Development of Methods for Protein and Peptide Analysis Applied in Neuroscience Utilizing Mass Spectrometry

BY

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Abstract
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This thesis describes the utilization of the matrix-assisted laser desorption ionization mass spectrometry (MALDI MS) and electrospray ionization (ESI) MS techniques for analysis of complex brain tissue samples.

Direct molecular profiling of biological samples using MALDI MS is a powerful tool for identifying phenotypic markers. MALDI MS-profiling of proteins and peptides directly on brain tissue sections was used for the first time to study experimental models of Parkinson’s disease (PD). The mass spectrometer was used to map the peptide and protein expression directly on 12 μm tissue sections in mass-to-charge (m/z) values, providing the capability of mapping specific molecules of the original sample, that is, localization, intensity and m/z ratio. Several protein and peptide expression profile differences were found in the dopamine denervated brains when compared to the corresponding controls, for example, calmodulin, cytochrome c, cytochrome c oxidase, and the neuroimmunophilin protein FKBP-12. The increased expression of FKBP-12 from the profiling experiments was supported by miRNA expression analysis and two-dimensional gel electrophoresis separation analysis. Multiple genetic deficits have linked impaired ubiquitin-conjugation pathways to various forms of familiar PD. This study showed for the first time an increased level of unconjugated ubiquitin specifically in the dorsal striatum of the dopamine depleted PD brain. The strength of the MALDI MS-profiling technique is that a minimum of sample handling and manipulation is necessary pre-analysis. This ensures preservation of the spatial localization of the biomolecules in the tissue section.

Biological liquid samples often contain high amounts of salt that is non-compatible with the ESI MS technique. A nano-flow capillary liquid chromatography (nanoLC) system coupled on-line with ESI-MS was used to study the metabolism of the peptide LVV-hemorphin-7 in the brain and blood using in vivo microdialysis. The microdialysis technique provides capabilities for very precise sampling in specific brain regions. The combination of on-line desalting and pre-concentration by nanoLC with ESI MS is a powerful tool to detect minute concentration of metabolic fragments and endogenous biomolecules.

The utilization of mass spectrometry in neuroscience applications provides a uniquely advantageous tool for the analysis of complex biochemical events that underlie the pathological symptoms expressed in different disease states. Furthermore, the MALDI-MS profiling technique shows great potential for the future with regards to proteome analysis and drug discovery.

Keywords: Mass spectrometry, Parkinson’s disease, Metabolism, Profiling Mass Spectrometry, Proteomics

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To my mother and father, for your endless support and always believing in me.
List of Papers

The following papers are included in this thesis.

I In vivo processing of LVV-hemorphin-7 in rat brain and blood utilizing microdialysis combined with electrospray mass spectrometry
Katarina Sanderson Nydahl, Johan Pierson, Fred Nyberg, Richard M. Caprioli and Per E. Andrén

II Molecular Profiling of Experimental Parkinson’s Disease: Direct Analysis of Peptides and Proteins on Brain Tissue Sections by MALDI Mass Spectrometry

III Increased levels of ubiquitin in the 6-OHDA-lesioned striatum of rats
Johan Pierson, Per Svenningsson, Richard M. Caprioli, Per E. Andrén
J. Proteome Res., accepted for publication

IV Increased striatal mRNA transcription and active protein expression of the immunophilin FKBP-12 in experimental Parkinson’s disease
Johan Pierson, Karl Sköld, Marcus Svensson, Richard M. Caprioli, Per Svenningsson, Per E Andrén.
Manuscript
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<tbody>
<tr>
<td>2-DE</td>
<td>two-dimensional gel electrophoresis</td>
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<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
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<td>ADP</td>
<td>Adenosine diphosphate</td>
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<td>ATP</td>
<td>Adenosine triphosphate</td>
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<td>CE</td>
<td>Capillary electrophoresis</td>
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<td>CI</td>
<td>Chemical ionization</td>
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<td>CNS</td>
<td>Central nervous system</td>
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<td>COX</td>
<td>Cytochrome c oxidase</td>
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<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<tr>
<td>Da</td>
<td>Dalton</td>
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<td>DHB</td>
<td>Dihydroxybenzoic acid</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>EI</td>
<td>Electron impact</td>
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<td>ESI</td>
<td>Electrospray ionization</td>
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<td>FAB</td>
<td>Fast atom bombardment</td>
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<td>GC</td>
<td>Gas chromatography</td>
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<td>GSH</td>
<td>Glutathione, reduced</td>
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<td>GSSG</td>
<td>Glutathione, oxidized</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<td>HUGO</td>
<td>Human genome project</td>
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<tr>
<td>HUPO</td>
<td>Human proteome project</td>
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<tr>
<td>LC</td>
<td>Liquid chromatography</td>
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<tr>
<td>L-DOPA</td>
<td>Levodopa</td>
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<tr>
<td>m/z</td>
<td>Mass-over-charge</td>
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<td>MALDI</td>
<td>Matrix-assisted laser desorption ionization</td>
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<td>MS</td>
<td>Mass spectrometry</td>
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<tr>
<td>MCP</td>
<td>Micro-channel plate</td>
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<tr>
<td>mw</td>
<td>Molecular weight</td>
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<tr>
<td>OCT</td>
<td>Optimal cutting temperature compound</td>
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<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
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<tr>
<td>PMF</td>
<td>Peptide mass fingerprinting</td>
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<tr>
<td>PTM</td>
<td>Post-translational modification</td>
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<td>Q</td>
<td>Quadrupole</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RP</td>
<td>Reversed phase</td>
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<tr>
<td>Sinapinic acid</td>
<td>3,5-dimetoxy-4-hydroxycinnaminic acid</td>
</tr>
<tr>
<td>SNC</td>
<td>Substantia nigra pars compacta</td>
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<tr>
<td>SPE</td>
<td>Solid phase extraction</td>
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<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TOF</td>
<td>Time-of-flight</td>
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<tr>
<td>UV</td>
<td>Ultra violet</td>
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<tr>
<td>α-cyano</td>
<td>α-cyano-hydroxycinnaminic acid</td>
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Introduction

Mass spectrometry
A mass spectrometer is an instrument that gives a very precise weight measurement of molecules in gas phase.

It all starts back in 1897 with J.J. Thomson’s pioneering work in “Theoretical and experimental investigations on the conduction of electricity by gases” which led to the discovery of the electron. He was awarded the Nobel Prize in 1906 for this research, and in 1911 he constructed the first mass spectrometer, which he called a “parabolic spectrograph”. Ions were separated by their different parabolic trajectories after passing through an electromagnetic field and hitting a fluorescent screen or a photographic plate.

The mass spectrometer have since then undergone a huge transformation into the sophisticated instruments that are in use in modern laboratories today. The basis for a MS is still the same; molecules are transferred into gas phase and the mass determined by a mass analyzer.

As a generalization, a mass spectrometer consists of three different parts; ion source, mass analyzer and detector (Figure 1).

The two main ionization techniques are electro spray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI). However, there are also several other ionization techniques used, such as fast atom bombardment (FAB), electron impact (EI) and chemical ionization (CI). In this thesis ESI and MALDI have been utilized.
The most commonly used mass analyzers are so called “time-of-flight” (TOF) or quadrupole (Q) mass analyzers. Mass spectrometry (MS) today is a common technique in different research areas, such as medicine, physics, chemistry and biology. The instrument that up till recently was a “machine” to be handled by experts is now rapidly turning into another instrument in the lab that everyone can learn to use after proper training.

Even though the mass spectrometer is a tremendously powerful instrument that can resolve molecules from each other according to mass, very often some sort of separation is needed in order to avoid unwanted effects as ion suppression and/or overlap in mass due to almost similar nominal mass.

Several different types of on-line pre-separation can be used in combination with MS such as gas chromatography (GC), capillary electrophoresis (CE) and high performance liquid chromatography (HPLC). The different separation techniques offer different prerequisites on the sample, and the choice depends on the application and the physical and chemical properties of the sample. GC coupled to MS has for long been the predominant technique in pharmaceutical research and is optimal for thermostable and small hydrophobic molecules. Protein and peptide research have gained a massive interest as several organisms have had their complete genome sequenced. HPLC coupled to ESI is well suited for analysis of thermolabile, medium sized to large molecules, such as proteins and peptides. This is reflected in the number of publications where LC-MS has been used show a drastic increase the last 10 years (Figure 2).

![Figure 2](Image)  
**Figure 2.** The number of publications over 40 years reflects the increase in popularity of the combination LC-MS. The search was performed at www.sciencedirect.com.
Proteins and Peptides

Proteins and peptides play a major role in our everyday life. They regulate numerous physiological functions and processes such as blood pressure, sexual arousal and differentiation in fetus among other.

In neurological diseases, several proteins and peptides have been found to be altered in expression or functionality. In Parkinson’s disease (PD), the proteins Parkin and α-synuclein among others have been found to be affected. The current methodology includes immunological and radiological methods that targets one protein / analysis. Mass spectrometry is therefore an effective tool to study changes in expression of a number of proteins and peptides in a single analysis.

Proteins make up about 15% of our body mass and are the driving force beyond all the biochemical processes that makes our body function. They can function as antibodies, e.g., they can recognize and defend against different types of infections. They can also be catalytically (enzymes), regulatory (hormones) or structural (skin, hair and nails). All proteins are made up of building blocks, e.g. amino acids. The amino acids are then combined in a sequence. As there are 20 different amino acids, a string of 10 amino acids can have $10^{20}$ different sequence variations. To complicate the matter further, any protein can be post-translational modified (PTM) e.g., the protein is chemically modified. The amino acid-sequence is the same but another chemical moiety is added that change the function of the protein. The implication of this is that the expression “one gene, one protein” is not altogether correct, rather “one gene, one amino acids-sequence” is more correct as one sequence can have many different functions depending on type and amount of PTM.

Peptides are rather similar to proteins. A general definition of a peptide is that the molecular weight is below 10000 Da. Classical peptide hormones and neuromodulators are traditionally considered to derive from specific processing of larger protein precursors by the action of particular enzymes. However, in the past two decades, some attention has been drawn to potentially bioactive peptides formed by catabolic degradation of other functional proteins. For example, peptides with opioid activity have been isolated from enzyme digests of milk protein (β-casomorphins), mitochondrial cytochrome b (cytochrophins) and the blood protein hemoglobin (hemorphins).

Peptides are most often regulatory. They can bind to a receptor and thereby change for example the level, affinity or stability of a protein or create a cascade of events through interaction with a receptor.

Proteins and peptides can be processed enzymatically to achieve different functionality than that of the original protein / peptide. In order to study such events in vivo, microdialysis is an excellent tool. The microdialysis probe is a hollow metal tube surrounded by a semipermeable membrane that allows...
liquid to be pumped through and equilibrated with the surrounding fluid. As the membrane can be selected with different cut-off ranges, it is possible to exclude large molecules such as proteins, and only equilibrate against the surrounding small molecules and peptides.

Microdialysates from body fluids contains low concentrations of endogenous molecules. To be able to analyze them with MS, some form of pre-concentration is needed. By injecting the microdialysate onto a reversed phase (RP) C18 column under conditions with low organic content, most molecules adsorb to the RP media. This is an effective way of concentrating and desalting samples to avoid adduct formation and accumulation of salts at the ESI spraying needle and orifice of the mass spectrometer. The organic content in the mobile phase is then rapidly increased. This will have the effect that virtually all molecules equilibrate over to the mobile phase and elute.

“Mass spectrometry is a tricky business”

A. Ingvast
Methods

Electrospray ionization mass spectrometry

The basis behind ESI is the formation of charged molecules in a solution and then transferring them into gas phase under atmospheric pressure. The first reports on ion formation driven by an electric field in atmospheric pressure came in 1968 [9]. Later on it was later developed into the technique as we know it today [54]. ESI is considered a "soft" ionization technique as it does not induce fragmentation in the ionization process. It is widely used in industry and academia for both routine analysis and research projects. The ability to form multiply charged ions [39] has been, and still is of great value and has made ESI the most commonly used ionization technique in atmospheric pressure today.

The basic process in ESI is that electrolytes in solution are transferred into gas phase by means of an electric field and an electric potential is applied to a tip of a capillary or through a liquid junction, containing a solution of electrolytes (Figure 3).
Figure 3. Schematic view of the electrospray process. Positively charged species accumulate at the tip of the spraying needle and oxidation of negatively charged species occurs. A spray is generated as the drag from the electric field overcomes the surface tension of the solute. The droplets undergo further reduction in size and finally molecules are transported into the mass spectrometer. Picture kindly provided by Andreas Dahlin.

When a positive potential is applied on the tip, positively charged molecules will accumulate at the meniscus at the tip of the capillary as oxidation of negative electrolytes will occur. The concentration of positive electrolytes increases at the meniscus of the solvent and starts to destabilize the meniscus, and a spray is generated. The actual process is still under discussion and two different theories exist on the mechanism [9,23].

Numerous physiological states, biochemical processes and organisms have been investigated with ESI-MS e.g., body fluids [41], biomarkers from bacteria [38], insect brains [29] and defects in glycosylation events in heart failure [26].

Several reports on the combination of microdialysis and ESI have been published, including studies on release of neurotensin in discrete rat brain regions [2,11], basal levels of acetycholine in rat brains [22] and determination of free-form amphetamine in rat brain [13].
In vivo microdialysis

The microdialysis technique was introduced in 1974 [52], and has since then been commonly used in CNS research and to monitor patients with various disease states [42], as well as drug penetration through the skin [37].

Basically, a dialysis membrane surrounds a hollow steel tube and a fluid is slowly pumped through the steel tube. When the microdialysis probe is placed in extracellular fluid in vivo, the artificial extracellular fluid inside the probe is slowly equilibrated with the surrounding media due to the concentration gradient (Figure 4).

Figure 4. Molecules within the membrane cut-off range can diffuse in and out over the dialysis membrane (dotted lines) and equilibrate with the surrounding fluid.

Peptides and other small molecules diffuse down their concentration gradient over the semi-permeable membrane. The perfusion fluid is then collected and used to determine the extra cellular content of molecules within the cut-off range of the semipermeable membrane in the structure where the probe is placed. The microdialysis probe can also be used to administrate a substance into a tissue or fluid.

The mw cut-off on the semi-permeable membrane is typically low (20 kDa) and only molecules of a certain size limit will pass over the membrane. Large proteins and other molecules will not pass the membrane and no further enzymatic degradation of peptides and protein will therefore occur after the analyte has passed over the membrane to the fluid inside the probe.

Microdialysis samples contain high amount of salt, normally 0.9% (w/w). The high amount of salt means that the conditions for ESI are far from optimal. High salt content will interfere with the ionization process as salts, in general, are non volatile. The salt molecules will accumulate in the rapidly shrinking droplets generated by the ESI process and adhere to the charged analytes as they enter gas phase. The salt/analyte-complex will be detected as an adduct, e.g. a molecule species with another molecule attached to it. Also, accumulation of crystallized salt on the interface of the mass spectrometer will cause a significant reduction in sensitivity due to lowered ion transmission.
Several different methods, such as dialysis, solid phase extraction (SPE) and liquid-liquid extraction can be utilized in order to desalt a sample [14,15]. A very elegant and efficient way of desalting and concentrating a sample is by using a C$_{18}$ column with a small volume coupled on-line to a mass spectrometer. Samples like microdialysates, which can contain minute amounts of peptides, can be trapped, concentrated and desalted with a minimum of handling. The high salt content sample can be applied directly onto the column through an injector and desalted and concentrated. The sample is then eluted directly into the mass spectrometer.

Matrix-assisted laser desorption ionization mass spectrometry

The introduction of MALDI-MS for use of analysis of large biomolecules was a big breakthrough for the field of protein analysis.

The first paper regarding analysis of large biopolymer utilizing soft laser desorption, later known as matrix assisted laser desorption, was done by Tanaka et al in 1988 [28]. Later the same year Hillenkamp and Karas published an article in the same field [20]. They also developed the technique further to make analysis of high molecular weight biopolymers a standard procedure.

**Figure 5.** Schematic view of the MALDI process. The analyte molecules, (large spheres, M) are imbedded in the matrix (light grey). As a laser pulse irradiates the co-crystal energy is absorbed in the crystal lattice and ions and matrix molecules are ejected. In the expanding gas plume ions and matrix molecules are ionized. Picture kindly provided by Andreas Dahlin.
The most common laser in commercial MALDI instrument is generally a nitrogen laser emitting at 337 nm. Matrix and analyte molecules are rapidly volatilized by the laser pulse under high vacuum and allowed to travel in a field free flight tube until impacting the detector (Figure 5).

A general MALDI-TOF-MS experiment consists of an analyte that is co-crystallized with an organic molecule that act as an UV-absorption matrix. This is generally (optimally) done in a 5000:1 ratio between matrix molecules and analyte, but should be optimized depending on analyte [21]. For analysis of protein and peptide the three most commonly used MALDI matrices are α-cyano-hydroxycinnamic acid (α-cyano), 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), and 3,5-dihydroxybenzoic acid (DHB) (Figure 6).

![Figure 6. The three most commonly used MALDI matrices. a) α-cyano-4-hydroxycinnamic acid (α-cyano), b) 2,5-dihydroxy benzoic acid (DHB), c) 3,5-dimetoxy-4-hydroxycinnamic acid (sinapinic acid).](image)

The matrix molecules are dissolved in an organic solvent, typically acetonitrile, ethanol or isopropanol, and the pH is adjusted, commonly with trifluoroacetic acid (TFA) in order to achieve an efficient protonation of the analyte. The mixture of analyte/matrix is then deposited onto a MALDI target plate and dried. The solution evaporates and the analyte co-crystallizes with the matrix molecules to give a non-volatile crystal. Desorption of the analyte takes place as the laser ablates the co-crystal and proton transfer occurs in the gas-phase. The actual process involved is not completely understood, for a review on the subject, see [25,27].
MALDI profiling mass spectrometry

The MS profiling and imaging technique was first described in 1997 [6]. The technique utilizes MALDI MS to determine the spatial distribution of peptides and proteins in biological samples in situ. Applications range from low-resolution images of peptides, proteins, drugs and their metabolites 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 1000-50,000 [6]. Individual m/z values can then be assembled from the mass spectra to produce selected m/z images.

When performing MALDI-MS experiments directly on tissue sections, several things have to be taken into consideration. The choice of matrix and solvent influence the spectra [8]. Biological tissue, e.g., rat brain or liver have a high content of salt and lipids. In the case of brain tissue, the condition for crystallization is far from optimal. Also, if the purpose is to analyze the whole cell content, care should be taken to ensure that the cells are not intact. Otherwise only secreted proteins might be analyzed.

In general, the tissue is snap frozen in liquid nitrogen as soon as possible when dissected out of the animal in order to minimize protein degradation. The tissue is then mounted in a cryostat with optimum cutting temperature (OCT) compound fluid (Figure 7). It is of importance to keep the tissue section clean of any OCT as this will interfere with the analysis and produce interference peaks. Different types of tissue require different cutting temperatures in the cryostat to achieve sections that do not shred and are easily handled in frozen form.

![Figure 7. Photograph of a frozen mouse brain mounted with OCT. The two following pictures (right) represents section mounted on a target plate and a section mounted on a target plate and coated with matrix for an imaging experiment, respectively.](image)

The most commonly used tissue thickness is 12 µm. This section thickness insures that as many cells as possible are cut open, and that the intracellular content is freely available for matrix crystallization. The sections are then transferred to a MALDI target plate kept at the same temperature as the tis-
sue section and thawed by placing the thumb under the plate, or by transferring the plate to room temperature. When the section has thawed, the plate is transferred onto dry ice. The sample plate is then placed in a desiccator to remove the remaining moisture from the tissue section.

There are different ways to apply the matrix onto the tissue depending on the type of analysis that will be performed. If a profiling experiment is to be performed, small droplets of matrix solution are placed onto areas of interest and allowed to dry either in room temperature or in cold room. If matrix application is performed with a pipette the smallest droplets that can be pipetted are in the 0.1 µl range. A rapid spotter can also be deployed to deposit drops of matrix solution. This method provides capabilities for smaller droplets in the µl range, and multiple droplets are deposited on top of each other to ensure that there will be a build up of crystals of appropriate size. The rapid spotter is controlled by a computer that can be programmed to deposit arrays of matrix spots over a given area. This provides numerous points for analysis and larger data set can be obtained compared with matrix application done with a pipette (Figure 8).

Figure 8. Photographs that display the difference in spot size using the rapid spotter technique or the manual pipette technique. The rat brain has been spotted with matrix manually (0.1 µl) and the smaller mouse brain has been spotted automatically with the rapid spotter.
As different tissue, such as liver and brain, vary in the composition with regard to lipid composition, care has to be taken when choosing appropriate matrix solvent. Experiments have shown that 50/50 (v/v) isopropanol/water 0.1% TFA and 20 mM octylglucoside works very well on brain tissue and gives small spots with a narrow radius. When applying the same matrix on liver, the droplets have a tendency to spread across the tissue and not being confined to a narrow radius, and produces spectra of poor quality. Tissue sections from different organs vary in composition and different solvents and matrices might fit different types of tissue (Figure 9).

![Figure 9](image.png)

Figure 9. Different matrices give different spectra even though the crystallization parameters were kept the same (cold room and with 1%TFA and 50/50 acetonitrile/water). From top to bottom, 3,5-dimethoxy-4-hydroxycinnamic acid, dihydroxybenzoic acid and D-cyano-hydroxycinnamic acid. All spectra were acquired on liver tissue sections.

Also, even if the crystals look fine to the eye and are contained within a small radius, different solvents produce spectra of various qualities. As a guideline, ethanol, acetonitrile and isopropanol together with sinapinic acid give spectra of good quality, and produces peaks over a broad mass range [43].

"The only source of knowledge is experience"
Albert Einstein
Experimental model of Parkinson’s disease

6-hydroxydopamine (6-OHDA) acts as a neurotoxin and induces degeneration of nigro-striatal dopamine neurons. 6-OHDA is administrated through intracerebral injections into the substantia nigra pars compacta (SNc) [51] as it can not pass the blood-brain barrier. To induce Parkinson’s disease (PD) in animal models, 6-OHDA and MPTP is widely used both in rats, mice and primates.

The 6-OHDA induced lesions might either be unilaterally or bilaterally. Rats administered with 6-OHDA unilaterally displays a tendency towards rotational behavior to the lesioned side, which indicates a depletion of dopamine in the dopamine denervated side of the two hemispheres. Specific catecholamine transporters transport 6-OHDA into the catecholamine neurons where it induces degeneration of both dopamine and noradrenaline neurons [40].

There are two major pathways in which 6-OHDA acts, both by generating reactive oxygen species (ROS) and by interfering with the respiratory chain in mitochondria. The combination of monoamine oxidase activity, auto-oxidation and elevation of free iron is responsible for the high ROS production after 6-OHDA treatment [16]. It has been shown that intrastratial injections of 6-OHDA produce apoptosis in developing animals [30]. 6-OHDA inhibits complex I and IV of the electron transport chain in the mitochondria which will have a severe effect on the oxidative phosphorylation of adenosine diphosphate (ADP) to adenosine triphosphate (ATP). The increase in ATP/ADP ratio will affect the possibility to maintain the calcium homeostasis in the cell and lead to an increase in $[\text{Ca}^{2+}]$ [7].

It has previously been shown that the ratio of reduced glutathione and oxidized glutathione (GSH/GSSG) is decreased due to oxidative stress, which further promotes radical formation [44]. Increased levels of free radicals and depletion of GSH have been shown to induce apoptosis in neurons [32].

For a review on the subject, see [10,17,47].
Aim of This Thesis

Two different mass spectrometry techniques were utilized to study peptides and proteins in the brain. In vivo microdialysis and ESI MS in combination with nanoLC was used to characterize peptide metabolism. Peptides are responsible for a large number of processes in the central nervous system acting as neuromodulators and signaling substances.

The MALDI-MS profiling experiments were aimed at understanding the neurochemical basis for initiation and progression underlying the pathophysiology in experimental Parkinson’s disease.

Specific aims of the thesis were:

- To develop a nanoLC ESI MS method for studying the in vivo metabolism of the opioid peptide LVV-hemorphin-7 in the brain and the blood using microdialysis.

- To develop the MALDI MS profiling technique directly on brain tissue section without any prior sample handling in an animal model of Parkinson’s disease.

- To locate spatially different expression patterns of proteins and peptides in 6-OHDA-induced parkinsonism.

- To identify and relatively quantify differences in protein and peptide expression in 6-OHDA-induced parkinsonism.

- To correlate expression alterations of the neuroimmunophilin protein FKBP-12 obtained with MALDI-MS profiling in experimental Parkinson’s disease with other well established techniques, such as 2D-GE and in situ hybridization.
Results and discussion

The papers included in this thesis will be discussed and summarized. For a more detailed description of the experimental procedures the reader is referred to the original articles.

*In vivo* processing of LVV-hemorphin-7 in rat brain and blood utilizing microdialysis combined with electrospray mass spectrometry (paper I)

Several reports on the metabolic activation of precursor proteins into biological active peptides have been published. The conversion of proteins or neuropeptides into bioactive fragments is important in the neuromodulation mechanism [19]. Processing of neuropeptides and proteins might produce biological active fragments with different specificity other than the precursor and it is important to study these events (Figure 10).

Exogenous (d8)LVV-hemorphin-7 were dissolved in artificial CSF and infused by microdialysis probes implanted in the rat striatum. To be able to study the metabolism of the LVV-hemorphin-7 precursor into smaller fragments we employed nano-LC ESI-MS.

![Mass spectrum of (d8)LVV-hemorphin-7 fragments produced *in vivo* in rat striatum after 30 min.](image)

**Figure 10.** Mass spectrum of (d8)LVV-hemorphin-7 fragments produced *in vivo* in rat striatum after 30 min.
We also infused the aminopeptidase inhibitor amastatin to be able to distinguish unspecific endopeptidase N- and C-terminal degradation from potentially specific enzymatic cleavage (Figure 11).

**Figure 11.** Relative levels of (d8)LVV-hemorphin-7 fragments produced *in vivo* after infusion of (d8)LVV-hemorphin-7 (1.0 pmol/mL) (grey bars) or (d8)LVV-hemorphin-7 with co-infusion of the aminopeptidase inhibitor amastatin (1.0 pmol/mL) (black bars) 30 min (A) and 60 min (B) after the infusion. (A) 30 min after the infusion of (d8)LVV-hemorphin-7, the relative formation of C-terminal fragments 2–10, 3–10, and 4–10 was decreased when amastatin was co-infused with (d8)LVV-hemorphin-7, but the fragments 5–10, 1–9, 1–8 and 1–6 were increased. (B) 60 min after the infusion of (d8)LVV-hemorphin-7, only the fragments with increased relative concentration after the amastatin co-infusion could be detected.

The extracellular metabolism of (d8)LVV-hemorphin-7 in striatum produced several fragments, 2–10, 3–10, 4–10, 5–10, 6–10, 7–10 and the C-terminal fragments 1–9, 1–8 and 1–6.
Infusion of the aminopeptidase inhibitor decreased the metabolism of the N-terminal fragments to 2-10, 3-10 and 4-10 in the 30 min samples. Meanwhile the relative concentration of fragment 5-10 together with the C-terminal fragment 1-9, 1-8 and 1-6 in the 60 min samples increased (Figure 11). Amastatin inhibits aminopeptidase A (cleaves N-terminal amino acids from peptides) [50] and aminopeptidase B (cleaves N-terminal amino acids Arg and Lys from) [5], leucine aminopeptidase and microsomal aminopeptidase [53].

![Figure 12.](image) Fragments produced over time as percent of LVV-hemorphin-7 infused into the jugular vein for 30 min (without amastatin).

However, the specific enzymes responsible for the generation of LVV-hemorphin-7 and other shorter hemorphin fragments in vivo remain to be determined. Further, the pattern of fragments found for the in vivo degradation of LVV-hemorphin-7 in the striatum and blood is probably due to the action of more than one enzyme, since both N- and C-terminal fragments with overlapping sequences were found. This can indicate a possible, so far unknown exopeptidase interaction.

**MALDI profiling on tissue section of experimental Parkinson’s disease (paper II)**

This paper is the first publication where MALDI-MS profiling is used to analyze proteins and peptides directly on brain tissue sections using an experimental of Parkinson’s disease (PD). Several proteins were either up or down regulated when the 6-OHDA treated hemisphere were compared with the corresponding untreated control side. All of these findings could be relatively quantified by normalization to two proteins that showed constant intensity ratio with each other, independent of lesioned or unlesioned hemisphere.
The protein profiles obtained were reproducible. Several different areas were compared in an untreated rat in order to verify the reproducibility of the protein profiles in-between hemispheres (Figure 13).

![Figure 13](image)

**Figure 13.** Six spectra from different brain regions that show the reproducibility of the technique. Spectra 1 & 6, 12 & 9 and 10 & 7 are all corresponding spectra from the left and right hemisphere of an untreated rat brain. All corresponding spectra display similarities as well as differences in-between areas within the brain.

Rats were given unilateral intracerebral injections of 6-OHDA into the SNc in order to induce Parkinsonism. The brains were then dissected out and placed in a cryotome and 12µm thick sections were placed on MALDI target plates.

The sections were dried in a desiccator and matrix was applied on areas of interest and subsequently analyzed. Several different proteins were found to differ in-between the hemispheres, such as cytochrome c, calmodulin and cytochrome c oxidase.
Several proteins were identified and some of these were used as internal calibrants (Figure 14, Table 1). We also detected differences in several unidentified proteins, and proteins with potential post-translational modifications, such as acetylations.

Table 1.

<table>
<thead>
<tr>
<th>MW (Da)</th>
<th>protein</th>
<th>Swiss-Prot acc.no</th>
<th>remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>4936</td>
<td>Thymosin β-10</td>
<td>P13472</td>
<td>N-term acetylated&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4964</td>
<td>Thymosin β-4</td>
<td>P20065</td>
<td>N-term acetylated&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>6649</td>
<td>COXJ</td>
<td>P35171</td>
<td>N-term acetylated&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>8565</td>
<td>Ubiquitin</td>
<td>P02248&lt;sup&gt;a&lt;/sup&gt;</td>
<td>N-term acetylated&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>9939</td>
<td>Acyl-CoA binding protein</td>
<td>P11030</td>
<td>N-term acetylated&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>9979</td>
<td>Cyt c oxidase</td>
<td>P56391</td>
<td>N-term acetylated and two disulfide bonds&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>11308</td>
<td>Histone H4</td>
<td>P02304</td>
<td>N-term acetylated and heme group&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>12135</td>
<td>Cytochrome C</td>
<td>P00009</td>
<td>N-term acetylated and heme group&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>13778</td>
<td>Histone H2B1</td>
<td>P00715</td>
<td>Same as mouse</td>
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<tr>
<td>15200</td>
<td>Hemoglobin α</td>
<td>P01946</td>
<td>N-term acetylated</td>
</tr>
<tr>
<td>16793</td>
<td>Calmodulin</td>
<td>P02593</td>
<td>N-term acetylated</td>
</tr>
</tbody>
</table>

<sup>a</sup> = Positive identification with MS/MS but modification not confirmed

In the present study cytochrome c showed decreased levels in the 6-OHDA treated hemisphere. This is at first eye a contradiction as release of cytochrome c to the cytosol is described as a characteristic step in the initiation
of apoptosis. When released into the cytosol, cytochrome c together with other proteins is responsible for activating a caspase reaction [24] which promotes apoptosis. Yet, it may be speculated that the time-period after the initial cell death, the released cytochrome c have been degraded after being released from the mitochondria.

We also detected changes in the levels of calmodulin. Calmodulin is a major Ca\(^{2+}\) binding protein in the brain and regulates several Ca\(^{2+}\) dependent pathways such as protein kinases and phosphatases. Normally, calmodulin activity is tightly controlled and free calmodulin is sequestered to regulate interaction and availability of calcium/calmodulin-dependent proteins in the cell [46].

Calmodulin showed a significant decrease in the 6-OHDA/L-DOPA treated hemisphere compared to the untreated hemisphere. In the group that only had 6-OHDA treatment, there were no statistically significant changes, but there was a trend towards increased levels in the 6-OHDA treated hemisphere.

Cytochrome C subunit IV also showed decreased levels in the 6-OHDA/ L-DOPA treated hemisphere. In the group treated with 6-OHDA only, there was a trend towards increased levels.

The role of cytochrome c oxidase (COX) is to be a driving force for the oxidative phosphorylation of ADP to ATP. COX catalyses the electron transfer from reduced cytochrome c and mediates proton transfer to form water from oxygen. In this process COX consumes protons from the mitochondria and contributes to membrane potential.

Decreases in cytochrome c oxidase activity in muscle cells have been reported in human patients with idiopathic PD treated with L-DOPA [4].

Oxidative stress is an expression for the reaction of substances derived from O\(_2\), such as hydrogen peroxide (H\(_2\)O\(_2\)) and hydroxyl radicals (OH\(^*\))[31]. It has previously been shown that the ratio of reduced glutathione and oxidized glutathione (GSH/GSSG) is reduced by oxidative stress, which further promotes radical formation [44]. Oxidative stress impairs the structural integrity of the lipids in the cellular membranes, due to lipid peroxidation. It has been shown that PD brains have a decrease in polyunsaturated lipids and an increase in other lipid peroxidation indicators [12]. Increased levels of free radicals and depletion of GSH have been shown to induce apoptosis in neurons [32].

Several other proteins/ peptides were also found to have altered expression between the different groups. As of today we do not have any positive identification on these proteins. We also detected possible post-translational modifications on proteins. Post-translational modifications are regulatory events, and responsible for recognition, signaling, degradation and protection. Acetylations have been showed to be responsible for protein stability and protection from N-terminal degradation from proteases.
Increased levels of Ubiquitin in the 6-OHDA-Lesioned Striatum of Rats (paper III)

Multiple genetic deficits have linked impaired ubiquitin-conjugation pathways to various forms of familiar Parkinson’s disease. Genetic studies have identified mutations in the genes coding for several proteins involved in the proteasome degradation pathway, such as alpha-synuclein, parkin and ubiquitin C-terminal hydrolase L1. Lewy bodies have been detected in many cases of PD, but the roles of these are still unclear. Major components of these are ubiquitin and ubiquitinated protein. As human PD progresses over many years, it is likely that the build-up of Lewy bodies is a slow process that is difficult to replicate in 6-OHDA and MPTP animal models. Treatment with 6-OHDA and MPTP destroys dopaminergic neurons rapidly [18].

Our present data show that there is a build up of monomeric ubiquitin and we detected a statistically significant increased level of unconjugated ubiquitin specifically in the dorsal striatum of the dopamine depleted hemisphere. No similar changes were found in the intact hemisphere or in the ventral striatum of the dopamine depleted hemisphere or the L-DOPA treated group. (Figure 16).
Figure 16. The level of ubiquitin was increased in the dorsal striatum (upper panel) in comparison to the ventral striatum (lower panel). This is in accordance with the efficiency of the 6-OHDA lesion, as the dopaminergic neurons projecting in the dorsal part are more sensitive to 6-OHDA induced degeneration.

In an experimental model of PD striatal glutathione (GSH) was significantly depleted following 6-OHDA lesion [49]. Reduced GSH levels could cause decreased activity of the thiol-containing ubiquitin activating enzyme (E1) since the thiolgroup that normally binds ubiquitin becomes oxidized and unable to bind and activate ubiquitin [36]. This could lead to a build up of monomeric ubiquitin as the activation and subsequent transfer of ubiquitin is halted.

Other possible reason for the build-up of ubiquitin is energy depletion as a result of an increase in the ATP/ADP ratio. As 6-OHDA is known to cause oxidative stress and interfere with complex I in the mitochondria, it is conceivable that the activation of ubiquitin is a consequence of the decrease in ATP levels.

Most publications present results on increased levels of ubiquitin in Lewy bodies. The most common techniques used are immunological techniques based on antibody-antigen recognition. The mass spectrometer has the ability to distinguish between metabolic fragments and post-translational modification and is a powerful complement to common immunological techniques, where the molecular recognition is based on antigen-antibody binding and may not distinguish between metabolic product and post translational modifications.

Further, most antibodies that have been used in immunocytochemical studies to assess the presence of ubiquitin in PD-associated Lewy bodies are directed towards a protein-bound form of ubiquitin, because free monomeric ubiquitin is not immunogenic in most mammalian species used to produce antibodies [34,35].
However, these antibodies are also capable of cross-reacting with free monomeric ubiquitin [34] why it is unclear in which form ubiquitin is measured using these immunocytotoxic techniques [1].

A recent study also highlights the problems involved in using fluorogenic substances for assaying the proteasome complex as these are also degraded by the proteasome complex in an ubiquitination manner. As such these substances can detect alterations in the proteolytic efficiency of the proteasome complex, but not alterations in the levels of free ubiquitin.

Increased striatal mRNA transcription and active protein expression of the immunophilin FKBP-12 in experimental Parkinson’s disease (paper IV)

FKBP-12 is one among over 20 FKBP’s and they all mediate correct folding of proteins and are involved in neural development and repair. The presence of FKBP-12 in the growth cones of developing neurons suggests an involvement in neural growth [45].

Our data show an up-regulation of FKBP-12 in 6-OHDA dopamine denervated rats. In situ hybridization was used to examine the mRNA levels of FKBP-12 (Figure 17).

A homogenate of the striatum was also analyzed and separated on 2D-GE and proteins identified with MALDI-MS peptide mass fingerprinting (PMF). The spot corresponding to FKBP-12 was enzymatically degraded with trypsin and identified by MALDI-TOF PMF. The peptide fragments were compared and matched against the annotated database Swiss-Prot (Figure 18).
Figure 18. MALDI-TOF peptide mass fingerprint from FKBP-12. Several peptides in the m/z range 1100-2100 were matched against theoretical peptides in the swiss-prot database. The search was conducted with the Profound V4.10 search engine.

The MALDI-MS profiling data was acquired at the same level (consecutive sections) as the in situ hybridization was performed and showed an up-regulation of FKBP-12 in the dorsal, middle part of the striatum, but not in the ventral parts (Figure 19). Our previous work on ubiquitin in paper III showed the same pattern and this may be due to the difference in lesion efficiency.

Figure 19. Relative intensity of FKBP-12 (m/z 11 791) in the unilateral dopamine depleted rats. The immunophilin FKBP-12 showed significant increased levels in both dorsal (a) and middle (b) but not the ventral part (c) of striatum after 6-OHDA treatment (black bars).
FKBP-12 binds to the ryanodine receptor, which is an interacellular calcium channel. The ryanodine receptor is located in the sarcoplasmic reticulum of muscle and in the endoplasmatic reticulum in the brain, where it among other things is involved in Ca\(^{2+}\) storage. It is also involved in exitation-contraction in muscle [33].

FKBP-12 can bind to FK-506, an immunosuppressant drug, that will create a complex that interferes with the calcineurin phosphatase activity that is mediated by Ca\(^{2+}\)-calmodulin [55]. In situ hybridization has showed that FKBP-12 is expressed up to 50 times higher in the brain than in the immune system [48].

The function of the immunophilins in lesioned nerve endings is unknown, but possibly it plays a role in correct protein folding and/or binding to an unknown natural ligand [3]. The highest levels of FKBP-12 are found in the substantia nigra. High levels were also detected in rat corpus striatum and in substantia nigra. In patients with PD there is also an increase of FKBP-12 in caudate, putamen and globus pallidus [3]. The levels of FKBP-12 have been shown to decreased in dying nerve endings, but elevated in less severely damage neurons. It has also been shown that FKBP-12 is co-localized with α-synuclein in Lewy bodies in clinical samples from PD patients. This further implicates involvement of FKBP-12 in the complex biochemical events that lead to degradation of damaged and misfolded proteins by the proteasome complex.
Future aspects

The mass spectrometer has become something of a workhorse in proteome studies. The rapid development of new bench top instruments with lower detection limit and higher resolution gives good promise for the next generation of mass spectrometers.

The successful effort of genome sequencing has showed that technological evolution is of great importance. The technologies for DNA sequencing evolved rapidly, the speed increased and more and more kilobits of genes were sequenced. Massive parallel and high throughput analysis systems were constructed and the seemingly endless task of deciphering the blueprint of life suddenly looked doable.

This highly illustrates the need for sophisticated, automated and high speed scanning equipment for the exploration of the new frontier; the proteome. As new analytical software is developed with the capability to interpret, and in a user friendly way display the massive information from the mass spectrometers, new hypotheses can be asked and tested.

Currently instrument vendors are most likely in the process of building dedicated imaging mass spectrometers. These instruments will hopefully be the first in line that is dedicated to the technique of creating mass spectra and images directly from tissue. It will most likely be some degree of automation built into it and larger sets of tissue samples can be processed and in turn generate more data that can be analyzed with various statistical models data with higher degree of accuracy, precision and statistical significance.

New matrices and sample handling protocols will probably be developed. Investigations have been made on different solvent/matrix compositions to be able to achieve the highest sensitivity and resolution. The spectra quality is dependent on several factors such as ion intensity, background noise and overall sensitivity in a broad mass range. Basic research will have to be done in order to investigate what types of proteins are detected at different conditions. Different subsets of proteins such as membrane bound integral and peripheral integral proteins, cytoplasmic and secreted proteins all require different sample handling protocols in order to be studied effectively. The size of the proteins and peptide varies in an extreme range up to several hundred thousand Da. This broad mass range will demand different requirements on both the type of instrumental parameters and the sample handling protocols used depending on the mass range and type of protein of interest.

“Science…never solves a problem without creating ten more”

George Bernard Shaw
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This is the hardest part of the thesis to write, afraid to forget someone!!

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I have probably missed out on someone here that I feel should have a sentence or two of acknowledgement, but don’t feel forgotten. If you know me well enough you know it’s just a common glitch in my neuronal memory circuit that wasn’t working properly………as usual!

Tight Lines

George Kelson, 1835- 1920
References


A doctoral dissertation from the Faculty of Pharmacy, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy. (Prior to July, 1985, the series was published under the title “Abstracts of Uppsala Dissertations from the Faculty of Pharmacy”.)