Molecular Profiling and Imaging of Peptides, Proteins and Drugs in Biological Tissue using Mass Spectrometry

ANNA NILSSON
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Abstract

Biological functions within cells and organisms are mainly carried out by the translational products; proteins and peptides. The analysis and characterization of these biomolecules are of great importance for the progress in disease research and biomarker and drug discovery. The term peptidomics was introduced to describe the comprehensive analysis of peptides (e.g. neuropeptides) in biological tissues. In this thesis, a peptidomics approach using nanoflow liquid chromatography coupled to electrospray mass spectrometry (MS) has been developed for detection, identification, and quantification of neuropeptides in different disease models. A thoroughly controlled sample preparation technique and targeted neuropeptide sequence collections have been used to improve sample quality and to increase the number of identified neuropeptides. In particular, neuropeptide changes in experimental models of Parkinson’s disease (PD), with or without L-DOPA treatment, and the effect of antidepressant treatment on neuropeptide expression have been investigated. Several novel, potentially bioactive, neuropeptides have been identified and a number of peptides derived from precursors such as secretogranin-1, preproenkephalin-B, and somatostatin have been found differentially expressed. Some of them represent novel findings, not previously associated with PD or treatment with antidepressants.

In addition, MALDI imaging MS (IMS), a technology that permits detection and spatial distribution determination of endogenous compounds and/or administered drugs directly on tissue sections, has been used in both small protein and drug applications. MALDI IMS on tissue samples from experimental models of PD revealed differential expression patterns of two small proteins involved in calcium regulation, PEP-19 and FKBP-12. Biomolecular interaction analysis was performed on FKBP-12 using surface plasmon resonance together with MS and several potential binding partners were identified.

In a second approach, MALDI IMS was used to study the distribution of the anticholinergic bronchodilator tiotropium in rat lung following inhalation of the drug. The distribution of the drug was monitored in both MS and MS/MS mode and the levels where linearly quantifiable in the range of 80 fmol – 5 pmol.

Conclusively, in this thesis mass spectrometry based technologies have successfully been developed to detect, identify, and characterize small proteins, peptides, and drugs in various tissue samples.

Keywords: peptidomics, mass spectrometry, liquid chromatography, MALDI imaging, neuropeptides, drugs, brain

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To my family
List of Papers

This thesis is based on the papers listed below, referred to in the text by the roman numerals I-V


V  Tracking distributions of inhaled compounds within tissue compartments using MALDI imaging mass spectrometry. A. Nilsson, T. Fehniger, M. Andersson, K. Kenne, L. Gustavsson, G. Markovarga, and P. E. Andrén. In manuscript

Reprints are published with kind permission from American Chemical Society.
Related papers not included in this thesis


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PD and experimental models of PD

Neuropeptides and depression

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<tr>
<td>2D-GE</td>
<td>two-dimensional gel electrophoresis</td>
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<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
</tr>
<tr>
<td>ACN</td>
<td>acetonitrile</td>
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<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
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<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
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<td>BIA</td>
<td>biomolecular interaction analysis</td>
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<tr>
<td>CCK</td>
<td>cholecystokinin</td>
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<tr>
<td>CHCA</td>
<td>alpha-cyano-4-hydroxy cinnamic acid</td>
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<td>CID</td>
<td>collision-induced dissociation</td>
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<tr>
<td>CRF</td>
<td>corticotropin-releasing factor</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>DA</td>
<td>dopamine</td>
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<tr>
<td>DHB</td>
<td>2,5-dihydroxy benzoic acid</td>
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<tr>
<td>ECD</td>
<td>electron capture dissociation</td>
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<tr>
<td>ESI</td>
<td>electrospray ionization</td>
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<tr>
<td>FKBP-12</td>
<td>12 kDa FK506-binding protein, Peptidyl-prolyl cis-trans isomerase FKBP1A</td>
</tr>
<tr>
<td>FTICR</td>
<td>Fourier transform ion cyclotron resonance</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptors</td>
</tr>
<tr>
<td>GPe/i</td>
<td>globus pallidus externa/interna</td>
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<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>ICAT</td>
<td>isotope-coded affinity tag</td>
</tr>
<tr>
<td>IFC</td>
<td>integrated micro-fluidics cartridges</td>
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<tr>
<td>IMS</td>
<td>imaging mass spectrometry</td>
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<tr>
<td>iTRAQ</td>
<td>isobaric tag for relative and absolute quantitation</td>
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<tr>
<td>LC</td>
<td>liquid chromatography</td>
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<tr>
<td>L-DOPA</td>
<td>3,4-dihydroxy-L-phenylalanine</td>
</tr>
<tr>
<td>LTQ</td>
<td>linear trap quadrupole</td>
</tr>
<tr>
<td>m/z</td>
<td>mass-to-charge ratio</td>
</tr>
<tr>
<td>MALDI</td>
<td>matrix-assisted laser desorption/ionization</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
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<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>NA</td>
<td>noradrenaline</td>
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<tr>
<td>NGFI-A</td>
<td>nerve growth factor-induced protein A</td>
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<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
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<tr>
<td>PEP-19</td>
<td>Purkinje cell protein 4 / Brain-specific polypeptide PEP-19</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>PET</td>
<td>positron emission tomography</td>
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<tr>
<td>POMC</td>
<td>pro-opiomelanocortin</td>
</tr>
<tr>
<td>PPE-B</td>
<td>preproenkephalin-B / beta-neoendorphin-dynorphin</td>
</tr>
<tr>
<td>Q</td>
<td>quadrupole</td>
</tr>
<tr>
<td>RPC</td>
<td>reversed phase chromatography</td>
</tr>
<tr>
<td>SA</td>
<td>3,5-dimethoxy-4-hydroxycinnamic acid</td>
</tr>
<tr>
<td>SCX</td>
<td>strong cation exchange</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<tr>
<td>SNr</td>
<td>substantia nigra pars reticulata</td>
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<tr>
<td>SPR</td>
<td>surface plasmon resonance</td>
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<tr>
<td>SPU</td>
<td>surface prep unit</td>
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<tr>
<td>sst1</td>
<td>somatostatin type-1 receptor</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
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<tr>
<td>TMAB</td>
<td>trimethylammoniumbutyrate</td>
</tr>
<tr>
<td>TOF</td>
<td>time-of-flight</td>
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<tr>
<td>TRH</td>
<td>thyrotropin-releasing hormone</td>
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INTRODUCTION TO PEPTIDOMICS

Extensive knowledge about the molecular basis of disease and molecular functionality has been obtained with DNA oriented techniques. However, it is mainly the translational products, peptides and proteins, that are functional within cells and organisms [1, 2].

Proteomics can be defined as the systematic analysis of proteins present in a cell or tissue at a given time point [2, 3]. The goal is to determine their identity, quantity, function, and structure. This task, however, is not trivial. The complexity of the proteome is enormous, predominantly due to processes like alternative splicing, and various posttranslational events including proteolytic cleavage or post-translational modification. It is believed that almost all proteins undergo some kind of post-translational modifications, and that more than 300 different post-translational modifications exist [4-7].

Remarkable advances in proteomic technologies have been made in the past decade. Among proteomic techniques, mass spectrometry (MS) has emerged as the preferential method for analysis of the production and function of proteins in native systems [8-10]. Successful combination of soft ionization techniques [11-13], improved mass analyzers, and the coupling of chromatographic techniques to MS have made it possible to analyze biomolecules at zeptomole levels [14, 15]. Today, in addition to protein identification, functional characterization of protein complexes and protein pathways has been made possible [16].

Traditionally, the analysis of proteins is carried out using two-dimensional gel electrophoresis (2D-GE) together with MS based identification methods. This technology is still widely used but due to its limitations, such as limited reproducibility, extensive analysis time, and difficulty in automation, other MS compatible separation methods have gained interest [17-19]. These include liquid chromatography and capillary electrophoresis. Recent developments have also led to the introduction of new miniaturized and chip based technologies [20-23]. A major drawback of the conventional gel-based proteomic techniques is that biomolecules below 10 kDa are lost in the 2D-GE separation. Hence, the biologically interesting fraction including neuropeptides, peptide hormones, and small proteins is not covered.
The term peptidomics, introduced in 2001 [24-26], is analogous to proteomics and refers to the analysis of peptide content within an organism, tissue, or cell. Even though this definition also includes a pool of peptides that are transient protein degradation products, most studies performed are focused on bioactive peptides. The definition of a true bioactive peptide is, however, not trivial. A common view is that a bioactive peptide should fulfill some criteria including presence of signal peptide, conventional cleavage sites, and sequence conservation [27]. Traditional peptide research has been dependent on bioassays that functionally define a peptide and extensive laboratory work has been needed to establish the peptide sequence. Peptides were typically purified from large amounts of tissue sample, tested in bioassays and the pure product was subjected to peptide sequencing for identification [28]. Currently, hundreds of peptides can be detected and sequenced from less than mg amounts of tissue using liquid chromatography coupled to mass spectrometry [29].

In contrast to proteomic studies, no enzymatic digestion is needed in peptidomic studies. Instead, the endogenous peptides can be extracted and analyzed directly in their native state by MS. The identification process, however, is often more difficult in peptidomic studies, as the aim is to identify all peptides present in a sample by its sequence. In proteomic studies, the mass or sequence from just a few tryptic peptides can be enough to assign the identity of a protein.

One of the most challenging tasks within the field of proteomics and peptidomics is to accurately compare protein or peptides levels between different physiological or diseased states. Knowledge acquired from these types of studies is important and may lead to the discovery of novel biologically relevant peptides, biomarkers of disease, or novel functionally important biological pathways.

**Neuropeptides**

The first neuropeptide was discovered in the early thirties by von Euler and Gaddum [30]. The unidentified depressor substance was isolated from horse brain and intestine and was referred to as substance P. The structural identity of the peptide was however not described until forty years later [31] when it was sequenced by Edman degradation and carboxypeptidase treatment. By this time there were several other peptides that had been structurally characterized, with the term neuropeptide being introduced by de Wied in the early seventies [32].
Neuropeptides include peptide neurotransmitters and peptide hormones. Peptide neurotransmitters are involved in cell-to-cell communication between neurons and play key roles in a great number of physiological processes such as sleep, feeding behavior, anxiety, learning, and reward mechanisms. The peptide hormones often act more distant to their release site and are involved in e.g. homeostatic mechanisms, including regulation of glucose levels, water retention, and food intake [27]. Both peptide neurotransmitters and peptide hormones act through binding to cognate receptors.

![Figure 1](image1.png)

**Figure 1.** Schematic overview of peptide processing. Prohormone convertases cleave at dibasic sites like KR and RR. Carboxypeptidases remove the C-terminally remaining amino acids.

Bioactive peptides are produced from larger precursors by the action of specific peptidases. After synthesis in the endoplasmatic reticulum, the precursors are packed in the Golgi apparatus before being transported to their release sites. The precursor molecules are stored in so called large dense-core vesicles or secretory granules together with processing enzymes until release [33]. The active processing enzymes are predominantly endopeptidases (prohormone convertases), and carboxypeptidases (Figure 1). Typically, the endopeptidases cleave at dibasic sites like KR, and RR [34] but cleavage may also occur when the basic amino acids are separated by 2, 4, or 6 amino acids [35, 36]. The carboxypeptidases then remove the C-terminal remaining amino acids [35, 36]. Additionally, some peptides are produced from non-basic sites but the enzymes responsible for this cleavage are not well characterized [37].

Neuropeptide precursors may contain several copies of the same neuropeptide, e.g. thyrotropin-releasing hormone (TRH) [38], or several different neuropeptides, e.g. the corticotropin-lipotropin precursor (also called POMC) [39] (Figure 2).
Figure 2. Neuropeptides are synthesized from proneuropeptide precursors by the action of specific proteases. The precursors may contain multiple copies of the same active neuropeptide such as the prothyroliberin precursor (Mus musculus) that contains five copies of TRH (A). The corticotropin-lipotropin precursor contains different neuropeptides within the same precursor (B). These are produced in a tissue specific manner. ACTH and β-lipotropin is predominately produced in the anterior pituitary lobe while further processing to α-MSH, β-endorphin, and γ-lipotropin occurs in the intermediate lobe [27].

For the peptides to become active or more stable, additional enzymes often add post-translational modifications such as C-terminal amidation, N-terminal acetylation, phosphorylation, and N-terminal pyroglutamation [40-42]. The processing of peptides can vary in different cell types or even in the same cell under different conditions [27].

Exocytosis, the formation of fusion pores between membrane bound vesicles and the presynaptic membrane, is triggered by a rise in cytosolic calcium concentration [43]. Neuropeptides often coexist with neurotransmitters within the same neurons and sometimes the same vesicles [44], exerting their action by binding to receptors, predominantly G-protein coupled (GPCRs). The first neuropeptide receptor was cloned in 1987 [45] and today there is evidence that multiple subtypes of receptors exist for different neuropeptides, e.g. tachykinins, and somatostatins [46, 47]. Among the 550 GPCRs in the human genome there are still about 25% that are orphans, i.e.
lack known endogenous ligands, and it is believed that many of these unknown ligands might be peptides [48, 49].

**Analysis of neuropeptides**

Traditionally, bioactive peptides have been analyzed with sensitive immunoassay methods. These approaches suffer from some limitations. Their selectivity is sometimes compromised due to cross-reactivity with structurally related peptides. They are also time-consuming as peptides have to be pre-isolated for the development of specific antibodies. Additionally, the number of peptides that can be analyzed simultaneously is limited [50, 51]. The combination of capillary liquid chromatography and mass spectrometry has proved to be a both sensitive and selective technology for peptide analysis and is offering the possibilities for discovery of previously uncharacterized peptides while simultaneously detecting a great number of peptides.

A reduction in sample complexity is often needed to obtain a successful mass spectrometric analysis of peptidomic samples. This can be performed in- or off-line with the mass spectrometer. If a sample is too complex the MS analysis can be compromised due to ionization competition [52-54]. The resolution of the mass spectrometer also affects the degree of complexity that can be analyzed. Overall it has been shown that a chromatographic separation of the sample before MS analysis improves the analysis and yields more peptide identifications [18].

**Sample preparation**

The quality of the sample and the sample preparation technique are of great importance in neuropeptidomics. During dissection of tissue and extraction of peptides, a small amount of protein degradation often occurs. Since most bioactive peptides are typically present only at low levels relative to the major proteins, the breakdown of even a small fraction of the major proteins can overwhelm the weaker signals from endogenous neuropeptides [29, 37, 55]. However, all proteolytic fragments may not always be produced post-mortem. Proteolytic peptides can occur naturally in vivo, e.g. in blood or other body fluids [56]. These peptides might not be biologically active but they can serve as indicators for disturbed physiological processes in disease states, so called biomarkers. Nevertheless, neither neuropeptidomics nor peptide biomarker discovery can be efficiently performed if large amounts of proteolytic peptides are present in the sample [55, 57-59]. A sample preparation technique that prevents post mortem proteolysis is therefore crucial for success in neuropeptide detection and identification. Several different approaches have been undertaken to circumvent the interference of degradation
products. Some have chosen to work with tissues that are relatively high in peptide contents and low in degradative enzymes, such as pituitary [60, 61] while others have specifically isolated only C-terminally amidated peptides [62]. Fricker et al. have used a strain of mutant mice that lack carboxypeptidase E activity (Cpe(fat) / Cpe(fat)) as their source of neuropeptides [63]. The neuropeptides from this mutant are extended by the basic amino acids lysine and arginine due to inactivity of carboxypeptidase E and can be separated from un-specific proteolytic fragments on an anhydrotrypsin column. This approach, however, is limited to the Cpe(fat) / Cpe(fat) mice and specifically to neuropeptides that are cleaved from conventional neuropeptide cleavage sites.

Our laboratory [29, 57] have showed that the rapid denaturation of proteases and peptidases by microwave irradiation or conductive heat transfer greatly improved the detection of neuropeptides. Additionally, a short N-terminal fragment of stathmin has been found to correlate with the level of degradation and can hence serve as a marker for sample quality [57].

Liquid chromatography

Liquid chromatography is a technique used for separating analytes in a liquid mobile phase. The separation is performed in a column system and is based on physiological properties of the analytes. The most common way of separating peptides is via hydrophobicity on a reversed phase column system. The column dimensions have decreased during the past years in order to achieve better sensitivity and separation efficiency [64]. Microcolumns (<100μm i.d.) and nano-liter / minute flow rates are commonly used for this purpose. Such flow rates are typically not reached with conventional pumps, but require flow splitting. In the nano-liter flow range, almost 100% ionization efficiency can be obtained with electrospray [65]. Furthermore, capillary columns need small amounts of sample which is beneficial as the available amount of biological samples is often limited. Especially, endogenous bioactive peptides are often present in very low concentrations and a pre-concentration step on a trap column might therefore improve their detection.

When analyzing more complex mixtures an additional dimension of separation can be introduced. This is often based on separation by charge and strong cation exchange (SCX) columns are commonly used either off-line, in a multi-dimensional LC system, or packed into the same column as the reversed phase separation media (MudPit, [66]).
Mass spectrometry

Initially, mass spectrometry was predominantly used within the field of physics, but with the development of soft ionization techniques in the 1980’s it became more suitable for biological applications [67]. Mass spectrometers measure the mass-to-charge ratios (m/z) of ions in the gas phase and can hence determine the mass of a molecule if the charge is known. This is achieved by manipulating the ions in electric/magnetic fields or by measuring their time-of-flight. A mass spectrometer basically consists of three different parts: a source, where ionization of analytes take place, mass analyzers, where the analytes are separated, and a detector that registers the analytes presence [67, 68]. The detected signal is proportional to the amount of the peptide present in the sample but the technique is not absolutely quantitative as the ionization efficiency of different molecular ions varies depending on chemical properties of the particular molecule.

Electrospray ionization (ESI)

ESI was introduced into the biological field by Fenn et al. in the late 1980’s [11]. ESI is a method where ions are ionized from a solvent. The analyte ions are introduced into the mass spectrometer by applying a high voltage potential between the inlet of the mass spectrometer and the spray emitter. This potential causes the ions to be dispersed into a spray of fine droplets that are desolved upon entering the mass spectrometer (Figure 3). There are two models that try to explain this phenomenon, the charge residue [69] and ion evaporation model [70].

![Figure 3. The ESI process. High voltage is applied to the liquid and the counter-electrode, resulting in a spray. The droplets evaporate as they move towards the entrance of the mass spectrometer and free charged analyte molecules, which can be analyzed for their mass-to-charge ratio, are produced.](image)

One of the typical features of electrospray ionization is that multiply charged ions are produced. This makes it possible to analyze biomolecules with high molecular weight (up to 100kDa) in a small m/z window (around 2000 m/z
The presence of multiple charged species of the same molecule enables full mass deconvolution. Additionally, multiple charged peptide ions yield more fragment ions, improving sequence coverage in MS/MS experiments.

**Matrix-Assisted Laser Desorption Ionization (MALDI)**
MALDI was developed in the mid 1980’s [12, 13] and is today extensively used for analysis of biomolecules. The sample to be analyzed is co-crystallized with an organic matrix on a MALDI target plate. The matrices α-cyano-4-hydroxy cinnamic acid (CHCA) and 2,5-dihydroxy benzoic acid (DHB) are mostly used for peptide analysis, while 4-hydroxy-3,5-dimethoxy cinnamic acid (SA) is preferable for protein analysis [72]. The sample analytes are ionized when a laser beam is fired onto the UV absorbing matrix crystals. This generates a plume of matrix and analyte ions which are accelerated into the mass spectrometer by an electric field (Figure 4). In contrast to ESI, MALDI is relatively tolerant to contaminations like salt and detergents but clean up steps prior to analysis are preferred to improve the sensitivity, resolution, and accuracy of analysis [72, 73].

![Figure 4. The MALDI process. A plume of matrix and analyte ions is produced when the laser beam hits the UV absorbing matrix, which is co-crystallized with the analytes. The ions formed in the process are accelerated into the mass spectrometer by an electric field.](image)

**Mass analyzers**
The common feature of mass analyzers is to separate the analyte ions in time or space. This can be performed with a wide range of different mass analyzers including the quadrupole (Q), time-of-flight (TOF), ion trap, and Fourier transform ion cyclotron resonance (FTICR) [68]. Often two or more of the analyzers are combined in one instrument to improve the analysis or to en-
able MS/MS analysis (e.g. Q-TOF). Typically, in an MS/MS experiment, the ions are separated in one analyzer, fragmented, and subsequently separated in a second analyzer. The analyzers of the mass spectrometers used for this thesis work will be described briefly; more extensive information can be found elsewhere [68, 74].

In a TOF mass analyzer, ions are separated by traveling time in a field free region (Figure 5). Their m/z ratios are determined by measuring the time it takes for the ions to move through the flight tube to the detector. Flight time can be measured in either linear or in reflectron mode. The reflectron acts as an ion mirror by deflecting the ions and sending them back through the flight tube, thereby improving the ion resolution [75]. The TOF analyzer is well suited to the pulsed nature of laser desorption ionization [74].

![Figure 5. TOF analyzer. The ions are accelerated into a field free region and their mass-to-charge ratios are determined by the time it takes for the ion to reach the detector. The reflectron acts as an ion mirror that deflects the ions and sends them back through the flight tube.](image)

A quadrupole mass filter consists of parallel circular or, ideally, hyperbolic rods (Figure 6). Complex static and oscillating fields are applied to select an ion in a small or a wide mass (m/z) window [76]. Quadrupoles are often used as ion transmission cells and/or as collision cells [77].

![Figure 6. The quadrupole analyzer. Four voltage-carrying rods serve to give ions which travel between them oscillations. Only ions with the right m/z can undergo these oscillations without hitting one of the rods and are hence transmitted to the detector.](image)
The linear ion trap (present in the LTQ) [78] is a mass analyzer well suited to do MS/MS analysis, with large ion capacities and scan rates [78, 79]. However, the mass resolution of MS data acquired with the LTQ does not allow determination of the charge state of multiple charged peptide ions [80], which makes it less suitable for peptide profiling experiment. In a linear ion trap, the ions are maintained in the trap by application of appropriate RF and DC voltages. For precursor ion selection the voltage is adjusted so that all ions except for the one of interest are eliminated from the cell. Ions are fragmented, for example by collision-induced dissociation (CID), and the fragments are ejected from the trap for detection by adjustment of the applied voltages. In the LTQ, ions are ejected and detected in two directions which increases the sensitivity of the analysis.

The FTICR MS is an MS instrument with a very high mass accuracy and resolution. Ion cyclotron resonance uses a strong magnetic field to separate ions [81]. Ions are trapped in the mass analyzer in a circular movement pattern. The angular frequency of the circular motion depends on the m/z value of that ion and the magnetic field. The ions are excited and the induced oscillating current can be translated into a basic angular frequency using Fourier transformations. The whole process is performed in a nondestructive way and an ion can therefore be maintained within the cell enabling MS^n experiments of the same. Fragmentation of the ions can be achieved using CID, and electron capture dissociation (ECD) [82-84]. The high mass accuracy obtained with FTICR instruments is beneficial for the peptide identification process as it narrows the number of possible hits in database searches [85].

Peptide sequencing/identification

Edman degradation was traditionally used to determine the amino acid sequence of peptides and proteins [86]. Some obvious limitations of this procedure are the analysis time needed, the purity of the sample needed, and the difficulties in analyses of N-terminally blocked peptides/proteins [87]. Today, sequencing of peptides is routinely performed by mass spectrometry and the most generally used fragmentation technique is CID [88]. This type of fragmentation occurs when peptide ions collide with an inert gas, generating predominately b- and y-ions.

The assignment of confident identities to detected endogenous peptides from MS/MS data is not a trivial task. Most tools available for this procedure are designed for identification of proteins from tryptic peptide digests. The most common way of assigning identities to peptides from MS/MS spectra is by searches against virtually cleaved protein databases using search engines.
such as X!Tandem or Mascot [89]. As cleavage sites for endogenous peptides vary for different peptides, the available protein databases have to be searched with unspecific cleavage settings. This creates a huge search space giving rise to high cut-off scores for significant identity hits. The presence of possible post-translational modifications increases the possibilities even further. Additionally, many tryptic peptides have optimal size and charge state for producing high quality MS/MS data and it is often enough to have sequence data from a couple of peptides to deduce the identity of a specific protein. Regarding endogenously cleaved peptides, all peptides have to be targeted for MS/MS analysis and the fragmentation has to be informative enough to deduce the peptide sequence.

In order to improve the identification process of neuropeptides, databases targeting endogenously produced peptides have recently been developed by our laboratory [90, 91]. The SwePep database contains information about peptide precursors and their processed peptides. This information is derived from UniProt, experimental data produced in our laboratory, and peer-reviewed publications. Three targeted sequence collections: SwePep precursors, SwePep peptides, and SwePep predicted, have been constructed from the SwePep database and the use of these have enabled a fast, specific, and sensitive identification of endogenous peptides from mouse brain samples. Three times as many endogenous mouse peptides were significantly identified using the sequence collections, in comparison with searches against the entire mouse proteome [91].

Instrument scan speed and increased mass accuracy are other features that improve peptidome sequence coverage, strengthen the confidence in peptide identifications, and facilitate discovery of post-translational modifications [16].

**Peptide quantification**

Differential quantification between samples derived from different physiological states is one of the most important but also most challenging task within the neuropeptidomics field. Most research on detection and quantification of neuropeptides has utilized specific radioimmunoassays. These assays are sensitive but unable to define the detected neuropeptide sequence. They are also expensive and time-consuming when trying to analyze several peptides at the same time. Conversely, mass spectrometric based techniques are well suited for multiplexing.

The first differential peptidomics MS approach was reported by Jiménez et al. in 1997 [92], where a reference peptide was used to semi-quantitatively determine differences in peptide concentration in neurointermediate lobe
homogenates from rats using MALDI-TOF MS. Currently, there are a range of MS based techniques used for quantification of differences between samples. These are either based on stable isotope labeling schemes or label-free approaches.

Labeling schemes are usually developed for proteomics applications and in most cases are not optimal for true peptidomic approaches. For instance, the commonly used isotopically coded affinity tag (ICAT) [93] specifically targets Cys residues. These residues are known to be infrequently present in proteins [94], and less than a third of the mouse peptides present in SwePep contain cysteins. Labels that are more suitable for peptidomic approaches are those targeting the N-terminus and the epsilon-amino group of lysine residues, such as the isotope-coded protein label (ICPL) [95], the isobaric tag for relative and absolute quantitation (iTRAQ) [96], and H9- and D9-trimethylammoniumbutyrate (H9- and D9-TMAB) [60, 97]. Among these, the H9- and D9-TMAB have successfully been used for the differential analysis of endogenous mouse peptides in several studies [60, 98, 99]. However, many known bioactive peptides are blocked at the N-terminus (e.g. by pyroglutamation or acetylation) and hence escapes this type of labeling.

The alternative approach of using label-free quantification is appealing because the whole peptidome is targeted and the workflow is fairly simple compared to labeling based techniques. Two main strategies have been employed; the measurement and comparison of mass spectrometric signal intensities, and the counting and comparison of number of fragment spectra identifying peptides of a certain protein [100]. The latter is hence more adapted to suit proteomic studies. There are studies indicating that the label-free approach is less accurate compared to stable isotope labeling [101] and it is therefore of great importance to keep the experimental steps to a minimum and to carefully consider the experimental parameters that affect the analytical accuracy of quantification by ion intensities. These include for example the mass accuracy of the mass spectrometer, the reproducibility of the chromatographic profile, and the balance between acquisition of survey and fragment spectra [101]. When conducting profiling experiments it is advantageous to have a well defined chromatographic profile for each detected peak. This criterion is compromised if conducted on an instrument that constantly switches between MS and MS/MS mode. However, by performing profiling and identification experiments on separate MS instruments, which are best suited to each task, this dilemma can be overcome.

Advantages of the label-free approach are that it is less time-consuming, less costly, and there is no limit to how many experiments/samples that can be compared. Additionally, there is evidence that label-free approaches provide higher dynamic range of quantification than stable isotope labeling [101]. In
a study on the initiative of the Association of Biomolecular Resource Facilities (ABRF) Proteomics Research Group (PRG), conducted by 52 independent laboratories, the label-free peptidomics quantification approaches performed as well as, or even better, than stable-isotope labeling approaches [102].

The data analysis of a peptidomics profiling experiment is a challenge. Quantitative data has to be extracted from the acquired spectra, peptide identifications have to be performed and matched to the profiling data, and appropriate statistical analyses have to be employed. There are currently a number of commercially and freely available software packages (e.g. DeCyderMS [103, 104], and MSight [105]) that help in automating the described processing steps. Typically, the data is log-transformed (assuming that the data is lognormal-distributed) and normalized before quantification and statistical analysis [101].

MALDI imaging MS

MALDI imaging MS (IMS) is a technique that allows for the on tissue detection of a wide range of endogenous compounds, including proteins, peptides, lipids, and administered pharmaceutical compounds. Two-dimensional ion density maps of the analytes are obtained and their distribution can be mapped to specific tissue compartments (Figure 7). The first paper utilizing this technology was published by Caprioli et al. in 1997 [106] where both direct tissue analysis and blotted target analysis were described. Subsequent developments of imaging computer algorithms, that allowed instrument control as well as data acquisition and processing, provided the tools necessary for obtaining images of endogenous compound distributions within thin tissue sections [107, 108].

Since its introduction, tremendous effort has been put into the field, attempting to increase spatial resolution [109], and improve sample preparation, reproducibility, and sensitivity [110, 111]. Initially, only MALDI-TOF instruments were used but currently the application has spread to include MALDI coupled to FTICR [112, 113], Q-TOF [114], and QIT instruments [115].

The MALDI IMS technology has numerous different applications, ranging from low resolution profiling of peptide and protein in brain tissue sections [116], to high resolution images of drug compounds and metabolites in whole-body animal sections [117, 118]. Additionally, MALDI IMS has been shown to have great potential as a diagnostic tool for cancer [119, 120]. The method requires minimal sample manipulation and has the unique feature of
not only enabling comparisons of relative levels of endogenous compound or drugs but also revealing their spatial distribution within tissue sections. Other imaging technologies (e.g. autoradiography, magnetic resonance imaging (MRI), and positron emission tomographic (PET)) are often limited in resolution, as well as by the requirement of specific labeling with multiplexing being time-consuming and difficult [121, 122].

Figure 7. Schematic overview of the MALDI IMS process. In a typical MALDI imaging experiment, thin tissue sections (12-20 μm) are cut in a cryomicrotome, and thaw-mounted onto MALDI target plates. The tissue sections are then either coated or spotted with a suitable MALDI matrix. Spectra are acquired from each location (pixel) over the surface of the tissue. 2D ion intensity images can then be reconstructed from individual peaks in the spectra.

Matrix application
The application of matrix is one of the most crucial steps in the experimental workflow of MALDI IMS. Careful sample preparation and handling is critical to achieve high-quality images. The tissue sections are often washed before matrix application in order to reduce the salt and lipid content of the tissue and hence improve IMS protein signal sensitivity [123]. DHB and CHCA are commonly used for monitoring drugs and peptides, whereas sinapinic acid is more amenable to the detection of proteins (>2,000 Da) [106, 124, 125]. However, it is important to test different matrices and to optimize the solvent system for a specific target analyte.
For profiling applications, the matrix is often applied manually in volumes of 0.1-0.5 μl. The recent development of robotic MALDI matrix spotters allows discrete volume deposits in the picoliter range. This enables extraction of endogenous compounds from smaller areas, allowing targeting of specific structures. Automatic robotic spotting has also increased the sensitivity and reproducibility of the tissue analysis [126]. Two main technologies for robotic matrix spotting exist: acoustic deposition [126] and inkjet printing [127] (Figure 8). Even though the individual droplet sizes are in the range 100-200 pL, approximately 10-20 nL have to be deposited on the tissue to obtain good quality spectra which typically results in a resolution of above 200 μm [126, 128]. This can be considered a limitation for high resolution imaging applications.

![Figure 8](image)

Figure 8. A) The chemical ink-jet printer available from Shimadzu is a piezoelectric device that delivers matrix solutions from a glass capillary with an inner diameter of 55 μm. B) The Portrait 630™ reagent multi-spotter (LabCyte) uses a nozzle free acoustic droplet ejection to deposit matrix solutions. Ultrasonic acoustic energy is focused into a fluid sample to eject small droplets of liquid from open wells. Published with permission from Shimadzu and LabCyte.

To increase the spatial resolution, the tissue sections can be coated with homogenous matrix layers. This can be achieved either manually using e.g. a thin-layer chromatography (TLC) or airbrush sprayer or automatically using recently developed robotic spray deposition devices (Image Prep, Bruker Daltonics). To obtain high-quality images, the matrix must be deposited on the surface of the tissue as a homogenous layer. This should be performed so that the tissue becomes wet enough to allow co-crystallization between proteins and the matrix, but not so wet that delocalization of proteins across the tissue occurs. For imaging applications, the laser and the crystal size are the limiting factors determining image resolution. The advantage of using an automated device, such as the Image Prep, for matrix application is that the matrix layer builds up slowly and the wetness of the tissue can be monitored in real time.
Imaging data acquisition
Following matrix application, the coated tissue is analyzed by acquiring mass spectra in a raster set at a fixed resolution. All mass spectra acquired from the tissue constitute an image dataset analogous to pixels in a digital photograph. A specific m/z value can be extracted from all the collected spectra and the relative abundance of that ion in each pixel can be visualized by a color intensity scale in a 2D map (Figure 7). Commercial or custom software can be used to generate images depicting the localization and relative intensities of hundreds of ions in a single tissue section.

Typically, over 400 distinct mass signals are observed between m/z 2000-70000 with the majority of peaks (~90%) being below m/z 30 000 [129]. Current MALDI-TOF systems are usually equipped with a solid-state Nd:YAG laser functioning at repetition rates up to 200 Hz [130]. This has greatly reduced the time needed for data acquisition compared to early MALDI-TOF systems which functioned at only a few Hz.

Peak identification
The most tedious process accompanying MALDI IMS experiments is the determination of the identities of the detected peaks. Typically, these are established by extraction of proteins from tissue samples followed by fractionation by HPLC [131, 132], and subsequent screening of the fractions by MALDI MS. The fractions containing the targeted molecular weight compounds are digested by trypsin and analyzed by MS/MS. However, the identities of small peptides and lipids have been determined through MS/MS analysis directly on tissue sections [113, 115, 133] and a recent approach utilizing on tissue trypsination have successfully revealed the identity and distribution of some endogenous proteins [134].

Drug imaging applications
One of the emerging applications of MALDI IMS is the analysis of drug and drug metabolite distribution in different organs or whole-body tissue sections of dosed animals [114, 135, 136]. This approach offers the ability to differentiate between the distribution of a drug and its metabolite within a tissue section, which previously has not been possible with conventional methods such as PET or autoradiography. Recently, a number of different laboratories have successfully analyzed the localization patterns of a wide range of pharmaceutical compounds, systemically administered to different animal models, directly in tissue sections (See reviews: [135, 136]). Targeted drug/metabolite analyses are often performed in MS/MS mode, as this increases both the specificity and the sensitivity of the analysis. Additionally, several studies have shown MALDI signals to be proportional to the densities of pharmaceutical compound and linear responses have been obtained.
from tissue sections [137, 138], indicating that qualitative as well as quantitative measurements could be obtained.

Even though MALDI IMS drug applications are at a very early stage, there is considerable evidence indicating that this technology will provide a powerful tool for the investigation of in vivo distribution of new chemical entities. Additionally, the effect of a particular drug candidate or its metabolite/s on the targeted tissue could yield important toxicological information.

Protein interaction studies using Surface Plasmon Resonance (SPR)

Biological functions of most macromolecules depend on their ability to interact with other molecules. Biomolecular interaction analysis (BIA), a label-free biosensor based technology able to detect interactions between biomolecules, is hence a very useful tool for understanding protein function. The BIA technology is based on the optical phenomenon of surface plasmon resonance (SPR). The angle of the reflected light between two media is measured and depends on the refractive index of the material on the non-illuminated side i.e. an increased sample concentration on the sensor chip surface increases the refractive index and hence the measured SPR angle [139]. Typically, the molecule of interest is immobilized on the sensor chip and the analyte solution is passed over the chip in a microfluidic system (Figure 9). When binding occurs, the refractive index and hence the SPR angle is altered. The signal is measured in resonance units (RU) and is proportional to the amount of analyte bound (Figure 10), with 1000 RU corresponding to 1 ng bound protein / mm² [139, 140].

![Figure 9. Schematic overview of a biomolecular interaction analysis. When binding to the immobilized protein occur, the increase in sample concentration on the sensor chip surface causes a corresponding increase in refractive index. This increase alters the SPR angle which is measured and can be correlated to the amount of protein bound to the surface.](image-url)
A microrecovery method for the bound material has been developed [141]. This method enables recovery of the bound analytes in a very low sample volume as the recovery solution is handled in between two air segments (Figure 10). The recovered proteins can subsequently be identified by trypsination and mass spectrometric analysis. Typical amounts of material isolated from sensor surfaces is 10-50 fmol, hence multiple cycles of recovery are often needed for successful detection and identification of potential binders. Currently, a number of studies have utilized the SPR-MS combination in ligand fishing-like experiments [142-146].

![Figure 10. Sensorgram from a recovery experiment. The difference in resonance units between A and B represents the amount of protein bound to the surface during injection. The difference between B and C corresponds to the amount of protein recovered in the sandwich elution step.](image)

Obvious advantages of SPR/MS based methods for the study of protein-protein interactions are that no tags or labeling are needed and the interactions can be monitored in real-time. Once binding partners are found, interaction characteristics such as kinetic information can be studied in great detail.

Yeast-two-hybrid screening, affinity chromatography, and affinity pull-down experiments represent other methods that are commonly used to study protein-protein interactions [147, 148]. However, compared to SPR/MS based strategies, these approaches require either targeting of proteins to the yeast nucleus or the availability of specific tags or antibodies. Moreover, it has also been suggested that the sensor surface displays less nonspecific interactions compared to chromatographic beads [142].
PD and experimental models of PD

PD is one of the most common neurodegenerative diseases, affecting approximately 1% of the population over the age of 65, with cardinal symptoms including bradykinesia, rigidity, and resting tremor [149]. The pathology is characterized by degeneration of dopaminergic neurons in the substantia nigra, subsequent loss of dopamine in the striatum, and the formation of inclusion bodies, called Lewy bodies, in the surviving dopaminergic neurons [150, 151]. Current therapies aim at restoring the DA levels by administration of the dopamine precursor levodopa (L-DOPA) and/or dopamine agonists [152].

Neuropeptides, however, might also be part of the pathophysiological process [153, 154]. Many of the dopaminergic neurons also contain neuropeptides (e.g. CCK, and neurotensin) and the medium spiny neurons in the striatum that receive input from the nigrostriatal dopaminergic neurons contain different populations of peptides depending on their subtype. Substance P and dynorphin are coexpressed in the GABAergic neurons in the direct pathway projecting to GPi/SNr while enkephalin is expressed in the indirect pathway projecting to the GPe [33, 153].

There have been numerous studies over the years reporting changes in neuropeptide levels in experimental models of PD or post-mortem material from PD patients [155-160]. Many of these involve the opioid peptide systems (i.e. peptides derived from proopiomelanocortin, proenkephalin or prodynorphin) and/or the tachykinins (substance P, neurokinin A, and B). The general trend in current literature indicate that PD is associated with increased expression of enkephalin and decreased expression of dynorphin and tachykinin [161, 162], even though some studies have reported no change [156, 157, 159].

Additionally, there are studies that indicate that some neuropeptides possess a protective or regenerative effect in PD. Administration of a synthetic ACTH 4-9 analogue, ORG2766, accelerated spontaneous recovery in 6-OHDA lesioned rats [163]. Furthermore, neurotensin increased the expression of tyrosine hydroxylase in dopaminergic neurons in the substantia nigra after retrograde transport from its injection site in the striatum [164, 165].

Animal models of PD play an essential role in the understanding of PD pathophysiology and the effect of PD therapies. An ideal animal model of PD should show features including selective and gradual loss of dopamine neurons that exceeds 50%, and the formation of Lewy bodies. The model should display detectable motor deficits including the cardinal symptoms of PD, and should preferably have a relatively short disease course of a few
months, allowing rapid and less costly screening of therapeutic agents [166]. Two of the most commonly used models are induced by using toxins, namely 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA), that specifically target and degrade dopaminergic neurons [167].

MPTP was accidently discovered when young addicts developed severe parkinsonism following injection of a synthetic heroin analogue (pethidine) contaminated by a byproduct, MPTP [168]. Today, MPTP represents the most frequently used parkinsonian toxin applied in animal models [166, 169]. MPTP is mainly used in non-human primates and in mice, as rats have shown to be less sensitive to the toxin [170]. MPTP has the ability to cross the blood brain barrier and is most often given systemically. It is converted by monoamine oxidase B (MAO-B) to its active metabolite, 1-methyl-4-phenylpyridinium (MPP+), which is taken up specifically by dopamine transporters and concentrated in mitochondria, where it inhibits complex I of the electron transport chain [166, 171]. This inhibition reduces ATP generation and increases free-radical production subsequently leading to cell death [172, 173].

The toxin 6-OHDA, specifically taken up by DA and NA transporters, [174] also targets the function of the mitochondrial complex I leading to the production of free radicals and oxidative stress [167]. This hydroxylated analogue of natural occurring dopamine fails to pass the blood brain barrier and consequently an experimental model of PD was developed by intracerebral injection of the substance [175]. 6-OHDA is typically injected unilaterally into the substantia nigra, the medial forebrain bundle or the striatum [176, 177]. Unilaterally lesioned animals display quantifiable motor behavior induced by systemic injection of either dopaminergic receptor agonists or dopamine releasing drugs [178]. Following stimulation of postsynaptic receptors, e.g. apomorphine, the animals rotate contralaterally, whereas compounds that stimulate the release of dopamine, e.g. cocaine, makes the animals rotate ipsilaterally. The 6-OHDA model has been successfully used in rats, mice, cats, and primates [166].

Limitations of these two toxin-based models include the induction of acute effects, which significantly differ from the slow progressive pathology of human PD, as well as a failure to produce Lewy bodies [166, 179].

**Neuropeptides and depression**

Unipolar depression can be characterized by three primary core symptoms: anhedonia (loss of pleasure or interest in most activities), depressed mood,
and decreased levels of energy or fatigue [180]. Despite the progress in fundamental neuroscience and many decades of study, the pathophysiology of anxiety disorders and depression remains elusive and no revolutionary therapies have reached the clinic in the past decade [181]. Current antidepressants therapies have a delayed onset and the mechanism(s) underlying their therapeutic actions remain largely unclear [181, 182]. Collected evidence have suggested several neuropeptides to be involved in depression, including substance P, corticotropin releasing factor (CRF), neuropeptide Y, vasopressin, and somatostatin [183].

CRF has been found to be increased in patients with stress-related depression. Clinically effective antidepressants often normalize CRF levels and it has therefore been speculated that glucocorticoid receptors might serve as good therapeutic targets. Another argument for the latter possibility is that many depressed patients have a dysfunctional feedback inhibition system in the hypothalamus-pituitary-adrenal cortex axis. A substance P receptor antagonist has also been shown to have clinical efficiency as an efficient serotonin selective reuptake inhibitor in depression [184, 185]. Additionally, there is consistent evidence that somatostatin levels are decreased in the CSF of depressed patients compared to healthy controls [186-188] and that somatostatin affects the hypothalamic-pituitary-adrenal axis by inhibiting the stimulated release of ACTH from the pituitary [188].

It is naturally difficult to reproduce the complex symptomatology of depression in a rodent. However, rodents often exhibit both anxious and depressive behaviors in behavioral tests thus reflecting clinical features of the diseased state. The criteria for an animal model of depression was outlined more than 35 years ago [189]. Desired features of the model included symptomology reasonably analogous to the human condition; behavioral changes that could be objectively monitored; resultant behavioral changes that could be reversed by therapies effective in humans; and reproducibility of the model [189, 190]. The most common used rodent models of depression are the forced swim test, tail suspension test, olfactory bulbectomy, and learned helplessness [189, 190].
AIMS OF THE THESIS

The aim of the current thesis has been to develop mass spectrometric methodologies suitable for detection and quantification of neuropeptides, small proteins and drugs in experimental models of disease. In particular, the focus has been:

- To develop a quantitative neuropeptidomic approach using nanoflow liquid chromatography electrospray mass spectrometry.

- To develop MS based strategies to identify endogenous neuropeptides from peptide brain extract of different brain regions, such as the mouse striatum and hypothalamus.

- To apply the neuropeptidomics approach to study changes in neuropeptide expression in experimental models of PD and following antidepressant treatment.

- To further validate and characterize some of the differentially expressed protein/peptide findings from the global proteomic/peptidomics studies by e.g. in situ hybridization, MALDI imaging MS, and biomolecular interaction studies using SPR MS.

- To develop MALDI IMS protocols suitable for analysis of peptides, small proteins and drugs in tissue sections.

- To determine the distribution and quantify the levels of tiotropium in rat lung following drug administration by inhalation.
MATERIALS AND METHODS

This section contains a summary of the materials and methods used in paper I-V. More detailed descriptions can be found in the papers included in this thesis.

Animals

Mice
Adult male C57Bl/6 mice were used in paper I, III-IV. In paper I, the mice were subcutaneously administered MPTP.

In paper III, mice were unilaterally lesioned with 6-OHDA. A noradrenaline uptake inhibitor (desipramine) and the monoamine oxidase B inhibitor (pargyline) were co-administrated to the animals prior to lesioning in order to maximize the selective dopamine depletion and spare NA neurons. The lesioned animals were administered saline or L-DOPA for two weeks, four weeks after surgery.

In paper IV, mice were administered the antidepressant imipramine in an acute or chronic manner. Subsequent experiments involved the co-administration of the somatostatin type 1 receptor antagonist, SRA880.

In paper I, III-IV, mice were euthanized by focused microwave irradiation and the brain regions of interest were rapidly dissected and stored in -80°C.

Rats
Male Sprague Dawley and Wistar rats were used in paper II and paper V, respectively.

In paper II, unilateral 6-OHDA lesions were performed. The rats were co-administrated desipramine and pargyline prior to lesioning in order to maximize the selective dopamine depletion and spare NA neurons. The efficacy of the lesion was monitored by observing the number of contralateral rotations following apomorphine administration.
In paper V, rats were administered tiotropium through an inhalation protocol utilizing a two-stage nose-only flow-past chamber. The animals were euthanized by decapitation and the tissue of interest was rapidly dissected out and stored in -80°C.

Sample preparation

Brain protein extracts
In paper II, brain protein extracts were prepared for the 2D-GE analysis, the Western blot experiments, and the SPR-MS ligand fishing experiments. The extracts were prepared by sonication in different sample buffers. For the gel experiments, denaturizing agents, such as urea and SDS, were included in the sample buffers. These agents were excluded in the preparation of brain extract for the SPR-MS experiments as it was important to keep the proteins in their native states. In paper I, III, brain protein extracts were prepared by sonication in SDS based sample buffers for Western blot analysis.

Brain peptide extracts
All brain peptide extracts (paper I, III-IV) were prepared by sonicating the tissue samples from the brain regions of interest in 0.25% acetic acid. The cell debris was removed by centrifugation and the peptide fraction was isolated through centrifugation through a molecular weight cut off filter (10kDa). In paper III and IV, an extra incubation step in 95°C was introduced to enhance inhibition of proteases, and in paper III deuterated met-enkephalin was added to the sample buffer.

Tissue sectioning
In paper I-V, tissue sections of 12-20 μm were cut using a cryomicrotome. The sections were either mounted on indium tin oxide (ITO) coated glass for subsequent MALDI analysis or on poly-L-lysine coated slides for in situ hybridization experiments.

Analysis

2D gel electrophoresis
In paper II, striatal brain protein samples from 6-OHDA lesioned side and intact side were compared by two-dimensional gel electrophoresis. The first dimension was separation by isoelectric focusing between pH 6-11 and the second dimension separation, based on size, was carried out in a 14% poly-
acrylamide gel. Differentially expressed spots were excised from the gels, trypsinized and identification was carried out by MALDI-TOF MS analysis.

Western blot
Western blot experiments were performed in paper I-III, according to protocol described in each paper. The experiments were made to verify the efficacy of the experimental parkinsonian models by targeting tyrosine hydroxylase, an enzyme present in dopaminergic neurons and the rate-limiting step in DA synthesis. In paper II, a monoclonal antibody against FKBP-12 was used to compare the levels of FKBP-12 on lesioned versus intact side of the brain.

Ligand fishing SPR-MS
In paper II, the SPR technology was used in combination with MS in order to identify binding partners to FKBP-12 from cytosolic brain extracts. Briefly, FKBP-12 was immobilized on a CM5 chip through amine coupling and cytosolic brain extracts were injected over the surface. Proteins binding to FKBP-12 were recovered in 0.25% TFA and subsequently trypsinized and analyzed by nano-LC ESI MS/MS. Control experiments on blank chips were performed in order to control for unspecific binding.

Peptide profiling and identification
Capillary nano-flow liquid chromatography coupled to either ESI Q-TOF MS or LTQ MS/MS was used for profiling and identification of peptides in paper I, III-IV. In paper I and IV, the chromatographic separation was performed on commercially available columns (pre-column and analytical column from LC Packings). In paper III, an in-house packed spray emitter was used as the analytical column. This significantly increased the resolution of the chromatography and the number of peptides detected. A column switching setup was utilized in the nano-LC. This enabled sample loading and desalting at a higher flow rate, typically 10 µL/min, decreasing the analysis time. The peptides were then eluted onto the analytical column and separated during a 40 min gradient at a flow rate of approximately 190 nL/min. The nano-LC was coupled directly to the ESI Q-TOF MS or LTQ MS for the peptide profiling and identification experiments, respectively.

MALDI tissue analysis
MALDI tissue analyses have been performed in paper I-II, and V. In paper I-II, the tissue sections were washed/ixed in ethanol before matrix application. This procedure was not performed for the drug application in paper V.
In paper I, sections from one hemisphere of MPTP lesioned mice and saline treated controls were spotted with MALDI matrix using an acoustic robotic spotter. The spots were placed at a resolution of 280 μm and the diameters of the spots were approximately 190 μm. Semi-quantitative measurements of PEP-19 were made between sections from saline and MPTP administered animals, placed on the same MALDI target.

In paper II, the expression of FKBP-12 was investigated in striatal regions of 6-OHDA unilaterally lesioned rats. The MALDI matrix was hand-spotted onto the tissue sections rendering spots of approximately 1 mm in diameter. Spectra were collected from three different locations in the striatum and the expression of FKBP-12 was compared between lesioned and intact side of the brain.

In paper V, the distribution of tiotropium was measured in lung tissue sections from rats administered the drug by inhalation. The rat tissue sections were coated with MALDI matrix (CHCA) using the Image Prep (Bruker Daltonics) and the distribution of tiotropium was determined both in MS, and MS/MS mode, at a resolution of 200 μm. Quantitative measurements of tiotropium in dosed tissue were obtained by comparing the tiotropium levels on dosed tissue to those on control tissue sections spotted with different concentrations of tiotropium standard.

**In situ hybridization**

In situ hybridization experiments have been performed in paper I-II, IV according to protocols described in each paper.
RESULTS AND DISCUSSION

Animal models of PD

Experimental rodent PD models were used in Paper I-III. These disease models were validated using both biochemical and behavioral measurements. The 6-OHDA rat model in paper II was validated post mortem by Western blot analysis of the TH expression and by DA transporter binding assay (Figure 11). TH is an enzyme involved in DA synthesis and is specific for DA neurons [191]. The amount of binding of the selective radioligand, 3H-CFT, to the DA transporter was measured in striatal tissue sections, and this was strongly decreased (>90%) on the lesioned side of the brain (Figure 11). The efficacy of the lesion was also assessed by examining the rotational behavior to apomorphine 21 days after lesioning. Apomorphine is a DA agonist, that induces contralateral rotations in unilaterally lesioned animals, due to hypersensitive receptors [167]. The number of contralateral rotations was counted during a 60 min period and all animals that showed > 1.7 turns / min were included in further analysis.

![Figure 11. A) A microphotography showing the efficacy of the 6-OHDA nigrostriatal lesion. The binding of the dopamine transporter ligand, 3H-CFT is strongly reduced in the denervated striatum. B) Histogram of the TH protein level in the intact and lesioned hemisphere.](image)

In paper I, the murine MPTP model was used and validated by the expression of TH in the striatum post mortem. The TH expression in MPTP lesioned animals were reduced by 78 % compared to saline treated controls. The 6-OHDA mice in paper III were validated by TH expression in striatal regions and by rotational behavior in response to apomorphine and L-DOPA. The 6-OHDA lesioning caused a near complete reduction of striatal TH.
Calcium regulating proteins and PD

In paper I, a nano-LC profiling study led to the identification of a small acetylated protein called PEP-19, which was significantly down-regulated in the mouse MPTP model compared to saline administrated controls. PEP-19 was identified through accurate mass matching (nano-LC FTICR MS data) against small protein and peptide masses present in SwePep and subsequent MS/MS analysis of three PEP-19 derived tryptic peptides, including the acetylated N-terminal fragment, confirmed the identity. MALDI IMS experiments (Figure 12) and in situ hybridization further showed that PEP-19 was pre-dominantly localized to the striatal part of the brain and both technologies confirmed the down-regulation found in nano-LC MS experiments.

PEP-19 is a 6.7 kDa polypeptide that belongs to a family of proteins known to be involved in calmodulin-dependant signal transduction [192]. It contains an IQ motif responsible for binding to calmodulin and an acidic residue mo-
tif responsible for modulation of Ca\textsuperscript{2+} binding to the C-domain of calmodulin. Pep-19 is highly expressed in the basal ganglia and it has been shown to suppress the apoptotic process in PC-12 cells, suggesting a potential regulatory mechanism for pathways leading to cell death [193].

The combination of nano-LC profiling and MALDI imaging approaches offers great advantages in that not only can the differential expression between physiological states be monitored but so can the distribution of the protein of interest. In paper I, both the nano-LC experiments and MALDI imaging MS showed similar alterations in expression of PEP-19.

In paper II, the experimental unilateral 6-OHDA model was used to investigate protein differences in the striatal rat brain. Initially, the differential expression was investigated using a conventional 2D-GE protocol. The software image analysis and subsequent MALDI MS analysis of tryptically digested spots revealed an up-regulation of FKBP-12 on the lesioned side of the brain. This up-regulation was further examined by MALDI profiling MS, showing a more pronounced up-regulation in the dorsal part of the striatum. *In situ* hybridization experiments revealed that FKBP-12 transcripts also were elevated in the lesioned striatum.

FKBP-12 is known to be involved in calcium homeostasis through its interaction with two intracellular calcium receptors, the inositol-1,4,5-triphosphate (IP3) receptor and the ryanodine receptor [194] and it is speculated that FKBP-12 might play an important role in neuroprotection [195, 196]. To further characterize the function of FKBP-12, studies to identify cytosolic binding partners to FKBP-12 were performed. For this purpose, an SPR-MS ligand fishing protocol was developed. This approach revealed several new binding partners to FKBP-12 in both mouse and rat brain extracts. Interestingly, the identified proteins 14-3-3-zeta/delta, pyruvate kinase isozyme M1/M2, 1-cys peroxiredoxin, and heat shock protein-70 had all previously been reported to be affected in neurodegenerative diseases [197-199].

The ligand fishing SPR-MS approach is a fairly new technology enabling both characterization of binding and subsequent identification of binding partners to a specific protein. The amounts recovered in each cycle, however, are close to the detection limit of MS [141] and careful optimization strategies were undertaken to maximize the recovered material while minimizing the unspecific binding. The recovery experiments can either be performed in the integrated micro fluidics cartridges (IFC) or in an external surface prep unit (SPU). Experiments performed in the IFC allow for real-time monitoring of binding to the sensor chip and four parallel flow cells can be utilized. The advantages of using the SPU are that a larger area (approxi-
mately 3.5 times larger) is available for immobilization and the short injection and recovery channels minimize nonspecific interactions that give rise to carry over from the injected sample to the recovered material [142].

Label-free quantitative neuropeptidomics

In papers III and IV, the global peptidomics approach was applied to investigate changes in neuropeptide expression in an experimental model of PD, and following antidepressant therapy, respectively.

Neuropeptidomics of experimental PD

In paper III, the 6-OHDA mouse model was used. The lesioned animals were administered either L-DOPA or saline four weeks post surgery. All animals were euthanized by focused microwave irradiation and the striatal part of the brain was dissected. The peptide fraction was isolated as described previously [29, 200] and analyzed by nano-LC ESI Q-TOF MS for profiling and by nano-LC ESI LTQ MS/MS for identification. In total, 90 peptides were identified through database searches and mass matching. Among these there were 12 previously uncharacterized peptides derived from seven different peptide precursors. Many of these potentially novel neuropeptides had cleavage sites and/or post-translational modifications characteristic for neuropeptides. These observations support the possibility of biological relevance.

![Figure 13](image-url)

*Figure 13. The experimental set-up of the nano-LC MS analysis. One sample from each group of animals (saline-intact, saline-lesioned, L-DOPA-intact, L-DOPA lesion) was analyzed in between the reference samples in a randomized fashion.*

The experimental set-up was designed so that a reference sample was analyzed as every fifth sample and one sample from each group of animals was analyzed in between the reference samples (Figure 13). This set-up was used in order to monitor if there where changes in the experimental conditions over time. The reference samples were also included to validate different normalization strategies prior to statistic analysis. Additionally, all samples were spiked with 40 fmol/μl deuterated met-enkephalin already in the extraction buffer. Following normalization the spiked deuterated met-enkephalin was used to calculate the endogenous levels of met-enkephalin. Over all, the samples levels of met-enkephalin were 250±10 fmol/μl (mean±SEM) and the reference samples contained 260±6 fmol/μl
(mean±SEM). This correlated very well with previously measured levels of striatal met-enkephalin [201, 202].

After validation of different normalization strategies, a linear regression normalization was applied to the data set (In manuscript). This normalization reduced the median coefficient of variance with 32 %. An advantage of using this kind of normalization is that single peaks of very high intensity do not influence the normalization as much as they would in total intensity distribution normalization.

After filtering the data, 283 peaks were submitted for statistical analysis. Two independent ANOVA models were applied: one that measured the within sample effect, hence the effect of the lesioning, and one that measured the effect between samples. In the latter case, the effect of the L-DOPA treatment is evaluated. In total, 15 peptides were found differentially regulated and 11 of those were identified with a significant score in the database searches. Most of the changes found were differences between lesioned and intact side (Table 1). The peptides found to be differentially regulated were predominantly derived from precursors such as secretogranin-1, somatostatin, cholecystokinin (CCK), and prodynorphin (PPE-B). An interesting aspect is that peptides derived from the same precursor were almost always found co-regulated (Figure 14).

Table 1. Striatal peptides found differentially regulated in the 6-OHDA mice model of PD. Peptides in italic are only identified by mass matching. CCKN: Cholecystokinin, SMS: somatostatin, SCG: secretogranin, PDYN: prodynorphin, PCSK1: ProSAAS, CBLN1: Cerebellin-1, PEBP1: Phosphatidylethanolamine-binding protein 1, COLI: Corticotropin-lipotropin, CART: Cocaine- and amphetamine-regulated transcript protein.

<table>
<thead>
<tr>
<th>PRECURSOR</th>
<th>PEPTIDE</th>
<th>L-DOPA-LESION–</th>
<th>SAL-LESION</th>
<th>SAL-LESION–</th>
<th>SAL-INTACT</th>
<th>L-DOPA-LESION–</th>
<th>SAL-INTACT</th>
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<tr>
<td></td>
<td></td>
<td>(unpaired t-test)</td>
<td>(paired t-test)</td>
<td>(unpaired t-test)</td>
<td>(paired t-test)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCKN</td>
<td>Pyro-Glu(Q)/PYPVEATPVEQ²⁴</td>
<td>0.06</td>
<td>0.712</td>
<td>0.25</td>
<td>0.155</td>
<td>0.43</td>
<td>0.024</td>
</tr>
<tr>
<td></td>
<td>APSGRMSVKNQOQPDPRE²⁴</td>
<td>0.48</td>
<td>0.308</td>
<td>-0.38</td>
<td>0.336</td>
<td>1.33</td>
<td>0.005</td>
</tr>
<tr>
<td>SMS</td>
<td>SANPAPR²⁹</td>
<td>-0.51</td>
<td>0.042</td>
<td>0.54</td>
<td>0.016</td>
<td>0.05</td>
<td>0.803</td>
</tr>
<tr>
<td>SMS</td>
<td>Pyro-Glu(Q)/RSANSNAPMREP²⁸⁰</td>
<td>-0.63</td>
<td>0.084</td>
<td>0.71</td>
<td>0.006</td>
<td>0.23</td>
<td>0.304</td>
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<td>SCG1</td>
<td>PSSKEADFATVRGEL²³⁰</td>
<td>-0.24</td>
<td>0.255</td>
<td>0.51</td>
<td>0.010</td>
<td>0.03</td>
<td>0.881</td>
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<tr>
<td>SCG1</td>
<td>GGRGPEAGHSDTREE²⁸⁵</td>
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<td>0.340</td>
<td>0.68</td>
<td>0.017</td>
<td>0.63</td>
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<td>SCG1</td>
<td>NHPOSELESTANRGEETEE²⁶⁶</td>
<td>-0.17</td>
<td>0.549</td>
<td>0.38</td>
<td>0.130</td>
<td>0.65</td>
<td>0.016</td>
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<tr>
<td>SCG1</td>
<td>VPSKQWOE²⁶⁶</td>
<td>-0.32</td>
<td>0.033</td>
<td>0.24</td>
<td>0.115</td>
<td>0.10</td>
<td>0.477</td>
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<tr>
<td>SCG3</td>
<td>ELAERPLNEQIAEADK²⁰</td>
<td>0.52</td>
<td>0.120</td>
<td>-0.50</td>
<td>0.060</td>
<td>0.94</td>
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<tr>
<td>PDYN</td>
<td>SSEMARDEDGGQGDDQVGEDLY²³⁰</td>
<td>0.76</td>
<td>0.021</td>
<td>-0.03</td>
<td>0.920</td>
<td>0.23</td>
<td>0.415</td>
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<tr>
<td>PCSK1</td>
<td>LNPSPQPAP²⁵²</td>
<td>-0.33</td>
<td>0.230</td>
<td>-0.01</td>
<td>0.978</td>
<td>-0.58</td>
<td>0.011</td>
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<tr>
<td>CBLN4</td>
<td>SKVAFSAVRSTN²⁷</td>
<td>-0.12</td>
<td>0.504</td>
<td>-0.20</td>
<td>0.212</td>
<td>-0.42</td>
<td>0.020</td>
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<tr>
<td>PEBP1</td>
<td>Acetyl-AADISQWAGPL²¹²</td>
<td>0.03</td>
<td>0.895</td>
<td>-0.05</td>
<td>0.833</td>
<td>0.79</td>
<td>0.003</td>
</tr>
<tr>
<td>COLI</td>
<td>KNIKNKH²⁷²</td>
<td>0.08</td>
<td>0.814</td>
<td>0.66</td>
<td>0.020</td>
<td>0.72</td>
<td>0.013</td>
</tr>
<tr>
<td>CART</td>
<td>Pyro-Glu/QEDAELOPR²⁹⁶</td>
<td>-0.31</td>
<td>0.413</td>
<td>0.65</td>
<td>0.013</td>
<td>0.00</td>
<td>0.990</td>
</tr>
</tbody>
</table>
Several peptides derived from secretogranin-1 were found upregulated following 6-OHDA lesioning (Table 1, Figure 14). Secretogranin-1 has previously been found to be altered in a number of neurological brain disorders, such as schizophrenia [203], multiple sclerosis [204], and Alzheimer’s disease [205, 206]. Furthermore, the administration of reserpine to rats revealed an increase in secretogranin-1 mRNA levels in substantia nigra pars compacta [207].

![Figure 14. The expression profile of peptides derived from secretogranin-1. Peptides marked with (*) were differentially regulated. All secretogranin-1 derived peptides show a trend of being upregulated in the lesioned striatum.](image)

Two peptides derived from the somatostatin precursor were also upregulated on the lesioned side of the brain. This regulation was reversed following L-DOPA treatment. There are previous reports indicating a connection between somatostatin expression and the DA system. Intracerebroventricular injections of somatostatin have been shown to increase DA synthesis and release [208, 209] and administration of DA agonists and antagonist to rats affect the somatostatin levels. The administration of the D2 antagonist haloperidol resulted in increased somatostatin levels, whereas reduced somatostatin levels were detected following co-administration of the D1 and D2 agonists SKF38393 and quinpirole [210]. The increased levels of somatostatin peptides on the lesioned side of the brain found in our study might serve as a compensatory mechanism trying to restore the DA levels in the denervated striatum.

Peptides from both CCK and PPE-B were found to be affected after L-DOPA administration. On the lesioned side of the brain, the PPE-B derived peptide was upregulated following L-DOPA treatment compared to saline treatment, and the CCK derived peptides were elevated in the lesioned striatum compared to intact side following L-DOPA administration.
changes might be related as it has previously been demonstrated that the administration of CCK derived peptides produces an increase in the release of PPE-B derived peptides [211].

Overall, this work has shown a great number of endogenous peptides from striatal mice brain samples can be detected and identified. The label-free quantitative peptidomics approach of the experimental model of PD revealed several differentially expressed peptides. Some of these can be considered novel, while others correlated well with previous findings using different technologies.

Neuropeptidomics of antidepressant therapy

In paper IV, the global peptidomics approach was applied to study the effect of a monoamine based antidepressant on hypothalamic peptide expression. The pathophysiology of depression remains largely unknown but there is evidence indicating that chronic treatment with antidepressants can correct for some observed abnormalities [212-214]. The antidepressant treatment has also been shown to affect the peptidergic systems [49, 215].

The experimental set-up was the same as in paper III, except for the analytical column used. Here, the profiling MS experiments were performed on a commercially available column (75μm i.d., 15 cm) together with an unpacked spray emitter. Compared to the previous set-up, with a packed spray emitter, this renders a chromatographic resolution inferior to the one obtained using a packed-needle column, with peptide peaks eluting during 1 min instead of 0.35 min and with less peptides detected. Despite this, more than 60 peptides were identified and an additional 14 peptides were suggested by mass matching FTICR MS data against peptide masses present in SwePep.

Following statistical analysis, several peptides were found differentially expressed after acute or chronic imipramine treatment. The affected peptides derived from precursors such as somatostatin (Figure 15), secretogranin-1 (Figure 15), and cerebellins. Subsequent in situ hybridization experiments indicated that some of these regulations occurred also on a transcriptional level (Cbln-1) while no differences in mRNA levels were observed for some precursors (somatostatin, secretogranin-1, cerebellin-2,-4).

As somatostatin has previously been implicated in depression [183, 186, 216-218], additional investigations concerning its potential role were carried out. A selective somatostatin type 1 receptor (sst1) antagonist, SRA880, was co-administrated with imipramine in mice and a number of different biological measurements and behavioral studies were obtained. In accordance with
a potential role of the sst1 receptor as an autoreceptor, an increase of somatostatin mRNA was found in hypothalamus following SRA880 administration.

Figure 15. Some of the hypothalamic peptides found to be affected following acute or chronic imipramine treatment in mice. Four peptides from the secretogranin-1 precursor were downregulated following chronic imipramine treatment (panel A-D). Two peptides from the somatostatin precursor were upregulated following chronic administration of imipramine (panel E-F). ANOVA followed by Newman-Keul’s test for pairwise comparisons. *: acute vs saline, #: chronic vs saline, *, #: p<0.05, ##: p<0.01.

Further, we demonstrated that acute administration of imipramine increased the expression of the immediately early gene NGFI-A, in hypothalamus, and there was a significant potentiation of the anti-immobility effect of imipramine in animals that were challenged with imipramine in combination with SRA880. Additional evidence, from measurements on the levels of BDNF, favors the fact that SRA880 may enhance the antidepressant effect of imipramine.

In conclusion, this study has provided further evidence that neuropeptides are involved in the actions of antidepressants and has potentially identified the sst1 receptors as targets for the development of novel strategies for treatment of depression-like states.
Tracking drug distribution in rat lung tissue by MALDI Imaging MS

The administration of drugs by inhalation is standard therapy for common respiratory diseases [219-221] and is emerging as an alternative route of delivery in other indications such as diabetes [222]. In the last paper, a drug MALDI IMS approach was developed and applied to study the distribution of an inhaled compound in rat lungs. The rats were administered tiotropium, a muscarine antagonist, through an inhalation protocol.

Figure 16. The distribution of tiotropium in rat lung tissue visualized by the ion intensity distribution of the parent ion (m/z 392) or the fragment ions (m/z 152, 170). A, D) Photographic images of a rat lung sections from an animal dosed with tiotropium. The upper panel display the distribution of tiotropium acquired in MS mode before (B) and after (C) normalization by total ion current. The lower panel displays the distribution of the fragment ions of tiotropium: m/z 152 (E), and m/z 170 (F).

The MALDI matrix and the solvents system play an important role for the efficiency of detecting a drug compound in tissue. Hence, a number of different MALDI matrices and solvent systems were evaluated to optimize the efficacy of detection of tiotropium. The best suitable MALDI matrix was CHCA and the solvent system of choice was 50:50:0.1 ACN:H₂O:TFA. The
The limit of detection of tiotropium was estimated to about 40 fmol on MALDI target and 80 fmol on tissue. MS/MS analysis of the drug produced two dominant fragment ions at m/z 152 and 170.

MALDI imaging MS experiments were performed on lung tissue sections coated with matrix using the ImagePrep station (Bruker Daltonics). This instrument enabled monitoring of matrix layer thickness and tissue wetness during the coating procedure, making the procedure more reproducible. The distribution of tiotropium was determined using both MS and MS/MS mode and their overlay of consecutive sections correlated very well after normalization by total ion current in MS mode (Figure 16).

Additionally, a novel quantitative approach to determine the levels of tiotropium in the lung was proposed. Control lung sections, placed on the same MALDI target as lung sections from dosed animals, were manually spotted with different amounts of tiotropium. Both sections were then coated with MALDI matrix and analyzed in either MS or MS/MS mode. The result from three different areas on the tissue section containing low, medium or high intensity pixels corresponded to levels in the range between 0.2 pmol and 1.3 pmol (Figure 17), which was supported by levels obtained from LC-MS analysis of tissue extracts.

Figure 17. A) Intensity distribution of the tiotropium fragment m/z 152 on a lung tissue section from a rat dosed with tiotropium. B) Intensity distribution of the tiotropium fragment m/z 152 on a control lung tissue spotted with different amounts of tiotropium. Spot 1-10 represents 10 pmol, 5 pmol, 2.6 pmol, 1.3 pmol, 640 fmol, 320 fmol, 160 fmol, 80 fmol, 40 fmol, 20 fmol, respectively. Both tissue sections were simultaneously coated with matrix using the ImagePrep station (Bruker Daltonics) and analyzed by MALDI imaging MS/MS. Average intensities from the tiotropium spiked regions were calculated and a standard curve from these values was constructed. The average intensities in three different areas (representing high, medium, and low intensity regions) on the lung tissue section from a rat dosed with tiotropium were calculated and the values were matched onto the standard curve. These regions corresponded to 1.3 pmol (I), 0.7 pmol (II), and 0.2 pmol (III) amount of tiotropium.
Monitoring drug distribution in MS/MS mode provides a more sensitive and specific analysis than in MS mode. The presence of endogenous compounds in tissue sections is very complex. An endogenous compound might therefore interfere with the drug compound by possessing the same m/z value, making the two indistinguishable in MS mode. Displaying the distribution of the drug compound in MS/MS mode enables separation between the compounds of same precursor mass. Although, there are advantages of analyzing tissue sections in MS mode. The distribution of endogenous compounds and the drug can be monitored simultaneously, also enabling detection of patterns correlating to the distribution of the drug.

Additionally, we showed that the MALDI imaging MS approach can be successfully performed using a great variety of MALDI matrix application protocols and different MALDI MS platforms.
CONCLUSIONS AND FUTURE PERSPECTIVES

Despite many years of intense research, it has been difficult to define the exact role of neuropeptides in the central nervous system. Pharmacological experiments, especially with specific high affinity antagonists, have revealed much of what we know about the function of classical neurotransmitters. Similar approaches have not been available to the same extent for neuropeptides as peptide based agonists/antagonists are often limited by their biodegradability and inability to cross the blood-brain barrier. This thesis work has brought new insight to the field of neuropeptidomics. A label-free quantitative peptidomic approach, to investigate changes in neuropeptide expression in different disease models or following drug administration, has been developed. When applying this methodology to study experimental models of PD and effects of antidepressant treatment, several differentially regulated peptides and/or peptide families were revealed. Some of these are novel findings that previously have not been associated with PD or antidepressant therapy.

In addition, the use of controlled sample preparation methods together with nano-LC MS has enabled the detection and identification of several novel potentially bioactive peptides in striatal and hypothalamic mouse brain samples. MS based methods alone can not reveal their true function in the central nervous system. Additional biochemical and behavioral characterizations are needed. However, differential neuropeptidomics approaches towards different pathophysiological states might help in suggesting roles for these potential neuropeptides. Furthermore, the ability to simultaneous monitor several peptide families and/or several peptides from the same precursor might give clues on how they interact. A complementary technique such as MALDI IMS can also provide further information about regional distribution and co-localization patterns that might be of use for interpretation of the functionality of a specific neuropeptide.

An improved understanding of the complex relationship between neuropeptides and their receptors in relation to for example dopaminergic drugs, would enable the development of novel therapies that would hopefully lead
to an improved quality of life for PD patients, and reduced side effects associated with the current L-DOPA therapy.

As part of this thesis work, an SPR-MS based methodology was developed to study protein-protein interactions. This set-up allowed us to investigate the interaction network of FKBP-12, a small protein found to be upregulated in an experimental model of PD. These types of studies are important as the functions of proteins are often defined by their ability to interact with other proteins.

MALDI imaging MS is a promising technique for the investigation of distributions of endogenous compounds, drugs, and biomarkers of disease. In the present thesis, MALDI IMS has been used as a complement to nano-LC MS based methods as well as a stand alone approach for the investigation of drug distribution in tissue sections. Both qualitative and quantitative results have been obtained and the results clearly show that it is possible to map endogenous components to specific compartments on tissue sections. Future directions could be to link MALDI IMS to non-invasive technologies such as PET and MRI for both pre-clinical and clinical applications. The complimentary data would most likely improve diagnostic precision. The recent development of tissue treatment protocols enabling MALDI IMS analysis of formalin-fixed paraffin-embedded tissues [223-225] are about to open the door to a huge archive of clinical specimen derived from various diseases. It has already been shown that MALDI IMS has great potential as a tool in toxicological studies [226], where differential protein expressions have been linked to nephrotoxicity [226]. Additionally, the distribution of drugs and their metabolites have been investigated in a number of studies [114, 117, 118, 135, 136, 227-229]. These applications will likely prove to be of great value to the pharmacological and drug development industry in the near future. The ability to compare drug levels between different tissue compartments or organs directly from tissue sections, both qualitatively and quantitatively, as proposed in paper V, would further increase the potential for the technology.
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