The principal causative pathology of Parkinson disease is the progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta projecting to the striatum in the brain. The information regarding the expression of neuropeptides in Parkinsonianism is very limited. Here we have elucidated striatal neuropeptide mechanisms in experimental parkinsonism using the unilateral 6-hydroxydopamine model to degenerate dopamine neurons. A thoroughly controlled sample preparation technique together with a peptidomics approach and targeted neuropeptide sequence collections enabled sensitive detection, identification, and relative quantitation of a great number of endogenous neuropeptides. Previously not recognized alterations in neuropeptide levels were identified in the unilateral lesioned mice with or without subchronic 3,4-dihydroxy-L-phenylalanine administration, the conventional treatment of Parkinson disease. Several of these peptides originated from the same precursor such as secretogranin-1, somatostatin, prodynorphin, and cholecystokinin. Disease-related bioransformation of precursors into individual peptides was observed in the experimental model of Parkinson disease. Several previously unreported potentially biologically active peptides were also identified from the striatal samples. This study provides further evidence that neuropeptides take part in mediating the central nervous system failure associated with Parkinson disease. Molecular & Cellular Proteomics 8:1094-1104, 2009.

Parkinson disease (PD)\(^1\) is characterized by progressive loss of dopaminergic neurons in the substantia nigra pars compacta projecting to the striatum in the brain (1). PD symptoms are treated with dopamine (DA) replacement by administration of 3,4-dihydroxy-L-phenylalanine (L-DOPA) and other drugs that stimulate dopaminergic neurotransmission (2, 3). Besides the specific loss of DA content in the striatum, alterations in the transcript and translation of neuropeptides have also been described (4).

Changes in peptide expression may be important in the generation of abnormal basal ganglia activity and the generation of PD symptoms. It is well known that the regulation of opioid peptide synthesis in the basal ganglia is disturbed in Parkinsonism. The striatal RNA message for the primary precursor protein of enkephalin, preproenkephalin-A (PPE-A), is increased in Parkinsonism, whereas the precursor for dynorphin, preproenkephalin-B (PPE-B), is decreased (5–7). However, the information regarding the expression of neuropeptides from other precursors is very limited in PD.

An important question is the precise identity of which peptides are utilized as co-transmitters in the striatum and how their production is modified in Parkinsonism. Almost all previous studies have focused on the regional expression of the mRNA encoding the neuropeptide precursors using in situ hybridization. Very little is known regarding the precise identities of the peptide products themselves. This information is important because large precursor molecules (e.g. pro-opiomelanocortin) can be processed to produce a variety of neuroactive peptides. If the neuropeptide phenotype of individual striatal output cells can change, this could have important implications for therapeutic strategies because the possible peptide products may act on different receptor types.

The peptidome represents proteolytic processed entities that are derived from protein precursors. These endogenous peptides are often post-translationally modified, typically by C-terminal amidation and/or N-terminal pyroglutamic acidification or acetylation. Conventional techniques like radioimmunoassay (RIA) or Western blot have been used for measuring the differential expression of a number of neuropeptides, such as preproenkephalin A; PPE-B, preproenkephalin B; RIA, radioimmunoassay; GABA, \(\gamma\)-aminobutyric acid; PEG, polyethylene glycol; CV, coefficient of variance; CSF, cerebrospinal fluid.
in different neurological disorders (8–10). However, although these techniques are sensitive and robust, they are unable to measure several peptides simultaneously, and they can often not distinguish between different neuropeptide variants such as longer or shorter peptide forms or post-translationally modified species.

6-Hydroxydopamine (6-OHDA), a hydroxylated analogue of the natural dopamine neurotransmitter, is one of the most common neurotoxins used to experimentally model nigral dopaminergic degeneration (11, 12). 6-OHDA is commonly injected unilaterally into the ascending medial forebrain bundle, destroying nigral dopaminergic neurons and depleting the ipsilateral striatum of DA levels, hence reproducing the physiopathological features responsible for motor impairments in PD. The model was initially used on rats (13–15) and proved to present clinical features associated with PD also in mice (14, 16, 17).

In the present study, the unilateral 6-OHDA experimental mice model of PD and a peptidomics approach were used to perform a systematic regional analysis of striatal peptides. The striatum is the major input region of the basal ganglia because it receives the majority of afferents from other regions of the brain, especially the cerebral cortex, thalamus, and substantia nigra pars compacta (18). The striatum gives rise to two distinct inhibitory GABAergic projections to the globus pallidus, the so-called direct pathway to the medial globus pallidus and the indirect pathway to the lateral pallidal segment. There is also a direct inhibitory GABAergic pathway from striatum to substantia nigra pars reticulata. Both the direct and indirect pathways also contain specific peptide neuromodulators. The direct pathways contain substance P and opioid peptides derived from PPE-B, and the indirect pathway contains enkephalins derived from PPE-A (for reviews, see Refs. 19 and 20).

Our results showed that a number of identified striatal peptides from different precursors, such as secretogranins, somatostatin, PPE-B, and cholecystokinin (CCK), were significantly altered in unilaterally 6-OHDA-lesioned mice with and without subchronic L-DOPA administration. Additionally several previously unreported potentially novel peptide candidates were identified among the striatal peptides. These peptides had cleavage sites characteristic for neuropeptides and contained post-translationally modified species such as phosphorylation and pyroglutamic acid.

**EXPERIMENTAL PROCEDURES**

**Animals, Surgery, and Pharmacological Treatment**—The experiments were approved by the local ethics committee at Karolinska Institute (N282/06) and followed the European Communities Council Directive of November, 24 1986 (86/609/EEC). The animals (adult male on a C57BL/6 background (C57BL/6NCr, strain code 027), n = 12, B&K Universal, Sollentuna, Sweden) were housed in air-conditioned rooms (12-h dark/light cycle) at 20 °C and a humidity of 53%. Mice were anesthetized with ketamine (80 mg/kg, intraperitoneal; Parke-Davis)/xylazine (5 mg/kg, intraperitoneal; Bayer, Kiel, Germany), pretreated with desipramine (25 mg/kg, intraperitoneal; Sigma-Aldrich) and pargyline (5 mg/kg, intraperitoneal; Sigma-Aldrich), placed in a stereotaxic frame, and injected with 6-OHDA (1 μl of a 3 mg/ml solution dissolved in 0.01% ascorbate; Sigma-Aldrich) into the median forebrain bundle of the right hemisphere. The coordinates for injection were: anterior-posterior −1.1 mm, medial-lateral −1.1 mm, and dorsal-ventral −4.75 mm relative to bregma and the dural surface. The injection was at a flow rate of 0.5 μl/min. The injection cannula was left in place for an additional 5 min before being slowly retracted.

Two weeks after unilateral 6-OHDA lesioning, mice were administered apomorphine (1 mg/kg, intraperitoneal; Sigma-Aldrich), and their contralateral rotations were measured over 30 min. Only mice rotating >50 turns/30 min were included in further experiments. One animal did not pass this criterion and was hence excluded from the study. Four weeks after surgery, mice were treated with saline (n = 6) or L-DOPA/benserazide (n = 6; 50/12.5 mg/kg, intraperitoneal; Sigma-Aldrich) once daily for 14 days. The number of ipsi- and contralateral rotations were counted by inspection for 30 min. All mice were killed by focused microwave irradiation using special equipment (Muramachi Kikai, Tokyo, Japan) 15 min after the 15th L-DOPA injection. Striata (left and right) were dissected out for biochemical analyses and stored at −80 °C until further analysis.

**Western Blotting**—The efficacy of the dopamine denervation was verified post mortem by measurements of the striatal levels of tyrosine hydroxylase as described previously (21). Briefly microwaved frozen tissue samples were sonicated in 1% SDS, transferred to Eppendorf tubes, and boiled for an additional 10 min. Small aliquots of the homogenate were retained for protein determination by the BCA protein assay method (Pierce). Equal amounts of protein (20 μg) were loaded onto 12% acrylamide gels, and the proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (0.2 mm) (Schleicher & Schuell) by the method of Towbin et al. (22). The membranes were immunoblotted using antibodies raised toward tyrosine hydroxylase (Sigma-Aldrich, product number T2928). The antibody binding was revealed by incubation with goat anti-mouse horseradish peroxidase-linked IgG (Pierce, product number 31430, 1:6000–8000 dilution) and the ECL immunoblotting detection system (GE Healthcare).

**Sample Preparation**—The sample extraction buffer used was 0.25% acetic acid spiked with deuterated Met-enkephalin YGGF (F = Phe-d3), synthesized and purified by HPLC by the Department of Pharmaceutical Chemistry, Organic Chemistry, Uppsala University, Sweden, at a concentration of 40 fmol/μl. The striatal tissue samples, saline-administered intact side (SAL-INTACT, n = 6), saline-administered lesioned side (SAL-LESION, n = 6), L-DOPA-administered intact side (LDOPA-INTACT, n = 6), and L-DOPA-administered lesioned side (LDOPA-LESION, n = 6) were prepared as described previously (23, 24) with minor modifications. Briefly the peptidergic fraction of the striatal part of the brain was extracted by sonication in 95 °C sample extraction buffer (7.5 μl/mg of tissue) followed by incubation at 95 °C for 1 min. The cell debris were spun down by centrifugation for 40 min at 14 000 × g at 4 °C. The supernatant was transferred to a filtering device (Microcon, YM-10) with a cutoff limit of 10 kDa, and the peptide fraction was isolated through centrifugation for 60 min at 14 000 × g at 4 °C and stored at −80 °C until further analysis.

**Liquid Chromatography**—The peptide mixture was analyzed on a nano-LC system (Ettan MDLC, GE Healthcare) coupled to an electrospray Q-ToF2 (Waters) or a linear trap quadrupole (LTQ) (Thermo Scientific, San Jose, CA) mass spectrometer. The sample was injected and desalted on a precolumn (300-μm inner diameter × 5 mm, C18 PepMapR, 5 μm, 100 Å; LC Packings, Amstardam, The Netherlands) at a flow rate of 10 μl/min for 10 min. A 15-cm fused silica
emitter with a 75-μm inner diameter and a 375-μm outer diameter (Proxeon Biosystems, Odense, Denmark) was used as the analytical column. The emitter was packed in house with a slurry of reverse-phased ReproSil-Pur C18AQ 3-μm resin (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) dispersed in methanol using a pressurized packing device (Proxeon Biosystems). The mobile phases used were Buffer A (0.25% acetic acid in water) and Buffer B (84% ACN and 0.25% acetic acid in water). The peptide samples were separated and eluted during a 40-min gradient from 3 to 60% Buffer B at a flow rate of ~170 nl/min.

Peptide Profiling of the Striatum—The profiling analysis of the peptide samples were performed on the Q-TOF MS instrument. The mass spectrometer was calibrated according to the mass spectrometer manufacturer’s recommendations using a PEG solution (PEG 200, 23 ng/μl; PEG 400, 48 ng/μl; and PEG 600, 70 ng/μl; Fluka) delivered from a syringe pump at a flow of 300 nl/min during 2 min. The contact closure feature was used to start the data sampling, and the MS data were acquired in continuous mode in the m/z range 300–1000 with a mass resolution of 11,200 (full-width half-maximum) at m/z 560.37 (z = 3). 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. Data were collected during 50 min. A reference sample of pooled material was analyzed as every fourth or fifth sample, and in between these runs, one sample from each group of animals (SAL–INTACT, SAL–LESION, LDOPA–INTACT, and LDOPA–LESION) was analyzed in a randomized fashion. Each group of four to five samples was considered as one block in the statistical analysis.

The raw MS data were converted into an ASCII file using the Data Bridge module available in the MassLynx software (Version 3.5, Micromass). These text files were then imported into DeCyder MS2.0 (GE Healthcare) and processed using the import and batch module. Peptide masses were automatically detected in DeCyderMS with the following parameters: peak elution time, 0.4 min; peak resolution, 8000; accepted charge states, 1–12; a signal-to-noise cutoff of 5; and background-subtracted quantification (smooth surface). Manual verification of the detected peptides was performed, and some peptides were removed or added manually in the PepDetect module. The time alignment feature was used to correct differences in retention times for the samples allowing an adjustment up to 5 min. The aligned intensity maps were then used to match the PepDetect files with a match window of ±1 min and ±0.2 Da. The log2 intensities from the matched result were exported, and peaks with mass <500 Da or retention time >42 min were removed prior to normalization. On average 655 ± 13 peaks were found in each sample after filtering.

Relative Quantification of Peptides—Statistical analysis was performed in the language R (Version 2.6.0) (25) using the R/maanova (Version 1.5.1) Bioconductor package (Version 2.1) (26, 27). Linear regression normalization was applied by constructing a median reference file of all the medians of the peak intensities and then applying least squares regression to the scatter plot for each peptide profiling run; this is similar to the approach described by Roy and Becker (28). After normalization only peaks that were detected in at least five of six samples in the four treatment groups were included for further analyses (283 peaks). Missing values were imputed using k-nearest neighbors (k = 10). On average each sample contained 1.7% missing values prior to imputation.

For each detected peptide, a linear mixed model was utilized using restricted maximum likelihood estimation. Two types of analyses were performed. First, the treatment effect within individuals was estimated. Because the effects of the 6-OHDA lesion can be measured within the same individual, an ANOVA model where samples were set as fixed factors and blocks were set as random factors was used (ANOVA1). This way the individual variation of treatment effects is taken into account. Second, to compare all different treatment effects the data were analyzed using an ANOVA model with the four treatment groups as fixed factors and the samples and blocks as random factors (ANOVA2). Pairwise t testing between (SAL–LESION) and (SAL–INTACT), and between (LDOPA–LESION) and (LDOPA–INTACT), and t testing between (LDOPA–LESION) and (SAL–LESION) were performed on peptides significant in the ANOVAs. The reference samples were included for estimation of block effects. Significance testing was performed using random permutations of the samples (27, 29) to estimate p values for the ANOVA and t tests. The function matest() available in the R/maanova package was used. The number of permutations was set to 1000. The estimation of the error variance was accomplished using the James-Stein shrinkage concept, and a test statistic called Fs was used (30). The Fs test has been proven to be robust and perform well under a wide range of assumptions about variance heterogeneity (30). In gene expression studies using microarrays it has been demonstrated to perform acceptably when the variances are truly constant as well when the variances vary from gene to gene (30). This also makes it suitable for LC-MS data. For comparison tabulated p values for the observed data using classical F and t statistics (only using data from individual peptides) are presented in the supplemental information. The level of statistical significance was set at p < 0.05.

Peptide Identification—Nano-LC linear trap quadrupole MS/MS analyses were performed on all the samples for identification of peptides. The same LC setup as above was used, and the MS/MS data were collected in a data-dependent manner. The acquisition was set up to continuously switch between full MS scan (m/z 300–2000), zoom scan (most intense peak in full scan), and MS/MS scan (most intense peak in zoom scan) where the most intense peak can be picked twice in a time window of 40 s before being put on an exclusion list during a 150-s period.

The tandem mass spectra generated in the nano-LC-MS/MS run were converted into dta files using Xcalibur (Version 2.0 SR2), and the dta files were then combined into mgf files by using an in-house developed script. Tandem mass spectra were assigned sequences by searching them against three targeted sequence collections: SwePep precursors (123 protein entries), SwePep peptides (245 peptide entries), and SwePep prediction (3,413,034 peptide entries) as described previously (31) using X!Tandem (Version 2006.9.15.4) (32, 33). These targeted sequence collections have been developed using the SwePep database (34) and provide fast, specific, and sensitive identification of endogenous peptides (31). To confirm the identities of previously unreported peptides (not previously identified with mass spectrometry), their corresponding tandem mass spectra were searched against UniProt (48.8 mouse (10,431 protein entries) to check that their tandem mass spectra were not assigned to another sequence in a larger database. The reference sample was searched against UniProt to confirm the sample quality (35).

Tandem mass spectra where one of the three most abundant peaks could correspond to a neural loss of a phosphate group (36) were collected for identification of phosphorylated peptides. The spectra were searched against SwePep precursors allowing for phosphorylations of serine, threonine, and tyrosine, and all hits were manually verified.

The SwePep precursor and the SwePep peptide sequence collections were searched using unspecific cleavage, and the precollected SwePep predicted sequence collection was searched using no cleavage. The databases were searched using a peptide mass tolerance of ±2 Da and a fragment mass tolerance of ±0.7 Da. The data set was searched with a number of possible post-translational modifications (N-terminal acetylation, N-terminal pyroglutamic acid of glutamine and glutamic acid, C-terminal amidation, and oxidation and dioxygenation of methionine and tryptophan). The refinement function in
Fig. 1. A, reduction of tyrosine hydroxylase (TH) in the 6-hydroxydopamine-lesioned side of the striatum compared with intact striatum. The observed reduction was 94%. B, number of contralateral rotations during 30 min following saline or l-DOPA administration. Data are expressed as mean ± S.E. **, p < 0.01; ***, p < 0.001 (paired t test). Error bars represent standard error of the means.

All samples were spiked with 40 fmol/μl deuterated Met-enkephalin. This allowed for an absolute quantitation of endogenous Met-enkephalin. The endogenous levels of Met-enkephalin (260 ± 16 fmol/μl, 1.15 μg/g of tissue) calculated from the levels of spiked deuterated Met-enkephalin correlated well with levels previously measured in striatum (42, 43).

Post-mortem Sample Quality Verification—A peptide from stathmin (stathmin-(2–20)) known to be present in samples where post-mortem degradation has occurred was used as a quality marker in the present study (35). The reference samples were searched against UniProt, and as stathmin-(2–20) was not identified in the database search, the samples were considered to be devoid of protein degradation.

Identified Peptides in Striatum—From the tandem MS analysis of all analyzed samples, there were in total 68 peptides identified by searches against the targeted sequence collections (supplemental table). These peptides were derived from 21 peptide precursors, most of them known to contain neuropeptides. The identified peptides contained several post-translational modifications such as phosphorylation, C-terminal amidation, pyroglutamic acidification, and N-terminal acetylation. Among the identified peptides five were previously unreported (Table I).

Additionally two N-terminal peptides from elongation factor 1-β and two peptides from cerebellin-4 were identified in searches against UniProt. These precursors were not part of the SwePep sequence collections, and therefore the peptides were not identified in those searches.

By matching the experimental masses from the Q-TOF analysis to theoretical peptide masses in SwePep another 19 peptide identities were suggested at the mass accuracy threshold of 0.5 Da (supplemental table). These peptide sequences have so far not been identified above the significance threshold in database searches.

Differentially Expressed Striatal Peptides—To examine how the peptide expression differed in striatum of unilaterally 6-OHDA-lesioned mice treated with saline or l-DOPA, two


### Table I

| UniProt accession no. | Protein name | Peptide name | Sequence with adjacent cleavage sites | Mass (M + H) | Comment |
|-----------------------|--------------|--------------|---------------------------------------|--------------|---------|
| P22005                | PENK_MOUSE   | PENK_MOUSE 87-97 | KD [87]SSKQDSSHLA [87]KK            | 1214.601     | N-terminal part of synenkephalin |
| P16014                | SCG1_MOUSE   | Phosphorylated SCG1_MOUSE 313–330 | KD [177]SHQESTNNDNEDM [184]SKR | 1621.603 | Part of propeptide |
| P60041                | SMS_MOUSE    | SMS_MOUSE 87-100 | EL [87]Pyro-Glu(Q)RSANSNPAMAPRE [100] | 1511.702 | N terminus of a propeptide |
| P09240                | CCKN_MOUSE   | CCKN_MOUSE 21–34 | LA [24]Pyro-Glu(Q)PVVPAEATDPVEQ [25]RAEEAR | 1462.706 | |

### Table II

**Striatal peptides found to be differentially regulated**

Significant regulations \((p < 0.05)\) are marked in bold. Peptides in italic were only identified by mass match. SAL-INTACT, saline-treated intact side; SAL-LESION, saline-treated lesioned side; LDOPA-INTACT, L-DOPA-treated intact side; LDOPA-LESION, L-DOPA-treated lesioned side. lin, linear.

| Precursor Peptide | p value ANOVA1 | Fold change (LDOPA-LESION) – (SAL-LESION) (unpaired t test) | p value | Fold change (SAL-INTACT) – (SAL-LESION) (paired t test) | p value | Fold change (LDOPA-INTACT) – (LDOPA-LESION) (paired t test) | p value |
|-------------------|----------------|-------------------------------------------------|---------|-------------------------------------------------|---------|-------------------------------------------------|---------|
| P09240 CCKN_MOUSE | 0.038          | 0.06 1.05 0.712                                 | 0.25 1.19 0.155 | 0.43 1.35 0.024 |
| P09240 CCKN_MOUSE | 0.014          | 0.48 1.39 0.308                                 | 0.38 0.77 0.336 | 1.33 2.51 0.005 |
| P60041 SMS_MOUSE  | 0.052          | −0.51 0.70 0.042                                | 0.54 1.45 0.016 | 0.05 1.03 0.803 |
| P09240 CCKN_MOUSE | 0.016          | −0.63 0.65 0.084                                | 0.71 1.63 0.006 | 0.23 1.17 0.304 |
| P16014 SCG1_MOUSE | 0.035          | −0.24 0.85 0.255                                | 0.51 1.42 0.010 | 0.03 1.02 0.881 |
| P16014 SCG1_MOUSE | 0.011          | −0.24 0.85 0.340                                | 0.68 1.60 0.017 | 0.63 1.55 0.027 |
| P16014 SCG1_MOUSE | 0.026          | −0.17 0.89 0.549                                | 0.36 1.10 0.130 | 0.05 1.07 0.517 |
| P16014 SCG1_MOUSE | 0.234          | −0.32 0.80 0.033                                | 0.24 1.18 0.115 | 0.10 1.07 0.477 |
| P16014 SCG1_MOUSE | 0.003          | 0.52 1.44 0.120                                 | −0.50 0.71 0.060 | 0.94 1.92 0.002 |
| O35417 PDYN_MOUSE   | 0.715          | 0.76 1.69 0.021                                 | −0.03 0.98 0.920 | 0.23 1.17 0.415 |
| Q09X0 V0 Q0X0V0     | 0.038          | −0.33 0.80 0.230                                | −0.01 1.00 0.978 | −0.58 0.67 0.011 |
| Q8BM9 CBLN4_MOUSE   | 0.037          | −0.12 0.92 0.504                                | −0.20 0.87 0.212 | −0.42 0.75 0.019 |
| P02966 PEPB1_MOUSE  | 0.010          | 0.03 1.02 0.895                                | −0.05 0.97 0.633 | 0.79 1.74 0.003 |
| P01193 COLI_MOUSE   | 0.007          | 0.08 1.05 0.814                                | 0.66 1.58 0.020 | 0.72 1.65 0.013 |
| P56388 CART         | 0.044          | −0.31 0.80 0.413                                | 0.65 1.56 0.013 | 0.00 1.00 0.999 |

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**Secretogranin**—Secretogranins, also called chromogranins, are a class of acidic secretory proteins that occur in endocrine, neuroendocrine, and neuronal cells. Several known bioactive peptides are derived from this class of proteins (44). In the present study, 14 peptides from secretogranin-1, two from secretogranin-2, and one from secretogranin-3 were identified. From the ANOVA1s, four of the peptides from secretogranin-1 and the secretogranin-3-derived peptide were found to be differentially expressed (Table II). The GRGREGPAHSAALDREE peptide was increased on the lesioned side both in animals receiving saline (60%) and L-DOPA (55%). The unphosphorylated form of the potentially bioactive peptide PSPKESKEADVATVRLEGE was up-regulated by 42% on the lesioned side of the saline-treated animals, but following L-DOPA administration this increase was reversed. The peptide YPQSKWQEQE, which is part of the GAWK peptide (45), was also affected by the L-DOPA treatment, showing a decrease on the lesioned side of the brain following L-DOPA administration (Table II). The general pattern of expression for all the secretogranin-1-derived peptides was characterized by an increase on the lesioned side of the brain. This trend was especially true for the saline-treated animals, whereas the trend was somewhat more diverse following L-DOPA treatment (Fig. 2).

The peptide derived from the secretogranin-3 precursor displayed a different expression pattern. The level of this peptide was lower on the lesioned side compared with intact side in saline-treated animals, but following L-DOPA administration there was a significant up-regulation in the lesioned side compared with intact side (92%).
somatostatin-derived peptides were up-regulated on the lesioned side of the brain (45 and 63%, $p < 0.05$, respectively), and following L-DOPA administration the levels were reduced by 30% ($p < 0.05$) and 35% (non-significant) (Table II). Altogether three peptides derived from the N-terminal part of somatostatin-28 and one post-translationally modified prolonged form of the same peptide were identified, and they all had similar expression profiles (Fig. 3). Another somatostatin-derived peptide, AGCKNFFFWTFTSC, displayed a different expression pattern (Fig. 3). However, this peptide was only identified by mass matching. The lack of acceptable MS/MS data might be due to the presence of a disulfide bridge.

Preproenkephalin B—There were in total four peptides identified from the PPE-B precursor. One of these was differentially expressed according to the ANOVA. The peptide SSEGRMSVLKNQSLDPSHRISD displayed an up-regulation on the lesioned side following L-DOPA treatment (69%) compared with the saline-treated lesioned side. Also in this case all peptides derived from the same precursor had similar expression profiles except for YGGFL (Leu-enkephalin), which is a peptide that is derived from multiple precursors (Fig. 4). The general trend was that L-DOPA treatment increases the expression of PPE-B peptides.

Cholecystokinin—CCK is widespread throughout the central nervous system. It has been suggested to be involved in nausea and anxiety as well as satiating (46, 47). There were two peptides derived from the cholecystokinin precursor that were differentially expressed. These peptides, APSGRMSVLKNQSLDPSHRISD and pyro-Glu(O)PVPAEATD-PVEQ, were increased on the L-DOPA-treated lesioned side compared with the intact side of the brain (151 and 35%, respectively) (Table II).

In addition, significant changes in the levels of single peptides from the precursors of phosphatidylethanolamine-binding protein 1 (PEBP1), pro-opiomelanocortin-$\alpha$, and cocaine- and amphetamine-regulated transcript were found between the lesioned and intact tissue (Table II). Our result did not show any significant pattern of regulation of PPE-A-derived peptides. In line with previous findings, our data indicated a down-regulation of substance P on the lesioned side of the brain, but this change did not reach significance ($p = 0.058$).

**DISCUSSION**

In the present study a label-free quantitative peptidomics approach was applied to study the relative abundance changes of striatal endogenous peptides in an experimental model of PD with and without L-DOPA administration. The coupling of nano-LC to MS enabled detection and characterization of hundreds of endogenous peptide species simultaneously, and about 70 striatal neuropeptides were unambiguously identified and relatively quantified. Both known and previously unreported peptides derived from precursors such
as secretogranin-1, somatostatin, PPE-B, and CCK were found to be differentially expressed. The present study also addressed the effect on the biotransformation of peptide precursors to peptides, and variations were observed in several precursors after dopamine depletion and L-DOPA administration.

Previously we have measured levels of small proteins in parkinsonian experimental models using profiling and imaging MS technology directly on brain tissue sections (48–50). A number of unique protein expression profile differences were found in the dopamine-denervated side of the brain in specific brain regions when compared with the corresponding intact side, for example, calmodulin, cytochrome c, cytochrome c oxidase, FKBP-12, ubiquitin, and PEP-19. This technology allowed for the study of complex biochemical processes such as those occurring in experimental PD and provided unique information regarding the peptide and protein expression in selected brain regions. Furthermore we have also showed

![Graph](image)

**Fig. 3.** The expression profiles of the somatostatin-derived peptides. Each trend line represents one individual peptide. The graph presents mean fold changes (log₂) for the striatal peptides levels in the different treatment groups (SAL-LESION, LDOPA-INTACT, and LDOPA-LESION relative to saline-administered intact side SAL-INTACT, which is set to base level (zero)). Peptides showing significant differential expression (p < 0.05) are marked with **.

![Graph](image)

**Fig. 4.** The expression profiles of the PPE-B-derived peptides. Each trend line represents one individual peptide. The graph presents mean fold changes (log₂) for the striatal peptides levels in the different treatment groups (SAL-LESION, LDOPA-INTACT, and LDOPA-LESION relative to saline-treated intact side SAL-INTACT, which is set to base level (zero)). Peptides showing significant differential expression (p < 0.05) are marked with **.
that the in vivo biotransformation of dynorphin A-(1–17) in the striatum is disturbed in experimental parkinsonism (51).

The 6-OHDA lesion model of PD used in the present study induces a selective, reproducible, and dose-dependent degeneration of nigrostriatal DA neurons (12) and has therefore been extensively used to model PD. Most previous studies have focused on the regional expression of the mRNA encoding the neuropeptide precursors using in situ hybridization, but not much is known about the effect on neuropeptide alterations. As individual peptides might target different receptors or different regulatory pathways it is of importance to study the precise identities of the endogenous peptide products. Previous attempts to measure the levels of neuropeptides in parkinsonian experimental models or PD patients have been made using RIA with inconsistent results (9).

Our present results on peptide expression using the 6-OHDA lesion model of PD showed an increase of somatostatin-derived peptides in the DA-denervated striatum. Following L-DOPA administration this pattern of regulation was normalized. Somatostatin is present in substantial amounts in the striatum, particularly in dense plexus of fibers and nerve terminals as well as in cell bodies belonging to a striatal population of medium sized spiny neurons. It acts through somatostatin receptors SST1–5 in the brain (52).

There are several studies indicating a relationship between somatostatin expression and the dopaminergic system. Intracerebroventricular injections of somatostatin increased DA synthesis (53) and release (54), and somatostatin increased DA release from striatal slices (55). Studies on animal models of PD showed an increase of somatostatin mRNA in unilaterally lesioned primates and rats (56, 57). Our present results are in line with the significant increase of somatostatin-like immunoreactivity levels investigated in cerebrospinal fluid (CSF) from 23 patients with untreated early parkinsonian syndrome compared with control subjects (58). However, the opposite was found in CSF of patients with moderate or severe disease (59). Given our data, it may be possible that the increase in somatostatin is a compensatory mechanism trying to restore the DA levels. This is in line with previous work administering DA agonists and an antagonist to rats where increased somatostatin levels were observed following administration of the D2 antagonist haloperidol. Reduced somatostatin levels were detected following co-administration of the two D1 and D2 agonists SKF38393 and quinpirole (60). However, the exact mechanisms of how the different individual somatostatin peptides function remain to be fully characterized.

Furthermore four peptides originating from the secretogranin-1 precursor were differentially expressed. There was a general trend of increased expression in the DA-denervated striatum compared with the intact side. This pattern was then either maintained or reversed following L-DOPA treatment. Secretogranins, also referred to as chromogranins, are present throughout the neuroendocrine system and have been suggested to play an important role in the packaging of hormones and the formation of secretory granules (61, 62) but also to function as precursors of active released peptides (62–64). At least eight secretogranin-1-derived peptides have been identified from different species, and apart from the antibacterial function of secretolytin they all have, to our knowledge, unknown functions (44, 65). Secretogranin-1 has been implicated to be differentially regulated in a number of brain neurological disorders such as schizophrenia (66), multiple sclerosis (67), and Alzheimer disease (68, 69). Additionally rats administrated with reserpine, a drug used for producing an animal model of PD, showed an increase of secretogranin-1 mRNA in substantia nigra pars compacta (70). However, RIA measurements on a secretogranin-1-derived peptide, PE11, in CSF from PD patients did not reveal any alterations compared with control subjects (71).

Our study also showed an increase of the PPE-B-derived peptide, SSEMAREDGGQDGQVGHEDLY, following L-DOPA treatment in different animal models of PD (7, 72–75). It has been speculated that the increased expression of PPE-B is a key component in the process eventually leading to L-DOPA-induced dyskinesias, the severe side effect associated with L-DOPA treatment (7, 72, 73).

Two cholecystokinin-derived peptides were increased on the lesioned side of the brain following L-DOPA administration. The roles of their individual function remain uncharacterized. DA plays an important role in CCK release, and CCK mRNA levels in the rat striatum have been shown to be elevated following treatment with DA agonists (76). Previously CCK levels have been measured by RIA in 6-OHDA-lesioned rats with or without prolonged L-DOPA treatment, and the L-DOPA treatment increased the CCK immunoreactivity in the lesioned striatum (77). Additionally cholecystokinin peptides have been shown to produce a significant increase of the release of dynorphin B in the neostriatum of the rat and in 6-OHDA-lesioned rats (78). The up-regulation of both CCK- and PPE-B-derived peptides observed in our study following L-DOPA treatment might therefore be related.

The label-free nano-LC-MS quantification approach of peptides used in our study has been shown to perform well compared with methods based on stable isotope labeling (79, 80). There are several advantages of using a label-free method compared with labeling technology. Fewer steps of sample preparation are needed, hence minimizing the loss of peptides during extraction. The cost is reduced because no labeling tags are necessary. A larger part of the peptidome...
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can be measured because most labeling technologies target only a subset of peptides; e.g. ICAT targets Cys-containing peptides (81). Additionally, biologically active peptides are often N- and C-terminally modified and therefore not targeted by amine-specific tagging such as isobaric tags for relative and absolute quantitation (82). On the other hand, the use of stable isotope labeling enables multiplexing, and up to eight samples can be quantified in the same analysis, and ratios representing relative abundances can be extracted directly.

Tissue handling and sample preparation techniques are crucial for obtaining proper quality data in neuropeptide discovery and differential peptidomics. The presence of proteolytic peptides produced post mortem from high abundance proteins has been shown to greatly influence the result and reduce the reproducibility and the quantification (23, 35). A marker for sample degradation such as the peptide stathmin-(2–20) (35) is therefore a valuable tool in assessing the quality of the sample. Obtaining a sample that reflects the true peptidome (without proteolytic peptides produced post mortem) and the use of targeted peptide sequence collections such as those derived from the SwePep database are therefore of great advantage for identifying and quantifying known and new potentially bioactive peptides.

Several recently reported (83) and previously unreported peptides in the present study are likely to have biological function. The peptide PSPKESKEADATVRLGE derived from secretogranin-1 was found to be increased following DA derivation. It has basic cleavage sites at both the N and C termini and was identified both with (83) and without phosphorylation. Another interesting peptide identified was DADDSSVEKQVALLKALYGHGQISH derived from protachyklinin (83). It has basic cleavage sites adjacent to both the N and C termini and contains the N-terminal part of neuropeptide K, a peptide well conserved within mammals and known to be biologically active (84). A third previously unreported peptide with potential biological activity is the cholecystokinin-derived peptide pyro-Glu(Q)PVVPAEATDPVEQ, which is the N terminus of its propeptide. The peptide is N-terminally modified and has basic cleavage sites at the C terminus. Obviously MS information alone cannot determine whether a peptide possesses a specific biological function or receptor binding potential. Additional investigations are necessary to elucidate their individual functions and characteristics.

In conclusion, the present data demonstrate that the peptidomics approach used in this study shows a great potential of detecting, identifying, and quantifying the levels of a considerable number of endogenous peptides simultaneously in an animal model of PD. Several of these peptides were differentially regulated including a number of potentially novel peptides. Although alterations in the expression pattern of individual novel peptides or peptide families in different physiological situations can help in suggesting the possible function of a peptide, additional functional characterization studies are needed. Furthermore increased knowledge of the complex relationships among neuropeptides and their interaction partners may make it possible to manipulate the biological system. The aim would be to reduce the problems associated with L-DOPA therapy, such as dyskinesias, and to improve the quality of life of PD patients.

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