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Neuropeptidomics – Expanding Proteomics Downwards

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Abstract

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Biological function is mainly carried out by a dynamic population of proteins which may be used as markers for disease diagnosis, prognosis, and as a guide for effective treatment. In analogy to genomics, the study of proteins is called proteomics and it is generally performed by two-dimensional gel electrophoresis and mass spectrometric methods. However, gel based proteomics is methodologically restricted from the low mass region which includes important endogenous peptides. Furthermore, the study of endogenous peptides, peptidomics, is compromised by protein fragments produced post mortem during conventional sample handling.

In this thesis nanoflow liquid chromatography and mass spectrometry have been used together with improved methods for sample preparation to semi-quantitatively monitor peptides in brain tissue. The proteolysis of proteins and rise of fragments in the low mass region was studied in a time-course study up to ten minutes, where a potential marker for sample quality was found. When rapidly denatured brain tissue was analyzed, the methods enabled detection of hundreds of peptides and identifications of several endogenous peptides not previously described in the literature. The identification process of endogenous peptides has been improved by creating small targeted sequence collections from existing databases.

In applications of the MPTP model for Parkinson's disease the protein and peptide expressions were compared to controls. Several proteins were significantly changed belonging to groups of mitochondrial, cytoskeletal, and vesicle associated proteins. In the peptidomic study, the levels of the small protein PEP-19 was found to be significantly decreased in the striatum of MPTP administered animals. Using imaging mass spectrometry the spatial distribution of PEP-19 was found to be predominant in the striatum and the levels were concordantly decreased in the parkinsonian tissue as verified by immunoblotting.

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

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To my family

List of Papers

This thesis is based on the papers listed below, which are referred to in the text by the Roman numerals I-VI.

- I. A neuroproteomic approach to targeting neuropeptides in the brain. Sköld, K.*, Svensson, M.*, Kaplan, A., Björkesten, L., Åström, J., Andren, P.E. (2002) *Proteomics*, **4**, 447-454.
- II. Peptidomics-based discovery of novel neuropeptides. Svensson, M.*, Sköld, K.*, Svenningsson, P., Andren, P.E. (2003) *J Proteome Res*, **2**, 213-219.
- III. The significance of biochemical and molecular integrity in brain proteomics and peptidomics: Stathmin 2-20 and peptides as sample quality indicators. Sköld, K.*, Svensson, M.*, Norrman, M., Sjögren, B., Svenningsson, P., Andren, P.E. *Submitted manuscript*.
- IV. Neuropeptidomics: Strategies for reliable and sensitive identification of endogenous peptides. Fälth, M., Svensson, M., Sköld, K., Nilsson, A., Fenyö, D., Andren, P.E. *Submitted manuscript*.
- V. Decreased striatal levels of PEP-19 following MPTP lesion in the mouse. Sköld, K.*, Svensson, M.*, Nilsson, A.*, Zhang, X., Nydahl, K., Caprioli, R.M., Svenningsson, P., Andren, P.E. (2006) *J Proteome Res*, **2**, 262-269.
- VI. Changes in cytoskeletal and mitochondrial proteins in striatum following MPTP lesion in the mouse. Svensson, M.*, Sköld, K.*, Nordvarg, H., Sjöberg, S., Hedberg, J.J., Fredriksson, A., Pickering, J., Svenningsson, P., Andren, P.E. *Submitted manuscript*.

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Papers not included in this thesis

1. An automated method for scanning LC-MS data sets for significant peptides and proteins, including quantitative profiling and interactive conformation. Kaplan, A., Söderström, M., Fenyö, D., Nilsson, A., Fälth, M., Sköld, K., Svensson, M., Pettersen, H., Lindqvist, S., Svenningsson, P., Andren, P.E., Björkesten, L. (2007) *J Proteome Res*,
2. Neuropeptidomics: MS applied to the discovery of novel peptides from the brain. Svensson, M., Sköld, K., Nilsson, A., Fälth, M., Nydahl, K., Svenningsson, P., Andren, P.E. (2007) *Analytical Chemistry*, **79**, 14-21.
3. Normalization and expression changes in predefined sets of proteins using 2D gel electrophoresis: A proteomic study of L-DOPA induced dyskinesia in an animal model of Parkinsons disease using DIGE. Kultima, K., Scholz, B., Alm, H., Sköld, K., Svensson, M., Crossman, A.R., Bezard, E., Andren, P.E., Lönnstedt, I. (2006) *BMC Bioinformatics*, **7**, 475
4. SwePep, a database designed for endogenous peptides and mass spectrometry. Fälth, M., Sköld, K., Svensson, M., Norrman, M., Svensson, M., Fenyö, D., Andren, P.E. (2006) *Mol Cell Proteomics*, **5**, 998-1005.

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Abbreviations

2-D	two-dimensional
ACTH	adrenocorticotrophic hormone
AD	Alzheimer's disease
CID	collision-induced dissociation
DIGE	difference gel electrophoresis
ESI	electrospray
FT-MS	Fourier-transform ion cyclotron resonance mass spectrometry
IDM	ion desorption model
IEF	isoelectric focusing
IEM	ion evaporation model
IMS	imaging mass spectrometry
LC	liquid chromatography
LDCV	large dense core vesicle
LPH	lipotropin
<i>m/z</i>	mass-to-charge ratio
MALDI	matrix-assisted laser desorption/ionization
MPP ⁺	1-methyl-4-phenyl-2,3-dihydropyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MSH	melanocyte stimulating hormone
MudPIT	multidimensional protein identification technology
MW	focused microwave fixation
PAGE	polyacrylamide gel electrophoresis
PAM	peptidyl α -amidating mono-oxygenase
PC	prohormone convertase
PCR	polymerase chain reaction
PD	Parkinson's disease
<i>pI</i>	isoelectric point
PMF	peptide mass fingerprinting
POMC	proopiomelanocortin
PTM	post translational modification
Q	quadrupole
RPC	reversed phase chromatography
SDS	sodium dodecylsulfate
TOF	time-of-flight

Preface

During the last 50 years, life expectancy in Sweden has increased from 70 to 81 years of age. Part of this increase is ascribed to lifestyle improvements and part on advances in medical treatment such as drugs and vaccines ⁽¹⁾. In concordance, it has been reported that the proportion of the world population > 65 years has tripled during the same time period and is anticipated to triple once more until 2050 ⁽²⁾. The risk of being diagnosed with neurodegenerative disease, e.g., Parkinson's (PD) and Alzheimer's disease (AD) rise sharply with advancing age ⁽³⁾. It has been estimated that the approximate prevalence of PD and AD is 1.5-2% at the age of 65 years ⁽³⁻⁵⁾, and is doubling every four years for AD ⁽⁵⁾. Therefore, it is of utmost importance to find new approaches of studying these neurodegenerative diseases to find molecular markers and to better understand the mechanisms causing the disabling symptoms.

The cellular, molecular, and functional heterogeneity of the brain distinguishes it from other tissues as the most complex organ of higher organisms ^(6,7). Characterizing the molecular content of neural tissue is consequently a significant challenge for current analytical techniques. At the same time, the brain is of growing interest to medical research and pharmaceutical industry because of the economical and social impact of the more common neurological diseases ⁽⁸⁾.

As these inevitable medical challenges approach, every contribution to the collected efforts around sensitive analyses and early disease diagnoses is needed. Improved sample handling and technique developments come well in hand to meet the future demands on efficient and accurate treatments.

Introduction to proteomics

The proteome

Complementary to the DNA and RNA-associated genome there is a proteome. This field of research is dealing with the expressed products from translated genes of an organism. The complete genomes of several eukaryotic species have been described, such as the human^(9,10), and the mouse genome⁽¹¹⁾. Obviously, there is more to the understanding of an organism at the molecular level than genome studies alone can provide. Biological function is mainly carried out by a dynamic population of proteins which is determined by interactions of gene and protein regulation with extracellular influences^(12,13). The structure of a protein is incompletely defined by its gene sequence. Analysis of the expressed product is needed to detect the presence of differential splicing, posttranslational modifications (PTMs), or expression levels of the proteins participating in this functional interplay⁽¹³⁾. Thus, characterization of the proteins provides more information and an additional view to biological structures and signaling pathways.

Proteomics is the large scale study of proteins. It was originally defined as the protein complement expressed by a genome⁽¹⁴⁾. However, a proteome is a highly dynamic entity that is constantly changing in time and differs from cell to cell. This is well illustrated in the example of the butterfly and its caterpillar in which every somatic cell of two appearing individual organisms have identical genomes⁽¹⁵⁾. Considering this, it has been suggested that the proteome definition should include all protein isoforms and modifications and the specification of what cell type(s) at a certain time^(13,16).

Traditional proteomics methods usually involve separation by two-dimensional polyacrylamide sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (2D PAGE) prior to protein identifications by mass spectrometry (MS)^(14,17,18). Quantitative methods i.e., protein staining and scanning, can be used in the gel to analyze the protein expression profiles, hence enable the characterization of cellular events associated with e.g., disease progression. Indeed, some classes of proteins such as membrane proteins, extremely acidic or basic proteins, very large or small proteins are underrepresented or absent when using 2D PAGE^(19,20). An alternative technique is to use gel free proteomics which is based purely on liquid chromatography (LC) such as the multidimensional protein identification technology (MudPIT)⁽²¹⁾. Complete proteomes are enzymatically digested prior to LC separa-

tions and MS detection. The MudPIT approach relies heavily on bioinformatics tools to facilitate the interpretation of the tremendous amount of generated data. The protein quantitation in gel free proteomics is carried out either by labeling the proteins with stable isotopes (in vivo or in vitro) which are detected by MS ⁽²²⁾ or by label free quantitation ⁽²³⁻²⁵⁾ (**Papers I, III, and Paper V**). However, the 2D PAGE proteomic methodology remains the standard for separation, and characterization of complex protein samples ^(26,27).

While the upstream fractionation and separation techniques have started to change, MS has established itself as the method of choice for protein identifications ^(28,29). MS instruments are based on several different techniques with the common objective of high resolution separations of ionized analytes according to mass-over-charge ratio (m/z). The specific pattern produced from each analyzed molecule can subsequently conduce to its identification either by amino acid sequencing or by database searching protocols ^(30,31). The combination of electrospray ionization (ESI) or matrix-assisted laser desorption ionization (MALDI), tandem MS (MS/MS), on line reversed phase LC (RPC), and sequence database searching of collision-induced dissociation (CID) spectra has proven to be particularly apt for protein identifications following gel based separations ^(32,33).

Attempts to analyze every protein possible in a standard proteomic sample pose a significant dilemma regarding complexity. A mammalian genome e.g., the mouse (*mus musculus*), consists of ~30,000 protein coding genes ⁽¹¹⁾. Recent transcript expression analyses points to a substantial higher number of individual transcripts for protein expression ^(34,35). This elaboration of the transcription machinery has been attributed to the non-coding RNA and alternative splicing of the transcripts, which makes fine regulation and isoform gene expression possible ^(35,36).

After translation, it is believed that up to 80% of all proteins are subject to a range of more than 300 different types of PTMs ^(19,37-41). Via processing events, i.e., proteolytic cleavage or by addition of modifying groups, e.g., phosphorylation, glycosylation, acetylation, and ubiquitination, the protein properties are covalently changed to give it novel commissions or provide it with additional information ⁽⁴¹⁾. For example, acetylation of melanocyte stimulating hormone (MSH) products of proopiomelanocortin (POMC) may switch the activity between endorphinergic and melanocortinergic which have competing and often functionally antagonistic properties ⁽⁴²⁾. It has been estimated that 80-90% of the proteins synthesized in the cytoplasm of mammalian cells are N-termini acetylated ⁽⁴³⁻⁴⁵⁾. PTMs may be definite, e.g., proteolysis of peptide precursors to form hormones and neuropeptides ^(41,46), or of transient nature, e.g., phosphorylation regulation of kinase cascades ⁽⁴⁷⁻⁴⁹⁾. Other processing events associated with protein turnover/ageing degradation also contribute to increased complexity, primary as protein fragments in the lower mass regions. This problem is sometimes observed in connection with

the use of post mortem tissue or deficient sample preparation protocols and is caused by either enzymatic or non-enzymatic proteolysis⁽⁵⁰⁾.

Furthermore, the dynamic range of protein expression is heterogeneous. The concentrations of different proteins in serum vary by a factor of 10^{10} ⁽⁶⁾ and in tissue the range is likely to be lower but has been estimated to six orders of magnitude^(15,18,26). It is necessary to determine the protein level directly as there is no protein methods available equivalent to selected amplification by polymerase chain reaction (PCR). In addition, determining protein levels from transcribed genetic information is sometimes complicated since it may or may not provide results with a simple quantitative relationship to protein levels⁽⁵¹⁻⁵³⁾ as there is biological regulation between transcript and protein as well as regulation of protein degradation^(35,54,55).

Subproteomes

By its definition proteomics is a large scale venture. However, the fact that the analytical tools used at the end of the analysis chain (e.g., MS) have a definite speed and detection range, it is often strategic to pre-fractionate, or when possible, to use single cell⁽⁵⁶⁻⁵⁸⁾ or organell⁽⁵⁹⁾ extraction procedures to reduce the complexity of the sample. In the case of pre-fractioning, one may focus on selected populations of proteins e.g., those in different subcellular organelles such as the lysosomal⁽⁶⁰⁾ and mitochondrial proteome⁽⁶¹⁻⁶³⁾ or other subsets of proteins with common characteristics such as the phosphoproteome^(49,64). Partially purified preparations can in this way provide discrete and comprehensive subproteomes to be analyzed with reduced protein complexity⁽⁶⁵⁾.

It should be noted, however, that by including an additional step in the protocol additional uncertainty is introduced^(27,66) and comparative studies between incompletely pre-fractionated protein subsets or pre-fractionations of differently handled samples may in contrary to what was intended give rise to intricate distortions of the results^(67,68).

Peptidomics

Based on their physical size, the low molecular weight endogenous polypeptides in an organism, tissue, or cell may be called a subproteome (Figure 1). This rather heterogeneous entity contains small proteins, peptide hormones, neuropeptides, and transient fragments of protein degradation. Perhaps better describing, the expression peptidome and peptidomics came into use by several groups in 2001⁽⁶⁹⁻⁷²⁾, which in analogy with the proteomics technology, aims at the simultaneous visualization and identification of all peptides expressed in a cell or tissue.

Figure 1. The mouse proteome and peptide subproteome. 11903 mus musculus protein entries from the UniProtKB/SwissProt database, and 246 mus musculus peptide entries from the SwePep database⁽³⁰⁾ (indicated $\times$ and $\times$ respectively) plotted according to their isoelectric point (pI) and molecular mass. The dashed rectangle outlines the separation limits of frequently used 2D PAGE protocols (i.e., pI range of 3 to 10 and mass range of 15 to 200 kDa).

The levels of the endogenous peptides are likely to vary from one moment to another, reflecting information of the particular physiological status, i.e., sex, age, and stress^(73, 246, 74). By comparing the peptidome in samples of e.g., diseased tissue with those in normal tissue, differential expression patterns can be revealed. This may lead to the discovery of biologically relevant peptides as biomarkers for diseases or indicators of certain pathological/pharmacological events⁽⁷⁵⁻⁷⁸⁾. Biomarkers offer a widespread applicability in medicine and drug development by serving as diagnostic flags for diseases, or as indicators of drug efficacy and adverse events. Thus peptide expression patterns may contribute to more individualized therapy^(28, 79, 80).

However, the detection and quantification achieved by the traditional proteomics approach (i.e., 2D-PAGE techniques) is inadequate for peptidome characterization and alternative strategies have to be considered. In addition, comprehensive peptide analyses are restricted by the low levels of some peptides, especially in relation to the high levels of protein turnover/ageing degradation fragments masking their detection (**Paper I**)^(50, 81).

Endogenous neuropeptides

In 1931 von Euler and Gaddum discovered the neuropeptide Substance P. However, more than two decades passed before the first peptide hormones, vasopressin and oxytocin, were structurally characterized⁽⁸²⁾. In the early seventies the term neuropeptide was introduced by de Wied and colleagues to describe an endogenous peptide synthesized in nerve cells and involved in nervous system functions⁽⁸³⁾.

Today it is known that neuropeptides are present in several types of cells and have a vast range of functions^(84,85). As peptide hormones, signal transmitters/modulators these polypeptides of 3-100 amino acids influence physiological processes such as regulation of reproduction, growth, feeding, circadian rhythms, and affective states^(84,86). They can be abundantly produced in large neural populations or in trace levels from single neurons⁽⁸⁷⁾. For example, in striatal mouse tissue the levels of met-enkephaline was assessed to ~2 pmol/mg from a standard curve of three concentrations of a deuterium variant of the peptide included during homogenization (Nilsson, A., Svensson, M. et al., 2006, unpublished results).

The neuropeptide precursors are synthesized in the endoplasmatic reticulum and transferred to the Golgi network for packaging into large dense core vesicles (LDCV). In the LDCV the precursors are prepared by processing into their active form and stored until secretion⁽⁸⁶⁾. Their maturation into active neuropeptides often follows a general and evolutionary old multi step procedure. where the first step involves endoproteolytic cleavages by prohormone convertases (PCs) at sites containing combinations of the basic amino acids lysine (K) and arginine (R)⁽⁸⁸⁾. The basic C-terminus residue is subsequently removed by carboxypeptidase enzymes. The combinations KR and KK are the predominant processing sites whereas RK and K/R-X_n-K/R, where n is 0, 2, 4, or 6 are less frequent sites of processing^(89,90). However, single basic processing sites have been observed and reported (**Paper II**)⁽⁹¹⁾. Following the endoproteolytic processing some peptides are subjects to additional enzymatic modifications such as amidation or acetylation before they are released into the extracellular environment by membrane fusing of the LDCV. This occurs predominantly around the axon terminals but may also occur from the soma or dendrites^(84,92).

A number of neuropeptide precursors contain one or more sequences of both related and unrelated peptides as illustrated by the processing example of POMC⁽⁹³⁾ (Figure 2), and their differential generation varies in a tissue specific manner. POMC is expressed both in the anterior and intermediate lobe of the pituitary. In the anterior lobe the peptides generated include adrenocorticotrophic hormone (ACTH) and β -endorphin, whereas in the intermediate lobe these peptides are further processed to α -MSH and to acetylated and shorter forms of β -endorphin. These mutually exclusive sets of peptides have completely different biological activities⁽⁹⁴⁾.

Figure 2. Outline of the major processing steps of POMC leading to melanocortins and other neuropeptides. PC1 and PC2 cleave at dibasic amino acid residues and generate pro- ACTH and β-lipotropin (β-LPH). Pro-ACTH is further cleaved by PC1 to N-terminal POMC (N-POC), joining peptide, and ACTH. PC2 can cleave ACTH further to generate ACTH 1–17 and corticotropin-like intermediate lobe peptide (CLIP). PC2 can also cleave β-LPH to form γ-LPH and β-endorphin and cleave γ-LPH in turn to generate β-MSH. Mature α-MSH is generated by the removal of the C-terminal basic amino acids from ACTH (1–17) by carboxypeptidase E. The peptide is then C-terminally amidated by peptidyl α-amidating monooxygenase (PAM) to form ACTH (1–13) NH₂ or desacetyl-α-MSH. Desacetyl-α-MSH can then be α-N-acetylated by POMC N-acetyltransferase, and in some tissues, further acetylated to form N,O-diacetyl-a-MSH⁽⁹⁵⁾. Three forms of γ-MSH are formed by additional cleavage of N-terminal POMC. γ₁-MSH contains 11 amino acids and is C-terminally amidated. γ₂-MSH has an additional C-terminal glycine and is not amidated, and γ₃-MSH is C-terminally extended and contains 25 amino acid residues. Common amino acid sequences among the melanocortins is (i.e., Y-MEHFW), indicated by underscore.

Aim of this thesis

The objective of this project was in a first step to develop a method for simultaneous analysis of the endogenous peptide and small protein content from small quantities of brain tissue samples. In a second step the peptidome and proteome of parkinsonian tissue from 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) lesioned mice were to be analyzed by differential studies using the developed methodology together with traditional gel based methods.

During the course of the project it became apparent that the speed of proteolytic degradation of peptides and proteins had to be dealt with and examined, and an efficient method for mass spectrometric identification of endogenous peptides needed to be established.

Methods

Sample preparation

When analyzing neural tissue samples (or any other complex biological sample), the major concerns are changes of the polypeptide integrities and the reproducibility. To ensure the production of reliable proteomic data, it is critical to set high standards in the very beginning of the experiment, since the results greatly depend on the quality of original samples. In addition, a robust sampling methodology enables reduced number of specimens in each experiment which is fundamental when working with biological samples. Ideally, crude, unprocessed samples should be analyzed without any delay to avoid artificial losses or biases arising from sample collection, storage, and preparation. However, no method or instrument capable of such a single step operation exists.

To make use of the high resolution and detection provided by MS applications it is essential to use sample preparation protocols that minimize the post-sampling changes e.g., fragmentation of proteins and peptides which interfere with the analysis^(81,96-98). These proteolytic events can to some extent be delayed or minimized by the use of paraformaldehyde perfusion of the brain. Perfusion is commonly used in immunohistochemical methods for the detection of neurotransmitters⁽⁹⁹⁾ to produce excellent spatial information. Unfortunately, paraformaldehyde is somewhat intractable in combination with MS because of adduct formations and reduced sensitivity, probably because many peptides become cross-linked into the polymer matrix by fixation⁽¹⁰⁰⁾. Other techniques to reduce post mortem fragmentation include the use of protease inhibitor cocktails, transgenic animal models⁽¹⁰¹⁾ or heat inactivation of snap frozen samples^(81,97). However, when working rapidly with animal brain tissue under standard conditions of sampling and sample preparation, there will be time for enzymatic proteolysis during a delay of ~90 seconds (or even longer for a protease inhibitor to reach its agents or targets). The majority of gel based proteomic studies are not adversely affected by this delay when only a few percent of each protein is degraded but it is detrimental to peptidomic studies⁽⁵⁰⁾.

In **Papers II, III** and in **Paper V** in vivo focused microwave fixation (MW) was used to ensure high sample quality prior to MS analysis of brain peptides and small proteins. Also, in **Paper V** an instrument for conductive heat transfer was evaluated. By the use of these techniques the delay until

enzyme inactivation is avoided by raising the tissue temperature to 90 °C in < 2 seconds ^(81,102,103). The proteolytic events are thereby inactivated by irreversible heat denaturation and, in contrast to freezing methods, they permit further dissection and processing of the sample ⁽¹⁰⁴⁾. Instant heat inactivation improves the detection of neuropeptides and their modifications when using immunoassays ⁽¹⁰⁴⁻¹⁰⁷⁾ as well as when using MS ^(81,97,108,109). An advantage of the conductive heat method compared to MW is its uniform heat transfer on frozen samples. It is also important to stress that MW is one of the more humane methods of sacrifice, in comparison with other common methods in terms of animal stress and rapidity.

MPTP model of PD

Animal research plays a vital role of both human and animal health. Experimental animal models are important tools in medical science to better understand the pathogenesis of diseases and to test therapeutic approaches ⁽¹¹⁰⁾. Since post-mortem studies of human idiopathic samples or biopsies do not allow precise determination of the pathological onset, experimental animal models have been developed in which a predictable and reproducible progression of the sequence of events involved in e.g., neurodegeneration is obtained ⁽¹¹¹⁾.

There are several available models for neurodegenerative disorders such as PD, AD, Huntington's disease, and amyotrophic lateral sclerosis. In **Paper V** and **Paper VI** the MPTP mouse model of PD was used. The neurotoxin MPTP was discovered as a result of observations of parkinsonian symptoms caused by intravenous administration of contaminated meperidine in drug addicts ^(112,113). MPTP crosses the blood and brain barrier and is metabolized to its toxic metabolite 1-methyl-4-phenyl-2,3-dihydropyridinium (MPP⁺). This metabolite is selectively taken up by the dopaminergic neurons where its main effect is to inhibit complex I of the electron transport chain in the mitochondrion, leading to subsequent death of these neurons. The MPTP model mimics the pathological and functional alterations that characterize idiopathic PD, i.e., selective loss of the nigro-striatal dopaminergic neurons in the brain. However, it lacks the slow progression of the pathological process and might act through unrelated mechanisms. The brains of the MPTP model do not display so called Lewy bodies, a hallmark of idiopathic PD ^(114,115).

Mass spectrometry

Mass spectrometric detection has become almost indispensable in the field of proteomics^(19,116,117). The principal components of a mass spectrometer are the ion source, where the analytes are ionized and vaporized, the mass analyzer, where the molecules are separated according to their m/z , the ion detection system to measure the relative abundance of the resolved ions, and the vacuum system which maintain a very low pressure in the instrument (Figure 3)⁽¹¹⁸⁾. The modes of operation of these parts vary among different instruments with their individual advantages and disadvantages extensively summarized elsewhere⁽¹¹⁷⁻¹²⁰⁾.

Ion formation

Today, the two dominating ionization techniques in protein and peptide analysis are ESI and MALDI^(19,121) whose inventors were awarded the Nobel Prize in chemistry in 2002^(122,123). Both ESI and MALDI are soft ionization techniques which is essential to minimize source fragmentation during succeeding analyses of non-volatile macromolecules. A prerequisite for the success of these techniques in proteomics is the ease of which that the analytes (i.e., proteins and peptides) are ionized by acid/base chemistry or by adduct formation⁽¹⁹⁾.

ESI has become the technique of choice for converting molecules in solution to gas phase ions for the purpose of MS analysis. In **Papers I-III** and **Paper V**, positive ESI MS was used to for the analysis of endogenous peptides and small proteins. In **Paper V** and **Paper VI**, positive ESI MS was used for the analysis of tryptic peptides from digested proteins. ESI is based on the continuous dispersion of a dilute solution of analyte molecules as a fine spray of charged droplets at atmospheric pressure. In the experimental setup used in this thesis, acetic acid was added as modifier to the solution which was pumped from the RPC system through the spray needle. When a high voltage potential is applied between the spray needle and the inlet of the mass spectrometer the spray is produced. A Taylor cone⁽¹²⁴⁾ is formed at the needle apex due to charge separation in the electric field where positively charged droplets are directed to the inlet of the mass spectrometer. The theory behind the electrostatic dispersion in the Taylor cone can be found elsewhere⁽¹²⁵⁾. On the way to the mass spectrometer the solvent evaporates leading to an increase in charge density on the surface of the droplets. When the electrostatic repulsion becomes greater than the surface tension each droplet will repeatedly disintegrate and eventually render the analyte molecules ionized by one or more protons in gas phase.

There are two theories for the transfer of ions into gas phase which is not well understood⁽¹¹⁸⁾. The charged residue model was developed by Dole et

al.⁽¹²⁶⁾, and the ion evaporation model (IEM), also called ion desorption model (IDM), was developed by Iribarne and Thomson^(127,128). It has also been suggested that both mechanisms apply, depending on the types of analytes and solvents.

In MALDI, which is a pulsed ionization technique, the polypeptide sample is combined with a matrix material (e.g., α -cyano-4-hydroxycinnamic acid which is commonly used for peptides) for co-crystallization and formation of a 'solid solution'. A laser beam is focused onto the surface of the matrix/analyte, which causes rapid excitation and localized disintegration. Clusters of analyte molecules surrounded by matrix and salt ions eject from the surface. The matrix molecules evaporate away from the clusters to leave the free analyte in gas-phase. In the desorbed matrix-analyte cloud just above the surface, the photo-excited matrix molecules are stabilized through proton transfer to the analyte. Cation attachment to the analyte is also encouraged during this process. It is in this way that the characteristic H^+ , Na^+ , K^+ , etc. analyte ions are formed. The gaseous and ionized polypeptides are then extracted into the mass spectrometer for analysis.⁽¹²⁹⁾

MALDI predominantly produces singly protonated ion species which limits its combinations with certain mass analyzers due to the mass range restrictions⁽¹³⁰⁾. However, the mass spectra (i.e., MS/MS spectra) are easier to interpret.

Mass analyzers

The mass analyzer is central to MS and in proteomics its key parameters are sensitivity, resolution, and mass accuracy^(18,19,117). The most common types are the ion trap, time-of-flight (TOF), quadrupole (Q), and Fourier-transform ion cyclotron resonance (FT-MS) analyzers, all of which have been used in this thesis. They differ in design and operate on a variety of physical principles, but all separate ions according to their m/z before detection. The resulting ion spectra provide information of each ion and its relative abundance.

The combination of ESI/MALDI with one or more of the mass analyzers offers a large variety of analytic tools from simple robust arrangements to specialized MS instruments. For example, in MS/MS mode the Q-TOF and TOF-TOF hybrids as well as the ion trap can be used to further manipulate and fragment ions by CID (Figure 3). When the ions collide with an inert gas (e.g., argon) their kinetic energy is to some extent converted to internal energy. If high enough, the excess energy break chemical bonds and fragment the ion. The information from the resulting fragment ion spectra can be interpreted to determine the primary structure and to identify the polypeptide.

Figure 3. Outline of a quadrupole time-of-flight mass spectrometer in MS/MS mode. Gaseous ions are formed in the ion source and introduced to the quadrupole mass filter where specific m/z ratios are selected for transmission to the collision cell. The ions are dissociated by CID and the produced fragments analyzed further in the TOF. The whole system is kept highly evacuated by effective pumps.

Proteome analysis

Separation and visualization

The separation approaches of proteomic samples are generally based on electrophoretic and chromatographic techniques⁽¹³¹⁾. These may be used in multiple dimensions, e.g., 2D-PAGE^(132,133), 2D-LC⁽¹³⁴⁾, or combinations of both techniques, e.g., IEF-LC⁽¹³⁵⁾. The object is to reduce the complexity of the sample, to increase discrete concentration of each protein, and to achieve an analytically favorable environment in a downstream compatible matrix.

2D Polyacrylamide gel electrophoresis (2D PAGE)

The combination of isoelectric focusing (IEF) and SDS PAGE resulted in the advent of 2D PAGE^(132,133) which has been the principal technique for resolving proteomic samples over the past three decades⁽²⁶⁾. Thousands of proteins in a single sample can be resolved to enable identification of individual proteins in a tissue or sub-cellular fraction⁽¹³⁶⁾. However, there are intrinsic limitations of the 2D PAGE technique such as co-migrating proteins^(137,138), low solubility of preferentially membrane and nuclear proteins⁽²⁶⁾ and under representation or absence of pI extreme, very large, and small proteins^(19,26,139) (Figure 2).

In addition, the protein visualization and following identification poses another issue of traditional 2D PAGE. Silver staining⁽¹⁴⁰⁾, which is more sensitive than colloidal Coomassie blue staining⁽¹⁴¹⁾ and commonly used for high sensitivity protein visualizations, is non-favorable for protein identifica-

tion methods based on mass spectrometry and has a limited dynamic range^(142,143). The more recent alternatives of fluorescent stains such as Sypro Ruby and Deep Purple⁽¹⁴⁴⁻¹⁴⁶⁾ offer a higher dynamic range, and are easier to use compared to silver staining^(142,147).

Despite numerous cumulative improvements on the electrophoretic reproducibility in 2D PAGE such as the introduction of immobilized pH gradients for the IEF dimension⁽¹⁴⁸⁾, matching of corresponding proteins prior to relative quantitation in e.g., control and diseased sample comparisons have been difficult⁽¹⁴⁹⁾.

2D Difference Gel Electrophoresis (DIGE)

DIGE which is a more recent development from 2D PAGE, was used in **Papers III** and **Paper VI**. This technique is based on the electrophoresis principals of its predecessor, with enhancements in terms of gel-to-gel reproducibility, sensitivity, and dynamic range⁽¹⁵⁰⁾. By differential labeling of the samples with different fluorescent cyanine dyes (Cy2, Cy3 and Cy5) prior to PAGE, the DIGE technique allows multiple samples e.g., one standard, one control, and one treated to be simultaneously separated under identical conditions in one gel. These fluorescent dyes bind covalently to part of the lysine (minimal labeling) or all cysteine (saturation labeling) residues of the sample proteins⁽¹⁴²⁾. In a fluorescent scanner, multiple images of the samples are captured and visualized using the specific excitation wavelengths of the fluorescent dyes. The images are then superimposed, and quantitative differences between them determined. The standard in each gel may then be used for inter gel relations to minimize confounding gel-to-gel variations in the analysis process^(151,152). The Cy dyes have a linear response to variation in protein amounts down to 0.5 fmol over a protein concentration range of four to five orders of magnitude^(151,153).

Peptidome analysis

Separation and detection

Peptides obviously have different physico-chemical properties from proteins. Their small size and high motility together with low ability to bind different stains make them impractical to focus and visualize in gels^(72,154). To address the inability of traditional proteomics methods to characterize the peptidome, an alternative method was developed (**Paper I**) which was used (**Papers II-III** and **Paper V**) to analyze the small protein and peptide content in brain tissue. The method and detection system for these analyses needed to fulfill several analysis criterions: It must be sensitive and specific to allow small sample amount analyses (i.e., milligram quantities from sub-regions of

mouse brain tissue). The method had to allow for semi-quantitative comparisons of patterns among samples by simultaneous detection of the polypeptides in one experiment. Finally, it should make further analyses and identifications of polypeptides that differ in abundance above normal biological variation possible.

Nanoflow LC-MS

RPC has become the prime technique for separating peptides. The generally low hydrophobicity of peptides enables them to dissolve in aqueous systems without the use of detergents^(70,154). However, the hydrophobicity is sufficient for the peptides to adsorb to the stationary media (e.g., C18). A gradient of an organic modifier (e.g., acetonitrile) elutes the adsorbed peptides from the RP media which makes RPC a straightforward application suitable for ESI and MS coupling^(155,156). On line LC-MS offers automation of desalting, concentrating, and separation of the peptides prior to MS analysis which improves reproducibility.

In LC, as in the ESI interface, miniaturization is essential. This is certainly the case in neuropeptide analyses where the sample amounts often are small and the sensitivity is an important issue. Capillary nanoflow LC provides higher relative peak concentrations which is important for low-abundance species⁽¹⁵⁷⁾. In that way more sensitive MS detection, better use of the MS dynamic range, and reduced discrimination during ionization is achieved^(158,159). Nanoflow further decrease limit of detection due to an increased ion transmission over the LC-MS interface^(117,155).

When injecting large sample volumes (~5 µl) on nanoflow LC columns, (i.e., 75 µm diameter) on-column analyte focusing (**Papers I-III**) or a column switching setup is necessary (**Papers V and Paper IV**). An advantage of the latter approach is the possibility of higher flow rates during application and desalting of the sample. In this way experiment time is reduced and the column is spared through a certain level of sample clean-up on a rather expendable pre-column. However, in the case of neuropeptides, analyses are often subsequent to partial separation (e.g., centrifugation, filtration) and thus relatively clean, but desalting is mandatory.

Not many solvent delivery systems can readily reach the necessary low flow gradient by direct pumping. For such applications, the use of flow splitting is a convenient solution⁽¹⁶⁰⁾. By using a T-connector and restrictor capillaries, accurate and reproducible nanoflows can be generated. In Figure 4, the splitted nanoflows in the range of 140-210 nl/min under isocratic and gradient conditions from the LC setup in **Paper I** and **Paper II** is shown. The setup compensates for most of the viscosity changes during gradient elution and keeps the post column flow linear independent of the mobile phase composition⁽¹⁵⁶⁾.

Figure 4. Microflow splitting by T-connector and restrictor capillaries. The left graph describes post column flowrate (gray triangles) and pump pressure (black squares) responses with increased pump flowrate at 30% acetonitrile. The right graph describes the post column flow rate (gray triangles) and pump pressure (black squares) responses during a 5-95% acetonitrile gradient (Conc B).

Imaging MALDI MS (MALDI IMS)

The technique of mass spectrometric tissue imaging^(161,162) was used in **Paper V**, where peptides and proteins in thin (12 μ m) tissue sections were analyzed in situ. The sections are typically coated with a raster of matrix (e.g., sinapinic acid for peptide analyses) before an ordered array of mass spectra is acquired from the matrix spots where each spectrum represents the local molecular composition at known x,y coordinates. Image profiles of selected peptides and proteins in the section are generated by extracting their corresponding m/z ranges. Not only does this approach require less sample manipulation, but it also informs of the spatial localization of the peptides and proteins in the analyzed tissue. However, brain tissue has high concentrations of salt which make washing in ethanol needed⁽¹⁶³⁾. There is a possibility of analyte dilution and dispersion in this washing step. Also, the spatial resolution is limited by the ability to apply tiny amounts of discrete matrix spots in a repeatable manner with sufficient accuracy⁽⁸⁷⁾. Applications of imaging MALDI MS (IMS) range from low resolution peptide and protein profile images of selected areas in mouse brain⁽¹⁶⁴⁾ to single neural cell peptide profiling analyses⁽¹⁶⁵⁾ and high resolution imaging of proteins and drug metabolites in whole rat sections⁽¹⁶⁶⁾.

Polypeptide identification

The potential of MS is recognized in its production of broad dynamic range data. However, in order to fully appreciate the huge amount of generated information, techniques for data management and evaluation have to be incorporated. The part of proteomics dealing with polypeptide identifications is as fundamental to a successful analysis as proper sample preparation⁽¹²¹⁾.

Proteins are commonly identified from MS data by peptide mass fingerprinting (PMF) and from MS/MS data by MS/MS ion search. In PMF an algorithm is used to compare a set of masses from proteolytic peptides (e.g., trypsin digests) with sets of masses that arise from theoretical digestion of each entry in a database of protein sequences, and to assign a ranking score of the quality of each match. In **Papers III, V, and Paper VI** proteins were identified by the MS/MS ion search algorithm supplied by Mascot⁽¹⁶⁷⁾ where structural information from each peptide fragment is accounted for in the database search and scoring.

The identification of endogenous peptides from MS/MS data is typically more cumbersome than for tryptic fragments of proteins. Their on average larger sizes complicate the spectrum with their generally higher charge state⁽¹²¹⁾. However, the algorithms for MS/MS ion search offered by Mascot and X!Tandem⁽¹⁶⁸⁾ is applicable when specifying the use of non-enzymatic digest. This feature was utilized in **Paper IV** where strategies for better identification of endogenous peptides were investigated by using small alternative and targeted sequence collections extracted from existing databases such as UniprotKB⁽¹⁶⁹⁾ and SwePep⁽³⁰⁾. A prerequisite for a protein to be identified by PMF or MS/MS ion search techniques is that its sequence exists in the interrogated sequence database.

An alternative method to identify endogenous peptides is de novo sequencing. This method, which was used in **Papers I-III**, is intensively laborious but very potent because the peptide sequences can be revealed without previous knowledge of the precursor. Supportive software e.g., PepSeq (Waters), Peaks (Bioinformatics Solutions), and DeNovoX (Thermo Electron) in combination with homology searches (e.g., BLAST) make the assignment less time consuming. This process can be improved further by the use of mass mapping to neuropeptide sequence collections e.g., SwePep database, prior to de novo sequencing (**Paper V**). In this way the number of putative sequences are reduced significantly and the sequencing serve more or less as a confirmation of the proposed mass match⁽³⁰⁾.

Results and discussion

Proteomics technologies provide a useful repertoire for disease related applications such as diagnostics and therapy. The current collection of tools and their use is likely to expand to meet the need for rapid and accurate analyses⁽¹⁷⁰⁾. Particularly, the area of protein and peptide differential expression may lead to the discovery of novel biomarkers for monitoring of disease, predicting drug response, disease diagnosis, and subtype classification⁽¹⁷¹⁾.

The involvement of endogenous peptides in medicine either as drugs or as drug targets cannot be overestimated because of their wide spectrum of functions. An evident example and maybe the first, is the use of the peptide insulin for therapeutic treatment of diabetes⁽¹⁷²⁾.

One of the advantages of using MS when measuring tissue levels of endogenous peptides compared to immunoassays, is the specificity of the MS. Possible post-mortem degradation changes during the sampling or dissection procedure may cause unspecific cleavage of precursors. These peptides could cause cross reactions in the assay depending on the extent of such post-mortem events but would appear as completely different ion species in the mass spectrum. Another advantage of MS is that it is not necessary to have a priori knowledge of the identities of the studied endogenous peptides as needed in immunoassays. This enables broad and simultaneous peptide detection during an analysis.

In **Paper I** a method for analysis of peptides and small proteins in milligram amounts of brain tissue was developed. A simple aqueous extraction protocol combined with centrifugal filter separation and nanoflow RPC-MS made detection of an approximate 1,500 peptides possible from a single brain sample. The method enabled semi-quantitative comparisons of peptide profiles from different brain regions. However, among the 19 identified peptides the majority proved to be fragments from hemoglobin and other abundant proteins. The post mortem proteolytic actions during sample handling were sufficient to produce large number of non specific degradation products at high quantities. It is clearly difficult to perform peptidomic analyses on brain tissue without detecting these fragments since similar results have been reported by several other groups.^(96,173-175) Under standard conditions for sampling and processing of the sample, non-specific degradation produce an abundant layer of protein fragments that effectively mask the presence en-

ogenous peptides, and possibly also degrade the endogenous peptides as well.

An improved sample preparation protocol was developed in **paper II** to deal with the enzymatic degradation. By instant proteolytic inactivation of the sample using MW, the subsequent mass spectrometry analysis from 1 mg of brain tissue revealed a dramatic decrease in total number of observed peptides. The identification results showed 39 peptides all believed to be derived from specific processing. It is, of course, impossible to infer biological activity of a peptide from its sequence alone. Further studies e.g., receptor affinities, gene knocking, antisense blocking, and functional studies by administration of each peptide to cellular systems or live animals have to be conducted. There are, however, a few signatures present in many active peptide sequences to look for. First, more than one active peptide is often processed from one precursor protein ⁽⁹³⁾. Also, the processing of active peptides is frequently at specific sites carried out by dedicated peptidases ⁽⁸⁸⁾. Finally, active peptides are commonly modified by C-terminal amidation ^(86,176). Indeed, among the identified peptides, 20 were previously known classical endogenous peptides and 19 previously uncharacterized peptides from precursors known to contain neuropeptides, and others not previously reported peptides with the characteristic processing sites (Figure 5).

Figure 5. Selected part of the elution profile of a nanoflowLC-MS experiment of the peptide subproteome of rat hypothalamus. In the ranges of m/z 548.7-577.8 and elution time 19.2-29.6, five previously characterized neuropeptides and one not previously described peptide was identified. The uncharacterized peptide was found to be processed at dibasic amino acid sites from the known neuropeptide containing secretogranin 1 precursor.

The almost immediate termination of enzymatic activity caused by MW supplied the means for control samples in a time course study of the post mortem degradation of proteins and peptides in **Paper III**. It was found that the number of detected peptides increased rapidly with increased post mortem delay, whereas the levels of monitored neuropeptides decreased. In some cases the neuropeptides were relatively stable over the time-course, indicating hormone-like functions distant from the sites of release of these peptides⁽⁹²⁾. Modifications i.e., phosphorylations on mitogen activated protein kinase, were reduced already one minute post mortem on both peptides and proteins in concordance with other studies⁽¹⁰⁴⁾. The heat transfer inactivation method produced similar results on protein phosphorylations and the number of detected endogenous peptides as MW, indicating as rapid inactivation. Furthermore, using the gel based DIGE proteomics approach, it was found that 53 out of approximately 1,500 protein spots were significantly changed more than 50% between control and 10 minutes post mortem. Corresponding fragments from some of these proteins, e.g., dihydropyrimidinase-like protein 2, tubulin α , and actin were identified among the non-specifically generated proteolytic peptides. Interestingly, the level of a short N-terminal fragment of the protein stathmin was found to correlate with the general level of post mortem degradation. Stathmin is ubiquitously expressed and well conserved among many species making it a potential marker for sample quality.

Figure 6. Relative intensity of fragment 2-20 from stathmin. During the 10 minute post mortem time course the N-terminal fragment of the protein stathmin steadily increased.

Assigning identities to the detected endogenous peptides is presently a bottleneck in peptidomics^(30,50). Most tools and identification utilities are designed for identifying proteins from tryptic digests of proteomic samples. As the average size of a tryptical peptide is in the optimal range of most MS instruments a few fragments from most proteins can be detected. The goal is to identify a specified number of fragments from one specific protein to deduce its identity; it doesn't matter if several of its fragments remain undetected. In the case of endogenous peptides in peptidomics studies however, the objective is to characterize the primary structure and identify every present peptide in the sample since there is no redundancy to rely on. To increase the challenge, these peptides are typically larger than their average tryptic counterparts, leading to more complex MS/MS spectra. In **Paper IV** alternative strategies to automatically identify more endogenous peptides was investigated. It was shown that using existing tools and algorithms for protein identifications i.e., Mascot and X!Tandem, improvements could be made by experimenting with the interrogated databases. The identification results from three targeted sequence collections obtained from the SwePep database and from a prediction database were compared with results from standard searches against the entire mouse proteome. Combining the targeted sequence collections generated three times as many significant peptide identifications as the complete mouse database, indicating that the sequence collections used need to reflect the samples in a better way.

In an attempt to demonstrate the utility of proteomics, traditional gel based DIGE and the developed peptidomic methods were used to identify neuroadaptations in an animal model of PD. After dopaminergic neural depletion in substantia nigra caused by the neurotoxin MPTP the protein and peptide adaptations in the striatum were investigated.

In **Paper V** the peptidomic analysis revealed a significant decrease of levels of the N-terminally acetylated small protein PEP-19. Further analyses by MALDI IMS on coronal brain section confirmed the reduction and also showed the spatial distribution of PEP-19 to be predominant in the striatum. A non-mass spectrometric method (in situ hybridization) was used to investigate the mRNA levels of PEP-19, which also were in agreement with the decrease in the lesioned animals compared to control. This 7.6 kDa neuronally expressed polypeptide belongs to a family of proteins involved in calcium transduction through interactions with calmodulin. The IQ motif, which constitute more than half of PEP-19, has the ability to interact with and modify the affinity of calmodulin to calcium, thus, influencing the calmodulin-transduced calcium response^(177,178). Calcium ions play fundamental roles in cell signaling, 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 ⁽¹⁷⁹⁾.

In **Paper VI** the protein expressions in the striatum were investigated using gel based DIGE technology. The progression of neuroadaptations after dopaminergic depletion was characterized at different time-points up to 22 days following MPTP administration and compared to control. Out of approximately 800 matched spots, 31 significantly changed proteins were identified, belonging to groups of cytoskeleton, mitochondrion, oxidative stress, and vesicle associated proteins. These proteins represented early striatal adaptations caused by the loss of dopaminergic neurotransmission, which is a consequence of degeneration of dopaminergic nerve terminals in the striatum and provides further evidence that major alterations in the cytoskeleton and mitochondrial functions occur in PD.

Concluding remarks and future perspectives

Previously, the issues of MS research have mainly concerned the hardware employed and its development, whereas now more emphasis is put on sample preparation, data evaluation and interpretation⁽¹⁸⁰⁾. Clearly the quality of a sample is important. A standardized sample collection as well as the definition of proper storage conditions is necessary in order to obtain reliable and reproducible results with both peptidomics and proteomics. Processing of the generated raw data from the MS analysis and interpretation of the results to yield accurate identifications are equally important and an integral part of proteomics workflows.

In this thesis it has been shown that degrading proteins in inappropriately handled samples produce fragments primarily in the low mass region which make MS profiling of endogenous peptides unreliable or even impossible. Gel based protein expression mapping is also influenced by this fragmentation but to a lesser extent. Instant inactivation of the enzymatic activity enables a straightforward protocol for detecting, identifying, and monitoring several endogenous peptides, both previously characterized and novel. The identification process, which is commonly carried out by interrogating protein databases was improved. Targeted sequence collections extracted from existing databases yielded additional endogenous peptides to the identification list.

The peptidomics method, which is based on nanoflow LC-MS, allows for relative quantitation of endogenous peptides and small proteins between different sample groups. Together with MALDI IMS the levels of PEP-19 and its spatial distribution in parkinsonian tissue was determined and compared to control. Both methodologies showed significant reductions in the diseased tissue and predominantly in the striatum.

Considering the intense use and the amounts of gathered information from e.g., immunoassays, the significance of simultaneous monitoring of multiple endogenous peptides and small proteins could become enormous. Together with the proposed indicator of post mortem fragmentation, the sample quality could be ascertained to exclude distorting material from data analyses or better yet, omit the sample before analysis. In this way the results would become more reliable with improved statistics and it would save many hours of labor. It is anticipated that this methodology may well be used in the expanding field of biomarker discovery. The need for biomarkers which can be used for early detection of disease, for the identification of new

targets for therapeutics, or for more effective drug development through better monitoring of therapeutic effect and toxicity is obvious as the population ages and neurological diagnoses increase.

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