Striatal neuropeptides associated with L-DOPA-induced dyskinesia

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Abstract

Parkinson's disease (PD) is the most common neurodegenerative disease, with approximately 6 million sufferers in the world. The patients are usually diagnosed between the ages 50-70 years and as the disease progress more symptoms may occur. The cause of the disease is unknown, but the characteristic of PD is that it is a selective degeneration of dopaminergic neurons in the substantia nigra, which leads to an absence of dopamine release in striatum. This affects the motor system of the central nervous system, resulting in a movement disorder. It is not until 70% of the dopamine neurons have been lost that the patient will show the first symptoms of the disease. This will thereby complicate the treatment of the disease. When the motor symptoms are established, it is extremely difficult to stop or reverse the disease. The most effective treatment of patients with Parkinson's disease is L-DOPA that is converted to dopamine in the brain. Dopamine agonists have also been used as a treatment alternative in PD in attempts to avoid the motor symptoms. However, everyone who gets Parkinson's disease will eventually be required to start using L-DOPA as the disease worsen and dopamine agonists loses in efficacy. The majority of patients being treated with L-DOPA have disturbing adverse reactions during the treatment as involuntary, abnormal movements, known as dyskinesias may develop. Once you have got dyskinesias it will not disappear and cannot be effectively alleviated, and will therefore often impair the person's daily life. It is therefore a major focus in research to find new treatments focused on L-DOPA induced side-effects. In a previous study several unknown neuropeptides has been detected, which might have a strong correlation between the L-DOPA and dyskinesia. The main purpose of this study is to identify these neuropeptides and locate them in the striatum of high and low dyskinetic rats. This was performed by using MALDI imaging mass spectrometry and the computer program FlexImaging that provides a visualization of peptides and proteins and their distribution in tissue sections. Of the 76 peptide families that were found in rat brains, 33 of them were identified in this study, and some of them proved to be of high interest. From these 33, three neuropeptides; corticoliberin, P3(42) and cholecystokinin-39, were chosen for further investigation. We saw elevated levels of these three in both high and low dyskinetic rats. We were able to verify with antibodies their localization in normal brains in the striatum and hippocampus. In the future, these peptides will be studied in dyskinetic rats to verify if they are significantly elevated in dyskinetic animals. These three peptides together with the other identified peptides are very interesting in hopes to be able to cure or ameliorate L-DOPA-induced dyskinesias.
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Introduction

Parkinson's disease

Parkinson's disease (PD) is the most common neurodegenerative disease, with approximately 6 million sufferers in the world. The disease affects the extrapyramidal motor system of the central nervous system (CNS), giving the movement disorders. The typical symptoms of the disease include tremor, rigidity, akinesia and bradykinesia. The patients are diagnosed usually between the ages 50-70 years and the further course of the disease, the more symptoms may occur [1].

The cause of the disease is unknown, but the characteristic of the PD is that there is a selective degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNC), which leads to a absence of dopamine release in the dorsal striatum (caudate nucleus and putamen) [2].

Dopamine is a neurotransmitter produced in the dopaminergic neurons from tyrosine via DOPA. Dopamine is packed in vesicles, transported through axons and reacts when an action potential comes to the synapse. The dopamine which is released into the synapse may then be reabsorbed via the dopamine transporter into the cytoplasm and then packed into in vesicles that can be released again, or be degraded by monoamine oxidases. If the dopamine reuptake is blocked the dopamine will be methylated by catecholamine-O-methyltransferrases to 3-methoxytyramin and degraded [3].

When an action potential comes to the dopaminergic neurons in the SNC they will modulate glutamatergic excitatory signals to cortical, limbic and thalamic areas in the dorsal striatum via the dendritic branches of the GABAergic medium spiny neurons (MSN). MSN is the most common type of neurons in the striatum and can be easily distinguished by their connectivity to the basal ganglia output stations, nigra pars reticulata and globus pallidus pars interna (SNr-GPi). The basal ganglia are involved in motor activity and consist of subcortical structures in which the major components are the dorsal striatum and ventral striatum (nucleus accumbens). There are two types of MSN neurons that each has a signaling pathway that contains two different selective G-protein-coupled receptors, dopamine 1 receptor (D1R) and the dopamine 2 receptor (D2R). When dopamine is released in striatum, these two receptors will stimulate the two signal paths, the direct and the indirect signaling pathways [2, 4].
When the dopamine stimulates the dopamine receptors, the indirect pathway reduce motor activity and act as a brake, whereas the direct pathway will facilitate movement (act as a gas). In the indirect pathway, the GABAergic MSN will inhibit the release of GABA of the globus pallidus pars externa neurons, which in turn projects to the subthalamic nucleus (STN). The STN is glutamatergic and stimulates the globus pallidus pars interna (SNr-GPi). In the direct signal pathway, the D1-receptors inhibit SNr-GPi without any intermediate steps [4]. When these receptors are activated they will connect to different G-proteins that either increase or decrease the adenylyl cyclase enzyme (AC), which converts ATP to cAMP. Activation of the D2 receptors leads to Gαi/o-mediated inhibition of AC, which will then provide a reduction in the amount of cAMP. Activation of the D1-receptor provides a Gαolf-mediated stimulation of AC, resulting in an increase of amount cAMP. In a healthy person the SNr-GPi will get both stimulatory and inhibitory signals where GABAergic neurons in turn send inhibitory signals to the thalamus. Thalamus will then send signals to the cortex for stimulation of motor activity [2].

For patients with Parkinson's disease, the dopamine receptors will not be activated since no dopamine is released in the striatum. Globus pallidus will therefore not get any inhibitory signals from the two signal paths. The subtalamic nucleus will still stimulate the area which amplifies the inhibitory signals to the thalamus [4].

It is not until at 70% of the neurons that has been lost that the patient will show the first symptoms of the disease, since there are several compensatory mechanisms behind the degeneration of neurons. This may explain why the clinical diagnosis is so late and thereby complicate the treatment of the disease. When the motor symptoms are established, it is extremely difficult to stop or reverse the disease. [5]

As previously mentioned, the cause of the degeneration of dopaminergic neurons remains unclear some possible major factors may include mitochondrial impairment, calcium homeostasis, oxidative stress and ubiquitin-proteasome dysfunction [5].
L-DOPA-induced dyskinesia

The most effective treatment of patients with Parkinson's disease is the dopamine precursor L-DOPA, L-3,4-dihydroxy-phenylalanine. The treatment is very effective in the early stages of the disease but over time L-DOPA causes more motor symptoms including L-DOPA-induced dyskinesia (LID) [2,5,6].

As a drug, L-dopa has two major disadvantages, one is a short plasma half-life and two, an irregular absorption from the gastrointestinal tract. This leads to fluctuations of the drug from high to low plasma levels [5]. It has also been found that an increased pulsatile stimulation of dopamine receptors will increase the risk of development of LID [2].

The reason that the L-DOPA works best in the early stages of the disease is that dopamine can be stored in the remaining presynaptic dopaminergic terminals in striatum. In this way, the fluctuations in the plasma half-life but also the irregular absorption from the gastrointestinal tract will be avoided. These will disappear however the longer the disease progresses and more neurons are lost [5].

Dopamine agonists have been used as a treatment alternative in PD in attempts to avoid the motor symptoms. It has been shown that short-acting dopamine agonists have a higher risk than long-acting dopamine agonists to induce dyskinesias. But the short-acting dopamine agonists would have less propensity to induce dyskinesias if they were administered as a continuously infusion to avoid these fluctuations [5]. In the beginning, dopamine agonists reduce the motor problems. However, after using them a while, they will lead to the same motor symptoms as L-DOPA treatment as they are only delaying the onset of dyskinesias. This will also lead to that the patients will still have to start with L-DOPA treatment in order to obtain a sufficient therapeutic effect when the disease progress is getting worse [2].

The drug induction of dyskinesias may be due to a disorder of basal ganglia function due to the increasing fluctuation of drug plasma levels. The fluctuation that occurs with L-DOPA treatment alters the basal ganglia output (1, 2). Besides that it is a result of the L-DOPA therapy, it is therefore most likely that there is a combination of degeneration of neuronal cells in the substantia nigra and the defective signaling between the striatum and motor cortex that leads to dyskinesias [5]. It has also been shown that possibly the transcription of the neuropeptide dynorphin has been activated by several transcription factors in Fos family which would stimulate D1 receptors to increase cAMP and thereby lead to dyskinesia. It is therefore a major focus now to identify all the mechanisms that may lead to the development and expression of dyskinesias [7].
Proteomic analysis

An organism's genome is basically constant, but the genes are expressed differently in different cell types, and also changes from one period to another. After translation the proteins are chemically modified which are necessary for the protein's function. The mRNA analysis has also shown that there is no correlation between mRNA and the content of proteins [8,9]. Therefore, it is important to study protein structures and functions (proteomics). Proteomics studies are much more complicated than genomics studies because the proteome is the complement of both proteins and the changes made after translation to a specific set of proteins [10].

Proteomics consists of large-scale experimental analysis of proteins and are used in protein purification and mass spectrometry. For the identification of post-translationally modified (PTM) proteins it is possible for example develop an antibody that is specific for a certain modification and thereby determine the proteins that have undergone modifications which one is interested in. However, it is possible to find several PTM simultaneously with MS/MS analysis, which makes it more effective than the immunohistochemistry. Another way to determine posttranslationally modified proteins of interest is to subject a mixture of proteins in a "two-dimensional gel electrophoresis [11].

Animal model and lesion surgery

By injecting 6-hydroxydopamine (6-OHDA) into the nigrostriatal pathway of rats the pathophysiology of PD recreated, by inducing a specific and reproducible degeneration of dopaminergic neurons. After administration of L-DOPA the rats will even display similar motor symptoms, dyskinesia, as seen in patients with PD. However, not all rats will develop dyskinesia after L-DOPA therapy, just as not all the patients with PD do, which allows studying the difference between dyskinetic and non-dyskinetic rats. By recreating the pathophysiology the significant differences in protein and peptide expression can be investigated after degeneration of dopamine and the treatment with L-DOPA [12]. A lesion surgery/L-DOPA experiment was performed in a previous study by our research group and used in the analyses in this master thesis. The experiment followed the Swedish legislation on animal experimentation (Animal Welfare Act SFS1998: 56) and European Union legislation (Convention ETS123 and Directive 86/609/EEC) and was approved by the Uppsala animal ethical committee [13, 14].
Behavioral analysis
A cylinder test was performed a few weeks after lesion surgery to evaluate the rats' forelimbs. In the cylinder it was registered the number of times the rats touched the glass wall with its forelimb when they explored the new environment. The rats that used their contralateral forelimbs less than 30% of the time have lost more than 70% of the striatal dopamine. These rats were chosen for further studies. In dyskinesia test the rats were treated daily with L-DOPA. Their abnormal and involuntary movements were registered immediately after administration in time intervals for 3 hours every other day. By using a scoring system, the rats were then divided into two subgroups depending on the severity of these involuntary movements. After the last L-DOPA treatment, the rats were anesthetized and killed by decapitation. The extracted brains were frozen on dry ice and stored at -80°C [13,14].

Tissue preparation
A few brains from normal control rats had been pretreated with 4% paraformaldehyde (PFA) for 24 hours. Then they were incubated for another 24 hours in a solution of 20% sucrose in phosphate buffer (PB) before they were frozen. The frozen brains were cut in thin sections (striatum, substantia nigra and hippocampus) on a cryostat microtome at 18µm. The sections were directly thaw mounted on positive charged glass slides for later use in immunohistochemistry for frozen sections. For the free floating immunohistochemistry, sections were cut (40µm) and placed into an anti-freeze solution consisting 30% ethyenglycol and 30% glycerol in PB and stored in the freezer at -20°C [13,14].

MALDI imaging MS and identification of neuropeptides
Matrix-assisted laser desorption/ionization (MALDI) imaging mass spectrometry (IMS) is a spectrometry based image technology. MALDI-IMS is used for characterization, localization and even quantification of many different molecules in one biological tissue at the same time. Besides this great advantage the MALDI-IMS has a high sensitivity and a high molecular specificity [15]. In addition, it is one of the few methods that can detect hundreds of low molecular weight compounds, such as endogenous neuropeptides, in one single experiment. However, the disadvantage of MALDI IMS is that initially only the masses can be seen and therefore it must be combined with LC-MS/MS to identify peptides in MALDI IMS [16]. In MS/MS experiments the peptide collides with inert gas molecules and breaks at the peptide bond between amino acids, resulting in a fragmentation patterns that can be used for identification. Sometimes certain peptides are not fragmented as effective as others in the mass spectrometer and therefore are not detected. This may depend on chemical
properties or low intensity of the signal from the selected precursor ion. In order to better identify the fragmented peptides in MALDI IMS peptide sequences are compared with databases that contain the accurate masses for peptides of interest and their fragments. One of these databases is MASCOT that returns scores that reflects statistics over how the experimental data match the sequence in the database. The scores are based on probability which can be used to assess whether a result is significant or not. The higher the score, the better it is because there are more matching peaks. This help to filter out spectra below a score threshold of interest and thereby increasing the quality of their results [17, 18]. In a previous study, MALDI-IMS was used for the investigation and detection of striatal neuropeptides in a rat model of LID in PD. In the study, 12 µm striatal brain sections from both low dyskinetic (LD) and high dyskinetic (HD) rats were cut by a cryostat microtom which was then mounted on conductive glass that is suitable for MALDI-IMS. In the detection and imaging of neuropeptides by MALDI-IMS DHB (2,5-dihydroxybenzoic acid) was used as matrix for the MALDI. The peaks from the mass spectrometry and the imaging were then visualized in the computer program FlexImaging for identification of the neuropeptides [13]. However, MALDI-TOF MS/MS was not effective for identification of neuropeptides and therefore neuropeptides were extracted from the striatum of one normal intact rat and subjected to LC-MS/MS analysis as previously described [13,14].

**Aim**

In a previous study our research group has discovered hundreds of peptide peaks that show significantly increased or decreased levels in high dyskinetic animals compared to low dyskinetic animals [13]. A few peptides have already been identified, for example neuropeptides of the dynorphin and enkephalin family. The main purpose of this study is to develop a new method of neuropeptide identification in MALDI IMS and to locate them in striatum of high and low dyskinetic rats.
Materials and Methods

Materials

Positive glass slides (Superfrost® Plus, Menzel-Gläser, Brunswick, Germany) compatible with immunohistochemistry. The primary antibodies (Anti-beta Amyloid 1-42 antibody, Anti-Corticotropin Releasing Factor antibody and Anti-Cholecystokinin 8 antibody) used for immunohistochemistry were purchased from Abcam plc. (Cambridge, Massachusetts). The secondary antibodies anti-rabbit-Alexa Fluor 488 and Alexa Flour 555 were purchased from Invitrogen (Carlsbad, California). DAPI (4',6-diamidino-2-phenylindole) was purchased from Sigma-Aldrich (StLouis, Missouri) and the Vectashield from Vector Laboratories Inc. (Burlingame, California).

Method development for peptide classification

In the previous study, there were over 1,500 masses in the large compilation of LC-MS/MS-analysed peptides from rat brains [13,14]. In order to know which to priority first to investigate it needed a method development. By classifying the peptides in families and functions, it may be easier to choose the peptides that may be of interest to study. With the help of a database, the peptides can easily be classified. UniProt is a freely available database that provides a high quality protein sequence and functional information. UniProt contains large amounts of information about biological functions of proteins which are retrieved from reliable research literature. By inputting the peptide names, the peptides can be classified by attributes of interest [19].

Identification and localization with FlexImaging

FlexImaging Bruker is a computer program which is easy to use for visualization and localization of biomarkers such as peptides and proteins from tissue extracts, in this case, neuropeptides in the brain sections. The program is a necessary complement to MALDI-IMS since it provides a visualization of the distribution of peptides. In FlexImaging the entire spectrum from MALDI-IMS is displayed and can be read in the form of ion intensity images shown in the display. With the marker the peaks can be selected from the spectrum which will provide a visualization of peak intensity as color intensity. The peaks of interest can be selected for viewing on the display and thereby confirms the localization of the peptides of interest. In a result filter, the interesting signals can be added in which the degree of intensity and color can be modified. By selecting different peaks in the result filter, the
different masses can be overlaid. This will create a mixed color that can confirm if peptides from the same family have the same localization and the same distribution at different peaks. It is also possible to compare different tissues in order to see if there are differences in intensity of the peptides by visualizing peaks at the same absolute intensity.

**Immunohistochemistry**

Immunohistochemistry was used to verify neuropeptide distribution in rat brains by using antibodies of high specificity on neuropeptides of interest. Sections of the striatum, substantia nigra and the hippocampus were used in the both of immunofluorescence methods; free floating and frozen sections immunohistochemistry. Both methods were performed in basically the same way where the changes was only the choice of antibodies and in what concentrations. All washing steps were performed in the same manner with PBS for three times á 7 minutes.

**Immunofluorescent IHC, Free floating**

From the freezer free-floating sections were taken and washed in a scintillation container. The sections were pre-incubated for 60 minutes to reduce non-specific antibody incorporation. This was done by adding a solution consisting of 50% PBS-T (phosphate buffer saline with 0.3% Triton 100) and 50% horse serum solution (final concentration 2.5% horse serum in PBS). Thereafter the two primary antibodies were added to the solution and the dilution followed the specific instructions for each specific antibody. The sections with the primary antibodies were then incubated at room temperature overnight. The first secondary fluorescent antibody was added after the sections were washed. The sections were incubated in a dark environment for 60 minutes. After the sections were washed, the second secondary antibody was added and subjected to the same procedure. After the sections were washed one last time the sections were mounted on positive glass slides and left to dry in a dark environment for a few minutes. For the visualization of all cell nuclei the sections were covered at the end with a mixture of DAPI (4′,6-diamidino-2-phenylindole), 1/5000 in Vectashield mounting medium.
Immunofluorescent IHC, frozen sections

From the freezer thaw mounted fresh-frozen sections on glass slides were taken and left to dry for a few minutes. Then the sections were fixed with PFA for 60 minutes before they were pre-incubated with 2.5% horse serum in PBS for 60 minutes in a humidity chamber. In this method all the incubations take place in humidity chamber. Thereafter, the serum on glass slides were removed and then added the primary antibodies diluted in a solution consisting of 50% PBS-T and 50% horse serum solution (2.5% horse serum in PBS). The sections with the primary antibodies were incubated at room temperature overnight. Then the first secondary fluorescent antibody was added after the glass slides with the sections were washed. The sections were incubated in the dark humidity chamber for 60 minutes. After the sections were washed, the second secondary antibody was added and incubated. After the sections were washed one last time the glass slides with sections were left to dry in a dark environment for a few minutes. The sections were covered at the end with a mixture of DAPI (4’,6-diamidino-2-phenylindole), 1/5000 in Vectashield mounting medium.
Results

Peptide classification

By entering the entry names in Uniprot the peptides will be sorted into peptide families. The peptides from the large compilation were divided into 76 different peptide families. The table 1 shows the classification of the 17 most interesting peptides which was chosen by their functions and gene ontology (GO) based on specific keywords. GO is bioinformatics of the gene and gene product attributes. The keywords that had been used had some kind of impact on neuronal cell death or that can be connected to Parkinson's disease or dyskinesia.

Table 1: Classification of the most interesting peptides that were chosen for further analysis.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Entry name</th>
<th>Protein names</th>
<th>Entry</th>
<th>Entry name</th>
<th>Protein names</th>
</tr>
</thead>
<tbody>
<tr>
<td>P08592</td>
<td>A4_RAT</td>
<td>Amyloid beta A4 protein (APP) [Cleaved into: P3(42); P3(40)]</td>
<td>P14200</td>
<td>MCH_RAT</td>
<td>Pro-MCH [Cleaved into: Neuropeptide-glycine-glutamic acid; Melanin-concentrating hormone (MCH) (MCH_RAT 131-144)]</td>
</tr>
<tr>
<td>P43145</td>
<td>ADML_RAT</td>
<td>ADM [Cleaved into: Adrenomedullin (AM)]</td>
<td>P01186</td>
<td>NEU2_RAT</td>
<td>Vasopressin-neurophysin 2-copeptin (AVP-NPII) [Cleaved into: Copeptin]</td>
</tr>
<tr>
<td>P13205</td>
<td>ANFB_RAT</td>
<td>Natriuretic peptides B (Gamma-brain natriuretic peptide)</td>
<td>P13589</td>
<td>PACA_RAT</td>
<td>Pituitary adenylate cyclase-activating polypeptide (PACAP) [Cleaved into: Pituitary adenylate cyclase-activating polypeptide 38 (PACAP38)]</td>
</tr>
<tr>
<td>P01355</td>
<td>CCKN_RAT</td>
<td>Cholecystokinin (CCK) [Cleaved into: Cholecystokinin-39 (CCK39)]</td>
<td>P04094</td>
<td>PENK_RAT</td>
<td>Proenkephalin-A [Cleaved into: Synenkephalin; Met-enkephalin (Opioid growth factor); PENk(198-209); PENk(219-229); Met-enkephalin-Arg-Gly-Leu; Met-enkephalin-Arg-Phe]</td>
</tr>
<tr>
<td>P01143</td>
<td>CRF_RAT</td>
<td>Corticoliberin (Corticotropin-releasing factor) (CRF)</td>
<td>Q01177</td>
<td>PLMN_RAT</td>
<td>Plasminogen (EC 3.4.21.7) [Cleaved into: Activation peptide]</td>
</tr>
<tr>
<td>P04563</td>
<td>GAST_RAT</td>
<td>Gastrin [Cleaved into: Big gastrin]</td>
<td>P06302</td>
<td>PTMA_RAT</td>
<td>Prothymosin alpha [Cleaved into: Thymosin alpha]</td>
</tr>
<tr>
<td>P12969</td>
<td>IAPP_RAT</td>
<td>Islet amyloid polypeptide (Amylin)</td>
<td>P10362</td>
<td>SCG2_RAT</td>
<td>Secretogranin-2 [Cleaved into: Secretoneurin (SN)]</td>
</tr>
<tr>
<td>Q5PQL7</td>
<td>ITM2C_RAT</td>
<td>Integral membrane protein 2C [Cleaved into: CT-BR13]</td>
<td>P62329</td>
<td>TYB4_RAT</td>
<td>Thymosin beta-4 (T beta 4)</td>
</tr>
<tr>
<td>P01283</td>
<td>VIP_RAT</td>
<td>VIP peptides [Cleaved into: Intestinal peptide PHV-42]</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
FlexImaging

In this study, there were 6 available MALDI-IMS spectra for rat brains, a group of three high-dyskinetic and a group of three low-dyskinetic rats. The sections vary slightly in thickness and the spectra were only calibrated at acquisition and there is currently no software able to do a fine calibration after acquisition. In order to calibrate the spectra previously known peaks for known peptides were used. By taking their observed values in the spectrum as a function to their expected values an equation was calculated. For each brain the specific equation was used to obtain the expected values for the peaks. This would increase precision and the chances that the correct peptide was identified at the right peak.

Two of the brains, HD DHB5 and LD DHB2, gave clearer results than the others. These two sections showed a stronger signal of distribution and with higher intensity. Therefore these two sections were selected when the peptides were studied by using FlexImaging.

Figure 1: For the identification of peptides with FlexImaging striatal sections was used. The striatum is relatively easy to find in the brain (A) where possible significant differences of peptide levels can be seen directly. In FlexImaging a high dyskinetic rat brain, HD DHB5 (B), and a low dyskinetic rat brain, LD DHB2 (C) was used. These two sections gave the clearest visualization of the distribution of peptides in the brain.
Many peptides from different peptide families had the same m/z at their peaks which made the identification of them more difficult. To be able to distinguish between those we used MASCOT score but also the website Allen brain atlas which shows images of peptide and protein mRNA expression in a mouse brain. This may verify whether there is gene expression of a certain peptide in the area of the brain of interest [20]. In this study, we started by matching a specific MASCOT score in order to verify a peptide at a specific peak. However, this is something not always to rely on as the score only shows how big the probability is for the MS/MS peptide. Hereinafter are some examples of interesting peptides and their score.

Table 2: Two peptides from the same family.

<table>
<thead>
<tr>
<th></th>
<th>Met-Enkephaline-Arg-Phe</th>
<th>PENK 219-229</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed m/z DHB5</td>
<td>877,85</td>
<td>878,92</td>
</tr>
<tr>
<td>Expected m/z DHB5</td>
<td>877,74</td>
<td>878,68</td>
</tr>
<tr>
<td>Observed m/z DHB2</td>
<td>877,89</td>
<td>878,97</td>
</tr>
<tr>
<td>Expected m/z DHB2</td>
<td>877,62</td>
<td>878,56</td>
</tr>
<tr>
<td>Score</td>
<td>10,21</td>
<td>2,28</td>
</tr>
</tbody>
</table>

Figure 2: The Met-Enkephaline (A, B) shows a clear and well known pattern for the distribution of the peptide distribution, particularly for HD-section (A) but not for the LD-section (B). The pattern for the Penk 219-229 (C, D), is less evident and has a lower intensity in both HD-section (C) and LD-section (D). Their peak at m/z 878 indicates that this is an isotope for the Met-enkephaline. Met-Enkephaline has also a higher score (10.21) which can confirm the identification of the peptide.
The figure above shows the two peptides from the same family in which the suggested peak for Penk 219-229 is actually the second isotope peak for Met-Enkephalin. In a mass spectrum a peptide has at a specific m/z a higher intensity for the main peak than the second isotope and can show a similar distribution. The third isotope has a lower intensity than the second one, but the distribution may be not the same. In addition to the clear pattern of distribution, the MASCOT score (table 2) also shows that it is most likely that we see Met-Enkephalin at both peaks. Since these peptides are part of the same family, it is easier to identify peptides through their specific patterns. However, the identification of different peptide families in the same peak will be considerably more difficult since the score is not always to be trusted (table 3).

Table 3: Peptides from different families at the same peak along with their isotope.

<table>
<thead>
<tr>
<th>Peptide Family (Islet amyloid polypeptide, Secretoneurin, MetEnk-Arg-gly-Leu)</th>
<th>Big gastrin</th>
<th>GBNP</th>
<th>Islet amyloid polypeptide</th>
<th>Secretoneurin</th>
<th>MetEnk-Arg-gly-Leu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed m/z DHB5</td>
<td>900,85</td>
<td>900,85; 901,85</td>
<td>900,85</td>
<td>901,85</td>
<td>900,85</td>
</tr>
<tr>
<td>Expected m/z DHB5</td>
<td>900,85</td>
<td>900,88; 901,82</td>
<td>900,78</td>
<td>901,84</td>
<td>900,79</td>
</tr>
<tr>
<td>Observed m/z DHB2</td>
<td>900,87</td>
<td>900,87; 901,86</td>
<td>900,87</td>
<td>901,86</td>
<td>900,87</td>
</tr>
<tr>
<td>Expected m/z DHB2</td>
<td>900,73</td>
<td>900,76; 901,70</td>
<td>900,66</td>
<td>901,71</td>
<td>900,67</td>
</tr>
<tr>
<td>Score</td>
<td>1,31</td>
<td>5,76; 4,68</td>
<td>7,63</td>
<td>29,66</td>
<td>43,31</td>
</tr>
</tbody>
</table>

Figure 3: The figure shows how different peptide families may also have the same m/z and therefore the same pattern of distribution. Here is the MASCOT score also lower values for the isotope at m/z 901 (C, D) than for the isotope at m/z 900.85 (A, B). In both cases, it is visible pattern and stronger intensity at the high dyskinetic sections (A, C) compared to the low dyskinetic (B, D).
In the figure above, we see that different peptide families can show the same peaks in the spectrum and therefore the same pattern of distribution. At m/z 900.85, we have a specific pattern for the Met-Enkephalin where HD DHB5 (A) shows a higher intensity of the peptide and clearer localization than LD DHB2 (B). Even in this case the MetEnkephalin has the highest MASCOT score (43.31) which can verify that the particular peptide at that peak. Thereby, we will not be able to see the other peptides distribution at this mass. Secretoneurin have the same m/z as the isotope for Met-enkephalin. The peptide has also a very high score (29.66), which can complicate the identification of the peptide. In this case, we know that the main peak has a well-known pattern of the Met-Enkephalins location in an HD brain with a high MASCOT score, but we do not know if secretoneurin have similar patterns of distribution because they also have a high MASCOT score.

Table 4: Peptides from different peptide families.

<table>
<thead>
<tr>
<th></th>
<th>Copeptin</th>
<th>Thymosin alpha</th>
<th>GBNP</th>
<th>Intestinal peptide PHV-42</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Observed m/z DHB5</strong></td>
<td>1229,31</td>
<td>1230,27</td>
<td>1230,27</td>
<td>1230,27</td>
</tr>
<tr>
<td><strong>Expected m/z DHB5</strong></td>
<td>1229,28</td>
<td>1230,37</td>
<td>1230,25</td>
<td>1230,28</td>
</tr>
<tr>
<td><strong>Observed m/z DHB2</strong></td>
<td>1229,27</td>
<td>1230,30</td>
<td>1230,30</td>
<td>1230,30</td>
</tr>
<tr>
<td><strong>Expected m/z DHB2</strong></td>
<td>1229,34</td>
<td>1230,31</td>
<td>1230,19</td>
<td>1230,22</td>
</tr>
<tr>
<td><strong>Score</strong></td>
<td>0.38</td>
<td>2.98</td>
<td>4.29</td>
<td>26.93</td>
</tr>
</tbody>
</table>

Figure 4: For the HD-section (A) appear a clear pattern of peptides, both at m/z 1229, 31 and at m/z 1230.27. Also at LD-section (B) appear a clear pattern of peptides increased levels in the striatum.
In this case, we see that of those peptide families the Intestinal Peptide PHV-42 has the highest score (26.93) even though it is an isotope of the main peak. However, this is not an Intestinal Peptide PHV-42 at that mass, but it is the already identified Alpha neoendorphin (α-Neo) by its specific pattern in dyskinetic sections. However, the α-Neo peptide only has 7.91 in MASCOT score [13]. Here is a good example in which the MASCOT score does not always apply to the identifications of peptides. If it had not been Alpha neoendorphin here with its specific patterns it would be a risk of wrong identification of the peptide among these peptide families at these peaks.

In the previous study they managed to identify three peaks for proenkephalins, Penk 219-229 at m/z 1368, Penk 198-209 at m/z 1386 and Penk 219-229 at m/z 1467. All these had very high scores and had distinct patterns of high intensity [13]. In this study we successfully identified the same peptides but also managed to identify these peptides at the same m/z (1311.30). Penk 198-209 was also identified at the same mass as Penk 219-229, m/z 1368.35 and m/z 1467. They also had a very clear pattern of distribution, especially in the high dyskinetic sections (figure 5A, C, E and G). However, these two proenkephalins at the same peaks had the same peptide sequences. This may indicate that the MS/MS analysis must have done the same run twice and not distinguished between these two peptides. The sequence for Penk 198-209 at m/z 1387 is completely different from the other sequences of the two peptides. This may indicate that the sequences of the other peaks belongs to peptide Penk 219-229 [13,14].

What is interesting is that these peptides have the highest score of all the masses of all peptides in the large compilation of peptides, except Dynorphin B at the mass 1609 [13,14]. Of these proenkephalins the Penk 198-209 had the highest score of all those m / z they been identified for. At m/z 1311.30 Penk 219-229 has a MASCOT score of 48.56 while the Penk 198-209 has 53.62. At m/z 1368.35 the Penk 219-229 has a score of 41.95 while for Penk 198-209 it was at 53.63. Even at m/z 1467.55 the score is lower for Penk 219-229 (47.4) than for Penk 198-209 (52.89). The highest score in this compilation has the Penk 198-209 at m/z 1387.46, which are 68. This indicates, together with table 3, that the MASCOT score can be used roughly to indicate the correct peptide identification, but maybe not suitable above a certain value to rank for a certain peptide.

These are interesting peptides that should be investigated deeper, however, there were unfortunately no antibodies for immunohistochemistry for them at the time in order to be further examined in this study.
Figure 5: The figure shows the interesting Penk peptides with their very high score. At m/z 1311.30 (A, B) it shows the location of both Penk 219-229 and Penk 198-209. These peptides can also be seen at m/z 1368.35 (B, C) and at m/z 1467.55 (G, H). At m/z 1387.46 (E, F) it shows only the Penk-198-209.
Immunohistochemistry
Immunohistochemistry is a good way in order to confirm that certain peptides are found in specific brain areas. In this study, sections from the striatum and hippocampus in normal rat brains were used for verification of peptides of interest using antibodies. The results were, if possible, compared with other published reports.
At first free floating sections were used for the characterization of the cellular localization of the antigen as these are usually of higher quality. Thereafter the frozen sections were used for the characterization of antibodies to see if the antibodies showed a sufficiently high specificity to be used for testing on sections from high and low dyskinetic animals.
By using two different types of fluorescent antibodies together with DAPI it is possible to multiplex all three colors in the microscope. This results in a kaleidoscope of colors that can characterize an overlap in cellular localization of antigens. DAPI was used for labeling of all nuclei in blue, both neurons and glial cells.

![Figure 6: A venn diagram for interpretation of tricolor fluorescence.](image)

Corticoliberin
Corticoliberin, or more known as Corticotropin-releasing factor (CRF) is a peptide hormone that also can act as a neurotransmitter. The best known function it has is the involvement of the stress response. Nowadays we know that it also acts as a neurotoxic factor in for example cerebral ischemia and neurodegenerative diseases. Corticoliberin was particularly interesting here in that the low dyskinetic sections had a higher intensity of CRF compared to the high dyskinetic (figure 7). Despite two clear and relatively similar patterns at different m/z there was a high difference in their MASCOT score. The allen brain atlas was used to verify the expression of CRF in the brain in the striatum and hippocampus [20]. After some research, we also found that there are corticoliberin receptors in the substantia nigra but also in the striatum [21]. With these facts, it was decided to proceed with CRF and study them in more detail with immunohistochemistry. With the immunohistochemistry, we managed to confirm their presence in these areas in normal rat brains.
Figure 7: The figure above shows two interesting mass of Corticoliberin (CRF), at m/z 817.06 (A, B) and at m/z 833.04 (C, D). The low dyskinetic sections (B, D) have a clearer pattern in the striatum compared to the high dyskinetic sections (A, C). However, the score at m/z 833.04 (0.34) is much lower than at m/z 817.06 (7.88). In order to verify more the allen brain atlas was used to identify the localization of CRF expression [20]. In figure 6E the CRF expression is shown in striatum.
Cholecystokinin-39

Cholecystokinin (CCK) is a polypeptide found in the gastrointestinal tract but also in the brain. CCK’s brain functions are still unclear, although it is quite widespread in the brain, including in the striatum [20]. CCK can also be cleaved into smaller sequences, for example CCK39, which was identified in our study. CCK39 had an interesting distribution throughout the section especially around the striatum (figure 8). The intensity of CCK39 was high in both high and low dyskinetic brains at both masses. CCK39 also had fairly high MASCOT scores (15,25) especially at m/z 757.90 (C, D). Thereby it was decided to proceed with CCK39 for more detailed studies with immunohistochemistry. However, there were no antibodies against CCK39, and no reference literature for CCK39 for immunohistochemistry. Therefore, antibodies against CCK8 were used instead which is a smaller sequence from CCK39, and therefore it could also reflect changes in CCK39 immunoreactivity [19].

Figure 8: The figure shows two different m/z for Cholecystokin 39 (CCK39), m/z 724.47 (A, B) and m/z 757.90 (C, D). Both the high dyskinetic (A, C) and the low dyskinetic (B, D), showed an overall high distribution with high intensity over the whole brain section. In addition, the peptide showed at m/z 757.90 had approximately a three times higher MASCOT score (15.25) compared with the peak at m/z 724.47 (5.95).
**P3(42)**

P3(42) is a cleavage product of amyloid beta protein A4 (APP) and it is best known for the formation of plaques in Alzheimer's disease. APP is a cell surface receptor that performs numerous physiological functions on neurons, including neuronal growth. In the high dyskinetic section at m/z 1673.98 it displayed a pattern where the peptides are gathered in middle of the brain with a high intensity (figure 9). The pattern is consistent with the position of the septal nuclei in rat brain. The intensity of the peptide at that m/z was quite high, but also quite high at the low dyskinetic section (B). It was decided to proceed with the peptide for more detailed studies with used immunohistochemistry. For immunohistochemistry, an antibody against beta-amyloid 1-42 was used as no antibodies against the peptide P3 (42) were available. Since the P3(42) is part of the chain Amyloid beta 1-42 the immunoreactivity may reflect the levels of both peptides.

![Figure 9: The figure shows the P3 (42) which is one of the products when Amyloid beta A4 protein (APP) is cleaved. At m/z 1673.98 (A, B), the pattern is concentrated in the center where the high dyskinetic DHB5 section (A) has a higher intensity than the low dyskinetic DHB2 (B). However, MASCOT score at this m/z was low (0.95).](image)

Using immunohistochemistry, we found low levels of AB42 in the striatum of a normal control rat (figure 10) (A, B) and higher levels in the sepral nuclei in both neurons and non-neurons (C,D), which may possibly be glial cells. This was consistent with MALDI IMS when we tested the immunoreactivity of AB42 in a parkinsonian striatum (E,F) and observed reduced levels of AB42 compared to the striatum in a normal brain.
Figure 10: (A,B) We found low levels of AB42 immunoreactivity in the striatum of normal control animals. (C,D) In the septal nuclei, AB42 immunopositive neurons (arrows) were identified by neuron-specific NeuN immunolabeling. In addition several non-neuronal, probably glia, cells were also AB42 positive (arrowheads). (E,F) Reduced levels of AB42 immunoreactivity was observed in the parkinsonian striatum (F) compared with the normal intact striatum (E) of a low dyskinetic animal. Magnification 20X lens.
Discussion

The large peptide compilation from Ljungdal's and Hanrieder's study consisted of over 1500 peaks for 76 different peptide families. In this study 33 peptide families were identified, which means for each brain that was studied about the half of the peaks was identified from the MALDI IMS that was available in the compilation. With the MALDI IMS thousands neuropeptides can be detected and the distribution determined, which makes it a unique method with many advantages. With the MALDI IMS, it is possible to explore the proteome of complex tissues, such as the brain, with high specificity and in a cost effective manner. By visualizing different peaks, proteins and peptides can be located and thereby determine their spatial distribution in the brain. However, the method may fail when several different peptides have the exact the same m/z. In this study, we identified several peptides at the same peaks in the spectrum. The figure 3 is a good example where peptide families can show the same peaks in the spectrum and therefore indicate the same pattern of distribution. The high risk of misidentification of peptide identification highlights the importance of developing new methods for neuropeptide identification in MALDI IMS. For example, peptides from five different peptide families (Big gastrin, GBNP, Islet amyloid polypeptide, Secretoneurin and MetEnk-Arg-Gly-Leu) appears at m/z 900.85, but have a specific pattern for the Met-Enkephalin where HD DHB5 shows a higher intensity than the LD DHB2 of the peptide. The high MASCOT score of Met-Enkephalin also confirm that the pattern belongs to the Met-Enkephalin. In the figure 4 it is another good example of peptides from different peptide families has the same m/z. In this case, we see Copeptin, Thymosin alpha, GBNP and Intestinal peptide PHV-42 have a clear pattern at m/z 1229, 31 and m/z 1230.27 for both HD section and LD section. It would have been very difficult to know exactly which of these peptides is visualized in this peak unless we already knew that this is an already identified and well-known pattern of the peptide Alpha neoendorphin (α-Neo) in dyskinetic sections [13].

If there are several unknown peptides at a particular peak there are a number of methods that can determine which peptide whose distribution is visualized. One way is the use of knockout mice by knocking out just one of several genes that are present in striatal neurons. This will identify which peptides is visualized at the specific peak. In Ljungdal's and Hanrieder's study they used prodynorphin (Pdyn) knockout mice and compared with wild type control for identification and validation of dynorphin peptides. No dynorphin peptide peaks could be detected compared to the wild mouse which was detected and visualized characteristic high levels (Figure 11) [14].
Figure 11: In Ljungdal’s and Hanrieder’s study dynorphin peptides was validated in Pdyn knockout mice on striatal sections and compared to wild type control mice. In the striatum of Pdyn knockout mice were several prodynorphin peptides not detected, such as the dynorphin A (DynA), aNeo-endorphin (aNeo), dynorphin B (DynB) and the metabolite desTyr-aNeo. In the control mice characteristic distributions could be visualized. In that way it is known that these peptides occur at these peaks in MALDI IMS.

The second way to be able to identify the peptides is the on-tissue MS/MS analysis. In one study MALDI IMS were used directly on the tissue (on-tissue) which provides analysis of endogenous peptides but also peptides which have been cleaved by trypsins. By manually selecting the peaks in MS/MS analysis, peptides of interest may be analyzed without loss of spatial information of each tryptic fragment, i.e. peptides of interest. This provides a mapping of peptides distribution in brain tissue [22]. However, so far only a few peptides have been identified this way using MALDI-TOF MS/MS.

The identification of peptides at the same m/z can also be facilitated if the databases such as MASCOT were very reliable. In this study, we saw several examples of how the score could be consistent in one case but not in the other case. Met-Enkephalin-Arg-Phe and Penk 219-229 are two peptides belonging to the same family and were identified at m/z 877 and m/z 878 (table 2). As it shown, the pattern for the Penk 219-229 is less evident and has a lower intensity compared to the clear and well known pattern for the distribution of the Met-Enkephalin-Arg-Phe distribution. Here did the MASCOT-score work perfectly as the peak for Penk 219-229 is the second isotope peak for Met-Enkephalin which has a higher score (10.21) and can confirm the identification of the peptide.
Since these peptides are part of the same family, it is easier to identify peptides through their specific distribution patterns. In Table 3, it is another good example when the MASCOT-score actually works as we identified peptides from different peptide families at the same m/z. Even in this case the Met-Enkephalin has the highest MASCOT score (43.31) at m/z 900.85 and have the specific pattern for the Met-Enkephalin which can verify that the particular peptide at that peak. But on the other hand, secretoneurin also a very high score (29.66), but the peak has the same m/z as the isotope for Met-enkephalin. In the mass spectrum, the m/z 900.85 is the main peak since it has a higher intensity than the peak for the second isotope. Since the distribution is the same as in the main peak this indicates that we are seeing only one peptide, which in this case is Met-Enkephalin. If the intensity of the second isotope were as large as or larger than the main peak, it may instead be another peptide that is shown. This can complicate the identification of the secretoneurin because we do not know if secretoneurin have the similar patterns of distribution as the Met-Enkephalin. The peptide GBNP is identified at both m/z 900.85 and 901.85. In this case, the peptide's main peak is m/z 901.85, but has instead a deamination and can also be found at m/z 900.85. A peptide with a deamination has usually a higher MASCOT score (5.74) compared with a peptide without a deamination (4.68). In figure 4, we see that intestinal peptide PHV-42 have much higher MASCOT score (26.93) even though it really is an isotope of the main peak for the already identified Alpha neoendorphin (7.91). Here is a good example in which the MASCOT score does not always apply to the identifications of peptides. This highlight the high risk of false identification of the peptides without proper verification using other methods, such as immunohistochemistry.

In Ljungdal's and Hanrieder's study they identified three peaks for proenkephalins, Penk 219-229 at m/z 1368, Penk 198-209 at m/z 1386 and Penk 219-229 at m/z 1467. In this study we managed to identify the same peptides (figure 5) but also identified two different PENK peptides at the same m/z. These two proenkephalins had the same peptide sequences which may indicate that the MS/MS analysis must have done the same run twice and not distinguished between these two peptides. At the interesting m/z the peptides had very high MASCOT-score which can be used roughly to indicate the correct peptide identification, but maybe not suitable above a certain value.

Perhaps it is wrong to say that the peptides with the highest score has the highest probability to be present in MALDI IMS that it is precisely those peptides is visualized in the display. In addition to these databases are not complete, there are a large number of fragmentations that are not visible on the mass analysis because they have a low ionizaton efficiencies which reduces the matching with the databases [23].
To avoid the disadvantages of MALDI IMS, a research group studied the use of the new MALDI LTQ Orbitrap XL mass spectrometer on secretory neuropeptides. This is a method based on a combination of an optimized sample preparation and an ion trap-Orbitrap mass analyzer which can also create molecular images of distributions of neuropeptides. The principle is the same as MALDI IMS where you also can use DHB as matrix solution and where data can be obtained directly from the tissues. The difference is that in this method, a linear ion trap or an orbital trap detector receives the collected ions which then provides a high mass accuracy with high mass resolution spectra with significantly reduced chemical noise compared to the MALDI-TOF instrumentation. In order to analyze the results of signal intensities of peptides and their spatial distribution they used the software ImageQuest MSI. The major advantage over the MALDI IMS is that in this method, peptides with similar masses could be combined in one image by using different color codes where peptides can be located and identified individually. Different peptides at the same m/z will be presented in different colors and thereby the spatial distribution of the peptides and the identity in the tissue sample will be determined in one single experiment [24, 25]. Such a method can provide sufficient evidence in our study to identify different peptides at same mass in order to be able to produce new drugs against these molecular targets that may be involved in the LID.

Corticoliberin (CRF), cholecystokinin-39 (CCK39) and P3(42) are three other peptides that showed interesting distributions in both high and low dyskinetic brain sections which were then selected for further studies in immunohistochemistry. This was done for an additional validation that however is strongly dependent on antibody specificity for the peptide of interest. The corticoliberin had in MALDI IMS a higher intensity in low dyskinetic section compared to the high dyskinetic (figure 7). We managed to confirm the presence of CRF in the striatum and hippocampus by using immunohistochemistry. This is interesting because there has been reported that patients with Parkinson's disease have a reduction in CRF [27]. This has been confirmed reasonably in a study using animal models of Parkinson induced by MPTP which shown decreased number of CRF immunoreactive neurons both in the paraventricular nucleus of the hypothalamus and in the central nucleus of the amygdala [26]. In another study decreased levels of CRF immunoreactivity was found in the frontal, temporal, and occipital poles of the neocortex in PD patients with dementia. Dementias are also associated with dysfunction and death of neurons in different cell populations, where the reduction of levels of CRF may be involved [27].
Also in another and more recent study it was shown that stimulation of CRF may have a therapeutic use in PD. This was tested by having treated parkinsonian rats with CRF-related peptide, urocortin, which resulted in reduced levels of nigrostriatal damage in rats. Urocortin have therefore an ability to produce the recovery of the rats’ nigrostriatal neurodegeneration [28]. In this study we did not observe any significant increase of urocortin in MALDI IMS in both low and high dyskinetic rat brains, probably because of the neurodegeneration in our model is already completed weeks before L-DOPA treatment.

The CCK39 had an interesting distribution throughout the section with a general high intensity of CCK39 in both high and low dyskinetic sections. We managed to confirm the presence of this peptide in the striatum and hippocampus by using immunohistochemistry. This is an interesting peptide because they have already in a previously study successfully inhibited L-dopa-induced dyskinesias in parkinsonian monkeys by systemic administration of CCK-8. This peptide may thereby also be a potential drug candidate to study further with [29].

Using immunohistochemistry, we found low levels of AB42 in the striatum of a normal control rat. However, we found AB42 in both neurons and non-neurons, which may possibly be glial cells. When we tested the immunoreactivity of AB42 in a parkinsonian striatum of a low dyskinetic animal, we observed reduced levels of AB42 compared to the striatum in a normal brain. The optical density of the immunoreactivity gives only approximate quantification, but sufficient to obtain a fairly reliable estimate of the peptides density changes as previously shown [14].

There were no specific peptide antibodies against CCK39 and P3(42) for the immunohistochemistry so we used antibodies against peptides were the peptides are a part of the chain. In order to get even more specific immunoreactivity it should be tested with peptide antibodies for each peptide. This means that a completely new production of these immunospecific peptide antibodies would be required, which can take considerable time, and also be expensive to produce.
Conclusion

The cellular and molecular mechanism of dyskinesias in Parkinson patients is complicated to study which requires continuously new method developments. In this study, it may possibly need to have suitable animal models with down-regulating genes of interest that can study these complex mechanisms but also to distinguish the peptides that occur at the same m/z. In the future it would also be interesting to use the MALDI LTQ Orbitrap XL mass spectrometer which can facilitate our work to distinguish all the peptides that occur in dyskinetic brains. With such a method it may outcompete MALDI IMS but also the immunohistochemistry in the future. However, an Orbitrap is not current at this time since it costs about 10-12 million Swedish kronor, while existing antibodies costs on the other hand about 4000 Swedish kronor. If it would be necessary to produce new antibodies, it will costs around 25,000 Swedish kronor. So far, it is therefore more cost-effective to combine MALDI IMS and immunohistochemistry as we have worked with even though this method is a harder labor with a moderately specificity and sensitivity.

In conclusion, the immunohistochemical detection of P3(42) and the identified CRF, CCK39 and the proenkephalins can be worth further study in which these may possibly be a molecular target for blocking the development of L-DOPA induced dyskinesia.
References


