UPTEC X 03 019
AUG 2003

ISSN 1401-2138

ANNA NILSSON

A differential display peptidomics approach to study the brain

Master’s degree project

UPPSALA UNIVERSITET

Molecular Biotechnology Programme

Uppsala University School of Engineering

UPTEC X 03 019	**Date of issue 2003-08**

Author

Anna Nilsson

Title (English)

A differential display peptidomics approach to study the brain

Title (Swedish)

Abstract

The focus of this study was to develop a sensitive and specific analysis method to study the peptide expression pattern in the brain of an experimental model of Parkinson's Disease. A nanoflow liquid chromatography system was coupled to an electrospray ionization (quadrupol) time-of-flight mass spectrometer and the peptide levels in MPTP-lesioned mice (levodopa or saline treated) were compared against control mice.

Keywords

Electrospray mass spectrometry, nanoflow liquid chromatography, neuropeptides, peptidomics, Parkinsons Disease.

Supervisors

Per Andrén

Amersham Biosciences, Medical mass spectrometry research lab., Uppsala University

Scientific reviewer

Per Svenningsson

Karolinska Institute, Stockholm

Project name	Sponsors
Language **English**	Security **Secret until 2005-12-31**
ISSN 1401-2138	Classification
Supplementary bibliographical information	Pages **27**

Biology Education Centre Biomedical Center Husargatan 3 Uppsala

Box 592 S-75124 Uppsala Tel +46 (0)18 4710000 Fax +46 (0)18 555217

A differential display peptidomics approach to study the brain

Anna Nilsson

Sammanfattning

Parkinsons sjukdom är en av de vanligaste sjukdomarna som drabbar nervsystemet hos äldre människor. Den exakta bakomliggande orsaken är ej känd men sjukdomen karaktäriseras av nedbrytning av dopaminproducerande nervceller i hjärnan. Denna celldöd som leder till brist på dopamin ger symptom såsom skakningar, muskelstelhet och långsamma rörelser hos den drabbade patienten. Dopamin är en av många signalsubstanser som behövs för att nervceller ska kunna kommunicera med varandra. Vid Parkinsons sjukdom behandlar man patienterna med levodopa, ett förstadium till dopamin, som omvandlas till dopamin i kroppen. Andra viktiga signalsubstanser i hjärnan är neuropeptider. På senare år har en mängd olika neuropeptider identifierats och mycket forskning fokuserar på att kartlägga deras funktion.

Målet med denna studie var att kartlägga peptidmönstret i hjärnan hos en djurmodell av Parkinsons sjukdom. Möss som behandlas med ett nervgift, MPTP, utvecklar symptom som är analoga med Parkinsons sjukdom hos människa. Hjärnområden ifrån parkinsonistiska möss, levodopabehandlade eller obehandlade, jämfördes med friska möss med avseende på peptidinnehåll. Denna analys gjordes med hjälp av ett vätskekromatografisystem kopplat till en masspektrometer som detekterar och identifierar peptiderna. Skillnader i peptidnivåer mellan parkinsonistiska och friska möss skulle kunna ge indikationer på mekanismer bakom Parkinsons sjukdom.

Examensarbete 20 p i Molekylär bioteknikprogrammet

Uppsala universitet augusti 2003

TABLE OF CONTENTS

INTRODUCTION

Proteome projects involve the identification and characterization of all proteins expressed by a genome. Proteomics, the large-scale analysis of proteins, will contribute greatly to our understanding of gene function in the post-genomic era. However, classical proteomics methodology is restricted to the analysis of proteins with higher molecular masses (>10 kDa). Endogenous peptides, including well-characterized families of neuropeptide transmitters or neuropeptide modulators, hormones, and fragments of functional proteins (<10 kDa), all play a central role in many biological processes. The continuing search for new peptides and additional knowledge about the function of peptides is important as they may facilitate the treatment of diseases in the same manner as, for example, insulin in diabetes mellitus, growth hormone in dwarfism and hemoglobin in anemia [31].

Development of methods to analyze global peptide content would therefore be a critical advancement. This field of proteomics has been termed peptidomics and holds great promise for the investigation of endogenous peptide expression. Peptidomics complements molecular biological approaches in its ability to characterize amino acid sequences below 10 kDa. Also, it has the potential to directly describe changes in expression or post-translational modifications of peptides and small proteins.
However, analyzing neuropeptides is difficult because their concentration in tissue is extremely low, typically only subnanomolar concentrations [2] and they are often present in a very complex biological matrix at salt concentrations greater than 100mM. Consequently, the analysis of endogenous neuropeptides requires sensitive, specific and robust methods.

Aim of the study

The focus of this study was first to set up a sensitive and specific analysis system by combining capillary nanoscale reversed phase liquid chromatography (nano-RP-LC) with electrospray ionization time-of-flight mass spectrometry (ESI TOF MS).

Secondly, this project attempted to address the entire peptidome of the mouse striatum in an animal model of Parkinson's disease (PD). Parkinson's disease is a common neurodegenerative disorder that is characterized by the loss of nigro-striatal dopamine producing neurons [19]. The reduced level of dopamine in the striatum gives rise to symptoms like tremor, rigidity and slowness of movement in man. Although extensive research has been put into characterization of the disease, the actual cause of PD has not yet been elucidated.

Several animal models of PD exist including, for example, the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyrimidine (MPTP)-model. MPTP is a neurotoxin that degrades the dopamine producing neurons and produces symptoms analogous to PD in the mouse model [15,22]. Addition of the drug levodopa compensates for the lack of dopamine and a temporary reduction of the symptoms is obtained.

Here, the neuropeptide expression differences between untreated saline injected controls and MPTP lesioned mice (levodopa or saline treated) were studied. The relative concentrations of the peptides were compared and the differences were analyzed and identified by tandem mass spectrometry.

Mass Spectrometry

A mass spectrometer is an analytical tool used for measuring the masses of individual molecules that have been converted into ions, i.e., molecules that have been electrically charged. Mass spectrometers consist merely of three parts, the ionization source, the analyzer and the detector. The sample is introduced through the ionization source, where the molecules are ionized and extracted into the analyzer region. Here, the ions become separated according to their mass-to-charge (m/z) ratios. The ions are then registered at the detector and data of relative abundance measurements is stored. The analyzer and the detector areas are maintained under high vacuum, which allows for the molecules to travel without any hindrance from air molecules. Proteins and peptides are usually analyzed in a positive mode, detecting protonated ions ($M+H^+$).

There are different ionization methods available including e.g. atmospheric pressure chemical ionization (APCI), chemical ionization (CI), electrospray ionization (ESI) and matrix associated laser desorption ionization (MALDI). Also different mass analyzers are currently available including quadrupoles (Q), time-of-flight (TOF) analyzers and both Fourier transform and quadrupole ion traps. ESI-TOF and ESI-Q-TOF were used in this project and are therefore further explained.

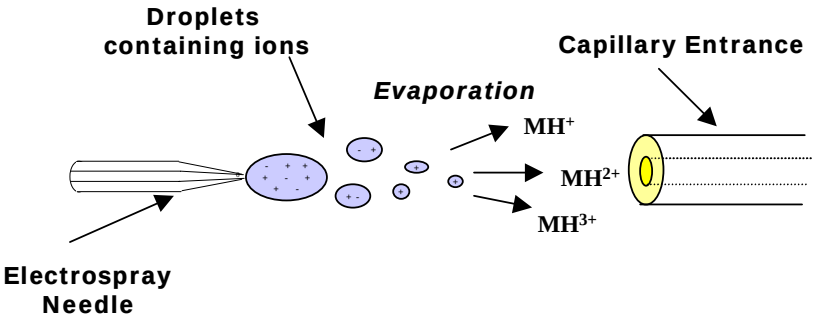


Figure 1. The principle for electrospray ionization.

In nanoflow ESI, the sample is pumped out through a thin needle, with an i.d. of about 10-50 μm (Fig. 1). By applying a high voltage, a strong electric field is created between the needle tip and the capillary entrance of the mass spectrometer. Because of this electrical field the sample is dispersed into an aerosol of highly charged droplets. These droplets diminish in size by solvent evaporation, sometimes assisted by a warm flow of nitrogen referred to as the drying gas. Eventually, only single sample ions exist and these are extracted into the capillary entrance and further into the analyzer region.

Time of Flight – TOF

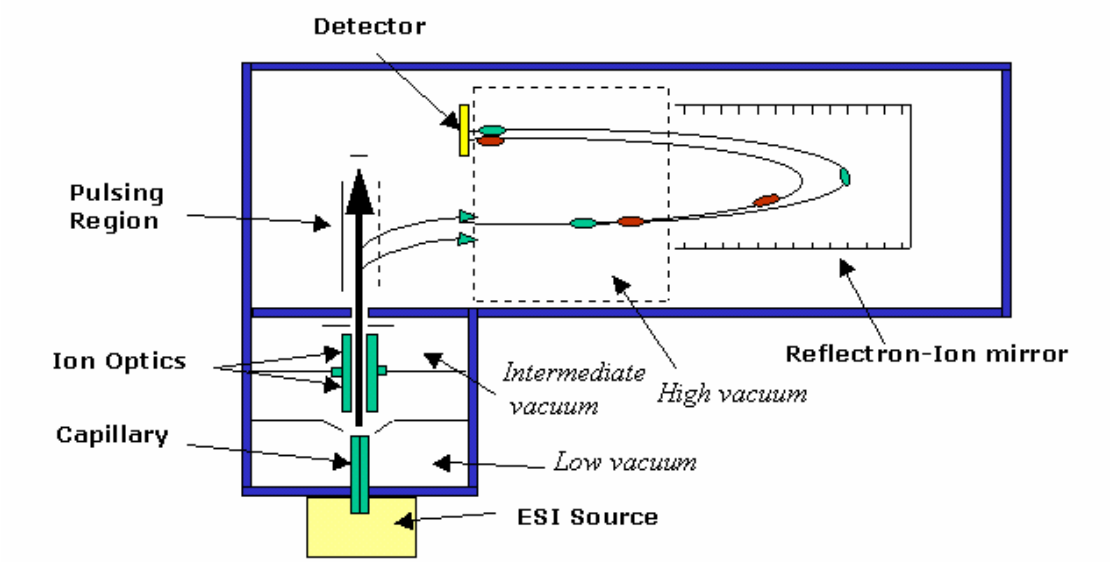


Figure 2. Schematic picture of the Ettan ESI-ToF mass spectrometer (Amersham Biosciences)

The function of the analyzer region is to separate and resolve the ions according to their m/z ratios. The ion beam is focused by a radio frequency (RF) hexapole before entering the pulsing region. In the Ettan ESI-ToF MS (Amersham Biosciences), this region can also be configured as a trap, accumulating the ions and releasing them in small packages. High voltage pulses in the pulser then accelerates the ions into the flight tube where they travel with constant velocity until they enter the reflectron (ion mirror). In the reflectron the ions are slowed down, their direction is turned and they are re-accelerated towards the micro-channel plate detector. The reflectron improves the resolution in two ways. First through time-focusing i.e., ions of the same m/z ratio but different acceleration energies arrive at the detector at the same time (Fig. 3A) and secondly by extending the flight path, which improves the separation of ions having the same energy but different m/z ratios (Fig. 3B). The ions are detected on the micro-channel plate detector, which converts the signal to a measurable current.

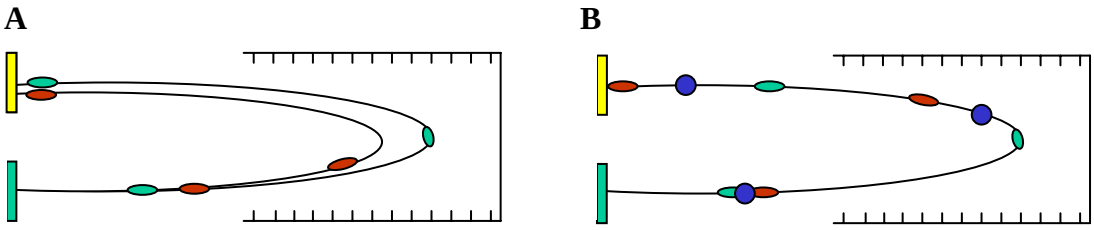


Figure 3. Two different ways in which the reflectron increases the resolution. A) Time- focusing; ions that have the same m/z ratio but different acceleration energies arrive at the detector at the same time because they are turned at different levels in the reflectron. B) The extended flight path increases the separation of ions having the same energy but different m/z ratios.

MS/MS using the ESI Q-TOF Mass Spectrometer (Micromass)

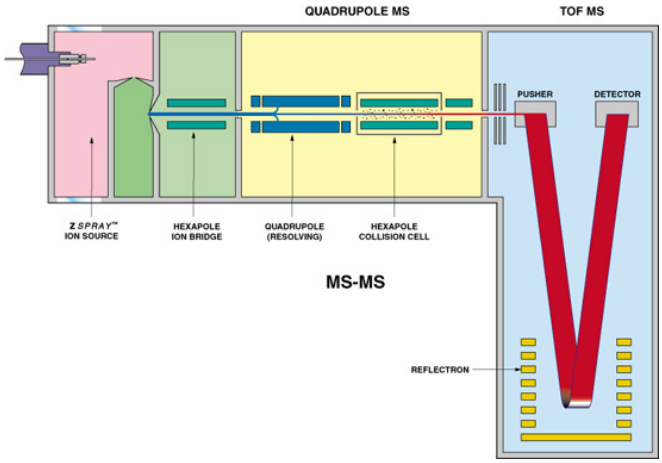


Figure 4. A schematic picture of the ESI Q-TOF mass spectrometer. This illustration was used with permission from Waters Corp.

A Q-TOF instrument (Micromass) was used to perform tandem mass spectrometry (MS/MS) in order to acquire primary structural information about the peptides and proteins. This is achieved by fragmentation of the ions inside the mass spectrometer. The Q-TOF has two analyzer regions, a quadrupole and a TOF region (Fig. 4). The quadrupole can be adjusted so that only the ions with a certain m/z ratio are allowed to pass through into the second hexapole, also referred to as the collision cell. Applying the right *ac* and *dc* voltages to the quadrupole rods will cause the ions of interest to resonate between the rods, while all other ions collide with them (Fig. 5).

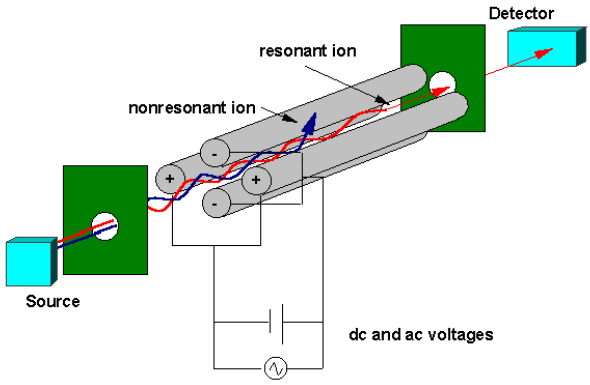


Figure 5. By applying the right ac and dc voltages, only certain ions are allowed to pass through the quadrupole and further into the collision cell. The ions of interest will resonate between the rods while the flight path of all other ions will be disrupted, as they collide with the rods. Illustration kindly provided by Marcus Svensson.

Once in the collision cell, the ions collide with an inert gas, e.g. argon, causing fragmentation of the ions. The m/z ratios of these fragments are measured at the detector and a spectrum of product ions is produced (Fig. 6).

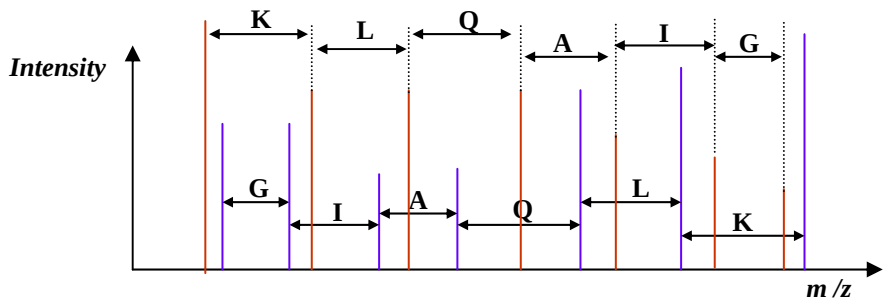


Figure 6. Simplified drawing of a product ion spectra, only displaying hypothetical peaks representing b- and y-ions.

There are three different bonds that can give rise to fragments along the amino acid backbone: the NH-CH, CH-CO and the CO-NH bonds. Each bond breakage produces two species, one natural and one charged. Only the charged species can be monitored by the mass spectrometer. Either one of the two fragments can be charged depending on the chemistry and relative proton affinity of the two species. Hence, each amino acid residue may give rise to six possible fragment ions as proposed by Roepstorff *et al.*1984 (Fig. 7)[29]. The so called a, b and c ions having the charge on their N-terminal fragment and the x, y, z- ions having the charge retain on their C-terminal end.

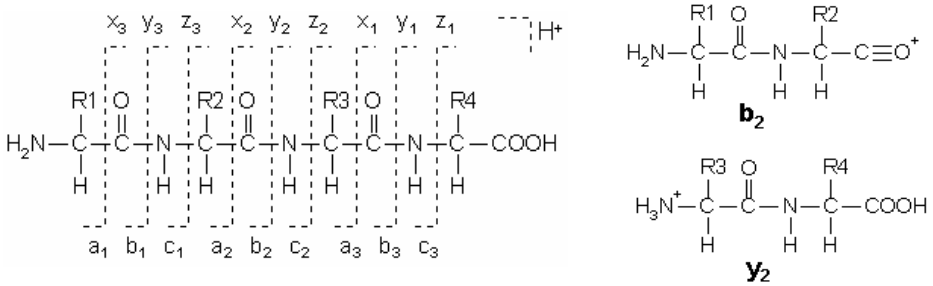


Figure 7. The nomenclature of the fragmentation of peptides as proposed by Roepstorff et al. (1984). The b- and y-ions are formed when the fragmentation occurs at the peptide bond. Illustrations used with permission from Matrix Science Ltd [1].

Ideally, the ions break at the peptide bonds producing smaller peptide fragments ions, namely the b- and y-ions (Fig. 7). The amino acid sequence of the precursor ions can be interpreted from the obtained mass spectra of the product ions, as the mass differences between the m/z peaks corresponds to the mass of an amino acid residue.
Additionally, immonium ions occur in the very low m/z range of the MS/MS spectrum. Each amino acid residue gives rise to a characteristic immonium ion, which can be useful for detecting and confirming the presence of an amino acid in the peptide. However, no information about the sequence position of the amino acid can be concluded from the presence of an immonium ion peak.

The processing and interpretation of the MS/MS spectrum can be automated to some extent but the formation of complex ion compositions and the presence of post-translational modifications make it somewhat difficult.

Nanoflow-Liquid Chromatography and Reversed Phase Chromatography

In order to get high-resolution separation of the peptides in complex biological matrices, the biological sample is separated with a high performance liquid chromatography (HPLC) system. The low flow system increases the resolution and the sensitivity and reduces the required amount of sample.

Molecules that possess some degree of hydrophobic character, such as proteins, peptides and nucleic acids, can be separated by reversed phase chromatography (RPC) with a very good recovery and resolution. The separation mechanism behind reversed phase chromatography depends on hydrophobic interactions between the solute molecule in the mobile phase and the immobilized hydrophobic ligand on the gel matrix i.e., the stationary phase. The initial binding conditions in RPC are primarily aqueous and a significant organization of water molecules exists around the solute molecules and the hydrophobic parts on the gel matrix. As the solute molecule binds to the hydrophobic ligands, the hydrophobic area exposed to the solvent is minimized, and consequently the extent of water organization is diminished leading to an increase in system entropy. In this way, it is favorable from an energy point of view for the solute molecule and the hydrophobic ligands to associate in an aqueous environment (Fig. 8B) [9,34].

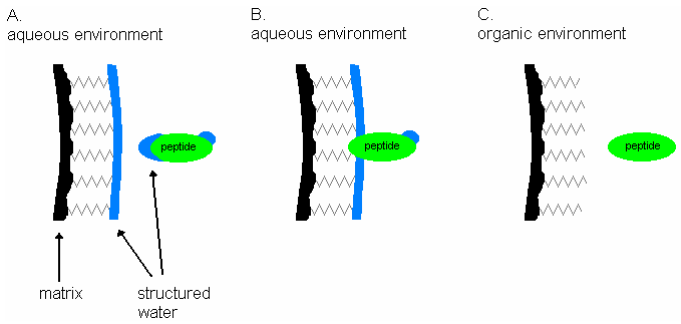


Figure 8. In an aqueous environment, it is favorable for the peptides and the hydrophobic ligands to associate. The peptides are then eluted according to their hydrophobicity as the organic content of the mobile phase is increased. Illustration kindly provided by Marcus Svensson.

The peptides/proteins can then be eluted by a decrease of the polarity of the mobile phase by increasing the percentage of organic modifier in the mobile phase (Fig. 8C). Depending on the varying extent of hydrophobicity on the solute peptides, separation of peptides is obtained in a gradient elution from aqueous to an organic solvent in the mobile phase.

Neuropeptides

Peptides are a sequence of amino acid residues connected by peptide bonds. The distinction between peptides and proteins is not exact, but if the molecules have a relatively small number of amino acids and lack substantial tertiary structure, they are considered to be peptides [6].

Peptides have been isolated from various organisms and serious and focused research on neuropeptides has been carried out for about 30 years [18]. A steady stream of peptides have been discovered and characterized. The discovery of novel peptides is continuos as improved sensitive analytical methods are developed [33]. This continuing search and additional knowledge about the function of peptides is important as they may facilitate the treatment of diseases.

The synthesis of neuropeptides is a complex process (see Eipper and Mains, 1999 [10]). Virtually all the bioactive neuropeptides are a part of larger, inactive precursor proteins. These proteins are stored in vesicles or secretory granules together with processing enzymes, so called convertases, which cut out the bioactive peptides. A precursor may contain several copies of the same peptide or several different peptides. For example, the mouse proenkephalin A precursor contains synenkephalin, leu-enkephalin, met-enkephalin-arg-ser-leu, met-enkephalin-arg-phe and four copies of met-enkephalin. Following release, the neuropeptides are often degraded in the extracellular space by peptidases [30].

Parkinson's Disease

Parkinson's disease (PD) is a chronic neurodegenerative disorder characterized by the loss of dopamine producing neurons in the *substantia nigra pars compacta* (SNc), thereby causing a decrease in striatal dopamine levels [19]. The four primary symptoms of PD are *tremor* or trembling in hands, arms, legs, jaw, and face; *rigidity* or stiffness of the limbs and trunk; *bradykinesia* or slowness of movement; and *postural instability* or impaired balance and coordination [11,27]. As these symptoms become more pronounced, patients may have difficulties in walking, talking, or completing other simple tasks. Although extensive research has been put into the field, the actual mechanisms of PD remain unknown. Theories suggest that oxidative damage, environmental toxins, genetic predisposition and accelerated aging could be involved [4,35-37].

Levodopa treatment

In the 1960s the precursor of dopamine, levodopa, was introduced as an effective drug against PD as it restores the dopamine level in the striatum. Levodopa is taken up by the remaining dopaminergic neurons and converted into dopamine, which is released by the dopaminergic terminals in the striatum [40]. Dopamine itself cannot be given because it does not cross the blood-brain barrier.

Initially levodopa treatment provides a symptomatic relief in Parkinson's disease. However, during disease progression the levodopa therapy is often associated with unpleasant side effects such as motor response fluctuations, dyskinesias and "on-off" phenomena [24,26]. More than 50% of the patients with long-term levodopa treatment develop dyskinetic movements that sometimes might become even more disabling than the disease itself [11,26].

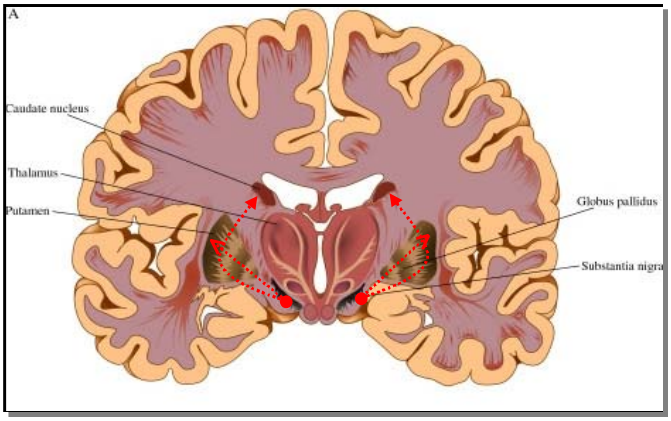


Figure 9. Schematic picture of a cross section of the brain. Red arrows indicate the nigro-striatal projection systems. Illustration was used with permission from Blackwell Publishing, UK. (Printed in Gary G. Matthews, Neurobiology Molecules, Cells, and systems., 2nd edition)

The pathogenesis for these motor fluctuations is poorly understood but it may involve imbalances in the direct and indirect striatal output pathways (Fig. 9,10) [38]. The major striatal projection systems arise from separate subpopulations of striatal projection neurons. The indirect pathway consists of neurons terminating within external segments of globus pallidus (GPe), while the neurons of the direct pathway end in internal segments of globus pallidus (GPi) and substantia nigra pars reticulata (SNr) [8,13]. The neurons of the direct pathway are primarily modulated by dopamine1 (D1) receptors (excitatory effect), containing gamma-aminobutyric acid (GABA) co-localized with the neuropeptides substance P and dynorphin. In contrast, the indirect pathway is primarily modulated by D2 receptors (inhibitory effect) and contains GABA, adenosine A2a receptors and enkephalin (Fig. 10) [12].

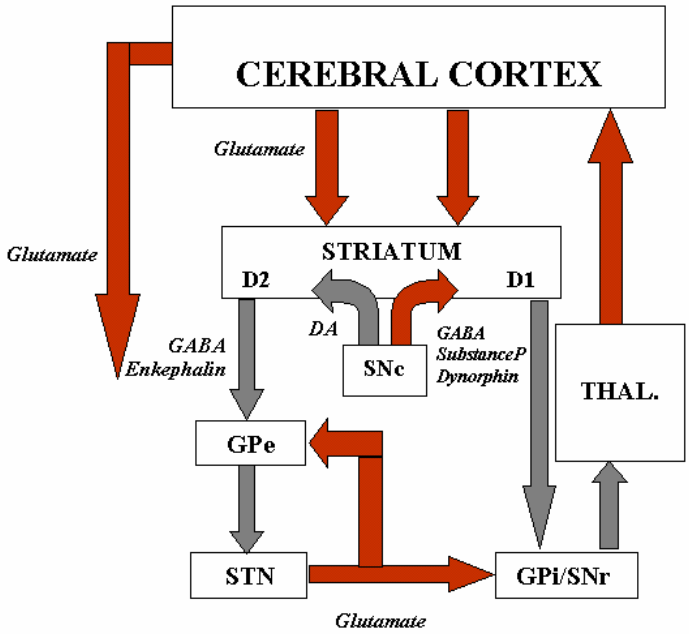


Figure 10. Simplified schematic drawing of neuronal pathways in the basal ganglia. Red arrows indicate excitatory pathways and grey arrows indicate inhibitory pathways. SNc/r = substantia nigra pars compacta/reticulata, GPe/i = globus pallidus externa / interna, STN = subthalamic nucleus.

It is commonly assumed that levodopa induces dyskinesia by excessive inhibition of neurons of the projection from the putamen to the external segment of the globus pallidus, and subsequent disinhibition of the GPe. This disinhibition leads, in turn, to overinhibition of the subthalamic nucleus (STN) and to subsequent hypoactivity in output neurons of the basal ganglia. The net effect of these imbalances would be reduced inhibition of thalamocortical neurons and over-activation of cortical motor areas [5]. There is multiple lines of evidence (see below) suggesting that the opioid neuropeptides, dynorphin and enkephalin are involved in the pathophysiology of levodopa-induced dyskinesias [16,23,25].

Animal models of Parkinson's disease

MPTP-induced parkinsonism

MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyrimidine) was first discovered in the late 1970s when several severe cases of parkinsonism were reported among young patients in California [7,21]. These patients had injected a meperidine analogue with heroin-like effects that was contaminated with MPTP. It was later shown that MPTP destroys the dopaminergic neurons of the SNc and induces symptoms analogous to PD in both primates and mice, which led to the development of a commonly used primate and mouse model of PD [15,22]. It is believed that in PD the neuro-degenerative process occurs over several years, while the most active phase of neurodegradation is completed within a few days following MPTP administration [20]. The sensitivity to MPTP follows the phylogenetic tree where the species most closely related to humans are the most vulnerable to the neurotoxin. Therefore, mice need a much higher dose than humans to produce significant SNc damage. Despite this, the use of mice as a model has become the most commonly used for both technical and financial reasons [27].

Animal models of PD indicate that differential alterations in the expression of neuropeptides are correlating with the changes in the neural activity in the basal ganglia [38]. Several studies on mRNA expression levels have shown that MPTP-induced destruction of the nigro-striatal pathway increases the level of Proenkephalin A precursor (PPE-A), the precursor of met-, leu- enkephalin and met-enkephalin /-arg-ser/-arg-ser-leu [3,14,17,39]. Decreased levels of PPE-B, the precursor of e.g. dynorphin, leu-enkephalin and beta-neoendorphin, and the Protachykinin 1 precursor (PPT), containing e.g. neurokinin A, and substance P have also been associated with MPTP-lesioning [3,14,38,39]. Following chronic treatment with levodopa, reports suggest that the decrease in PPT is reversed [38,39] and that the PPE-B mRNA level may increase over basal levels [17,38]. Different effects, both a decrease and an increase in the elevated PPE-A levels have been reported after levodopa treatment [17,39].

MATERIALS & METHODS

Animals and Tissue Sample Preparation

Mice (C57/BL6) were kept in a controlled environment and treated either by saline (n=3), MPTP (n=3), or MPTP and levodopa (n=3). The MPTP treatment was carried out for four weeks before the levodopa treatment started. The mice were sacrificed by focussed microwave irradiation and the different brain regions including, striatum, cortex, and hypothalamus, were dissected out. The brain tissue regions were suspended in cold sample buffer (0.25% acetic acid, 7.5 µl/mg tissue) and homogenized by microtip sonication (Vibra cell 750, Sonics & Materials Inc., Newtown, CT). Thereafter, the suspension was centrifuged at 20 000 g for 45 min at 4° C to sediment cell debris and undissolved material. The protein- and peptide-containing supernatant was transferred to a centrifugal filter device (Microcon YM-10, Millipore, Bedford, Massachusetts) with a nominal molecular weight limit of 10 kDa, and centrifuged at 14,000 g for 45 min at 4 °C. Finally, the peptide filtrate was frozen and stored at -80°C until analysis.

Experimental Set-up

A pilot study was performed using the Ultimate nano-LC system (LC Packings, Amsterdam, Netherlands) together with the ESI-Q-TOF (Micromass, UK) mass spectrometer. Three animals from each of the different treatment groups (saline, MPTP and MPTP/levodopa) were included.

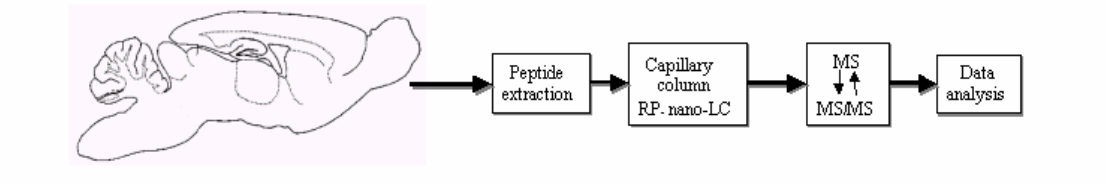


Figure 11. Flow-chart of the experimental set-up.

The Ultimate nano-LC system (LC Packings)

Five µl of peptide filtrate was injected into a 15 cm x 75 µm i.d. capillary column, packed with reversed phase particles with a diameter of 5 µm (Source 5RPC, Amersham Biosciences). The sample was desalted and concentrated by an isocratic flow of buffer A (0.25% acetic acid in water) for 35 min. The peptides were separated during a 60 min gradient from buffer A to buffer B (35% acetonitrile in 0.25% acetic acid) using the Ultimate nanoLC system (LC Packings). The sample was directly infused into the ESI Q-TOF mass spectrometer at a flow rate of approximately 300 nl /min. A 1.9 kV potential was applied to a 5 cm long fused silica spray emitter constructed in-house.

The spray emitter was made from a 75 µm i.d., 375 µm o.d., fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA). Approximately 0.5 cm of the polyimide coating was burned off and the capillary was then drawn in a micro torch flame to obtain a tapered spray emitter with an i.d. of approximately 10 µm and an o.d. of 20 µm. The high voltage was applied through electrical contact between the

solution and a metal zero dead-volume union (Valco, Houston, TX, USA) placed 5 cm from the emitter tip.

ESI Q-TOF MS

Data acquisition using the ESI-Q-TOF instrument was performed in the continuous mode and mass spectra were collected at a frequency of 3.6 GHz and integrated into a single spectrum each second. The time between each such spectrum was 0.1 s. Some of the parameter settings after tuning were set as follows: cone 39 V, extractor 3 V, RF lens 1.49, focus 0 V, ion energy 1.8 eV, collision energy 10eV, and multiple channel plate detector (MCP) 2100 V. The source block temperature was 80°C. The cone gas flow rate was set to about 100 L/h. In the wide bandpass quadrupole mode, mass spectra were collected in the *m/z* range of 300 –1000 with a mass resolution of 5800 at *m/z* 550.35.

For calibration of the Q-TOF instrument, a mixture of PEG 200, 400 and 600, with concentrations of 25, 50 and 75 ng/mL, respectively, in 2 mM ammonium acetate in 50% methanol (aq) with 0.2% formic acid, was infused with a syringe pump at a flow rate of 0.2 μl / min. At least 30 spectra were combined, smoothed and centered. To achieve an acceptable calibration for mass measurements (mean residual < mDa) a polynomial of order 4 was fitted according to the recommendations of the manufacturer.

Data Analysis

The raw data file from the mass spectrometer was converted into an ASCII file and further imported into analysis software under development by Amersham Bio-sciences. The peptides in each of the samples were extracted and visualized in 2- and 3-dimensional graphs. The peptides with the 10% highest intensity levels were included into further analysis.

Peptide levels from the MPTP-treated animals were compared against control animals. Peptides present in 5 of the 6 samples were selected and the Student's t-test was used to extract the peptides with the most differential expression.
Some known peptides were also extracted manually and differences in intensity levels were compared between the three experimental groups.

MS/MS

For MS/MS analysis and identification of the peptides, precursor ions were selected by automatic switching from MS to MS/MS mode to produce ion spectra by collision induced dissociation (CID) in the *m/z* range of 40 –1200 Da. The collision chamber was filled with argon with an inlet pressure set to about 15 psi. The collision energy was ramped from 23 –31 eV. Deconvoluted CID spectra interpretation was performed using the BioLynx and MaxEnt3 software (MassLynx 3.4, Micromass).

The peptide sequences suggested by the software were compared with the National Center for Biotechnology Information (NCBI) non-redundant database using the basic local alignment search tool (BLAST) "search for short nearly exact matches" to establish the peptide identities (http://www.ncbi.nlm.nih.gov/BLAST). Manual trials

to determine the amino acid sequence were also performed when the program failed to produce a relevant sequence.

Re-construction of a micro-LC system to a sensitive nano-LC system

Ettan micro-LC
Improvements of a liquid chromatography system were performed in order to get a functioning nano-LC flow system.
A microLC system (Ettan microLC, Amersham Biosciences) was rebuilt into a nano-LC system, by exchanging capillaries with i.d. of 75 μm to 20 μm, the calibrator loop was adjusted to increase the back pressure and the micro valve was exchanged for a nano valve. A pre-column was also introduced to the system to achieve a decreased sample application time and to make the process for desalting of sample more efficient.

A method for a nanoflow gradient was created using the Unicorn software and a summary of the different steps with approximate time intervals is presented below:
1) Pump priming, 5 min.
2) High flow capillary priming, 2 min.
3) Conditioning of the pre-column and the nano-column, 10 min.
4) Injection and desalting of sample (5μl, 12 min).
5) Nanoflow pressure adjusting, 2 min.
6) Gradient elution from 5% to 95% Buffer B (40% acetonitrile in 0.25% acetic acid) during 60 min.
7) Column cleaning in 95% acetonitrile, 5 min.
8) Re-equilibration back to 5% acetonitrile, 20 min.

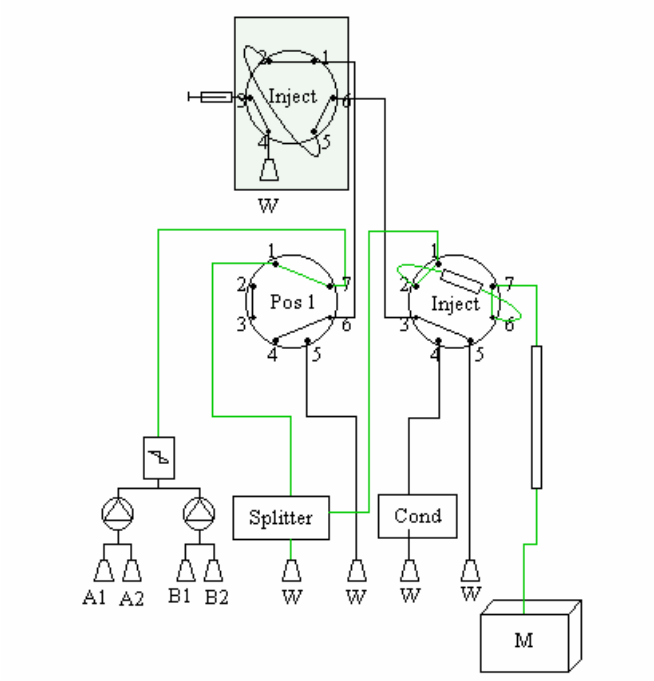


Figure 12. A schematic drawing of the liquid chromatography system after modifications for nano-flow applications. The green lines indicate the flow path during the gradient elution on the nano-column. Illustration kindly provided by Marcus Svensson.

The configuration of the flow scheme during gradient elution is visualized in Fig 12. The flow during the gradient was measured to 180 nl/min. The columns used for the test runs of the system were manufactured by LC Packings (Nano-column: PepMap™, 0.075x150 mm, 3µm and Pre-column: 5µm C18 100Å PepMap™, 300µm i.d. x 1 mm). The nano-column was mounted just upstream of the spray emitter.

Ettan ESI-ToF MS

The microflow-adapted interface on the ESI-ToF mass spectrometer was modified with an x-y-z table mounted on the door to the interface. A needle was attached onto a metal stick placed in line with the capillary entrance. The needle was connected to ground and an electric potential of approximately -1000 volts was applied to the capillary entrance to get a nebulizing spray (Fig. 13). The mass spectrometer was tuned for nanoflow applications and calibrated using a mixture of deuterated peptides (neurotensin, substance P(1-7,1-11), met-enkephalin).

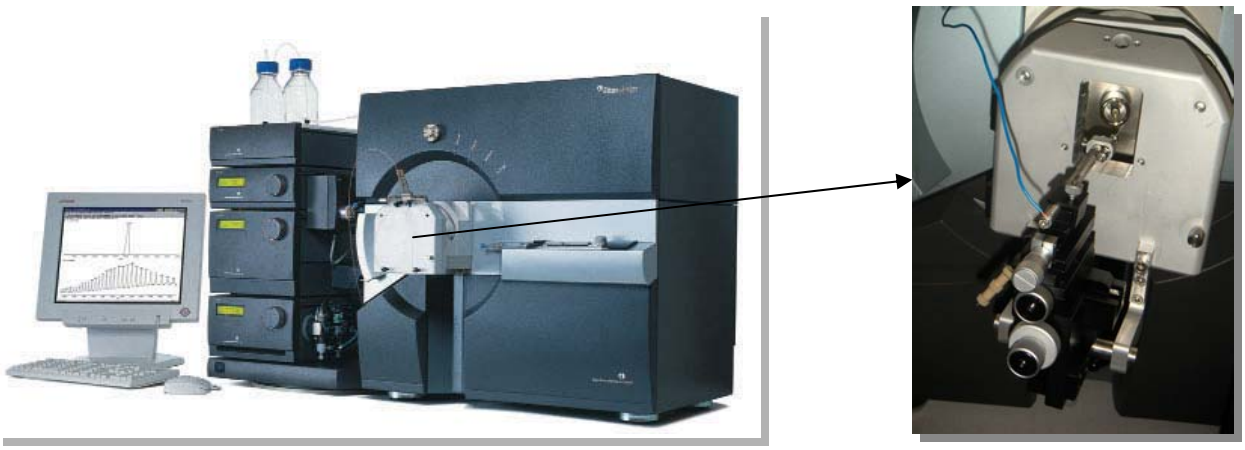


Figure 13. The interface of the mass spectrometer was modified for nano-flow applications. A hole was drilled on the door to the interface and an x-y-z-table was mounted as above (right). A metal stick, on which the needle was attached, was placed on the x-y-z table so that the needle end could be placed just in front of the capillary entrance.

RESULTS

Peptide extracts from the striatum of three different groups of mice, treated with saline, MPTP or MPTP/levodopa, were separated by reversed phase chromatography during a 60 min gradient and directly infused into the mass spectrometer. The total ion current (TIC) was recorded (Fig. 14A), representing the intensity levels of the detected peptides over time. The elution profile of a certain peptide can be visualized in an extracted ion chromatogram (EIC, Fig.14B) and the mass spectra of the same peptide ion can be obtained by combining several spectra over time (Fig. 14C)

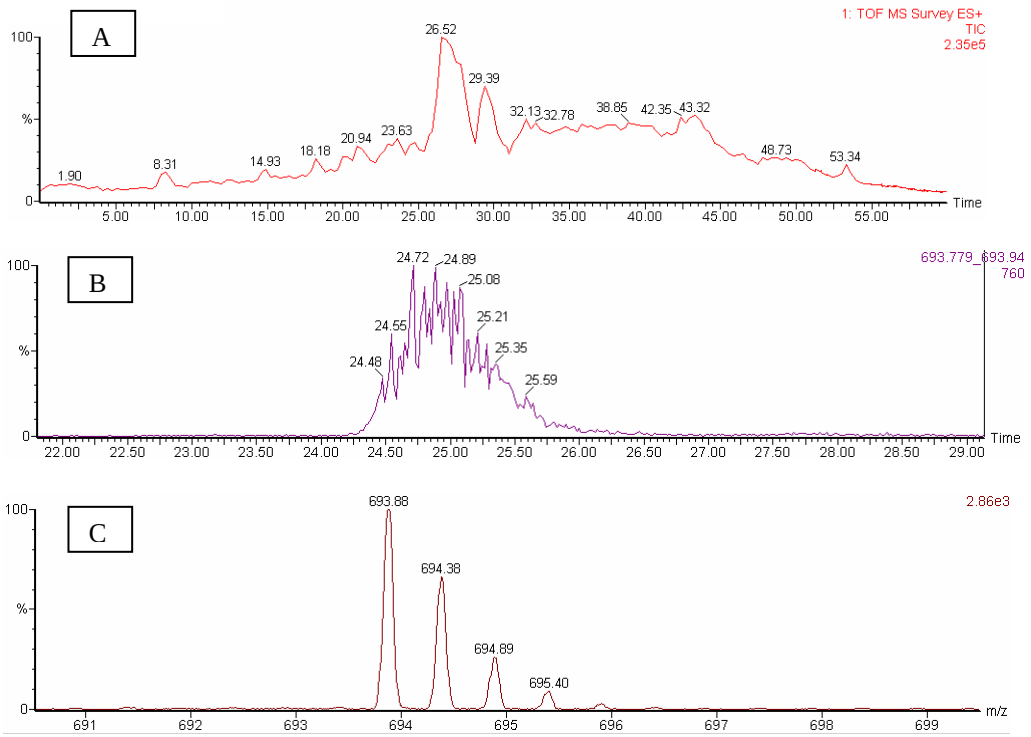


Figure 14. Examples of output data obtained from the mass spectrometer analysis. (A) represents the total ion current i.e. the sum of the intensities of all the peptide ions detected during the 60 min elution. (B) presents the EIC of the doubly-charged peptide ion with a m/z ratio of 693.88 Da displayed in the mass spectra in (C).

Detected peptides

In the different samples there were between 120-640 peptides detected during the 60 min elution. These results represent part of the striatal mouse peptidome and they can be visualized in 2-D and 3-D graphs using a proprietary software tool (Fig.15).

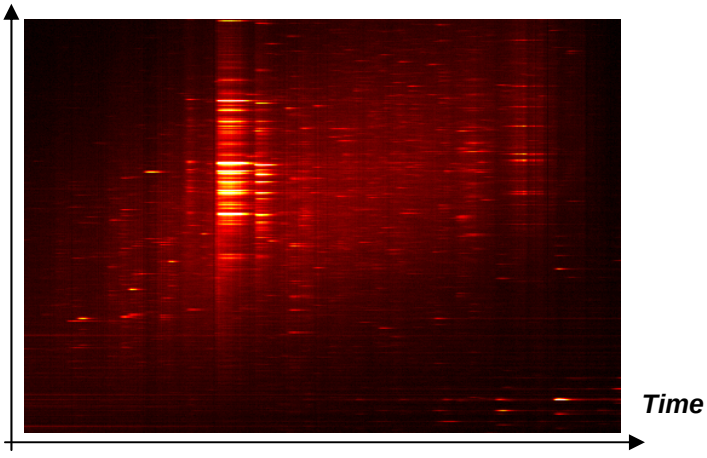


Figure 15A

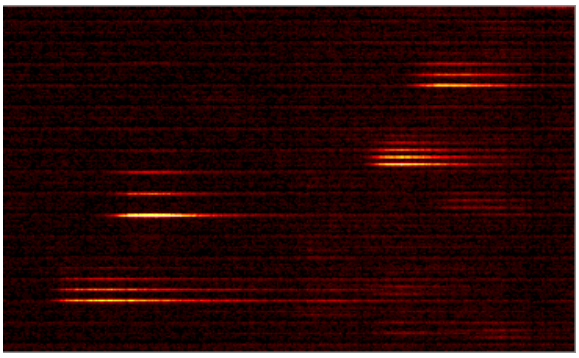


Figure 15B

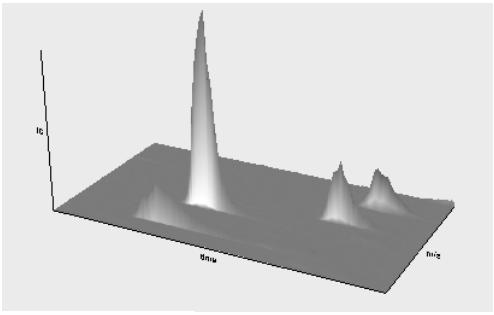


Figure 15C

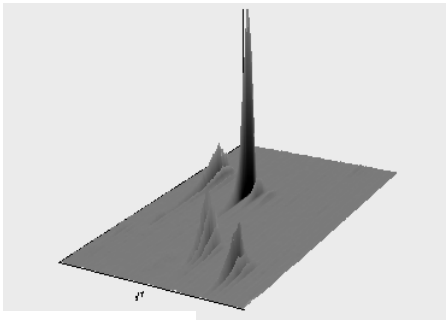


Figure 15D

Figure 15. (A) The peptidome map of the mouse striatum can be displayed in a 2-D graph. Peptide ions in the mass range of 300 –1000 Da are registered during the 60 min gradient. (B) When zooming in on a selected area, peptide peaks can be displayed showing the individual isotopes of the peptide. Graph C and D displays the same area as B but in 3 dimensions.

The peptides/protein fragments with the 10% highest intensities in each of the samples were extracted and peptide lists of the included peptides were exported to Excel (Microsoft) for further analysis. The numbers of peptides detected in the different samples is indicated in Table1.

Sample	Control1	Control2	Control3	MPTP1	MPTP2	MPTP3	MPTP+L1*	MPTP+L2*	MPTP+L3*
Included peaks	505	295	343	642	127	116	254	118	146

*L=levodopa

Table 1. Number of peptides detected in the different animals.

There were about 60 peptides that were present in all of the nine samples at the chosen detection level. Among these, there were several previous identified peptides originating from both known peptide precursors and potential novel precursors [33]. Some of these are listed in Table 2.

Mass (Da)	Precursor	Peptide
1385.8	Proenkephalin A precursor	(198-209)
876.5	Proenkephalin A precursor	Met-Enk-RF
929.6	Proenkephalin A precursor	Met-Enk-RSL
573.3	Proenkephalin A precursor	Met-Enk
555.3	Proenkephalin A precursor, prodynorphin	Leu-Enk
1812.0	Granin-like neuroendocrine peptide precursor	Little SAAS
1446.9	Pro-MCH precursor (Melanin Conc. Hormon)	Neuropeptide E-I

Table 2. Some of the peptides identified in several of the analyzed samples.

A fully automated analysis was also performed on the data from MPTP and control mice. Peptides present in five out of the six samples were included in the analysis. The fifteen most differentially expressed peptides are presented in Fig.16 together with the p-values from the Student's t-test, indicating the probability of getting the same result by chance. Three of the seven most significant peptides with a mass of 1385.8, 876.5 and 929.6 Da, respectively, derive from the biologically important opioid neuropeptide precursor Proenkephalin A.

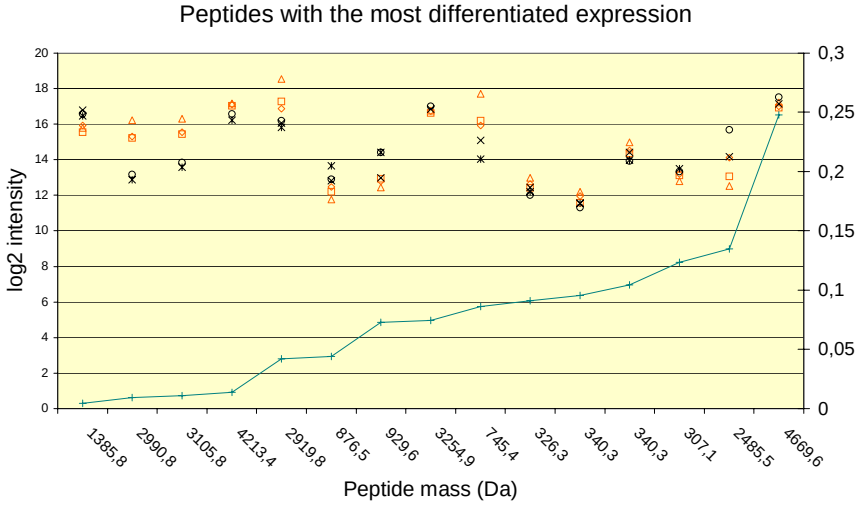


Figure 16. The most differentially expressed peptides when comparing MPTP lesioned mice to control. Peptides present in five of the six samples are included.

The proenkephalin A precursor also contains four copies of met-enkephalin. The automated analysis failed to detect the presence of this peptide in three of the samples but manual analysis showed a similar pattern of upregulation in MPTP-treated animals compared to control. Fig. 17 presents a selected region of the neuropeptide maps from the mouse striatum where the changes in met-enkephalin levels between the different treatment animal groups are displayed.

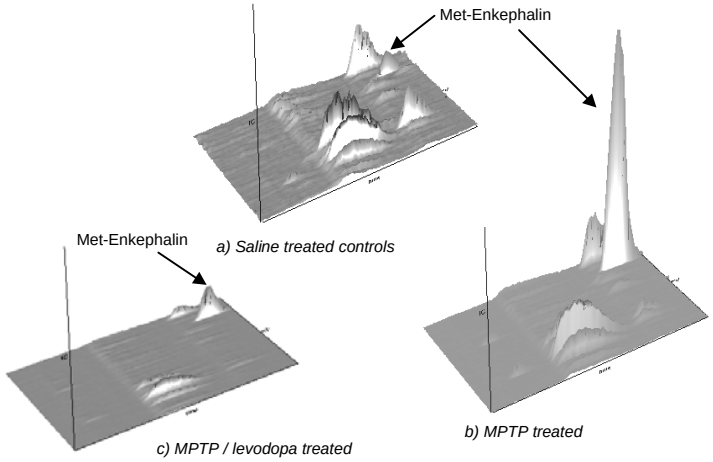
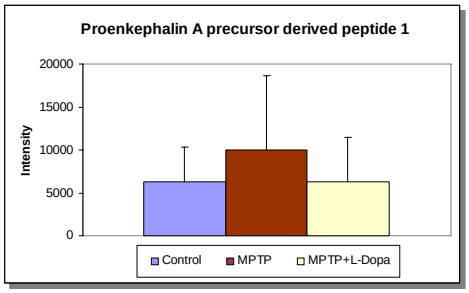
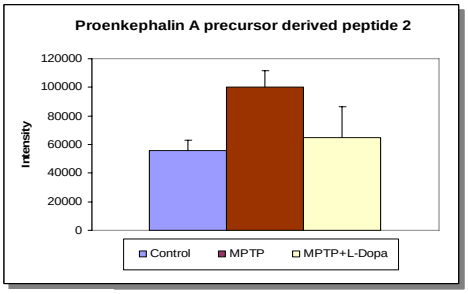


Figure 17. Three-dimensional graphs of the abundance levels of met-enkephalin in three mice treated with (a) saline, (b) MPTP or (c) MPTP/levodopa. There is a substantial increase in the level of met-enkephalin after MPTP treatment. This increase tends to be reduced following levodopa treatment.

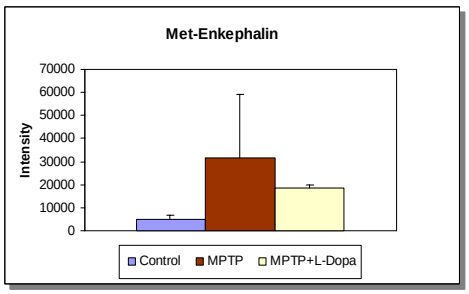
The intensity levels of four peptides derived from the proenkephalin A precursor are displayed in Fig 18. All of these peptides show a similar pattern of regulation. MPTP treatment increases the abundance levels while L-dopa tend to reduce this effect.



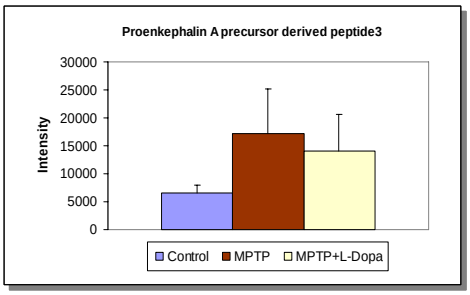
18A



18B



18C

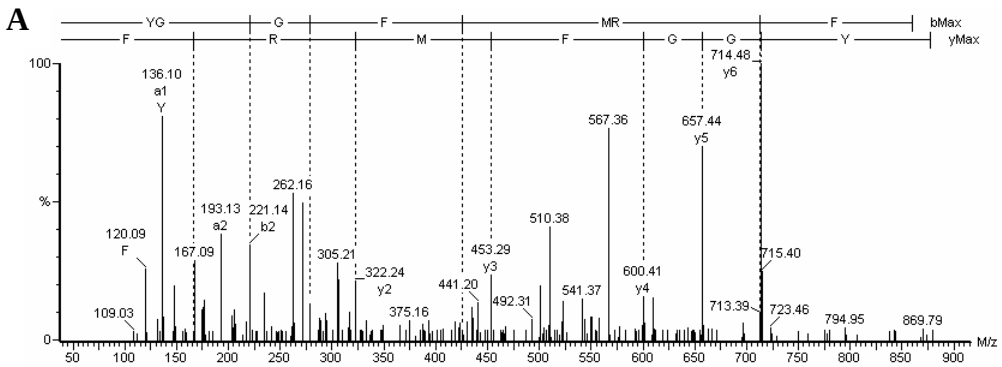


18D

Figure 18. Graphs displaying the abundance levels of four peptides derived from the Proenkephalin A precursor. All of them show a similar pattern of regulation, an up-regulation after MPTP-treatment and a reduction of this effect follows after levodopa distribution. The result is presented as mean values ± sd.

MS/MS analysis

A sample from one of the control mice was analyzed by tandem mass spectrometry. The precursor ions were selected by automated switching from MS to MS/MS mode to produce ion spectra by collision induced dissociation (CID) in the *m/z* range of 40 – 1200 Da. Spectra from selected precursor ions were analyzed by BioLynx Software (Micromass) using MaxEnt3, an algorithm that resolves multiply charged peaks onto a singly charged axis. Both automatic and manual sequencing was performed on the spectra of daughter ions. Some of the solved spectra are presented below (Fig. 19).



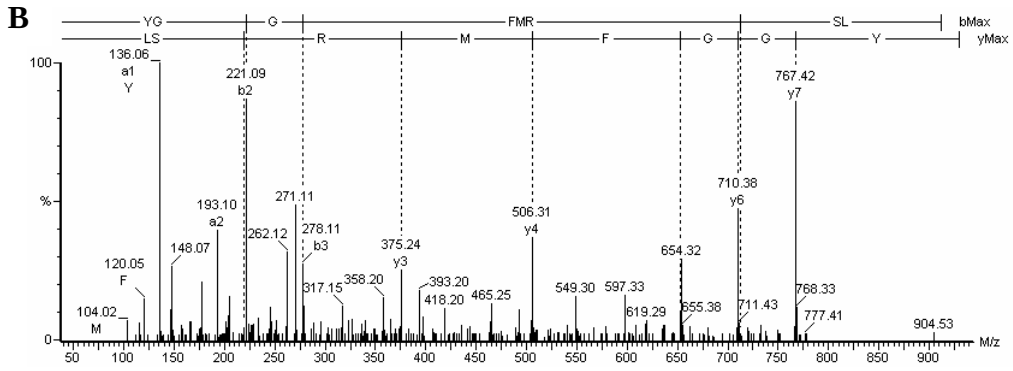


Figure 19. Tandem mass spectra of two neuropeptides derived from the pro-enkephalin A precursor. A) Met-enkephalin-RF B) Met-enkephalin-RSL

Some protein fragments were also identified among the detected peaks. As examples, there were fragments from Clathrin (AC:NP_058040) and Cytochrome C oxidase (AC:P56391) (Fig. 20).

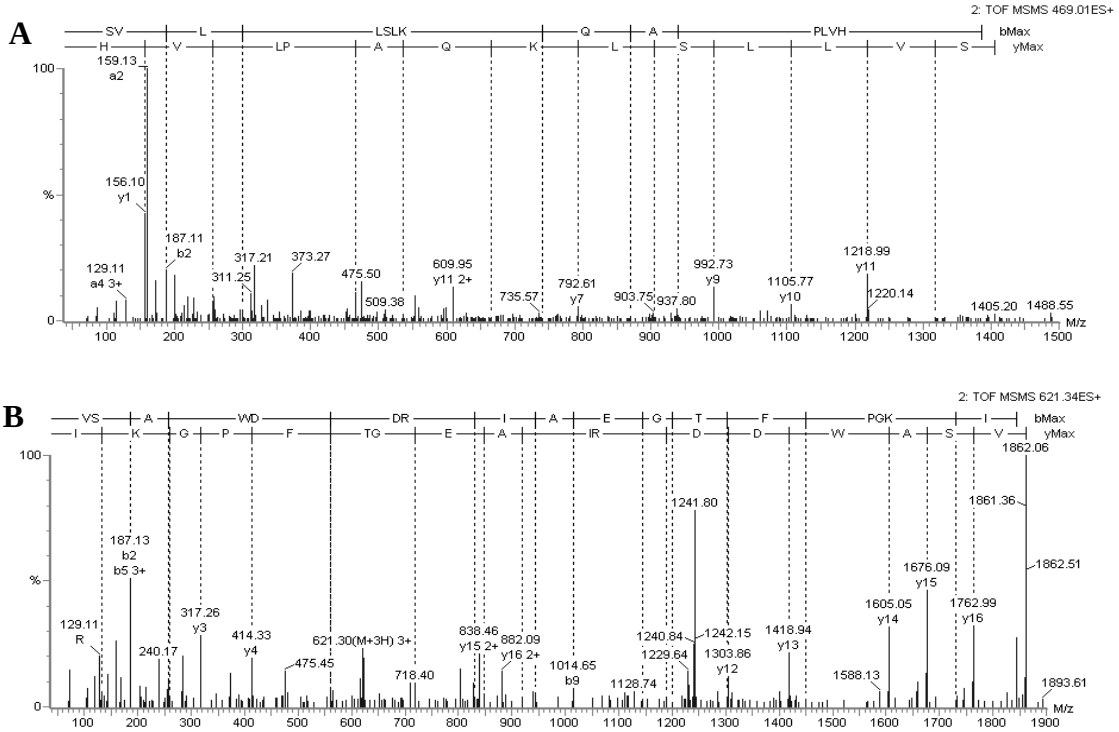


Figure 20. Tandem mass spectra of peptides identified as protein fragments derived from Clathrin(A) and Cytochrome C oxidase(B).

Preliminary tests on the Ettan nano-LC ESI-ToF MS system

Preliminary tests were performed on the Ettan nano-LC ESI-ToF MS. A sample that previously had been run on the nano-LC (Ultimate) ESI-Q-TOF was run on the Ettan system and the 2-D distributions of the peptide peaks (Fig. 21) were compared. The results indicate that the new chromatography system with the pre-column is performing better than the Ultimate system without the pre-column. This conclusion can be made because the peptide peaks are eluting during a shorter time period. However, comparing the two different mass spectrometry systems the resolution of the MS peaks seems to be lower in the Ettan system. This is visualized in Fig. 21C-D that presents a peptide with z = 2 from both the systems.

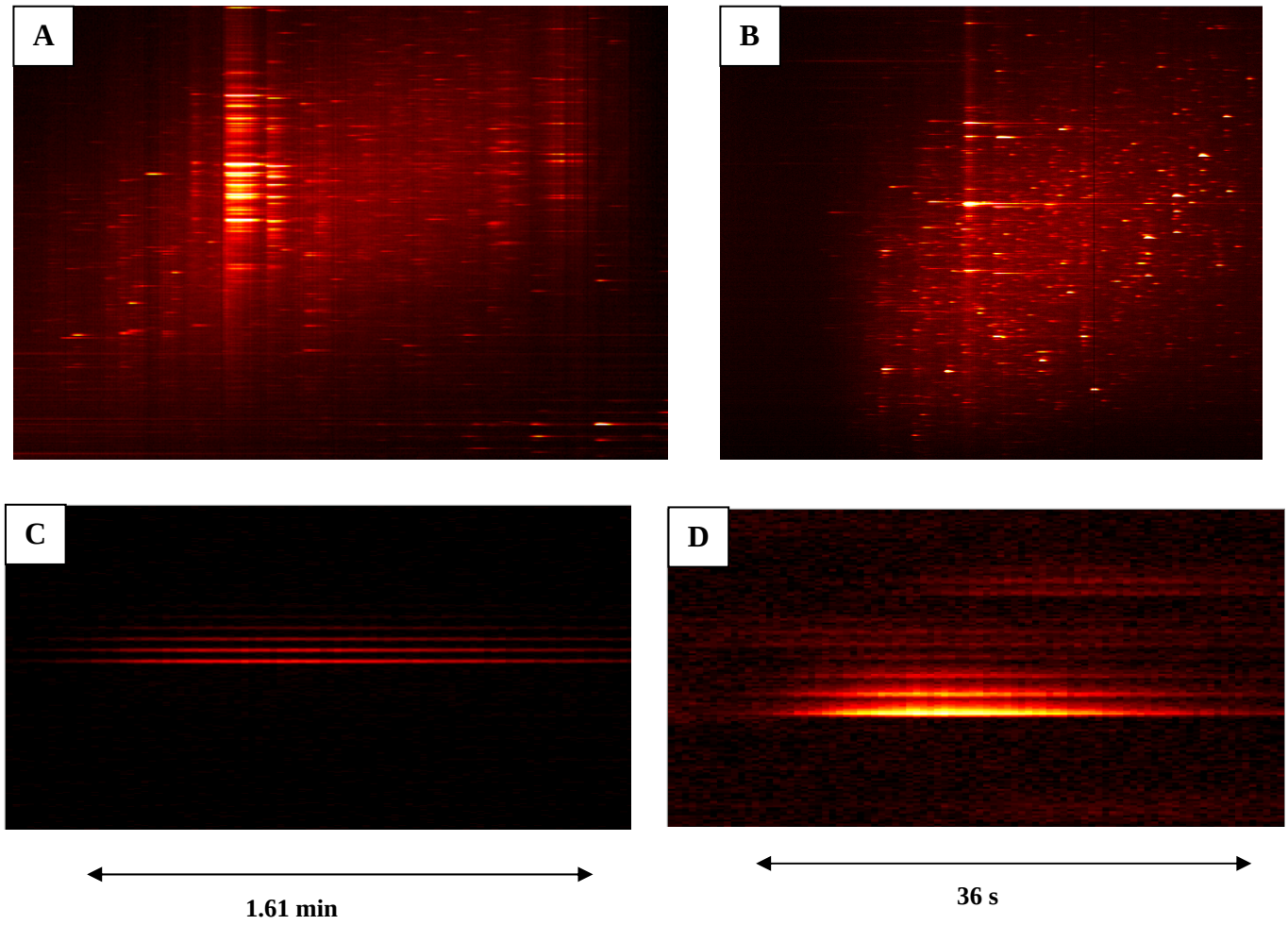


Figure 21. A comparison between the nano-LC (Ultimate) ESI Q-TOF MS system (panel A, C) and the Ettan nano-LC ESI ToF MS system (panel B, D). The same mouse peptidome is displayed in panel A and B. Both panel C and D present a doubly-charged peptide in a close-up view. The performance of the chromatography system is better in the new system (panel B, D) as the peptides elute during a much shorter time period. However, the resolution tends to be better when using the Q-TOF MS as a higher base-line separation of the peaks is obtained.

DISCUSSION

In this study it has been shown that nanoflow-LC together with electrospray Q-TOF is a very powerful technique for separating and detecting endogenous neuropeptides in brain tissue samples. Preliminary results obtained here indicate that significant changes in peptide pattern can be revealed when comparing MPTP- or MPTP / levodopa treated mice to control mice. However, the number of animals in each group in this preliminary study is not sufficient to enable good statistical interpretation. The present results should therefore be interpreted with caution.

Nevertheless, among the significant results, there are three peptides deriving from the proenkephalin A precursor (PPE-A) that are present in higher abundance levels after MPTP treatment. The levels of these peptides are then reduced after levodopa treatment. Similar upregulation of pre-proenkephalin A, following striatal lesioning, have been observed in a number of experiments on mRNA levels [14,17,28]. A reduction in the expression of PPE-A following levodopa treatment has also been observed at the mRNA level [39].

Considering the reproducibility and the quality of the samples, there are some concerns. The results show variation in the number of detected peaks between samples. This might be due to a variation in the effect of the microwaving procedure. Unpublished data by Skold *et al*. [32] have shown that there is a rapid post-mortem degradation of proteins when the proteases are not disarmed properly. A failure in protease deactivation would lead to a high abundance of protein fragments below 10 kDa. Such protein fragments could then interfere with the detection of native peptides. The MS/MS analysis supports this theory, as one of the samples with a high abundance of detected peaks was analyzed and fragments of known proteins were identified. This pattern of fragmentation was not present in the samples with less detected peptides. Another critical parameter that effects the fragmentation of peptides/proteins is the gas pressure in the collision cell. It was discovered, after running all the samples that the valve controlling the in-flow of argon gas had to be exchanged because of leakage. An increased flow of gas into the cell would result in fragmentation affecting the number of peaks detected in the sample, as one peptide would be fragmented into smaller parts.

Mass spectrometry is a very useful technique in the identification of amino acid sequences already present in databases available on the web. However, when trying to identify novel peptides and/or potentially modified peptides the identification process becomes somewhat difficult. Given the CID spectra of a peptide, its sequence has to be derived from *de novo* sequencing. In the ideal case, fragmentation only takes place at the peptide bonds, resulting in b- and y-ions. Unfortunately, this is not always the case. Other ions (a, b, x and z) may occur and peaks corresponding to a peptide that has lost a water molecule (-18 Da) or ammonia (-17 Da) are not unusual. The fact that almost all protein sequences go through some kind of post-translational modification also causes a problem. Often, sequencing programs only support a limited number of the possible post-translational modifications. However, once the sequence has been solved the corresponding peptide can often, in following experiments, be directly identified from its of m/z ratio and its elution properties.

So far, only half manual/half automated analysis methods have been used in the data mining procedure. The development and optimization of a fully automated analysis protocol would be of great advantage when considering a more high-through-put screening approach.

Despite the promising data obtained in this study, there are still some adjustments that have to be made before running biological samples on the Ettan nanoLC ESI-ToF system in a high throughput manner. However, preliminary tests give good indications for improvement of the system and that peptides can be detected with high confidence and at a high degree of separation. The system may have the potential to work better for screening applications than the ESI-Q-TOF system, as there are less sensitive parameters that could interfere. However, indications are that the resolution of the Ettan MS system is lower and that it might be more difficult to resolve highly charged peptides. Further tests have to be made to evaluate the system properly.

Future studies will be performed on an expanded group of animals that has gone through the same treatment protocol as above. Most likely, important information about the presence and regulation of endogenous neuropeptides can be extracted through this experimental set-up and hopefully this would lead to a greater understanding about the mechanisms underlying the degeneration of striato-nigral dopaminergic neurons in Parkinson's disease as well as L-DOPA-induced dyskinesias. This information would enable the development of novel therapeutic approaches to treat Parkinson's disease and its side-effects.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Per Andrén for invaluable comments on my report writing and for lots of scientific inspiration. I also want to thank Karl Sköld and Marcus Svensson for all the great help and support in the laboratory and Per Svenningsson for reviewing this work. Finally I would like to thank Amersham Biosciences for supporting this work and a special thanks to the people who contributed with lots of useful advice and fruitful collaborations.

REFERENCES

[1] "Peptide fragmentation, Sequence ions." 2000, Matrix Science Ltd., http://www.matrixscience.com/help/fragmentation_help.html (18 mar. 2003)

[2] Andren, P.E. and Caprioli, R.M., Determination of extracellular release of neurotensin in discrete rat brain regions utilizing in vivo microdialysis/electrospray mass spectrometry., *Brain Research*, 845 (1999) 123-9.

[3] Angulo, J.A., Christoph, G.R., Manning, R.W., Burkhart, B.A. and Davis, L.G., Reduction of dopamine receptor activity differentially alters striatal neuropeptide mRNA levels, *Adv Exp Med Biol*, 221 (1987) 385-91.

[4] Beal, M.F., Aging, energy, and oxidative stress in neurodegenerative diseases, *Ann Neurol*, 38 (1995) 357-66.

[5] Bezard, E., Brotchie, J.M. and Gross, C.E., Pathophysiology of levodopa-induced dyskinesia: potential for new therapies, *Nat Rev Neurosci*, 2 (2001) 577-88.

[6] Creighton, T.E., Darby, N.J. and Kemmink, J., The roles of partly folded intermediates in protein folding., *The Faseb Journal : Official Publication of the Federation of American Societies For Experimental Biology*, 10 (1996) 110-8.

[7] Davis, G.C., Williams, A.C., Markey, S.P., Ebert, M.H., Caine, E.D., Reichert, C.M. and Kopin, I.J., Chronic Parkinsonism secondary to intravenous injection of meperidine analogues, *Psychiatry Res*, 1 (1979) 249-54.

[8] DeLong, M.R., Primate models of movement disorders of basal ganglia origin, *Trends Neurosci*, 13 (1990) 281-5.

[9] Dorsey, J.G. and Cooper, W.T., Retention mechanisms of bonded-phase liquid chromatography., *Analytical Chemistry*, 66 (1994) 857A-867A.

[10] Eipper, B.A., Mains, R.E, Neuropeptide precursors. In G. Adelman, Smith, B.H. (Ed.), *Encyclopedia of Neuroscience*, Amsterdam, 1999, pp. 422.

[11] Fahn, S., The spectrum of levodopa-induced dyskinesias., *Annals of Neurology*, 47 (2000) S2-9; discussion S9-11.

[12] Ferre, S., Fredholm, B.B., Morelli, M., Popoli, P. and Fuxe, K., Adenosine-dopamine receptor-receptor interactions as an integrative mechanism in the basal ganglia, *Trends Neurosci*, 20 (1997) 482-7.

[13] Flaherty, A.W. and Graybiel, A.M., Input-output organization of the sensorimotor striatum in the squirrel monkey, *J Neurosci*, 14 (1994) 599-610.

[14] Gross, C.E., Ravenscroft, P., Dovero, S., Jaber, M., Bioulac, B. and Bezard, E., Pattern of levodopa-induced striatal changes is different in normal and MPTP-lesioned mice, *J Neurochem*, 84 (2003) 1246-55.

[15] Hallman, H., Olson, L. and Jonsson, G., Neurotoxicity of the meperidine analogue N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine on brain catecholamine neurons in the mouse., *European Journal of Pharmacology*, 97 (1984) 133-6.

[16] Henry, B. and Brotchie, J.M., Potential of opioid antagonists in the treatment of levodopa-induced dyskinesias in Parkinson's disease, *Drugs Aging*, 9 (1996) 149-58.

[17] Henry, B., Crossman, A.R. and Brotchie, J.M., Effect of repeated L-DOPA, bromocriptine, or lisuride administration on preproenkephalin-A and preproenkephalin-B mRNA levels in the striatum of the 6-hydroxydopamine-lesioned rat, *Exp Neurol*, 155 (1999) 204-20.

[18] Hokfelt, T., Broberger, C., Xu, Z.Q., Sergeyev, V., Ubink, R. and Diez, M., Neuropeptides--an overview., *Neuropharmacology*, 39 (2000) 1337-56.

[19] Hornykiewicz, O., Dopamine in the basal ganglia. Its role and therapeutic implications (including the clinical use of L-DOPA). *British Medical Bulletin*, 29 (1973) 172-8.

[20] Jackson-Lewis, V., Jakowec, M., Burke, R.E. and Przedborski, S., Time course and morphology of dopaminergic neuronal death caused by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, *Neurodegeneration*, 4 (1995) 257-69.

[21] Langston, J.W., Ballard, P., Tetrud, J.W. and Irwin, I., Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis, *Science*, 219 (1983) 979-80.

[22] Langston, J.W., Forno, L.S., Rebert, C.S. and Irwin, I., Selective nigral toxicity after systemic administration of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyrine (MPTP) in the squirrel monkey., *Brain Research*, 292 (1984) 390-4.

[23] Liminga, U., Johansson, P., Nylander, I. and Gunne, L.M., Intranigral infusion of enkephalins elicits dyskinetic biting in rats, *Psychopharmacology (Berl)*, 99 (1989) 299-303.

[24] Marsden, C.D. and Parkes, J.D., Success and problems of long-term levodopa therapy in Parkinson's disease., *Lancet*, 1 (1977) 345-9.

[25] McCormick, S.E. and Stoessl, A.J., Blockade of nigral and pallidal opioid receptors suppresses vacuous chewing movements in a rodent model of tardive dyskinesia, *Neuroscience*, 112 (2002) 851-9.
[26] Nutt, J.G., Levodopa-induced dyskinesia: review, observations, and speculations., *Neurology*, 40 (1990) 340-5.
[27] Przedborski, S., Jackson-Lewis, V., Naini, A.B., Jakowec, M., Petzinger, G., Miller, R. and Akram, M., The parkinsonian toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP): a technical review of its utility and safety, *J Neurochem*, 76 (2001) 1265-74.
[28] Quik, M., Police, S., Langston, J.W. and Di Monte, D.A., Increases in striatal preproenkephalin gene expression are associated with nigrostriatal damage but not L-DOPA-induced dyskinesias in the squirrel monkey, *Neuroscience*, 113 (2002) 213-20.
[29] Roepstorff, P. and Fohlman, J., Proposal for a common nomenclature for sequence ions in mass spectra of peptides., *Biomedical Mass Spectrometry*, 11 (1984) 601.
[30] Roques, B.P. and Noble, F., Dual inhibitors of enkephalin-degrading enzymes (neutral endopeptidase 24.11 and aminopeptidase N) as potential new medications in the management of pain and opioid addiction., *Nida Research Monograph*, 147 (1995) 104-45.
[31] Seiler, P., Standker, L., Mark, S., Hahn, W., Forssmann, W.G. and Meyer, M., Application of a peptide bank from porcine brain in isolation of regulatory peptides., 852 (1999) 273-83.
[32] Skold, K., Svensson, M., Kaplan, A., Bjorkesten, L., Astrom, J. and Andren, P.E., A neuroproteomic approach to targeting neuropeptides in the brain, *Proteomics*, 2 (2002) 447-54.
[33] Svensson, M., Skold, K., Svenningsson, P. and Andren, P.E., Peptidomics-based discovery of novel neuropeptides, *J Proteome Res*, 2 (2003) 213-9.
[34] Tanford, C.W., *Physical chemistry of macromolecules*, London, 1961.
[35] Tanner, C.M., Epidemiology of Parkinson's disease, *Neurol Clin*, 10 (1992) 317-29.
[36] Tanner, C.M., Occupational and environmental causes of parkinsonism, *Occup Med*, 7 (1992) 503-13.
[37] Tanner, C.M., Ottman, R., Goldman, S.M., Ellenberg, J., Chan, P., Mayeux, R. and Langston, J.W., Parkinson disease in twins: an etiologic study, *Jama*, 281 (1999) 341-6.
[38] Tel, B.C., Zeng, B.Y., Cannizzaro, C., Pearce, R.K., Rose, S. and Jenner, P., Alterations in striatal neuropeptide mRNA produced by repeated administration of L-DOPA, ropinirole or bromocriptine correlate with dyskinesia induction in MPTP-treated common marmosets, *Neuroscience*, 115 (2002) 1047-58.
[39] Zeng, B.Y., Jolkkonen, J., Jenner, P. and Marsden, C.D., Chronic L-DOPA treatment differentially regulates gene expression of glutamate decarboxylase, preproenkephalin and preprotachykinin in the striatum of 6-hydroxydopamine-lesioned rat, *Neuroscience*, 66 (1995) 19-28.
[40] Zigmond, M.J., Abercrombie, E.D., Berger, T.W., Grace, A.A. and Stricker, E.M., Compensations after lesions of central dopaminergic neurons: some clinical and basic implications, *Trends Neurosci*, 13 (1990) 290-6.