidomics, neuropeptide, bioinformatics

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This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.


III The significance of biochemical and molecular sample integrity in brain proteomics and peptidomics: Stathmin (2-20) and peptides as sample quality indicators. Karl Sköld, Marcus Svensson, Mathias Norrman, Sara Sjöberg, Jesper Hedberg, Benita Sjögren, Per Svenningsson, Per E Andrén. Manuscript

IV SwePep – A database designed for endogenous peptides and mass spectrometry. Maria Fälth, Karl Sköld, Mathias Norrman, Marcus Svensson, David Fenyö, Per E Andrén. Mol Cell Proteomics, 2006, 5, 998-1005

V An automated method for scanning LC-MS data sets for significant peptides and proteins, including quantitative profiling and interactive conformation. Anders Kaplan, Malin Söderström, David Fenyö, Anna Nilsson, Maria Fälth, Karl Sköld, Marcus Svensson, Harald Pettersen, Staffan Lindqvist, Per Svenningsson, Per E Andrén, Lennart Björkesten. Manuscript


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## Contents

Introduction.....................................................................................................9  
Background to peptidomics.................................................................10  
Neuropeptides and hormones .........................................................10  
Sample preparation..............................................................................13  
Issues using post mortem brain tissue .............................................14  
Inactivation methods ........................................................................14  
Denaturation ......................................................................................14  
Thermal denaturation.......................................................................15  
Proteomics denaturation methods.....................................................15  
Other inactivation methods ..............................................................16  
Protease inhibitors ..........................................................................16  
Analysis of brain peptides and proteins .............................................17  
Separation ..........................................................................................17  
Detection ...........................................................................................18  
Identification of neuropeptides ........................................................23  
Databases - SwePep .........................................................................24  
Aims of studies ..................................................................................26  
Methods .............................................................................................27  
Animals ...............................................................................................27  
Papers I-VI..........................................................................................27  
Sample preparation...........................................................................28  
Peptidomics .......................................................................................28  
Synthetic peptides............................................................................28  
Proteomics .......................................................................................29  
Separation............................................................................................29  
Peptidomics .......................................................................................29  
Proteomics .......................................................................................29  
In situ hybridization..........................................................................30  
Western blot.......................................................................................30  
MS detection and identification .......................................................30  
Peptidomics Q-ToF (papers I-VI)......................................................30  
Proteomics LTQ (papers III & VI)......................................................31  
Pep-19 identification..........................................................................31  
Imaging MALDI MS..........................................................................31  
Peptide database - SwePep...............................................................31
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2D-GE</td>
<td>Two-dimensional gel electrophoresis</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosin triphosphate</td>
</tr>
<tr>
<td>CID</td>
<td>Collision-induced dissociation</td>
</tr>
<tr>
<td>DIGE</td>
<td>Difference gel electrophoresis</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>FTICR</td>
<td>Fourier transform ion cyclotron resonance</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>LDCV</td>
<td>Large dense core vesicles</td>
</tr>
<tr>
<td>LMW</td>
<td>Low molecular weight</td>
</tr>
<tr>
<td>LTQ</td>
<td>Linear-quadrupole trap</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass-to-charge</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-assisted laser desorption ionization</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-methyl 1-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MS/MS</td>
<td>Tandem mass spectrometry</td>
</tr>
<tr>
<td>MW</td>
<td>Microwave</td>
</tr>
<tr>
<td>PC</td>
<td>Pro-hormone convertases</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>TOF</td>
<td>Time-of-flight</td>
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</tbody>
</table>
Introduction

The complete sequence of the human genome was published in early 2001, which marked a new era for biological research\cite{1, 2}. However, the genome gives only a relatively static overview of the functional potential of an organism and does not describe the immense dynamic processes that occur in a living organism\cite{3}. Therefore, effort has shifted towards functional genomic and proteomic studies. The name proteomics is derived from the term proteome, which by analogy to genome is defined as all proteins expressed by a cell or a tissue\cite{4}.

Protein structure and function can be predicted from the DNA sequences\cite{5, 6}. The genome alone does not provide enough information for an understanding of how protein function is regulated. Information about whether and when gene products are translated, the relative concentration of gene products, the extent of post-translational modifications of the gene products, and the effects of under- or overexpression may not be examined through the genome\cite{7}. Within the field of proteomics, the aim is to define the function and expression profiles of all proteins encoded within a given genome\cite{8}. From this follows a growing need of methods for separation, quantitation, and identification of a large number of proteins and peptides from biological samples.

Proteins in tissues, cells, or extra-cellular fluids such as blood, plasma, and urine are widely investigated by using two-dimensional gel electrophoresis (2D-GE), two-dimensional liquid chromatography (2D-LC), and mass spectrometry (MS)\cite{9}. An important part of the proteome is also the low molecular area of the proteome called the peptidome\cite{10}. Peptidomics is linked to the proteomics by regulating the maturation, activation and degradation of peptides\cite{11}. New improved preparation and separation methods in combination with the advances in MS have revolutionized peptidomics\cite{12} and made the analysis of low abundant proteins, hormones and neuropeptides possible.

The peptide and protein content in biological tissue is greatly influenced by the sample handling methods and by the time-interval between death and/or sampling to the inactivation of proteolytic enzymes\cite{13}. This puts great demands on sample handling and sample quality and will expose the fast degradation of some proteins. As presented in this thesis, methods for measuring
sample quality, retaining sample quality (proteins and peptides), identifying, and relatively quantifying neuropeptides have been developed. This thesis work has specialized on MS methods to analyze and characterize endogenous peptides of the central nervous system.

Background to peptidomics

Within the field proteomics, interest in the low molecular weight (LMW) peptides and proteins in pathophysiology continues to grow. Peptidomics is defined as the analysis of the peptide content within an organism, tissue, or even a cell\(^\text{(12)}\). Although this definition technically includes all peptides present in a system, including transient products of protein degradation, typical peptidomics studies are focused on bioactive peptides\(^\text{(13)}\). Peptides appear in all body fluids, cells and within tissues and have many different functions thus having a potential as diagnostic biomarkers\(^\text{(14)}\). Biomarkers serve as tools for detection and monitoring of disease and can be used to discriminate between different diseases with similar symptoms such as AD and vascular dementia\(^\text{(15)}\). Early detection and characterization of many diseases such as Parkinson’s disease (PD), Alzheimer’s disease (AD), cancer etc. are crucial for control and prevention\(^\text{(16-18)}\). Peptidomics applications also include studies in for instance pathophysiology, basic cell function and molecular organization, discovery of novel biologically active molecules and drugs, and discovery of novel drug targets\(^\text{(19)}\).

A number of research groups have analyzed peptide extracts from various tissues and body fluids using MS and relative quantitation of large number of peptides have been performed. However, because of the problem with post mortem protein degradation, many of the described peptides represent degradation fragments of proteins\(^\text{(20-24)}\).

Neuropeptides and hormones

The understanding of how the brain works is a huge challenge. To reduce the complexity of the problem, researchers try to break it into smaller pieces for systematic experimental analysis. Efforts are made in many different research areas, such as molecular, cellular, systematic, behavioral, and cognitive levels. Putting all these research areas together will eventually lead to a better understanding of the brain.

The brain receives signals from the sensory cells of the organism through nerve cells which are connected to the spinal cord. The signals are then processed by the brain and conversely, the brain controls movement through muscle control. The brain also exerts control through autocrine, endocrine
and exocrine excretion of hormones influencing cell populations, organs and glands throughout the body\cite{25}. The brain also reacts to hormones produced elsewhere in the body. The brain consists of a variety of structures containing different types of neurons and other brain cells. This thesis is focused on the endogenous peptides and hormones of the brain.

Vincent du Vigneaud and colleagues isolated the peptide hormones vasopressin and oxytocin and established their structures in the 1940s. They concluded that oxytocin contains only eight different amino acids, in contrast to proteins containing hundreds of amino acids. Vigneaud later received the Nobel Prize in chemistry 1955. In the early seventies, de Wied and colleagues coined the term ‘neuropeptide’ to describe an endogenous substance synthesized in nerve cells and involved in nervous system functions\cite{26}. Neuropeptides influence a wide range of physiological processes, including feeding and body weight regulation, circadian rhythms, sleep, pain, fear, anxiety, learning, and memory and reward mechanisms\cite{25}. Most peptides are released at the axon terminals of sets of neurons even if there is evidence of extensive peptide release from the dendrites\cite{27}.

The word hormone comes from the Greek ‘to set in motion’, and applies to peptides that communicate from one set of cells to another. Hormones reach their targets via the blood, or through other body fluids, and some are released into the brain\cite{28}. Even if the main function of neuropeptides and hormones seem to be that of messenger molecules, other functions such as antimicrobial and antifungal activity has also been reported\cite{29, 30}.

Neuropeptides generally originate from larger precursor proteins and are stored in large dense core vesicles (LDCV). The precursor proteins are synthesized in the endoplasmic reticulum, transferred to the Golgi apparatus where they are packed in LDCV, and transported to be stored or released\cite{31}. The processing of the precursor proteins take place in the LDCV and is performed in many steps. The pro-hormone convertases (PC) are responsible for the biosynthetic cleavages of the precursors which are usually followed by removal of the C-terminal basic residue(s) by carboxypeptidase E (Figure 1). The cleavage processes of the precursors are directed to specific sites. In most cases the sites consist of two sequential basic amino acids or two basic amino acids separated by two, four or six other residues, but even dibasic sites have been reported\cite{32}. The basic amino acid specific enzymes are known as PC1/3, PC2, furin, PC4, PC5/6, PACE4 and PC7\cite{33}.
Figure 1. Peptide precursor processing. The N-terminal signal peptide is removed by signal peptidases when the precursors enter the Golgi apparatus from the endoplasmic reticulum. In the Golgi apparatus the precursors are packed in LDCV. The precursors are thereafter processed in many steps in the LDCV. The basic sites in the precursor are processed by the prohormone convertases to introduce an irreversible hydrolysis of the peptide bond. After cleavage the remaining C-terminal basic amino acids are removed by carboxypeptidase E or D. Peptides with a C-terminal glycine are converted to form an amidation by the enzyme peptidyl-glycine α-amidating monooxygenase. The figure is modified from Fricker et al 2006[13].

In addition, some peptides are processed at sites with no basic amino acids[34-36]. These may be cleaved by novel proteases such as the recently described SKI-1 enzyme[37] or the NARC-1 enzyme[38] cleaving after the motifs R x (hydrophobic) x[37] and (V/I) F A Q[39], respectively.

Modifications of neuropeptides have multiple functions; they make the peptides more resistant to enzymatic degradation and regulate the binding affinity to receptors. Important modifications on neuropeptides include C terminal amidation, acetylation, phosphorylation, sulfation, and glycosylation[11]. C-terminal amidation is required for bioactivation of many neuropeptides and are specific to endogenous peptides[40].

Neuropeptides are regulated at many levels by proteases[11]. The intracellular processing of the peptides is followed by secretion, extracellular processing and finally a regulated degradation. The human genome contains approximately 500 protease encoding genes[11] thus the knowledge of peptide maturation and regulation will increase. The neuropeptidases responsible for extracellular processing and degradation of neuropeptides are in most cases
membrane bound, but may also be found as secreted proteins\cite{41}. Many are metalloendopeptidases such as angiotensin converting enzyme, nepriyisin, endothelin converting enzyme, and neurolysin with a wide specificity to different neuropeptide substrates\cite{41}.

Sample preparation

The advancement of analysis techniques provides new means to analyze protein and peptide content in tissues and cell cultures. This sets higher demands on the sample preparation. The purpose of sample preparation is to process the sample to get more accurate analytical results. The prepared sample should be free of interfering analytes and compatible with the method of choice.

To study proteins and peptides, the tissue or cell samples are usually disrupted by homogenization under certain specific buffer conditions. These buffers often contain ingredients that are supposed to cause a cessation of all protein activity, including protease activity\cite{42}. Unlike proteomics, the dynamic range of peptidomics analysis is often limited by protein degradation\cite{13}. Even if proteomic studies are affected by degradation during the post mortem period, a degradation of few percent of the tissue proteins will not completely impair the analysis as long as most of each protein remains intact\cite{13}. Nevertheless, it will certainly complicate or even preclude the possibility to carry out differential and comparable studies (see paper III). Many bioactive peptides are expressed at low levels relative to the proteins. The breakdown of even a miniscule fraction of the most dominant protein can overwhelm the detection of endogenous neuropeptides (paper I, II).

The most up-to-date reviews of protein sample preparation are based on two dimensional gel electrophoresis (2D-GE) studies\cite{43}. The proteomics sample preparation is different from that of peptidomics. The overall proteomics goal is often to extract as many proteins as possible. Due to the great diversity of chemophysical properties of proteins the sample buffers often contain mixtures of different substances (see Proteomic Denaturation Methods). Ideally the sample buffer will dissolve, disaggregate, and denature the proteins in the sample. Neuropeptides and hormones function as intercellular signaling molecules. Following intracellular processing they are secreted to the extracellular fluid. This knowledge can be used to compose the sample buffer to only dissolve the hydrophilic proteins, neuropeptides and hormones and leaving most of the hydrophobic proteins and cell membrane undissolved (paper I-VI).
Issues using *post mortem* brain tissue

Throughout the life span of cells, proteins are synthesized and degraded. This is a dynamic process which is extensively controlled by various mechanisms[44]. *Post mortem* enzyme activity has been shown to play an important role for the integrity of the peptide and protein content in the brain (paper I-III). Apparently, there will always be a low level of high abundant protein degradation peptides present in the sample as part of the protein-peptide homeostasis. In order to avoid protein degradation fragments, standardized methods such as instant denaturation of a sample is therefore necessary to accurately measure and quantify proteins and neuropeptides. A disease state may change this balance, and thus the peptide pattern can be used for the characterization of a disease[19, 45].

The study of tissues and cells requires removal of the sample from supportive environments. The disabling of the supply of oxygen and nutrients to the sample leads to anoxia. This is an area not well understood, and is described in literature almost only by the studies on brain ischemia. Brain cells utilize oxygen at a high rate but do not contain any reservoir of oxygen such as myoglobin in muscle cells[46]. The stores of oxygen in blood vessels can support normal oxygen consumption for only a few seconds[47]. Without oxygen, oxidative phosphorylation and subsequently ATP production is halted, causing deficiencies in cell functions[48]. There is an efflux of K+-ions from rat brain cortex immediately after induction of anoxia through cardiac arrest[49]. Studies of complete ischemia in rat cortex induced a rapid increase in intracellular Ca^{2+} levels after approximately 60 seconds[50]. After one to two min of ischemia, ATP energy levels are insufficient to support Na^+–K^+–ATPase activity, causing depolarisation and a flow of Na^+, K^+, Ca^{2+}, and Cl^- down their concentration gradients [49, 51]. The decrease in oxygen and increase in intracellular Ca^{2+} also has the potential to activate Ca^{2+} regulated phosphatases and kinases as reported in studies of kidney[52].

Inactivation methods

Denaturation

The intention of using rapid denaturation is to preserve cells and tissue constituents in an as close to a life-like state as possible. Protein denaturation is commonly defined as any noncovalent change in the structure of a protein[53]. This structural modification affects the secondary, tertiary or quaternary structure of the molecules. For enzymes, denaturation can be defined as the loss of enough structure to render the enzyme inactive[53].
Thermal denaturation

Thermal denaturation is the only denaturation procedure that can be performed directly on the tissue or body fluid without adding anything to the sample. Heat can be introduced in many ways, e.g. by conduction and convection. Biological samples are normally poor heat conductors. The time it takes for the heat to penetrate the tissue is of high importance. The tissue has to be formed in such a way that the heat can be introduced through an as large as possible area of the tissue. One way is to use microwave (MW) irradiation\[54]\() although it is difficult to optimize the temperature. MW introduces a temperature gradient between the surface and the tissue core. Other drawbacks are that frozen tissue can not directly be radiated\[55]. It has to be thawed before MW radiation to be sufficient heated. Contrary to what many believe the MW irradiation in itself does not hinder protein degradation\[56]. It is the heat generated from the MW that inactivates the proteins responsible for the degradation\[57]. Proteins exposed to increasing temperature lose their enzymatic activity\[58]. Exposure of most proteins to high temperatures results in irreversible denaturation\[59].

Proteomics denaturation methods

A variety of different agents are used to denature and solubilize proteins prior to proteomic studies. In order to avoid degradation, samples are often disrupted directly in a strong denaturizing buffer containing sodium dodecyl sulphate (SDS) and/or urea in combination with dithiothreitol (DTT)\[60]. SDS is a negative ionic surfactant that binds to the main peptide chain of the protein\[61]. This effectively imparts a negative charge on the protein\[61]. Since like-like charges repel each other, the proteins are caused to unfold, immediately rendering them functionless but still remaining in solution. Some quaternary structure may remain due to disulfide bonding or due to covalent and noncovalent linkages to other types of molecules\[42]. DTT and 2-mercaptoethanol are strong reducing agents. Their specific role in sample denaturation is to remove the last bit of tertiary and quaternary structure by reducing disulfide bonds\[42]. These denaturation agents are often used prior to 2D-GE separation, but the sample are not compatible with LCMS analysis unless they are removed or sequestered\[42].

However, regardless of which sample preparation that is chosen, it is of utmost importance to minimize protein modifications which might result in artifact spots on a 2D-GE map. Samples containing urea must not be heated as this may introduce considerable charge heterogeneity due to carbamylation of the proteins by isocyanate formed from the decomposition of urea. Generally speaking, samples should be subjected to as little handling as possible and kept cold at all times\[62].
Other inactivation methods

Formaldehyde is the most widely employed universal fixative\textsuperscript{[63]}. Glutaraldehyde, acrylic aldehyde, and malonaldehyde are examples of other aldehydes infrequently employed in fixative solutions\textsuperscript{[64]}. As aldehydes have a limited penetration capacity of the sample, only small blocks of tissue (1-2 mm\textsuperscript{3}) fix well at temperatures of 1-4 °C. The penetration can be accelerated by MW irradiation\textsuperscript{[65]}. Details of the fixing action of aldehydes are not known although the general principles are understood. It is thought that the aldehydes form cross-links between proteins, creating a gel, thus retaining cellular constituents in their \textit{in vivo} relationships to each other\textsuperscript{[66]}. Fixation by aldehydes is often used in pathology prior to tissue histology, as well as in electron microscopy. It is possible to extract and analyze nucleic acids after formaldehyde fixation\textsuperscript{[67]}. However the possibility to extract the covalent linked proteins is limited\textsuperscript{[68, 69]}.

If the pH of the tissue extract containing solution is reduced far below the isoelectric point of a specific protein, the protein will lose its negative charges and the positive charges will repel each other and cause unfolding of the protein\textsuperscript{[61]}. The effect on the protein is widely dependent on its specific properties\textsuperscript{[61]}.

Organic solvents such as methanol and ethanol have a role as fixatives. The denaturizing action on proteins is primarily due to disruption of the hydrophobic bonds which contribute to maintaining the tertiary structure of proteins\textsuperscript{[61]}.

Protease inhibitors

Individual protease inhibitors are active only against specific classes of proteases, so it is usually advisable to use a combination of protease inhibitors\textsuperscript{[42]}. Proteases are often characterized by the nature of their active center and they can be grouped into five main groups, i.e. serine, threonine, cystein, aspartic and metalloproteases\textsuperscript{[11]}. Serine proteases contain serine and histidine at their active center, whereas threonine proteases contain a threonine, and cystidine proteases contain a cystidine. Metalloproteases contain a cation such as Zn\textsuperscript{2+}, Ca\textsuperscript{2+}, or Mn\textsuperscript{2+} and aspartic contain an aspartic acid group\textsuperscript{[70]}. To inhibit these proteases, specific protease inhibitors, often directed towards one or more of these groups of proteases are used. A few examples of protease inhibitors are phenylmethylsulphonyl fluoride (PMSF), aminoethyl benzylsufonyl fluoride (Pefabloc), EDTA or EGTA, peptide protease inhibitors (leupeptin, pepstatin, aprtinin or bestatin), tosyl lysine chloromethyl ketone (TLCK), tosyl phenylalanine chloromethyl ketone (TPCK), benzamidine\textsuperscript{[42]}. Protease inhibitors have many disadvantages and
limitations. They are often expensive and some are toxic. Some protease inhibitors render the sample incompatible with certain applications after they are added. Peptide protease inhibitors are small peptides and thus cannot be used in peptidomic studies\[^{[42]}\]. Considering the limited knowledge on proteases it is almost impossible to know if all proteins are inhibited\[^{[70]}\].

Analysis of brain peptides and proteins

The bottleneck in the analysis of peptides has previously been the detection and identification process. During the last decade MS has gone from being a research tool to an analysis tool. Huge technical improvements have improved the use of MS\[^{[71, 72]}\]. This has lately shifted the focus towards improved sample handling, separation, and quantification methods.

Separation

Analytes in a complex sample should preferably be separated prior to detection. The most commonly used method for separation of native protein mixtures in proteomics today is the 2D-GE technique. This is a combination of isoelectric focusing (IEF) and SDS polyacrylamide GE\[^{[9]}\]. The proteins are separated according to their charges (isoelectric point) in one dimension and size in the second dimension\[^{[73, 74]}\]. This method suffers from limited dynamic range, poor reproducibility, difficulty to automatize, and the lack of on-line integration. This has led to the development of alternative approaches. Liquid chromatography (LC) of digested proteins in one or several dimensions has become more frequently used\[^{[75]}\].

Two dimensional difference gel electrophoresis (2D-DIGE) is a technology which circumvents some of the problems associated with traditional 2D-GE. This method relies on pre-electrophoretical labeling of the proteins. The so-called CyDyes (Cy2, Cy3, and Cy5) are used to label the primary amino groups, typically the terminal amino group of lysine side chains of the protein\[^{[76]}\]. The samples to be compared are labeled with either Cy3 or Cy5, whereas Cy2 is employed to label an internal standard comprising equal amount of all samples in the study. The samples are mixed prior to analysis by 2D-GE, and the internal standard can be used to avoid the gel-to-gel variations. This method is more sensitive compared to conventional 2D-GE when coomassie\[^{[77]}\] or silver\[^{[77]}\] staining are used to visualize the proteins. The detection limit of proteins labeled with 2D-DIGE is in the order of 150-500 pg compared to 1 ng of protein by silver staining\[^{[76]}\]. Furthermore the dynamic range using coomassie or silver staining procedures has a linear dynamic range of around one order of magnitude\[^{[78]}\] compared to over five orders of magnitude using DIGE\[^{[76]}\].
However, proteomic methods are not, for several reasons, applicable for analysis of the peptidome. 2D-GE does not cover the LMW area and is therefore excluded as a separation method for peptidomics. Analyzing endogenous peptides is complicated because they are found in a complex biological matrix with high salt concentrations (100 mM) and because of their low, typically subnanomolar concentrations\(^{79}\). Gel free methods, where digestion of the proteins is the first step, give rise to a large amount of protein derived peptides that obstruct the discovery of neuropeptides. As a consequence, methods specialized for peptidomic studies have been developed to divide the proteome from the peptidome and further to separate the peptidome to reduce the complexity (paper I-II).

The main physical property that can be used to divide the proteome from the peptidome is the size of its components. Size exclusion chromatography, gel based chromatography or cut-off filters can be used for separation. Even after the separation, the peptidome is fairly complex and needs to be further separated. Most peptidomics separation methods today need to be MS compatible. MS is the leading detection method and is either directly coupled to the LC or used to analyze the fractions after LC. The LC is used to desalt, concentrate, and separate the samples. Miniaturization of the chromatographic methods enhances sensitivity and allows small sample volumes\(^{80,81}\). Because of the low flow rate (typically 50-200 nl/min), precolumns are used to enable larger sample volumes, and to save time. Reverse phase (RP) is the most common LC mode today\(^{82}\). The most frequently used column matrix is octadecyl-bonded silica (ODS), often referred to as C18, or only an octyl bonded silica, referred as C8. The sample is attached to the columns stationary hydrophobic phase using a hydrophilic buffer and the elution of the peptides is achieved by an increased concentration of a more hydrophobic buffer such as methanol (MeOH) or acetonitril (ACN).

Detection

The usage of antibodies is the common method for detection of neuropeptides. Antibodies are synthesized in response to a specific antigen such as a protein or peptide. The detection of more than one predefined target is therefore limited. The use of antibodies as a discovery tool is limited even if the use of protein chips has improved this area\(^{83}\). Immunoassays have many advantages; they are good quantitative tools (western blotting) and can be used for collecting spatial information (immunostaining). Drawbacks are the limited knowledge collected from the molecule detected. Antibodies have not the constringency to see if the antigen has become modified or truncated outside the epitope.
Using MS techniques, hundreds of neuropeptides can be detected and measured from the same sample (paper II). Sequence information about the peptides can be achieved simultaneously by tandem MS (MS/MS) and by the use of imaging MALDI MS, spatial distribution can be attained\[84\]. MS is not solely a detection technique as it also separates the molecules analyzed with respect to their mass-to-charge (m/z) ratio. The MS technique has made advances over the past few years and is competing with immunoassays in specific research areas.

**Mass spectrometry**

There are many different types of mass spectrometers. The type of experiment to be carried out decides the properties needed of the MS. Important qualities of a mass analyzer include accuracy, scan speed, and sensitivity\[71, 85, 86\]. A mass spectrometer mainly consist of three parts, the ion source, the mass analyzer, and the detector (Figure 2a). The ion source is where the molecules are introduced to the mass spectrometer and ionized. There are many different ionization principles; matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) are the two main ionization techniques and discussed below. After the molecules are ionized they are separated according to their m/z in the mass analyzer. The separation is achieved in vacuum by the dynamics of charged particles in an electric and magnetic field. The different kind of mass analyzers include time-of-flight (TOF)\[87\], quadrupole\[88\], quadrupole ion trap\[89\], linear ion trap (Lit)\[90\], Fourier transform ion cyclotron resonance (FTICR)\[91\] and orbitrap\[92\]. The mass analyzers are used in the instrument in one or several copies, such as the TOF-TOF and the triple-quadrupole instrument, as well as in different combinations such as quadrupole-TOF or Lit-FTICR. This is to take advantage of the strength in each mass analyzer, i.e. the ability to perform tandem MS (MS/MS). One example is the triple quadrupole, where the first mass analyzer is used to isolate ion, a second to stabilize the ion while it collides with a gas to fragmentize (collision-induced dissociation, CID), and a third to separate the produced fragments. MS/MS can be performed in a single mass analyzer such as the quadrupole ion trap. The most common method to fragment ions is CID, but other methods should also be mentioned such as electron capture dissociation (ECD) and electron transfer dissociation (ETD).
Figure 2. a) Schematic drawing of a quadrupole time of flight mass spectrometer in tandem MS mode. Analytes are ionized by ESI and introduced into the mass spectrometer. The quadrupole function as a mass selective filter, only ions with a specific m/z ratio passes. The ions are thereafter fragmented in the collision cell and further separated in the TOF tube. In the flight tube, the ions are forced to change direction in the reflectron, to hit the detector. b) ESI in positive mode. A high electric field between the electrospray emitter and the MS force the analytes to move towards the tip to form a jet spray. Small droplets are formed from the spray and due to solvent evaporation the analytes move into gas-phase. c) MALDI in positive mode. The analytes are embedded in matrix on the target. Energy is introduced by a laser pulse to be absorbed by the matrix. Analytes and matrix are ionized and ejected from the target and accelerated by an electric field towards the inlet of the mass spectrometer (the grid).

ESI MS

Electrospray ionization (ESI) is used to introduce macromolecules to the mass spectrometer. The first reported experiment by ESI was performed by Fenn and coworkers[93]. Fenn was awarded by the Nobel Prize in Chemistry 2002 for his work.

Electrospray is performed at atmospheric pressure. By applying an electric potential between the liquid and the mass spectrometer, electrophoretic movement of the analytes will force the liquid to form a cone from a fine jet
spray emerge small charged aerosol droplets towards the MS (Figure 2b). Each droplet in the aerosol will in the beginning contain numerous molecules with equal charges. Repulsion of the molecules make the droplets break up and form smaller droplets as the molecules will be forced closer and closed due to evaporation. This process will be repeated until the molecules are free of solvent and the ions brought to gas phase. Two main theories are proposed for how the analytes will go from the charged droplets to the formation of gas phase ions. The charged residue mode (CRM) propose that each droplet formed by the spray will split into two equally in size droplets (even fissioning) or two disproportional droplets (uneven fissioning)\(^94\). The ion evaporation model (IEM) suggests that when a droplet has reached a certain radius (~10 nm), the force of Columbian repulsion is enough to push the molecules directly out of the droplet\(^95, 96\). One aspect of the ESI process is the production of multiple charged ions in contrast to MALDI were mostly single charged ions are produced.

**MALDI MS**

Matrix-assisted laser desorption ionization (MALDI) is similar in character to ESI both in relative softness and ions produced. The first reported MALDI experiment was performed by Franz Hillenkamp and Michael Karas and coworkers in 1985. They accomplished to ionize the amino acid alanine by mixing it with the amino acid tryptophan. Tryptophan was used to absorb the laser energy and thus enable the non-absorbing alanine to ionize\(^97\). The first work regarding the analysis of larger molecules utilizing MALDI was reported 1988 by Koichi Tanaka and coworkers\(^98\). Tanaka received the Nobel Prize in Chemistry 2002 for his discovery.

MALDI is most commonly performed under vacuum. The analytes are mixed with a matrix and co-crystallize when dehydrated. The matrix is chosen for its property to absorb laser energy. The matrix and the analytes are rapidly volatilized by the laser pulse and form gas phase ions (Figure 2c). Predominantly singly charged ions are produced in the process. This could be advantageous when it comes to CID produced MS/MS spectra. The spectra produced are less complex and more easily to interpret.

**Imaging MALDI MS**

The imaging MALDI MS technique was first described in 1997 by Caprioli\(^84\). The technique is used to determine the spatial distribution of peptides and proteins in biological samples *in situ*. Applications range from low-resolution images of peptides and proteins in selected areas of tissue to high-resolution images of tissue cross sections. Using a raster of mass spectra from a defined area, images of samples are produced in specific m/z values, or ranges of values. Each spot on the sample irradiated by the laser is approximately 25 µm in diameter and typically covers the m/z range 500-
80,000 Da\textsuperscript{99}. Individual m/z values can then be assembled from the mass spectra to produce selected m/z images. To obtain the identification of the proteins, it is possible to perform on-target digestion by trypsin. For this procedure, the sample is treated by trypsin and analyzed by MALDI MS for determination of the tryptic peptide fragments\textsuperscript{99}.

**MS quantification**

One way of direct quantification by mass spectrometry is to use internal standards\textsuperscript{100}. An internal standard is often a copy of the molecule containing one or more heavy stable isotopes. The addition of predefined concentrations of the internal standard to the biological sample can be used to obtain accurate quantification\textsuperscript{100}. However, in peptidomic studies a large number of peptides are detected in a single analysis. Direct quantification of every peptide present in the tissue would be labor-intensive and too costly. Instead, it is easier to examine the relative level of a peptide in two different samples.

The abundance of peptides in the brain is often distributed to specific areas. One example is neuropeptide S, which is located in a small area between the locus coeruleus and Barrington’s nucleus\textsuperscript{101}. The precise dissection of different brain areas is therefore crucial to obtain comparable results. Because of sensitivity problems, microdissection is seldom used in peptidomic studies.

Different methods to compare relative levels of peptides in samples have been proposed. The incorporation of a heavy stable isotope tag has been used in proteomic studies for a long time\textsuperscript{102, 103}. The peptides are labeled by tags of equal structure and sometimes even of equal weight after extraction of the peptides. The samples are mixed and the relative abundance of the peptides can be calculated from the ratio of the peak intensity or peak area of the two isotopic forms in MS spectra or if tags of equal weight have been used in MS/MS spectra. Drawbacks of these methods are that many of them are specially made for proteomics studies. The labeling reagents are directed to the N-terminal lysine (Lys) or arginine (Arg) after cleavage by trypsin, like the Mass-Coded Abundance Tagging (MCAT)\textsuperscript{104}. Other reagents react with different amino acids such as Isotopic Coded Affinity Tags (ICAT)\textsuperscript{105} that react with the sulfhydryl group of cysteines (Cys) residues or the Isobaric Tagging for Relative and Absolute protein Quantification (iTRAQ)\textsuperscript{106} that reacts with the side chain of lysine or the N-terminal of the peptides. However, not all neuropeptides contain Cys and/or Lys residues and many peptides are N-terminally (acetylation, pyrilidone carboxylic acid) or C-terminally (amidation) blocked (Table 1). All of these techniques require peptides in samples to be chemically modified before they are mixed, and these procedures have the potential to introduce sample preparation biases.
Table 1. Peptides containing the amino acids, Lys, Cys, and Trp and peptides with modified N-terminal and C-terminal. The number is collected from the neuropeptide database SwePep (paper IV). Using labeling methods directed to a specific amino acid such as Cys means that only 98 out of 312 human peptides have the possibility to be labeled.

Another method to incorporate isotopic labels to peptides is Stable Isotope Labeling by Amino acids in Cell culture (SILAC) [107]. Cell samples to be compared are grown separately in media containing either a heavy or native form of an essential amino acid [107].

It is possible to obtain a semi-quantitative measurement of two different samples, analyzed separately by MS without labeling [108-110]. By using label free methods there are no limitations regarding amino acid composition of the peptides to be analyzed.

Identification of neuropeptides

Edman sequencing [111] has been the method used for protein identification since the 1980. Edman sequencing employs step-wise chemical degradation of proteins or peptides from its N-terminus [111]. Modifications such as acetylation on the N-terminal hence block sequencing of whole proteins using this method. Therefore the development of enzymatic digestion of proteins following by separation of the resulting peptides provided the opportunity to generate amino acid sequences from internal peptides [112]. These advancement in combination with the usage of translated protein databases rendered from DNA or mRNA have enable the identification process and cleared the way for MS based methods [113]. Most proteomic studies involve enzymatic digestion of proteins. Proteins are cleaved in sequence specific patterns by trypsin or other proteases to generate fragments that can be readily sequenced by MS/MS or used for molecular mass fingerprint identification of the protein [114, 115]. It is not necessary to identify all peptides from a protein to confirm an identity in proteomic studies [114].

Peptidomics studies on the other hand are usually performed in the absence of enzymatic digestion, which makes it easier to prepare the sample. However, there are many complications to identify neuropeptides compared to proteolytically cleaved peptides. Because of their in vivo processing there is not much information known about their C-terminal amino acid. In contrast

<table>
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</tr>
</tbody>
</table>
to proteomics, where it is enough to identify a few tryptic peptides for identification of a specific protein, MS identification of endogenous peptides is based on a single peptide. Each neuropeptide derived peptide precursor may have a unique biological function, hence important to identify. Neuropeptides often have post-translationally modified amino acids and sometimes more than one. Low-abundance peptides are not present in sufficient amounts to provide good quality spectra. The most abundant peptides can suppress the signal of the less abundant peptides. Neuropeptides may complicate the data analysis because of their large size; 175 out of 312 human peptides in SwePep are larger than 2.5 kDa and 112 are larger than 3.5 kDa. Even small singly charged peptides are difficult to decipher because of the relatively few ions produced by MS/MS and because the positive charge is located at the N-terminal resulting mainly in b-ions[116].

Data interpretation
The development of new and more advanced mass spectrometers has sped up the identification process. The bottleneck is now the interpretation of the data produced. The efforts to make this an automatized process are so far focused to proteomic studies[117]. Interpretation of MS/MS spectra can be achieved manually but is a laborious process. A number of automated softwares for de novo sequencing are available such as PepSeq (Waters), DeNovoX (Thermo Electron), and Peeks (Bioinformatics Solution), although these require high quality data to work accurately. By taking advantage of the information stored in public available databases such as NCBI, UniProt, etc., de novo sequencing can be limited. Search engines such as Mascot (Matrix Sciences), Turbo sequest (Thermo Electron), the GPM (www.thegpm.com), or other programs search databases for positive matches. Many endogenous peptides originate from a precursor existing in databases; however cleavage sites and modifications are not often described.

When analyzing proteolytically cleaved proteins, search engines use the defined cleavage patterns and cleave the databases in silico for comparison. The cleavage patterns for neuropeptides are unknown, making it necessary to cleave the databases in all possible ways. This results in huge number of alternative masses which makes it computer intensive and time consuming. Even if the manual work is limited, peptides from unknown precursors will be missed making de novo sequencing the final remedy.

Databases - SwePep
Most databases act as repositories of sequences. GenBank Gene Product Data Bank (GenPept) produced by the National Center of Biotechnology Information (NCBI)[118] is an example of such a database containing only
sequence data translated from nucleotide sequences. GenPept has been created in collaboration with the DNA Data Bank of Japan (DDBJ) and the European Molecular Biology Laboratory (EMBL). Entrez Protein, produced by NCBI, from translated sequence data (DDBJ/EMBL/GenBank database) and/or sequences from Swiss-Prot is another example database that functions as a repository.

The UniProt database on the other hand is a fusion of the Swiss-Prot\textsuperscript{119}, Translate from EMBL (TrEMBL)\textsuperscript{119} and Protein Information Resource Sequence Database (PIR-PSD) in an attempt to form a central database of protein sequences with annotations of structural, biochemical and functional information\textsuperscript{120}. Even if this increase the knowledge of the protein function, even the peptide precursors, little is said about the processing and functioning of the endogenous peptides. Working with MS it is of interest to be able to search the databases by mass. To extract such information from the databases, start and stop positions and possible modifications of the peptides has to be annotated.

SwePep stores novel neuropeptides identified in-house (the Laboratory for Biological and Medical Mass Spectrometry, Uppsala University), annotated peptides from UniProt, and peptides from the literature. Information about the peptides, including mass, isoelectric point, sequence, precursor protein, species, modifications, and localization is also stored in the database. Technical information of the experiments performed to discover a specific peptide in the laboratory is also collected, such as tissue, instrument used, experimental conditions, and MS/MS spectra.

SwePep has many advantages when it comes to identification of peptides. Direct identification can be performed by using the mass information of peptides, with or without modifications, and their corresponding MS/MS spectra are stored in SwePep. Programs such as The GPM and Mascot search engines are used to search peptides and precursors stored in SwePep. By using this rather limited database a rather large amount of peptides can be identified in a short time. Other possibilities are to use the additional information stored such as pI and retention time (hydrophobicity) to exclude false positives. The peptides stored in SwePep are classified in three different categories, i.e. biologically active peptides, potential biologically active peptides, and uncharacterized peptides.
Aims of studies

The aims of this thesis were:

- To explore and further develop sample preparation methods for peptidomics
- To examine the effects of *post mortem* sample handling on peptides and proteins
- To develop software for peptide comparison, identification and visualization
- To develop methods for identification and semi-quantification of peptides using mass spectrometry
- To identify novel potential biologically active peptides in the brain
- To utilize the methods to investigate differences of peptide and protein expression in animal models of Parkinson’s disease.
Methods

Figure 3. Schematic description of the work flow.

Animals

Papers I-VI

Two different species of animals were used in the present studies, Sprague-Dawley rats and C57/BL6 mice.

I. Rats (n=4) were sacrificed by decapitation. Striatum, motor cortex and thalamus were thereafter rapidly dissected out.

II. Rats and mice were sacrificed by focused microwave irradiation using a small animal microwave. Hypothalamus was thereafter rapid dissected out. Snap frozen decapitated mouse striatum were rapidly fixated from frozen
II. Twenty mice were randomly divided into five groups (n=4) and sacrificed by cervical dislocation and exposed to microwave irradiation after 1, 3, and 10 minutes post mortem. As a control group mice were sacrificed by focused microwave irradiation. In the fifth group, mouse brains were frozen rapidly after decapitation and directly denatured using a proprietary tissue fixation instrument. All brains were rapidly dissected for hypothalamus, cortex and striatum. Protein studies were performed on cortical brain tissue from 10 minutes post mortem and compared to directly MW irradiated brain tissue.

III. Rats and mice were sacrificed by focused microwave irradiation using a small animal microwave. Hypothalamus was thereafter rapidly dissected.

IV. Rats and mice were sacrificed by focused microwave irradiation using a small animal microwave. Hypothalamus was thereafter rapidly dissected.

V. Reserpine was administrated to mice subcutaneously in one dose of 10 mg/kg. Reserpine is commonly used to induce and study parkinsonism in animal models[121, 122]. Mice were sacrificed by focused microwave irradiation at 1h, 6h, 12h, and 24h after injection. One group of mice received an injection of saline (control). Each group consisted of five to six animals from which the striatum was dissected out.

VI. Mice were used for this study. MPTP was injected subcutaneously in a dose of 20 mg/kg followed by a second dose after 4h. Injections were repeated the next day. Animals were sacrificed three weeks after the second MPTP injection and striatum rapidly dissected. Striatum from saline treated controls (n=3) and MPTP lesioned animals (n=3) were analyzed by nano-flow LC-MS analysis.

Sample preparation

Peptidomics
The brain tissue was suspended in cold extraction solution (0.25% acetic acid) and homogenized by sonication. The suspension was centrifuged to sediment cell debris and undissolved material. The supernatant was filtered through a 10 kDa cut-off filter by centrifugation (papers I-VI).

Synthetic peptides
In the experiments on synthetic samples (paper V), a background of a tryptic digest of eight proteins (1 pmol/µL total) was used and three peptides were added as internal standards (bradykinin, angiotensin I and neurotensin at 1.2 pmol/µL each). The peptide mixture was then spiked with different amounts
of a tryptic digest of equine apomyoglobin. To scan for significantly varying peptides, samples containing 50 fmol/µL / 100 fmol/µL and 500 fmol/µL / 1 pmol/µL of digested equine apomyoglobin and four additional peptides (angiotensin III, substance P, fibrinopeptide B, and ACTH 1–24) were compared. In the protein profiling experiment, the eight-protein mixture was spiked with different amounts of the myoglobin digest in the range between 50 fmol/µL and 3 pmol/µL. In the experiment to investigate the linearity of the method, the myoglobin digest was added in amounts from 10 fmol/µL to 10 pmol/µL. One µL of each aliquot was analyzed by nano-flow LC-MS.

Proteomics
2D-DIGE was used to examine the post mortem effect on proteins (paper III). Cortex was lysed by sonication and centrifuged and the protein concentration of each homogenate was determined. Fifty µg of protein was labeled with either Cy3 or Cy5. Cy2 was used to label the internal standard, which was prepared from pooled aliquots of equal amounts of the samples. Prior to isoelectric focusing (IEF) the labeled samples were mixed.

Separation
Peptidomics
Five microliters of peptide extract was injected directly into a RPC capillary column. The particle bound sample was desalted by an isocratic flow of buffer (0.25% acetic acid in water) and eluted over a 60-min gradient (35% acetonitrile in 0.25% acetic acid) directly coupled to MS (papers I-VI).

Proteomics
All 2-D separations were performed using standard 2-D polyacrylamide gel electrophoresis (PAGE) apparatus and reagents. Gel images were generated by scanning the gels. The Differential In-gel Analysis (DIA) module of the DeCyder analysis software was used for co-detection and direct comparison of spots. To allow mass spectrometric protein identifications, two preparative gels were run. The gels were stained using SYPRO Ruby Protein Gel. The protein spots in the analytical gels that met the defined statistical requirements were matched to the preparative gel spot maps. Spot picking, trypsin digestion and peptide extraction was done automatically and the tryptic mixtures were separated and infused directly to a mass spectrometer.
**In situ hybridization**

*In situ hybridization* was performed on five controls and seven MPTP lesioned mice (paper VI). $^{35}$S-labeled anti-sense and sense cRNA probes were prepared by *in vitro* transcription from cDNA clones corresponding to nucleotides 1-462 of the coding sequence of the rat PEP-19 gene. Tissue sections (12 μm thick) from the different groups of animals were cut using a cryostat. The sections were hybridized overnight with $10^6$ cpms of $^{35}$S-labeled probe. The slides were washed before being dehydrated in graded alcohols. The slides were then exposed on X-ray films for two to fourteen days\cite{123}.

**Western blot**

To investigate the effect on the dually phosphorylated mitogen-activated protein kinase (MAPK), striatum brain tissue was sonicated in 1 % SDS (paper III). The protein concentration of each homogenate was determined and equal amounts of protein were loaded onto SDS-PAGE gels. After separation proteins were transferred to nitrocellulose membranes. The membranes were immunoblotted using polyclonal antibodies against phospho-Thr202/Tyr205-MAPK or non-phosphorylated MAPK. Antibody binding was revealed by goat anti-rabbit horseradish peroxidase-linked IgG and the ECL immunoblotting detection system. Chemiluminescence was detected by autoradiography and levels of phosphorylated and total MAPK were quantified by densitometry\cite{36}.

**MS detection and identification**

**Peptidomics Q-ToF (papers I-VI)**

Mass spectra were collected in the m/z-ratio range of 300-1000 Da using a Q-ToF mass spectrometer. Several reruns were performed to receive MS/MS information of automatically selected precursors. The mass information was imported to DeCyder MS for detection and visualization of peak profiles. Peaks were counted and matched between the different samples and the peptide levels were compared. For peptide sequence identification the MS/MS spectra were deconvoluted using MaxEnt3 (MassLynx 3.4, Micromass) and interpreted by the BioLynx (MassLynx 3.4) software tools and/or manually by *de novo* sequencing. The proposed peptide sequences were compared with the nonredundant database of National Center for Biotechnology Information (NCBI) to establish the peptide identities using Basic Local Alignment Search Tool (BLAST).
Proteomics LTQ (papers III & VI)

Four MS/MS spectra of the most intense peaks were obtained following each full-scan mass spectrum using the LTQ mass spectrometer. The dynamic exclusion feature was enabled to obtain MS/MS spectra on co-eluting peptides. The sequences of the peptides were identified by searching the MS/MS against the UniProt database (Mus musculus) using Mascot\textsuperscript{124}. The criterion for positive identification of a peptide was a Mascot score of (P>30).

Pep-19 identification (paper VI)

For identification of PEP-19, one control mouse striatum was prepared and directly infused into a LTQ-FT MS for accurate mass measurement. The identity of PEP-19 was established by using the experimental mass as input to SwePep. To obtain sequence information of PEP-19 a 30 kDa fraction of the striatum was digested by trypsin over night. The digest were separated and directly infused to mass spectrometer.

Imaging MALDI MS (paper VI)

Imaging MALDI MS (IMS) was used to analyze PEP-19, directly on brain tissue sections. The tissue was cut into 12 µm sections and mounted directly onto gold-coated MALDI target plates. The matrix solution was deposited in a raster onto the tissue using a MALDI matrix spotter. The spotted tissue sections were analyzed using a Voyager DESTR MALDI-TOF mass spectrometer in automatic mode, according to a defined search pattern. The spectra from each of the matrix spots were processed and combined into an image file using a software (Department of Biochemistry and Mass Spectrometry research Center, Vanderbilt University School of Medicine). The image files were imported to an image processing application, which provides specific tools for MS image analysis. Spectra from the different experiments were also imported as ASCII files into OriginPro7.5 and processed to analyze differences in abundance of peptides/proteins in the animal groups.

Peptide database – SwePep (paper IV)

The database SwePep (www.swepep.org) was constructed using MySql and Java. The database is designed for storage of information regarding endogenous peptides such as mass, isoelectric point, sequence, post-translational modifications, and precursor protein. Because of the impossible task to differentiate between small proteins and peptides in experimental data, SwePep also contains 25,047 proteins with sequence length less than or equal to 120 amino acids. The information in SwePep is collected from three different
sources; experimental data, peptide information from UniProt (version 49.0) and peer-reviewed publications.
Result and Discussion

Sample preparation

The extraction of peptides in acidified water became the method of choice because the natural surrounding for bioactive peptides is the hydrophilic extracellular space. The use of methanol as a fixative and extraction buffer revealed fewer peptides and included an extra step to change buffer prior to separation with reverse phase. Using a simple protocol, the peptides were separated from the proteins centrifugation through a 10 kDa cut off filter. The peptides were further separated by nanoscale capillary reversed phase liquid chromatography (nano-flow LC) and directly infused into the mass spectrometer (Figure 3).

Identification of novel peptides

Approximately 1500 peptide peaks could be detected in one mg of brain tissue in the dynamic range amol to low pmol (paper I) in rat brain tissue (striatum, motor cortex and thalamus). Nineteen peptides were identified having masses from 890 to 3475 Da. The identities revealed high levels of fragments from abundant proteins, not likely reflecting the in vivo composition of peptides. Most identified peptides proved to be fragments from hemoglobin. In other publications these protein fragments were described as the tissue specific pool of peptides[125]. The relatively short post mortem time from decapitation to tissue preparation (3 min) was enough to produce peptides from fragmentation prone proteins, masking the detection of neuropeptides at low concentration, and to concurrently degrade some of the neuropeptides.

To investigate if the observed peptides were endogenous or if they were produced post mortem, a sample preparation technique comprising in vivo rapid heat inactivation was used in combination with the peptidomic approach. Denaturation by MW irradiation has been proven to be efficient for brain neuropeptides investigated by immunoassays[126-128]. Using this technique approximately 550 peptides were detected in mouse or rat hypothalamus brain tissue and more than 40 peptides were sequenced and identified. The identified peptides were either known biologically active endogenous neuropeptides, or previously not reported peptides from known neuropeptide
precursors, or peptides with processing sites corresponding to those of pro-
hormone convertase enzymes. Some peptides were identified both in mouse
and rat (Table 1). Peptides from fragmented high abundant proteins were not
identified, demonstrating that protein degradation is prevented by rapid heat
denaturation. The inhibition of proteolytic activity permits the detection and
identification of endogenous peptides at low concentrations. Furthermore,
post-translational modifications of the peptides were detected some at sites
previously not reported.

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<th>Sequence</th>
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</tbody>
</table>

Table 1. Novel peptides identified from hypothalamic tissue of rat and mouse using
nano-flow LC MS (paper II). a. The peptide has been identified in other publications
after the table was published 1, Pan et al., 2006[129] 2, Che et al., 2005[56] 3, Parkin et
al., 2005[130].

34
Post mortem time effects on peptides and proteins

As showed in paper II in vivo MW irradiation of the brain is an efficient method to prevent degradation and to ensure the quality of the sample. In addition to the in vivo deactivation using focused MW irradiation an alternative inactivation method has been proposed. This method was developed to enable the analysis of fresh or flash frozen samples (paper III).

The effect of post mortem degradation was investigated by analyzing the peptidome and proteome of mouse brain samples, subjected to different post mortem time intervals (Figure 4).

0, 1, 3, and 10 min MW post mortem delay

Cortex → 2D-GE

Striatum → Western blot

Hypothalamus → Peptidomics

Snap frozen, rapidly heated

Striatum → Peptidomics

Western blot

Figure 4. Different brain areas investigated by biochemical methods post mortem (paper III).

The effect of post mortem time on the hypothalamus peptide content of mice was investigated using the peptidomics approach. The number of peptides detected after different post mortem intervals rapidly increased by time. In the control group about 660 peaks were detected compared to over 2000 peptides in the 10 min post mortem group (Figure 5A). Altogether 24 neuropeptides, hormones, and potential biologically active peptides were identified. Most endogenous peptides were present in higher levels in the control group compared to 1, 3, and 10 minutes. However some peptides were not significantly changed through out the time course. For many peptides that are released in the brain, their long half-lives mean that they survive long enough to diffuse to sites distant from their release[27]. Dendritic release of peptides can generate a hormone-like signal in the brain. Even in the CSF, many peptides are present at concentrations high enough to activate the high-affinity receptors present in many brain regions[27].
The neuropeptide corticotropin-like intermediate lobe peptide (CLIP) was identified both with and without a phosphate group on serine 154. The level of the phosphorylated form decreased rapidly by post mortem time. The unphosphorylated form of CLIP also decreased but at a lower rate.

2D-DIGE was used to evaluate the post mortem influence on the proteins. Cortical brain tissue from 10 minutes post mortem was compared to directly MW irradiated brain tissue. Of about 1500 proteins detected on the gels, DeCyder images revealed 53 spots with a significant absolute abundance change (>1.5 fold) between any two groups (p<0.05). Twenty-eight of the regulated proteins were identified. The identified proteins belonged to a number of different protein families and were in concordance with the protein fragments detected in the 1, 3, and 10 minutes time study post mortem.

To investigate the effect on the dually phosphorylated mitogen-activated protein kinase (MAPK), western blot analysis was performed on striatal brain tissue. The time study by MW irradiating was complemented by striatal tissue heated directly after frozen. After 10 minutes post mortem the phosphorylations were decreased by 95% compared to the control group. No significant difference was detected between the groups of directly MW tissue and frozen tissue, directly heated. The levels of unphosphorylated MAPK were unchanged. Phosphorylation is involved directly or indirectly in all important cellular events and is the most studied modification of proteins\footnote{131}. It is estimated that 30% of all cellular proteins are phosphorylated, often at multiple sites and each phosphorylated event might have a distinct effect on protein function\footnote{131}. The phosphorylation is reversibly controlled by the combined action of two different classes of enzymes the protein kinases and the phosphatases. The detection of phosphorylations is difficult due to a number of reasons; only a small fraction of a given protein is phosphorylated, multiple phosphorylations, the low abundance of phosphoproteins, the existence of phosphatase enzymes which dephosphorylate protein unless special precaution are taken, and the dynamic nature of the phosphoproteome\footnote{132, 133}.

We speculate that the enhanced degradation in thawed samples\footnote{134} might be due to disrupted homeostasis caused by ice crystals penetrating the plasma membranes\footnote{135} caused by ions leakage.
Protein fragments as degradation markers

A method for determining the quality of a biological sample was described in paper III. Immediately inactivated biological samples contain low amounts of proteins fragments. With increasing sample handling time until inactivation the number of fragments and their abundance increase. This specific degradation can be used to discriminate samples of insufficient quality from samples with high integrity. The identity of proteins that are among the first to be degraded has been established. Among the identified proteins, the ubiquitously expressed phosphoprotein stathmin was found to decay in such a way that it produces a fragment that permits us to determine the quality of the brain tissue (Figure 5B).

Improved identification of peptides using SwePep

The identification of endogenous peptides is a labor and time consuming process. One of the reasons is the lack of easily accessible information in the public databases regarding endogenous peptides. SwePep contains more than 4000 annotated endogenous peptides from 394 different species. The peptides stored in SwePep are classified in three different categories, biologically active peptides, potential biological active peptides, and uncharacterized peptides. Using this classification we can store peptides identified by mass spectrometry in the database, even though their biological function is unknown. In Figure 6 peptides from rats separated by pI and mass are visualized.
Figure 6. Graph showing all 248 peptides, from rat tissue, described in SwePep. The peptides are separated in the graph by molecular weight (MW) and isoelectric point (pI). Blue dots display peptides identified in our laboratory (51 peptides).

In paper IV rat and mouse hypothalami were separated and detected as previously described (paper I-III). The MS spectra were automatically analyzed using DeCyder MS to attain a list of deconvoluted experimental peptide masses. The list was matched against the theoretical masses in SwePep for neuropeptide matches. Of about 400 detected peptide peaks concerning mice, 54 neuropeptide candidates were detected whereof 31 peptides were verified by MS/MS. This exemplifies the challenge of identifying neuropeptides and the fact that the chemical structures of neuropeptides are now elucidated before their biological significance becomes apparent[136].

DeCyder MS – differential display of peptides

By keeping the experimental conditions constant and randomize sample analysis order, label free semi-quantitative analysis of peptides was performed. The peptide signal intensity was measured by nano-flow LC-MS or MALDI IMS.

Searching for differences in peptide expression between samples using MS was initially performed manually. This procedure was very time consuming and not very exact. The MS analysis produces large amount of experimental data and several biological replicates are needed to get reliable and statistical significant results. In collaboration with GE Healthcare, Uppsala, a software
for MS data interpretation, DeCyder MS was developed. The software automatically detects peptides in MS raw data files and compares the peptide intensities between the different MS files. Peptide data generated by DeCyder MS can be directly searched against SwePep for identification.

DeCyder MS was used to study label free peptidomics by nano-flow LC-MS with automatic analysis of experimental data (paper V). Mixtures of tryptic and endogenous peptides of known composition in different concentrations were used. As a result a linear response over three orders of magnitude were achieved (Figure 7).

Figure 7. A mixture of tryptic peptides spiked by standard peptides (Angiotensin III (Ang III), fibrinopeptide B (GluFib) and ACTH 1-24) was automatically detected by DeCyder MS. Peak volumes of peptides are plotted as a function of spiked amount. All runs were repeated six times. All peptides were not observed at all concentrations due to the automatic detection mode of the mass spectrometer and the low concentrations. By visual inspection it could be concluded that the angiotensin III peak at 10 fmol/μl was disturbed by tailing from a high-intensity peak (see inset).

Animal models of Parkinson’s disease

The method for peptide extraction and detection previously described was applied to examine the neuropeptide content in striatum of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treated mice compared to control.
The neurotoxin MPTP is used to generate cell death in the nigrostriatal dopaminergic pathway in mice and to mimic the neuropathological, neurochemical, and clinical features in PD. The analysis by nano-flow LC-MS of striatal tissue revealed a significant decrease of PEP-19 protein level. Moreover, imaging MALDI MS showed that PEP-19 was predominantly distributed to striatum of mice and that PEP-19 was significantly reduced by 30%. By in situ hybridization, the levels of PEP-19 mRNA expression in MPTP lesioned animals were compared to control. In situ hybridization showed that PEP-19 mRNA is abundantly expressed throughout the brain with particularly high levels in striatum and a significant decrease of PEP-19 mRNA in striatum (42 % decreased, p<0.05). PEP-19 was identified by using the experimental exact mass from nano-flow LC FT-ICR MS and comparing this to the experimental masses in SwePep. Only two possible peptide matches were retrieved out of which one could be excluded. PEP-19 was confirmed by MS/MS spectra from three tryptic peptides constituting sequence coverage of 44% of the complete protein.

The brain specific polypeptide PEP-19 is a large peptide and rather defined as a small protein than a neuropeptide. PEP-19 is an N-terminal acetylated, IQ motif protein binding to calmodulin\textsuperscript{[137]} and it is directly translated from RNA and not further processed in vesicles as regular neuropeptides. PEP-19 has been reported to be regulated in neurodegenerative and neurocognitive disorders. Alcoholics have a reduced level of PEP-19 in the prefrontal cortex\textsuperscript{[138]} and PEP-19 levels are depressed in the basal ganglia in Huntington’s disease\textsuperscript{[139]}. Recently Dickerson el al. reported that PEP-19 is a substrate for protein kinase C and that the serine residue within the IQ motif is phosphorylated\textsuperscript{[140]}. They propose that this phosphorylation regulates PEP-19 activity.

Reserpine is a classical way to induce Parkinson symptoms to animal models\textsuperscript{[121, 122]}. The levels of neuropeptides was semi-quantitative measured in striatum of mice at different time-points (1h, 6h, 12h and 24h) after administration of reserpine treatment using nano-flow LC-MS. DeCyder MS was used to extract the peptide intensity. 18 peptides showed a significant altered expression according to ANOVA analysis and 12 of those were identified by searching their masses against SwePep (Figure 8).
Figure 8. Levels of two peptides originating from Secretogranin-1 (chromogranin B) precursor, at 1, 6, 12, and 24h post administration of reserpine. A) SFARAPQQLDL and B) LLDEGHYPV.

Reserpine is a substance known to deplete dopamine stores in the central nervous system through a competitive inhibition of monoamine uptake into intracellular vesicles of dopamine neurons\cite{141}. Not much is known about the influence of endogenous peptides. The processing of endogenous peptides is thought to be performed by the PC. PC2 has been shown to be inhibited by catecholamines \textit{in vitro} and therefore the increase in processing has then been suggested to be triggered by the depletion of dopamine caused by reserpine treatment\cite{142}. We speculate that the increase in peptide concentration at time point 1h and 6h is due to the depletion of catecholamines. The recovery of the peptide levels occur when the balance of catecholamines is recovered or that the increased secretion of neuropeptides leads to depletion of peptide precursors. This may also explain the evaluated levels of neuropeptide mRNA after reserpine treatment\cite{143,144}. 

![Figure 8: Levels of two peptides originating from Secretogranin-1 (chromogranin B) precursor, at 1, 6, 12, and 24h post administration of reserpine. A) SFARAPQQLDL and B) LLDEGHYPV.](image-url)
Conclusion

New improved methods for the study of endogenous peptides have been developed by combining the use of in vivo MW irradiation, molecular weight filtration and nano-flow LC-MS. Using these methods, we were able to detect and identify known peptides. Some of these peptides contained post-translational modifications not previously described for the particular peptide. Novel endogenous peptides were discovered in the brain originating from known neuropeptide precursors and with processing sites corresponding to those of prohormone convertase enzymes. Some of these peptides were identified in both mouse and rat.

A database, SwePep, was implemented to facilitate the identification of endogenous peptides. The database repository neuropeptides, hormones and small proteins and include both physical and biological information concerning the peptides. A program for automatic analysis of liquid chromatography tandem MS data, including scanning for, and further profiling of, biologically significant peptides and proteins was developed in cooperation with GE Healthcare. We show that peptides present in different amounts in groups of samples can be automatically detected using statistical tests.

The effects of post mortem time on endogenous peptides and proteins have been investigated in a time course study. The results showed that already after three minutes a substantial amount of high abundant degraded protein fragments emerged and some endogenous peptides were drastically reduced with increasing post mortem times. Of about 1500 proteins investigated by two-dimensional difference gel electrophoresis, 53 proteins were found to be significantly changed (>1.5 fold) between instant deactivated (fixed) samples and the 10 min post mortem samples. Moreover, the level of phosphorylation of the mitogen-activated protein kinase was monitored throughout post mortem time study using western blot. The phosphorylations were decreased by 95% in the 10 minutes post mortem samples.

New rapid denaturing sample methods have been developed in cooperation with Denator AB to complement in vivo MW irradiation. Using this method approximately the same number of peptides was detected as using in vivo microwave irradiation and no protein fragments were found. This denaturation method showed also that the levels of phosphorylation of the mitogen-
activated protein kinase were unchanged compared to MW irradiated samples.

A marker for sample integrity has been discovered. A fragment from the ubiquitously expressed phosphoprotein stathmin showed a characteristic and reproducible postmortem degradation profile. It could therefore be used to determine the quality of brain tissue.

The peptidomics method was used to investigate levels of peptides in two animal models of Parkinson’s disease. The small protein, PEP-19, was found to be significantly decreased in striatum of MPTP lesioned parkinsonian mice. The expression and localization of this protein was further investigated by imaging MALDI MS and by in situ hybridization. The peptidome of re-serpine treated mice were investigated at different time points after administration and displayed a number of significantly altered peptides, e.g. proenkephalin and secretogranin 1 (chromatogranin B) derived peptides in the brain region striatum.
Future aspects

The interpretation of data has become a limitation for MS analysis. New specialized databases such as SwePep, containing a large range of information, will play an important role in the future. These databases should contain for instance sequence, mass, tandem mass spectrum, anatomical location, post-translational modification, physico-chemical properties, LC retention time, as well as functional information. In combination with improved software for identification, annotation, normalization and differential display it will speed up the analysis and shift the bottle-neck to other areas.

It is possible that a number of endogenous biologically active peptides are still unidentified in the brain and around the body. To improve and possibly find new approaches and technologies to identify and characterize peptide is therefore of great importance. The sample handling is the key to analyzing neuropeptides utilizing a peptidomic approach. To avoid protein degradation fragments standardized methods such as instant denaturation of a sample is therefore necessary to accurately measure and quantify neuropeptides.

Because of the regulating function of many endogenous peptides and the direct link between the peptidome and the proteome, the peptidome is a considerable source for candidates of biomarkers for disease.
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53


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