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# Decreased Striatal Levels of PEP-19 Following MPTP Lesion in the Mouse

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PEP-19 is a neuronal calmodulin-binding protein, and as such, a putative modulator of calcium regulated processes. In the present study, we used proteomics technology approaches such as peptidomics and imaging MALDI mass spectrometry, as well as traditional techniques (immunoblotting and in situ hybridization) to identify PEP-19 and, specifically, to measure PEP-19 mRNA and protein levels in an animal model of Parkinson's disease. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) administration in mice resulted in a significant decrease in striatal PEP-19 mRNA. Capillary nano-flow liquid chromatography electrospray mass spectrometry analysis of striatal tissue revealed a significant decrease of the PEP-19 protein level. Moreover, imaging MALDI mass spectrometry also showed that PEP-19 protein was predominantly localized to the striatum of the brain tissue cross sections. After MPTP administration, PEP-19 levels were significantly reduced by 30%. We conclude that PEP-19 mRNA and protein expression are decreased in the striatum of a common animal model of Parkinson's disease. Further studies are needed to show the specific involvement of PEP-19 in the neurodegeneration seen in MPTP lesioned animals. Finally, this study has shown that the combination of traditional molecular biology techniques with novel, highly specific and sensitive mass spectrometry methods is advantageous in characterizing molecular events of many diseases, including Parkinson's disease.

**Keywords:** PEP-19 • Parkinson's disease • MALDI imaging MS • peptidomics • mouse • brain • striatum • immunoblotting • in situ hybridization

## Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder, affecting 1% of the population over the age of 60.[1] This slowly progressive disorder of the central nervous system (CNS) affects movements, muscle control, and balance. Symptoms of PD include resting tremor, rigidity, slowness of movement and postural instability.[2] Although the exact cause of PD is unknown, it is assumed that a combination of genetic susceptibility and environmental factors are involved.[3] Loss of dopamine neurons in the substantia nigra (SN) is one of the histological characteristics defining PD, leading to a marked reduction in dopamine function in the nigrostriatal pathway. There are to date no proven neuroprotective or neurorestorative treatments; instead current treatments of PD patients focus on relieving disabilities while minimizing side effects of medications.

PEP-19 (Purkinje cell protein 4; PCP 4) is a 6.7 kDa polypeptide that belongs to a family of proteins involved in calmodulin-dependent signal transduction.[4] PEP-19 binds calmodulin (CaM) via an IQ motif in a calcium independent manner.[5] CaM is a widespread and abundant transducer of calcium signaling in cells and, by complexing with calcium, it directly activates several types of intracellular enzymes, including kinases and phosphatases.[6] PEP-19, and presumably similar members of the IQ motif family of proteins (such as neuromodulin (GAP-43) and neurogranin), has the potential to alter the calcium-binding dynamics of free CaM as well as CaM bound to other target proteins.[5]

Immunohistochemical studies have shown that PEP-19 is highly expressed in the basal ganglia.[7] Initially, PEP-19 was thought to be restricted to the CNS,[8] but was later additionally found in the sensory cells of inner ear and the spiral ganglion,[9] the vestibular-,[10] trigeminal-,[11,12] and dorsal root ganglion.[13] PEP-19 is thought to be related to neurological development[8,14] and pathological mechanisms of certain diseases.[7,15] Further-

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more, it has been shown that PEP-19 can inhibit apoptotic processes in PC12 cells, suggesting a potential role in regulatory mechanisms in pathways leading to cell death.[16] Interestingly, PEP-19 is expressed in population of neurons that sometimes demonstrate resistance to degeneration (including Purkinje cells in cerebellum and granule-cell neurons in the dentate gyrus of hippocampus).[17,18]

In the present study, we investigated the levels of PEP-19 mRNA and protein after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment in the mouse. The systemic administration of the toxin MPTP in experimental animals replicates the neuropathological, neurochemical, and clinical features in PD, causing cell death in the nigrostriatal dopaminergic pathway by inducing oxidative stress.[19] MPTP-induced parkinsonism produces a clinical phenocopy of PD, though, pathologically, it destroys nigrostriatal neurons throughout the substantia nigra indiscriminately, and the characteristic features of PD, Lewy bodies, are not produced.

A range of different techniques, such as in situ hybridization, immunoblotting, nano-flow capillary liquid chromatography (nano-LC) electrospray ionization mass spectrometry (ESI MS) and matrix-assisted laser desorption ionization (MALDI) imaging MS (IMS) directly on tissue sections, were used to investigate the levels of PEP-19 mRNA and protein after MPTP administration. This study shows a significant decrease in the expression of PEP-19 in the MPTP lesioned striatum of the brain.

## Experimental Section

**Animals.** Adult male C57/BL6 mice (20−30 g) were used for this study. The animals were group-housed in a controlled environment with food and water available ad libitum. All animal procedures were approved by the local animal ethics committee and carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

**Drug Administration.** MPTP (Sigma Chemical Co., St. Louis, MO) was dissolved in saline and injected subcutaneously in a dose of 20 mg/kg followed by a second dose (20 mg/kg) after 4 h. The injections were repeated the next day (total amount injected 80 mg/kg). Animals were sacrificed three weeks after the last MPTP injection as previously described,[20] followed by rapid dissection on ice of the brain regions of interest. The tissue was kept at −80 °C until further analysis.

**Immunoblotting to Detect Tyrosine Hydroxylase (TH).** To verify the effectiveness of the MPTP lesion, immunoblotting experiments for TH were carried out as previously described.[21] Frozen striatal tissue samples were sonicated in boiling 1% sodium dodecyl sulfate (SDS) and boiled for 10 min. Small aliquots of the homogenate were retained for protein determination with a BCA-kit (Pierce, Rockford, IL), using bovine serum albumin as a standard. Equal amounts of protein (30 $\mu$g) were loaded onto 12% acrylamide gels, and the proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes (0.2 $\mu$m) (Schleicher & Schuell, Keene, NH).[22] The membranes were immunoblotted using a monoclonal antibody against TH (1:5000; Chemicon, Temecula, CA). Antibody binding was revealed by incubation with goat anti-rabbit horseradish peroxidase-linked IgG (Pierce Europe, Oud Beijerland, The Netherlands) and the ECL immunoblotting detection system (GE Healthcare, Uppsala, Sweden). Chemiluminescence was detected by autoradiography using DuPont NEN autoradiography film. TH levels were quantified by densitometry using National Institutes of Health IMAGE 1.61 software. Data were analyzed by Student's $t$-test with significance defined as $p \leq 0.05$.

**Nano-LC ESI MS Semiquantitative Analysis of PEP-19.** Frozen striatal tissue samples (50−100 mg) were suspended in cold sample buffer (0.25% acetic acid, 0.2 mg tissue/$\mu$L) and homogenized by microtip sonication[23] (Vibra cell 750, Sonics & Materials Inc., Newton, CT). 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 with a molecular weight limit of 10 kDa (Microcon YM-10, Millipore, Bedford, MA) 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.

Five $\mu$L peptide filtrate was injected and desalted on a C18 capillary column (15 cm, 75 $\mu$m i.d.; NAN75-15-03-C18PM, LC Packings, Amsterdam, The Netherlands) by an isocratic flow of buffer A (0.25% acetic acid in water). During a 60-min gradient from buffer A to B (35% acetonitrile in 0.25% acetic acid), delivered using an Ultimate LC system (LC Packings), the peptides were separated and directly infused into an ESI Q-TOF mass spectrometer (Q-TOF2, Micromass, Manchester, UK) at an approximate flow rate of 150 nL/min for analysis. A potential of 2 kV was applied over a 5 cm long fused silica spray emitter. The spray emitter was made from a 75 $\mu$m i.d., 220 $\mu$m o.d., fused silica capillary (Polymicro Technologies Inc., Phoenix, AZ). The tip of the capillary was drawn in a micro torch flame to obtain a tapered spray emitter with a diameter of approximately 10 $\mu$m. Acquisition of the ESI Q-TOF instrument was performed in the continuous mode. Mass spectra were collected at a frequency of 4 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 10 eV, and multiple channel plate detector 2100 V. The source block temperature was 80 °C. The cone gas flow rate was set to about 100 L/h. In the wide band-pass quadrupole mode, mass spectra were collected in the $m/z$ range of 300−1000 Da with a mass resolution of 5800 at $m/z$ 550.35 Da. The Q-TOF instrument was calibrated using a mixture of PEG 200, 400, and 600, with the concentrations of 25, 50, and 75 ng/mL, respectively, in 2 mM ammonium acetate in 50% methanol (aq) with 0.2% formic acid, according to the recommendations of the manufacturer. Mass spectra collected during the 60 min nano-LC gradient were exported and semiquantitatively compared between control and MPTP treated animals, using DeCyder MS (GE Healthcare).

**MALDI IMS of PEP-19 Directly on Brain Tissue Sections.** The tissue was cut into 12 $\mu$m sections at −15 °C using a microtome (Leica CM 3000, Leica Microsystems AG, Wetzlar, Germany) and mounted directly onto gold-coated MALDI target plates. Sections from different groups of animals were placed on the same target plate to be analyzed at the same time. The tissue sections were stored at −80 °C before matrix deposition.

The mounted tissue sections were thawed in a desiccator, fixed in ethanol (2 × 70% ethanol, 20 s, 1 × 100% ethanol, 15 s) and seeded with a fine powder of sinapinic acid (SA, Sigma). The matrix solution (SA, 25 mg/$\mu$L, 50:50 acetonitrile/water, 0.1% TFA) was deposited onto the tissue using a MALDI matrix spotter (RapidSpotter, Picoliter Inc., Sunnyvale, CA), where focused acoustic energy is used to eject small droplets of matrix

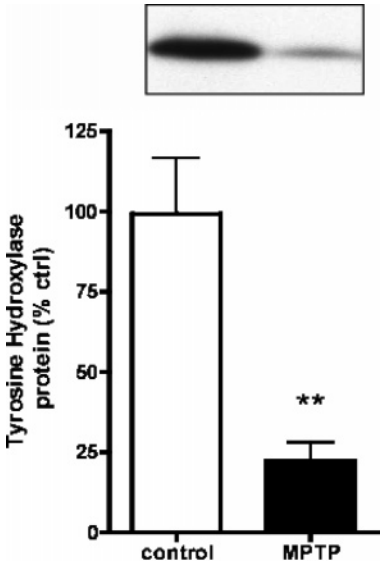


**Figure 1.** Regulation of TH levels by treatment with MPTP. The amounts of TH protein in tissue extracts from striatum were detected by immunoblotting and quantified by densitometry. Data represent means ± SEM for five to seven animals. *, $p < 0.01$ compared with control, Student's *t*-test.

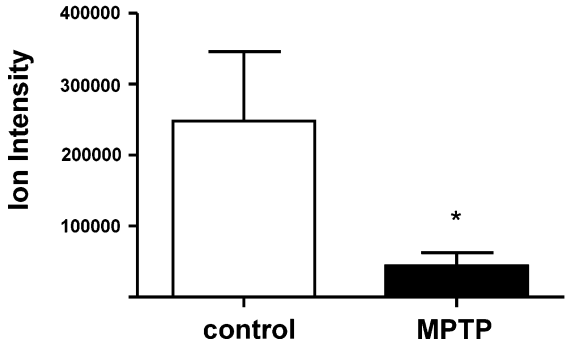


**Figure 2.** Ion intensity of PEP-19 in the striatum of mice after saline, MPTP and MPTP/L-DOPA administration. The ion intensities were measured by integrating specific mass-to-charge for PEP-19 during peak elution in the nano-LCMS profile using DeCyder software ($n = 3$ for all groups). *, $p \leq 0.05$ compared with control (Mann−Whitney test).

solution onto the tissue. Typically, the droplets were ejected at 10 Hz and 13 droplets were deposited per spot, two times repeatedly. The matrix spots were deposited at a resolution of 280 $\mu$m and the diameter of the spots were approximately 190 $\mu$m.

The spotted tissue sections were analyzed in a Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems, Framingham, MA) in automatic mode, acquiring and summarizing 5 × 25 laser shots per spectra from each matrix spot according to a defined search pattern. The acceleration voltage was set to around 19 kV and a delayed extraction of 150 ns was used. The spectra from each of the matrix spots were processed and combined into an image file using in-house written software. The image files were imported to an image processing application (BioMap, Novartis, Basel, Switzerland), which provides specific tools for MS image analysis.[24]

Spectra from the different experiments were also imported as ASCII files into OriginPro7.5 (OriginLab Corp., Northampton, MA) and processed to analyze differences in abundance of peptides/proteins in the animal groups.

**Accurate Mass Identification of PEP-19 Using ESI LCQ FT MS and SwePep.** Accurate mass data of PEP-19 was acquired from nano-LC ESI linear trap quadrupole Fourier Transform (LTQ-FT) MS (Thermo Electron, San Jose, CA). The identity of PEP-19 was established by using the obtained $m/z$ as input to the in-house developed endogenous peptide and small protein database, SwePep (www.swepep.com). This database consists of 3600 neuropeptides from 1600 precursor proteins and 25 000 small proteins (mw < 120 amino acids). It is specifically designed for neuropeptide research and searchable with relevant post-translational modifications. The accurate mass from the MS analyses together with the database information was utilized for sorting out the most possible peptide/small protein matches.

**MS/MS Identification of PEP-19.** Frozen brain tissue was suspended in boiling extraction solution (2 mg tissue/$\mu$L in 0.25% acetic acid) and homogenized by microtip sonication (Vibra cell 750). The homogenate was centrifuged at 20 000 × *g* for 45 min at 4 °C to sediment cell debris and undissolved material. The supernatant was transferred to a centrifugal filter with a molecular weight limit of 30 kDa (Microcon YM-30, Millipore), and centrifuged at 14 000 × *g* for 45 min at 4 °C. The peptide filtrate was incubated with trypsin overnight at 37 °C and lyophilized. The digest was dissolved in 10 $\mu$L 0.25% acetic acid and desalted on a trap column and eluted by a nano-LC system (Ettan MDLC, GE Healthcare) as described above, into an LTQ mass spectrometer (Thermo Electron). The spray voltage was 1.8 kV, capillary temperature was 160 °C, and 35 units of collision energy were used to obtain peptide fragmentation. Four tandem MS (MS/MS) spectra of the most intense peaks were collected following each full-scan mass spectrum. The dynamic exclusion feature enabled sequence information of as many different co-detected peptides as possible.

The information from the ESI−MS and ESI−MS/MS-spectra were correlated to protein and translated DNA sequence data in the NCBI database using the TurboSequest algorithm in the Bioworks 3.1 software package (Thermo Electron). The nonredundant sub database of *Mus musculus* was used with the following parameters: partial oxidation of methionine (+16 Da), peptide mass tolerance of 1.5 Da and fragment ion mass tolerance of 0.35 Da. Trypsin was specified as the digesting enzyme. The identified peptides were further evaluated using charge state vs cross-correlation number (Xcorr). The criteria for positive identification of peptides were as follows: Xcorr > 1.5 for singly charged ions, Xcorr > 2.0 for doubly charged ions, and Xcorr > 2.5 for triply charged ions.

**In Situ Hybridization.** In situ hybridization experiments were carried out as previously described.[25] Briefly, $^{35}$S-labeled anti-sense and sense cRNA probes were prepared by in vitro transcription from cDNA clones corresponding to nucleotides 1−462 of the coding sequence of the rat PEP-19 gene. The transcription was performed from 50 to 100 ng of linearized plasmid using [35S]UTP (>1000 Ci/mmol; Du Pont NEN) and T3 or T7 RNA polymerases. The probes were purified on Sephadex G50 column and precipitated in sodium acetate (0.1 vol.)/absolute ethanol (2.5 vol.). Tissue sections (12 $\mu$m thick) from the different groups of animals were cut with a cryostat (Leica CM 3000) and mounted directly onto poly-L-lysine coated slides. Cryostat sections were postfixed in 4% PFA for 5 min at room temperature, rinsed twice in 4 × sodium chloride-sodium citrate buffer (SSC) and placed into 0.25% acetic anhydride in 0.1 M triethanolamine/4 × SSC (pH 8) for 10 min

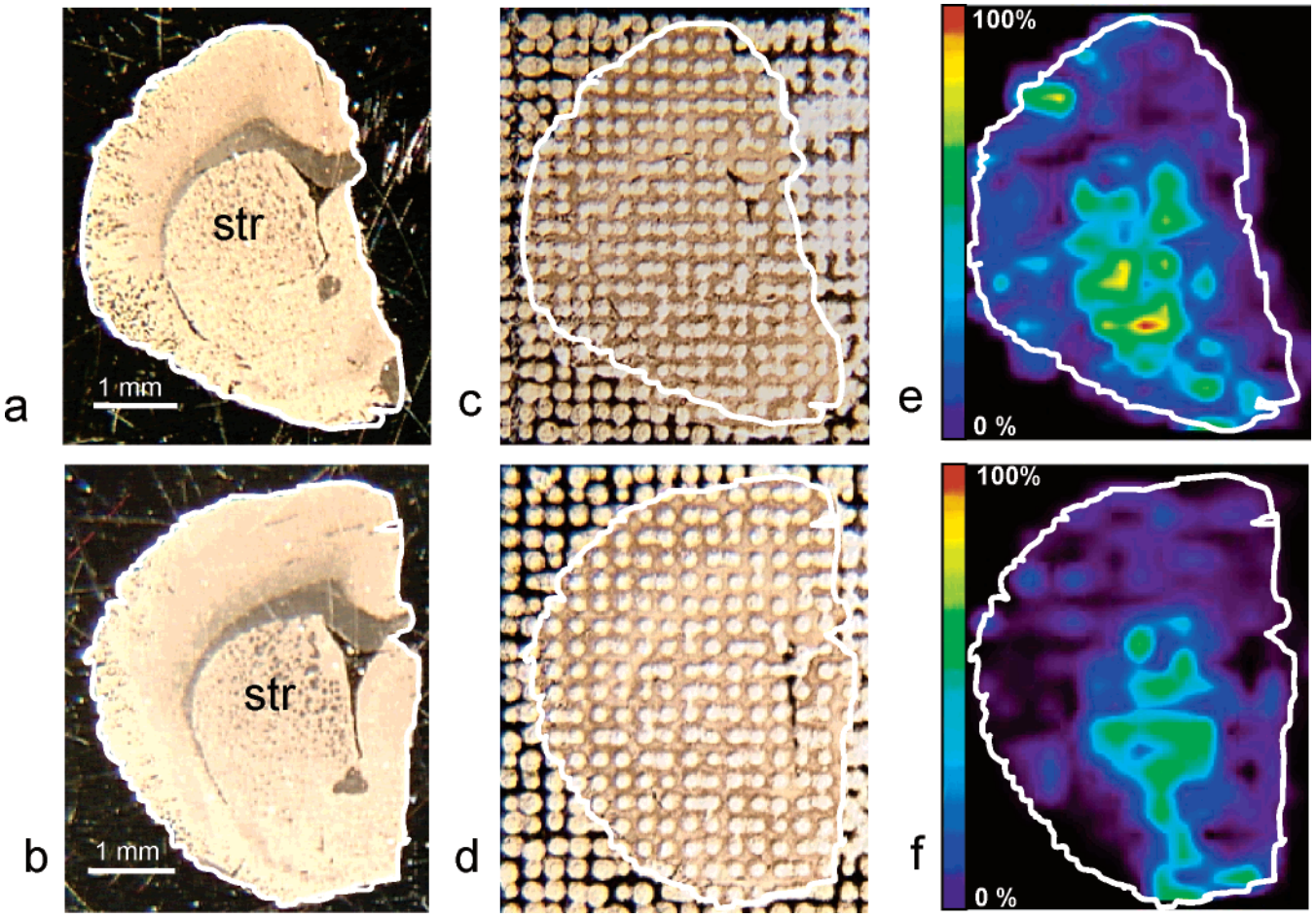


**Figure 3.** MALDI IMS of brain tissue sections following MPTP treatment. Panels a−b show the photographs of mouse brain hemispheres from one control and one MPTP treated animal, respectively, mounted on a gold-coated stainless steel plate (str = striatum). Panels c−d display the same tissue sections spotted with matrix solution (SA, 50:50:0.1 ACN:$H_2O$:TFA). The diameters of the matrix spots are about 190 $\mu$m and the distance from center to center is 280 $\mu$m. Panels e−f show the ion density of PEP-19. The distribution and relative intensity difference between control and MPTP treatment is representative for the analyzed tissue sections.

at room temperature. After dehydration in graded alcohols, the sections were hybridized overnight at 55 °C with $10^6$ cpm of $^{35}$S-labeled probe in 50 $\mu$L of hybridization solution (20 mM Tris-HCl/1 mM EDTA/300 mM NaCl/50% formamide/10% dextran sulfate/1 × Denhardt's/250 g/mL yeast tRNA/100 g/mL salmon sperm DNA/0.1% SDS/0.1% sodium thiosulfate). The slides were washed in 4 × SSC (5 min, four times), RNAse A (20 g/mL) (20 min, at 37 °C), 2 × SSC (5 min, twice), 1 × SSC (5 min), 0.5 × SSC (5 min) at room temperature, and rinsed in 0.1 × SSC at 65 °C (30 min, twice) (all washes contained 1 mM DTT), before being dehydrated in graded alcohols. The slides were then exposed on X-ray films for two to fourteen days.

## Results

**Lesion Verification.** To verify the efficacy of the dopamine denervation post mortem, striatal TH protein levels were examined. Previous work has shown that the TH levels correlates well with the degree of dopaminergic innervation.[26] Following treatment with MPTP (2 × 20 mg/kg for 2 days) there was a significant reduction (78% decrease) in striatal TH protein as determined by immunoblotting (Figure 1).

**Nano-LC ESI MS of PEP-19 Levels Following MPTP Administration.** Two groups of mice (saline controls and MPTP) were analyzed by nano-LC ESI MS to investigate the levels of PEP-19 in the striatum. In each chromatogram the ion intensity of PEP-19 was extracted and integrated over the nano-LC elution time using the DeCyder MS software. The calculated intensities of PEP-19 were then semiquantitatively compared between the groups. MPTP treated mice showed significantly reduced levels compared to control (Mann−Whitney; $p \leq 0.05$) (Figure 2).

**MALDI IMS Directly on Brain Tissue Sections.** The MALDI IMS experiment was performed by acquiring and summarizing 125 laser shots from each of the matrix spots into one spectrum. At a resolution of 280 $\mu$m between the matrix spots, about 350 spectra from each brain hemisphere section were generated. Typically, in the present experiment, about 400 distinct mass signals were detected in the mass range of 2−30 kDa.

Protein ion density maps (images) were obtained through processing in BioMap, where the intensities of specific $m/z$ values are displayed in two-dimensional space.

The molecular images of PEP-19 showed that the expression was predominantly defined to the striatum. After lesioning with MPTP, there was a significant decrease in the striatal expression. Figure 3a,b show one hemisphere of two mouse brains (one control and one MPTP lesioned animal, respectively), which are cut and mounted on the MALDI targets. Figure 3c,d show the same tissue sections spotted with matrix. In Figure 3e,f, the molecular images of PEP-19 are shown from one

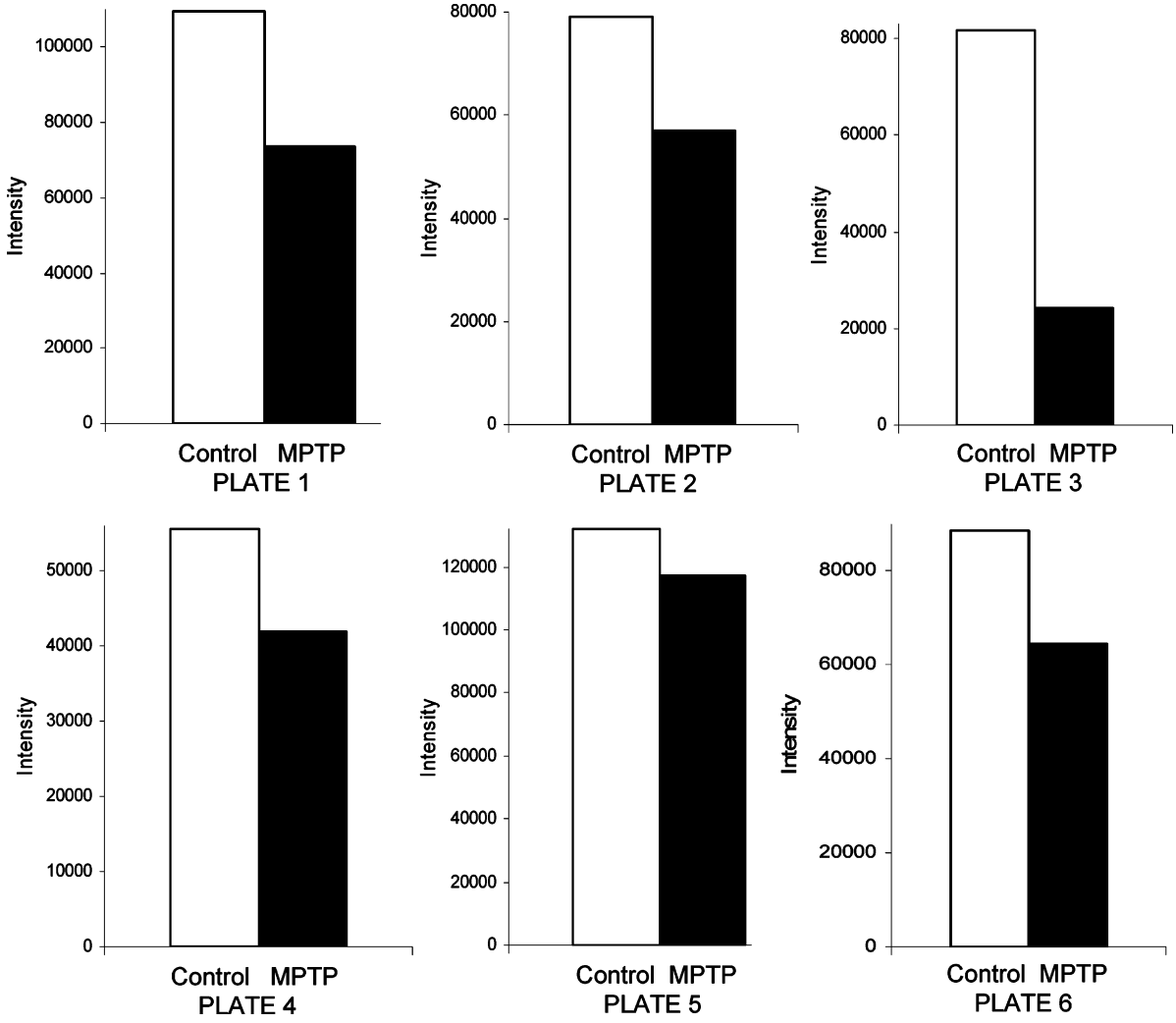


**Figure 4.** Individual signal intensities of PEP-19. Ion signal intensities of PEP-19 in the striatum were extracted and are visualized in these diagrams. In all sample plates there was a decrease (29−89%) of the striatal expression of PEP-19. The brain tissue sections on the different MALDI plates were analyzed on different occasions, with slightly different parameters; therefore, the relative expression changes are compared within each MALDI plate.

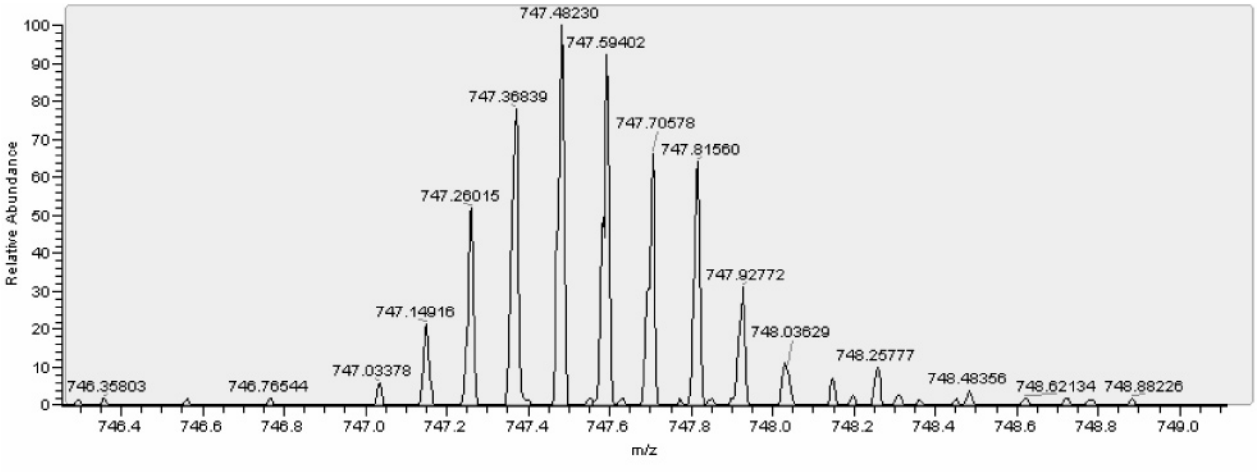


**Figure 5.** Accurate FT-ICR MS mass spectrum of PEP-19. The figure shows the ion convolute at monoisotopic *m*/*z* 747.0338 (charge state +9).

control and one MPTP lesioned animal, respectively; both images are representative of the total data set. The total ion intensity of PEP-19 was extracted from the striatum and the expression levels were found to be significantly decreased (Student's paired *t*- test, $p < 0.009$). The expression levels were reduced on an average of 30% (Figure 4).

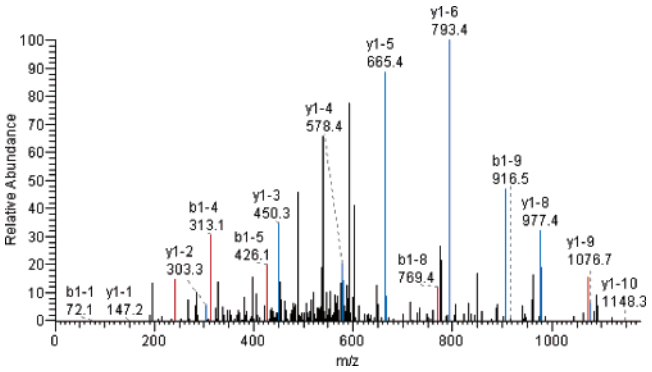


**Figure 6.** Tandem MS identification of PEP-19. MS/MS spectrum of one tryptic peptide from digested PEP-19 with the sequence AAVAIQSQFRK. Matching b- and y-ions are denoted in the spectrum.

**Identification of PEP-19 by ESI FT-ICR MS and SwePep.** Peptide extracts from mouse striata were injected onto a nano-LC ESI LTQ FT system and the MS data were compared to the SwePep database containing specific information regarding endogenous peptides and small proteins. The mass accuracy of the LTQ-FT MS is specified to less than 2 ppm by the manufacturer using external calibration.[27] To ensure all possible peptide matches, the SwePep database search was limited to 10 ppm. Two matches were retrieved from the search, i.e., the acetylated PEP-19 (mass 6714.2604) from mouse/rat and the small venom protein 1 precursor (mass 6714.2433) from parasitoid wasp. The mass was calculated from the most abundant charge state at $m/z$ 747.0338 (Figure 5).

**MS/MS Identification of PEP-19.** An extract of small proteins and peptides (<30 kDa) from brain tissue was digested by trypsin. The highly expressed low mass proteins/peptides, such as thymosin, ubiquitin, and PEP-19 generated the most abundant tryptic peptides. The digest was separated and eluted during a 60 min gradient and analyzed by nano-LC ESI MS/MS. The identities of thymosin, neuromodulin, neurogranin, superoxide dismutase 1, ubiquitin, diazepam binding inhibitor, and PEP-19, among others, were established by searching the NCBI nonredundant database of protein and translated DNA sequence data from *Mus musculus*. PEP-19 was confirmed by MS/MS spectra from three tryptic peptides constituting sequence coverage of 44% of the complete protein (Figure 6).

**Decrease in PEP-19 mRNA after MPTP Treatment.** In situ hybridization experiments showed that PEP-19 mRNA is abundantly expressed throughout the brain with particularly high levels in the striatum. There was a significant decrease in PEP-19 mRNA in MPTP lesioned mice compared to control (42% decrease, $p < 0.05$, Figure 7).

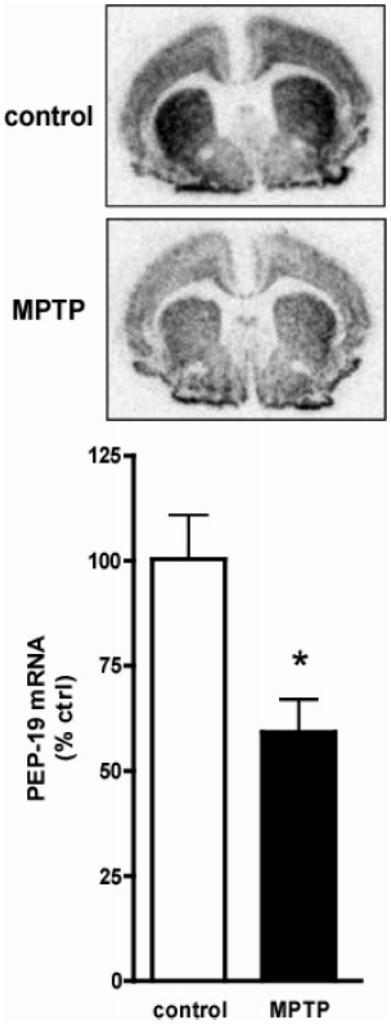


**Figure 7.** Relative levels of PEP-19 mRNA in the striatum of mice after saline and MPTP administration. The amounts of PEP-19 mRNA were determined by in situ hybridization and quantified by densitometry in striatum. Data represent means ± SEM for five to seven mice per group. *, $p < 0.05$ compared with saline-treated mice (Student's *t*-test).

## Discussion

Understanding the molecular processes that underlie neurodegenerative diseases is an ongoing challenge and an important goal of neuroscience today. To achieve this goal, there is a need for new scientific methods to analyze the complex nature of peptide and protein expression changes in such diseases. For example, new MS methods allow for unequivocal identification and quantification of proteins and peptides and MALDI IMS offers the ability to localize and characterize proteins and peptides in situ.[28]

In the present study we used a combination of analytical approaches, such as in situ hybridization, immunoblotting, nano-LC ESI MS and MALDI IMS, to examine the expression of PEP-19 on the mRNA and protein levels in an animal model of PD. We have used C57Bl6 mice, which is the most sensitive mouse strain to the MPTP toxin, and established protocols for MPTP administration.[29,30] Our results on TH levels, a 78% reduction, are compatible with the results obtained in these above mentioned studies. Although it would be interesting to compare the reduced expression of PEP-19 that we have observed in MPTP treated mice with other experimental animal models of PD disease, e.g., 6-hydroxydopamine (6-OHDA) injections. Unfortunately, at the moment, the nigral 6-OHDA-lesion model is associated with high postoperative mortality (ranging from 40 to 82% according to a recent study).[31] All techniques employed in our study show a significant reduction of PEP-19 in the striatum of MPTP lesioned mice. Moreover, MALDI IMS, directly on 12 $\mu$m tissue sections, demonstrates that the expression levels of PEP-19 protein are significantly reduced by 30% after MPTP treatment.

PEP-19 is a neuron-specific protein that binds CaM through an IQ-motif similar to the CaM binding domains of neuromodulin (GAP-43) and neurogranin (RC3).[4] This argues for a

role for PEP-19 in modulating calcium/CaM homeostasis. In recent years, a number of studies have been focused on calcium metabolism and homeostasis and its relationship with aging and neurodegenerative diseases.[32,33] Calcium ions play fundamental roles in cell signaling, helping to control processes such as muscle contraction, neurotransmitter release, transcriptional regulation, and cell death.[34] This occurs through the action of calcium pumps and several types of calcium-binding proteins. For example, CaM is a calcium modulated protein that is abundant in the cell cytoplasm of all higher cells and that has been highly conserved through evolution. Acting as an intermediary protein, CaM senses cell calcium levels and, in turn, relays signals to various calcium-sensitive enzymes, ion channels and other proteins.[35,36] Failure of calcium buffering or intraneuronal calcium homeostasis will contribute to calcium-mediated cytotoxic events in the pathogenesis of neurodegenerative diseases.[33]

Initially discovered as a marker protein for cerebellar development,[8] PEP-19 was subsequently localized specifically to the Purkinje-cell neurons in this area of the brain.[7] Further immunocytochemical studies have shown that PEP-19 is an abundant peptide in the nervous system; it is expressed in the cortex, putamen, olfactory bulb, retina, substantia nigra, hippocampus, dorsal-root and trigeminal ganglia.[7,9,13] A previous immunohistochemical study found no evidence that PEP-19 is expressed in dopamine neurons.[7] The methods used in our study, mass spectrometry and immunoblotting, do not provide the anatomical resolution to confirm whether the reduction of PEP-19 protein levels in striatum is somatodendritic or could originate from the loss of dopamine neurons projecting to striatum. However, the fact that we also found a significant reduction of PEP-19 mRNA in striatum strongly indicates that the reduction in PEP-19 protein levels depends on reduced production in striatal medium-sized neurons rather than dopaminergic neurons.

Interestingly, PEP-19 is one among the several proteins previously described to be regulated in neurodegenerative conditions, such as PD, Alzheimer's disease and Huntington's disease.[7,37] In this regard, a recent study showing that PEP-19 can inhibit apoptotic processes is of special interest. This study, by Erhardt and co-workers, showed that PEP-19, when expressed in PC12 cells, inhibited cell death following exposure to UV irradiation or treatment with staurosporine.[16] Other previously reported functions of PEP-19 include its ability to modulate calcium-dependent CaM kinase activity within cells.[38]

In a previous paper, Utal et al. used immunohistochemistry to examine the distribution of PEP-19 in human post mortem brain tissue. There were no apparent alterations on PEP-19 immunoreactivity in PD.[7] Post-mortem activity of proteases has been shown to play an important role on peptide and proteins concentration in the brain, as well as for detecting post-translational modifications.[20,23] Our earlier findings suggest that substantial protein and peptide degradation occurs in brain tissue within min post-mortem[23] and that instant deactivation of proteases enables neuropeptides to remain intact and minimizes degradation of proteins by proteolysis.[20] For this reason, the animals in the present study were sacrificed by focused microwave irradiation. Our recent experiments also indicate that PEP-19 is vulnerable to activity by proteases post-mortem (unpublished data). This may explain the discrepancy between our study and the previous study on PEP-19 and PD,[7] where no significant PEP-19 immunoreactivity changes were detected in striatum (3.5−6.25 h post-mortem delay). Other explanations may be differences in the phenotype between the animal PD model and PD, length of PD symptoms (weeks vs years), and specificity of the PEP-19 immunoreactivity.

The fact that MPTP lesioning lowers the levels of PEP-19 mRNA demonstrates that the down-regulation of PEP-19 occurs, at least in part, through an altered transcriptional activity in striatal neurons. It is known that several transcriptional factors, including members in the Fos/Jun family, CREB and zif268, are dysregulated in animal models of PD.[39−41] However, as the transcriptional regulation of PEP-19 is not well-described, further studies are needed to clarify the mechanism(s) whereby MPTP treatment reduces PEP-19 levels.

In recent years, MS has made highly significant advances in the area of direct molecular analysis of biological tissues. New MS methods allow for high-resolution detection of proteins and their characterization and quantification. Moreover, the MALDI IMS technique makes it possible to localize and characterize proteins and peptides in situ. MS offers a unique high-accuracy molecular specificity that will be invaluable in understanding the molecular events in this and other investigations of many disease states.

In summary, we have demonstrated specific, significant decreases in PEP-19 gene and protein expression in key brain areas affected by MPTP administration. Further experiments are needed to define the mechanisms behind the MPTP-induced decrease of PEP-19 and to be able to conclude whether the reduced level of PEP-19 in the MPTP model reflects compensation or is causative to a parkinsonian phenotype. It will also be interesting to investigate possible interactions between PEP-19 and other proteins known to be involved in neurodegenerative disease states. Finally, this study shows that the combination of traditional molecular biology techniques and novel MS techniques provide a powerful toolkit for sensitive and molecular specific detection of changes in proteins and peptides in experimental models of neurodegenerative disease.

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