

Identification of L-ferritin in Neuromelanin Granules of the Human Substantia Nigra

A TARGETED PROTEOMICS APPROACH*

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In the pigmented dopaminergic neurons of the human substantia nigra pars compacta the system relevant in iron storage is the polymer neuromelanin (NM). Although in most cells this function is mainly accomplished by ferritin, this protein complex appears not to be expressed in NM-containing neurons. Nevertheless the conceivable presence of iron-storing proteins as part of the NM granules has recently been discussed on the basis of Mössbauer spectroscopy and synchrotron x-ray microspectroscopy. Intriguingly by combining subcellular fractionation of NM granules, peptide sequencing via tandem mass spectrometry, and the additional confirmation by multiple reaction monitoring and immunogold labeling for electron microscopy, L-ferritin could now be unambiguously identified and localized in NM granules for the first time. This finding not only supports direct evidence for a regulatory role of L-ferritin in neuroectodermal cell pigmentation but also integrates a new player within a complicated network governing iron homeostasis in the dopamine neurons of the human substantia nigra. Thus our finding entails far reaching implications especially when considering etiopathogenetic aspects of Parkinson disease. *Molecular & Cellular Proteomics* 8:1832–1838, 2009.

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Received, January 6, 2009, and in revised form, March 24, 2009

Published, MCP Papers in Press, March 24, 2009, DOI 10.1074/mcp.M900006-MCP200

Neuromelanin (NM)¹ is a dark colored polymeric pigment produced in specific populations of catecholaminergic neurons in the brain (1). Unlike peripheral melanins, which are produced in specialized cells called melanocytes and may be transferred to other cell types, NM granules are believed to be stored in the neurons in which they are produced. NM granules display a unique, more heterogeneous appearance compared with peripheral melanins. Further unlike melanin, NM is traditionally thought to result from a non-enzymatic synthesis pathway with no known pathway for NM catabolism. More recent data, however, are indicative of some regulation of NM synthesis and turnover (1).

NM appears in greatest quantities in the human brain and in lesser amounts in some other non-human primates but is absent from the brain of many lower species. Interest in this pigment has seen a resurgence in recent years because of a hypothesized link between NM and the especial vulnerability of NM-containing neurons of the substantia nigra pars compacta (SN) for cell death in Parkinson disease (PD) (2, 3). In particular, the interaction between iron and NM has been a focus of research (4–8) because a marked accumulation of iron related to disease severity is reported in the parkinsonian SN (9–11). The cellular location of this apparent increase in iron is unclear, but a variety of changes in iron regulatory systems occur in PD (12–15).

A potential candidate for intraneuronal iron homeostasis in the SN, however, is NM. NM is able to bind a variety of metals; 7% (w/w) of isolated NM is reported to consist of iron, copper, zinc, manganese, and chromium (16, 17). Iron binding studies using NM isolated from the human SN demonstrated that NM contains high ($K_D = 7.18 \pm 1.08$ nM) and low affinity binding sites ($K_D = 94.31 \pm 6.55$ nM) for Fe(III) (18). Our recent data showed that a pure Fe(III) signal can be measured from intact frozen SN tissue using Mössbauer spectroscopy (18). These data indicated that iron is directly bound to NM granules in the SN (4, 16, 19) and that this signal is increased in PD (20). In addition, Mössbauer spectroscopy showed that iron binding

¹ The abbreviations used are: NM, neuromelanin; L, light; H, heavy; SN, substantia nigra pars compacta; PD, Parkinson disease; 1-D, one-dimensional; MRM, multiple reaction monitoring; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

sites in NM isolated from the human SN are similar to those of human ferritin and hemosiderin (21). Similar results were also reported recently in whole neurons from formalin-fixed and paraffin-embedded human SN sections using synchrotron chemical x-ray microscopy (22). Because ferritin, the main iron storage protein, is primarily located in glia rather than in neurons (23), it seems unlikely that it could regulate neuronal iron levels, and until today the exact iron storing mechanism in the NM-containing neurons of the SN was unknown.

The aim of the present study was thus to find direct evidence for the presence of L-ferritin in NM granules isolated from human post-mortem tissue of subjects with no history of neurological, neurodegenerative, or psychiatric diseases by using a targeted MS-based approach. Recently our group reported a method for the isolation of intact NM granules from the human SN to carry out the first protein profile of these organelles (24). The major findings were the identifications of numerous proteins closely associated with lysosome-related organelles originating from the endosomal system (24, 25). In our present study, we report for the first time the identification of L-ferritin as a component of NM granules, pointing to a ferritin-based iron storage mechanism in the NM-containing neurons of the SN, by using an approach combining one-dimensional (1-D) SDS-PAGE, reversed-phase nano-HPLC electrospray ionization tandem mass spectrometry (nano-LC-ESI-MS/MS and nano-LC-ESI-multiple reaction monitoring (MRM)-MS/MS), Western blot analysis, and immunotransmission electron microscopy.

MATERIALS AND METHODS

Isolation of NM Granules—Human SN tissue was obtained from the “Austrian-German Brain Bank” in Würzburg, Germany. The use of post-mortem human brain tissue was approved by the Ethics Committee of the University Clinics of Würzburg. The SN was dissected from post-mortem brains of subjects with no history of neurological, neurodegenerative, or psychiatric diseases within 36 h of death on a cool plate (-15°C). NM granules were isolated to high purity according to the method published previously (24).

Transmission Electron Microscopy Sample Preparation—The quality of the granule isolation was monitored by transmission electron microscopy as described previously (24). For immunodetection the isolated granules were fixed overnight in 0.1 M PBS containing 4% (v/v) paraformaldehyde, 0.1% glutaraldehyde, 2.5% (w/v) sucrose. The fixed specimens were washed in PBS (3×10 min), dehydrated in graded ethanol, and embedded in “LR White” resin (London Resin, London, UK) for 1 h. Fresh LR White was added for overnight incubation on 4°C prior to polymerization via UV irradiation (Philips TLD 15W/05; 300–460 nm; maximum, 365 nm) at 4°C for 2 days. Thin sections were prepared and mounted on nickel grids for subsequent immunodetection.

On-grid Postembedding Immunogold Labeling—Residual aldehyde groups present after aldehyde fixation were inactivated by 0.05 M glycine in PBS for 15 min. The ultrathin sections were blocked with PBS, 5% (w/v) BSA, 0.1% (w/v) cold water fish skin gelatin, 5% normal rabbit serum (Aurion, Wageningen, The Netherlands) for 30–45 min and washed in “incubation buffer” containing PBS, 0.1% BSA, 20 mM $\text{Na}_2\text{S}_2\text{O}_3$, pH 7.4 (3×5 min). The specimens were probed with a goat anti-L-ferritin antibody (1:400–800, v/v; Santa Cruz Biotechnology Inc., Heidelberg, Germany), washed in incubation buffer (6×10 min),

and incubated with a 10-nm colloidal gold particle-conjugated secondary rabbit anti-goat antibody (1:40, v/v; Aurion) for 2 h. The sections were washed, postfixed in 2% (v/v) glutaraldehyde, counterstained with uranyl acetate and lead citrate, and monitored in a LEO 912 electron microscope (Zeiss SMT, Oberkochen, Germany). For controls, the primary antibody was omitted from the incubation.

Sample Preparation for 1-D SDS-PAGE—Proteins of isolated NM granules were extracted with 16 mM 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate and SDS, mixed with reducing sample buffer containing DL-dithiothreitol, and heated for 10 min at 95°C .

1-D SDS-PAGE—The protein samples were separated electrophoretically on 10–20% Tricine gels (Novex, San Diego, CA) in an XCell II™ Mini Cell (Novex) using Tricine-SDS running buffer. Following electrophoresis a gel was either stained with colloidal Coomassie Brilliant Blue G-250 or further processed for Western blotting.

Antibodies and Materials—For Western blot analysis anti-human monoclonal antibody to L-ferritin was used (Santa Cruz Biotechnology Inc.), and horseradish peroxidase-linked secondary anti-rabbit antibody was obtained from Cell Signaling Technology Inc. (Frankfurt/Main, Germany).

Tissue Homogenate—To provide a positive control for the Western blot analysis, 0.5 g of SN tissue was disrupted in a Potter-Elvehjem homogenizer in “Lysis Buffer” containing protease inhibitor mixture (0.01%, v/v; Sigma), and proteins were extracted with 16 mM 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate (Calbiochem).

Western Blot Analysis—The separated proteins were transferred onto a nitrocellulose membrane (Invitrogen) using the XCell II blot module. Nonspecific binding was blocked with 5% (w/v) nonfat dried milk in Tris-buffered saline, pH 7.3 for 1 h at 20°C . Immunoblots were probed with primary antibody at the appropriate dilution at 4°C overnight. Membranes were washed in Tris-buffered saline containing 0.1% (v/v) Tween 20 (3×10 min) followed by incubation with the secondary antibody at 20°C for 1 h. Additional washing was performed with Tris-buffered saline containing 0.1% (v/v) Tween 20 (3×10 min), and the immune complexes were visualized by an ECL system (Roche Diagnostics).

In-gel Digestion—From the stained gel the region of interest was cut out and transferred into a separate quartz reaction tube (Sigma). The gel cubes were washed with 10 mM NH_4HCO_3 , pH 7.8 and 10 mM NH_4HCO_3 , pH 7.8, ACN (1:1, v/v) each for 10 min, respectively (23). The gel cubes were consequently reswollen by adding 2 μl of modified trypsin (Promega, Mannheim, Germany) (0.05 $\mu\text{g}/\mu\text{l}$ in 10 mM NH_4HCO_3 , pH 7.8) and incubated overnight at 37°C . 10 μl of 0.1% (v/v) TFA, ACN (1:1, v/v) were added to each gel slice followed by sonication for 10 min. This step was repeated twice, and the supernatants containing the extracted peptides were combined in separate quartz tubes.

Mass Spectrometry—Identification of L-ferritin was carried out as described previously (23). Peak lists were generated using Create DTA (extract_msn version 3.3, copyright 1997–2006, licensed to Thermo Electron Corp.) with the following parameters: general: thresholds: number of ions, 35; total ion current, 2×10^5 ; and MH^+ mass range, 450–5000 Da; grouping: mass tolerance, 1.5 Da; minimum allowed intervening scans, 0; number of adjacent scans to group, 1; and precursor charge state, ZSA; combine identical spectra (Comblon); determine charge state from MS/MS (ZSA); and correct MH^+ assignment (Correction). The analysis of the MS/MS data occurred automatically based on the Sequest™ algorithm (Turbo-SEQUEST, Parallel Virtual Machine (PVM) Slave v.27). The data were searched against the UniProtKB/Swiss-Prot (version 54.4, October 23, 2007, 272,212 entries, no species restriction) using the following parameters: average masses, variable modification of methionine (+16 Da) and cysteine (+71 Da), a mass tolerance of ± 1.5 Da for precursor and fragment masses, trypsin as a specific protease, and a

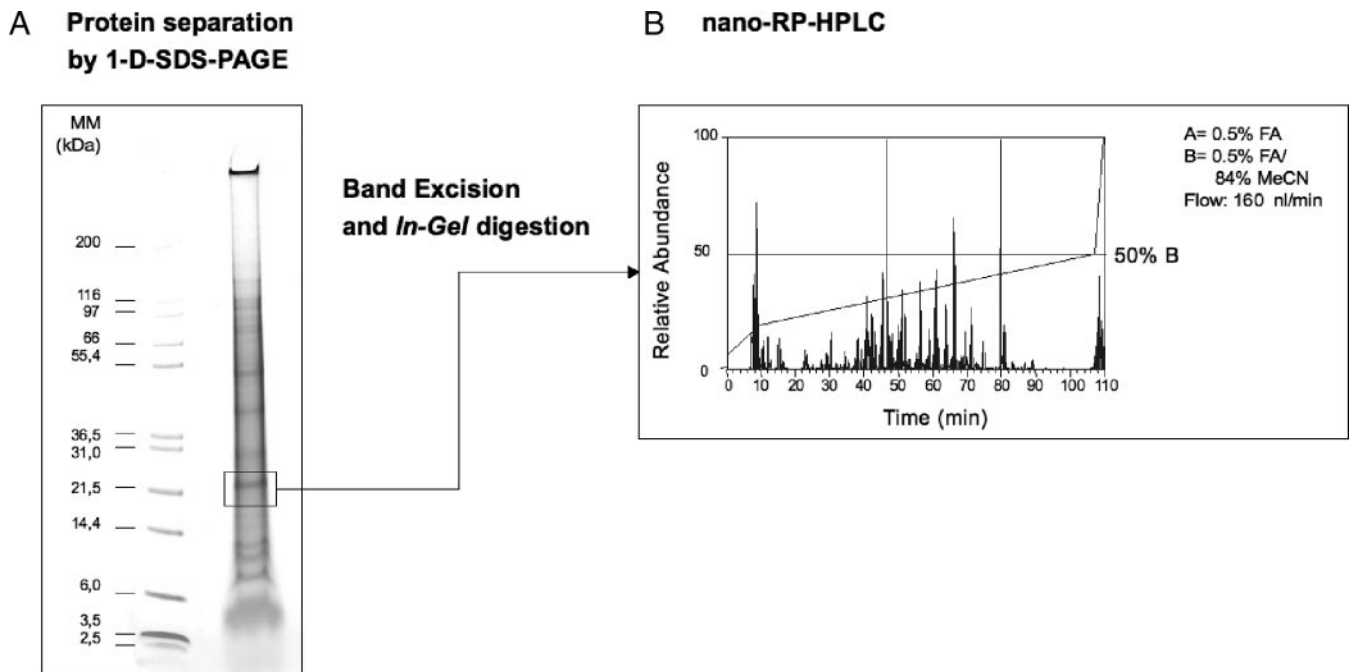


FIG. 1. A, proteins isolated from NM granules of human SN were separated by 1-D SDS-PAGE. The mass area containing L-ferritin was dissected from the SDS-PAGE and subjected to in-gel digestion prior to peptide separation by nano-reversed-phase (RP) HPLC (B) and mass spectrometric identification. FA, formic acid; MeCN, acetonitrile.

maximum of one missed cleavage site. In general, an Xcorr of greater than 2.0 and ΔCn greater than 0.1 were accepted for confident identification; manual inspection of the spectra was performed to confirm the Sequest results. Subsequent targeted MS analyses were performed using nano-HPLC of the tryptically generated peptides (24) followed by ESI-MRM. To verify the identity of the entity evoking the MRM signal the experiment was followed up by the acquisition of a tandem MS spectrum.

MRM analyses were performed using a 4000 QTRAP hybrid triple quadrupole linear ion trap mass spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with a Nanospray source. The MRM transitions were set up manually based on the MS/MS spectra previously acquired by ESI-MS/MS using the three most intense signals of the spectrum. Three transitions were selected to unambiguously identify the peptide. The MRM transitions used are listed in Table I. For the MS experiments (one MRM scan and three subsequent enhanced product ion scans), the needle voltage was adjusted between 2000 and 3200 V, and the needle gas (gas 1) was set between 25 and 35 p.s.i. The MRM dwell time was 50 ms, and the collision energy was set to 48 eV based on initial experiments. The MRM transitions were measured at unit resolutions of Q1 and Q3. The enhanced product ion experiment was triggered when the intensity of the MRM event exceeded 200 counts/s. This scan was performed with a scan rate of 4000 amu/s within a mass range from 100 to 1500 m/z . The linear ion trap fill time was set to 150 ms, and for each experiment two scans were summed with Q0 trapping activated. Resulting spectra from the same precursor mass were merged using the Analyst software (Applied Biosystems).

RESULTS

NM granules were isolated from the human SN by a *top-down* density gradient approach that yields specimens virtually free of contaminants, e.g. from glia and organelles (24). Extracted proteins were separated by 1-D SDS-PAGE.

The respective region was dissected from the gel followed by tryptic in-gel digestion. Peptides were analyzed by nano-LC-ESI-MS/MS (Fig. 1) as well as MRM to specifically confirm the presence of L-ferritin. The previously conducted nano-LC-ESI-MS/MS experiments unambiguously identified three different peptides of L-ferritin (Fig. 2). One of these peptides, LGG-PEAGLGEYLFER, corresponding to the amino acids 169–154 of ferritin light chain, could additionally be detected using LC-ESI-MRM (Fig. 3 and Table I). Basic local alignment search tool (BLAST) analyses (25) confirmed this peptide to be unique for human L-ferritin (Swiss-Prot accession number P02792). Finally the localization of L-ferritin in NM granules was confirmed by Western blotting and immunogold labeling for electron microscopy. Clusters of three to five gold particles are visible within the protein matrix of NM granules and are in line with the L-ferritin protein shell consisting of 24 subunits (Fig. 4).

DISCUSSION

At the outset of the study the pigment NM had been considered as the only endogenous iron-binding molecule in pigmented neurons (for a review, see Ref. 1). Mössbauer spectroscopy and synchrotron chemical x-ray microscopy experiments, however, had shown iron binding sites in NM similar to those present in human ferritin and hemosiderin (22). Nevertheless these spectroscopic methods are not suitable to identify proteins. In this study we thus used mass spectrometry coupled to gel-based protein fractionation to prove the presence of the iron storage protein L-ferritin in NM granules.

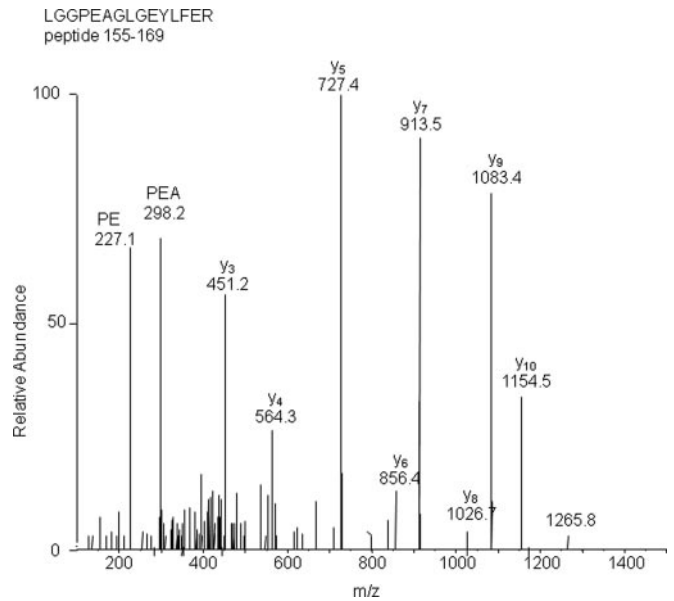
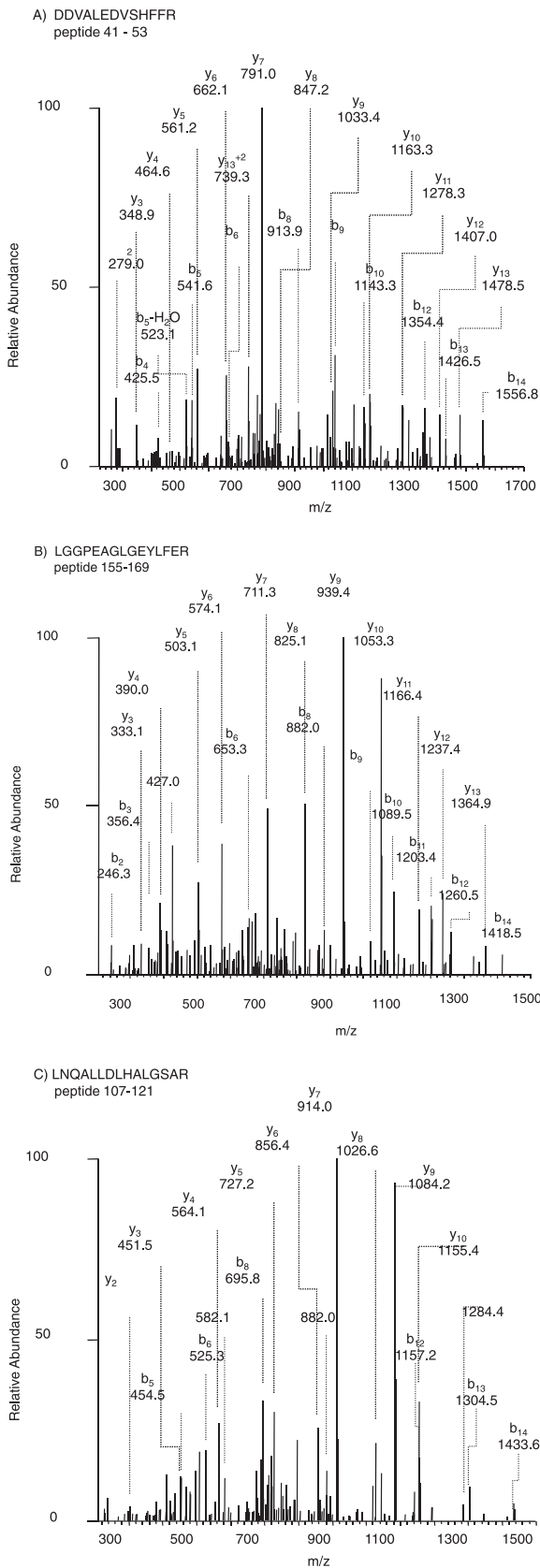


FIG. 3. ESI-LC-MRM spectrum of the L-ferritin-derived peptide LGGPEAGLGEYLFER.

TABLE I

Identification of L-ferritin by multiple reaction monitoring based on three transitions

Transition	Ion
804.4 \rightarrow 913.4	$[M+2H]^{2+} \rightarrow y7$
804.4 \rightarrow 1083.6	$[M+2H]^{2+} \rightarrow y9$
804.4 \rightarrow 1154.4	$[M+2H]^{2+} \rightarrow y10$

To unequivocally identify L-ferritin in NM, we used both Western blotting and targeted mass spectrometry. We used this 2-fold approach to remove any doubts on unambiguous identification that might arise in the case of antibody-based detection.

For target compound analysis in complex mixtures, triple quadrupole mass spectrometry operated in multiple reaction monitoring mode is today the method of choice. This method is currently more and more applied for the identification and quantification of proteins (26, 27). Using a two-stage mass selection technique, the specificity of detection can be highly increased to home in on target proteins even in a high background of other proteins. In this regard, MRM can be regarded as “mass spectrometry-based Western blotting” (28), circumventing problems of antibody specificity. Despite the high sensitivity of spectrometric mass detection, a reduction of the complexity of the sample is still required (29) especially when dealing with human brain tissue (30). Fractionation steps in the presented work flow

FIG. 2. Spectra (A–C) and sequences of peptides (C) that identify L-ferritin as a protein in human neuromelanin granules isolated from the substantia nigra are shown. A, DDVALEDVSHFFR, peptide 41–53, from L-ferritin. B, LGGPEAGLGEYLFER, peptide 155–169. C, LNQALLDLHALGSAR, peptide 107–121.

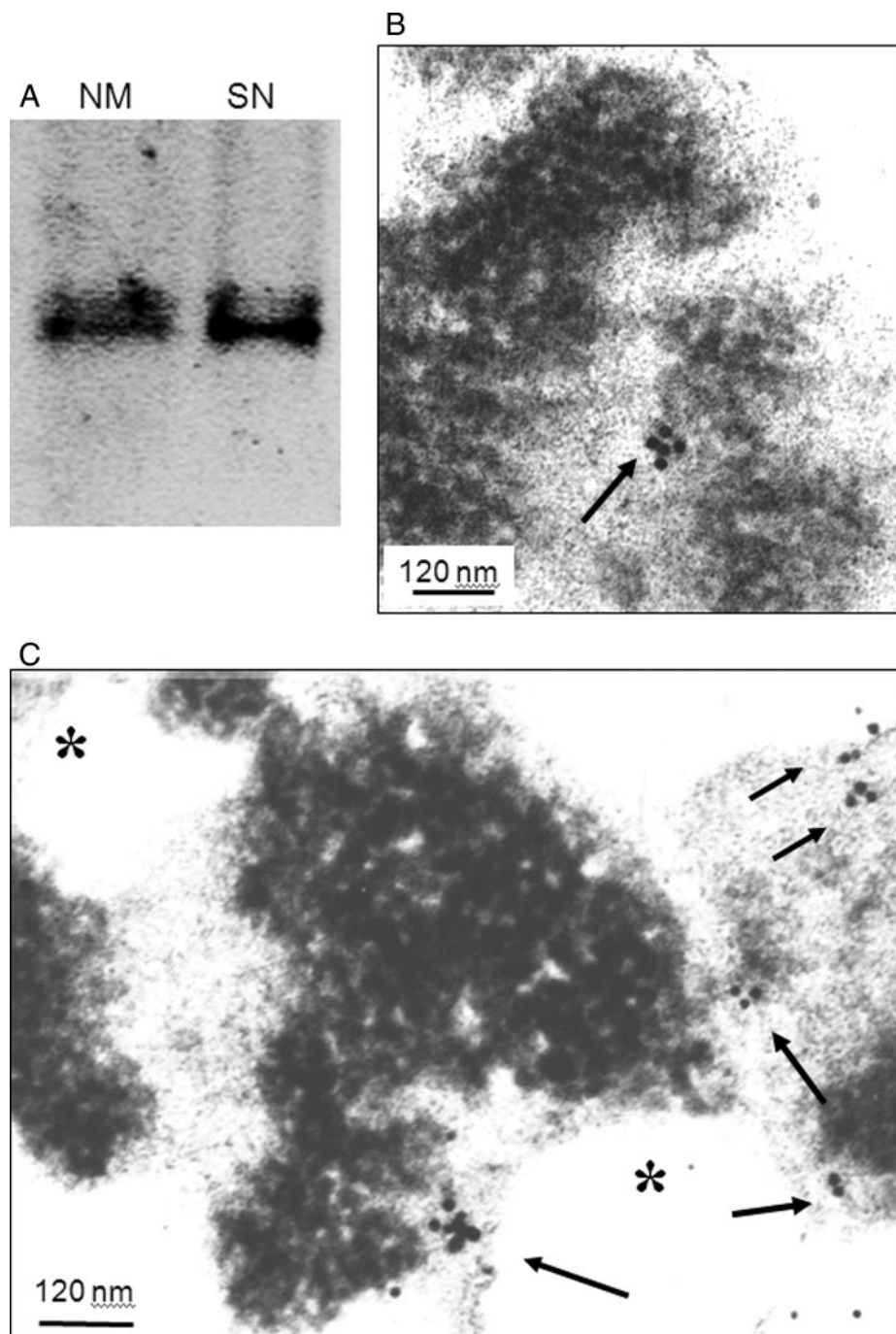


FIG. 4. A, identification and localization of NM granules isolated from human SN tissue. Western blot was performed and probed against L-ferritin comparing isolated NM granules with total SN that served as positive control. B and C, additionally localization of L-ferritin was performed by immunogold labeling for electron microscopy. 10-nm gold particles (*arrows*) demonstrate the presence of L-ferritin in NM granules. *, lipid bulbs, which are still attached to the granule.

thus included tissue disaggregation and isolation of the pigmented neurons, the isolation of the NM granules followed by protein extraction with SDS, and further separation by 1-D SDS-PAGE. Using this approach of several steps of prefractionation, L-ferritin could unambiguously be identified in NM using both the immunological and the mass spectrometric approaches.

In addition to that we demonstrated the localization of L-ferritin in NM granules by immunoelectron microscopy. The previous unsuccessful detection of L-ferritin in NM granules

via conventional immunohistochemistry may be due to inherent limitations to distinguish reddish to brownish dyes from the dark brown pigment NM.

Ferritins consist of 24 subunits of two types (heavy (H) and light (L)) that form a soluble hollow shell with an 80-Å-diameter cavity capable of storing up to 4500 Fe(III) atoms as an inorganic complex (31). These forms differ both in the function and in the cellular distribution in the brain. Although the H-ferritin form has a ferroxidase center that converts toxic Fe(II) into the less toxic Fe(III) and is involved in rapid uptake and

reutilization of iron, the L-form of ferritin is associated with long term iron storage. Based on immunohistochemical studies, e.g. neurons in the human cerebral cortex contain more H-ferritin than L-ferritin, and microglia contain more L-ferritin (32). This observation is consistent with current thinking about iron utilization in these cell types: microglial cells are mostly scavenger cells, whereas cortical neurons require a reusable supply of iron. In contrast, in the pigmented neurons of the SN the level of iron is significantly higher than in any other brain region and is concentrated within the NM granules (4).

Our finding supports the idea that NM granules are iron storage organelles (4, 21). It is, however, surprising that two different systems of iron storage, the NM-based system and an additional protein-, L-ferritin-based iron storage system, are both integrated in the NM granules, which mirrors the importance to face those exceptionally high levels of iron in the SN. The presence of both systems in the same organelle may provide a synergistic regulation of iron homeostasis, which guarantees effective neuroprotection from detrimental actions of iron via oxidative stress.

It was demonstrated *in vitro* that iron-depleted human NM decreases peroxidation of membrane lipids and attenuates membrane damage in the presence of Fe(III). Because of its affinities for iron, NM exerts antioxidant cell protection as long as its binding capacity is not exhausted (33), and in the case of iron saturation NM dramatically increases cell damage. Nevertheless cell damage is still prevented at a high load status of NM if an additional chelator for Fe(III) is present. L-ferritin is such an iron chelator.

Interestingly it has recently been reported that down-modulation of L-ferritin results in a depigmentation of human metastatic melanoma cells due to an influence on the proper maturation of the tyrosinase protein (34). Tyrosinase (EC 1.14.18.1), the key enzyme converting 3,4-dihydroxy-L-phenylalanine to oculocutaneous melanin, requires iron as a co-factor (35). In contrast to the skin, tyrosinase is not expressed in the brain (36, 37), but a tyrosinase-like activity has been suggested to form NM from dopamine (38).

A hypopigmented neuronal phenotype has so far been reported in 3,4-dihydroxyphenylalanine-responsive dystonia, a disease with parkinsonian features in early childhood (39, 40), in the Rett syndrome (41), and in Alzheimer disease (42, 43). Although the hypopigmentation in PD primarily results from the degeneration of pigmented neurons, pigment aberrations are found in the remaining intact neurons: indeed less pigment is found in the surviving neurons in PD (2), the NM structure is believed to be changed (44, 45), and the iron clusters in the vicinity of NM are smaller (46). An impact of L-ferritin and hypopigmentation in the human SN neurons has yet to be investigated. In fact, we are currently approaching these studies by comparing NM isolated from controls and PD with regard to chemical/biochemical characteristics as well as protein composition.

Despite the major application of proteomics to delineate the proteome of complex samples, e.g. tissue or cells, which results in large protein lists, this approach also holds the potential to investigate the presence of one specific protein in a focused manner. All in all, this first positive proof of L-ferritin localization in NM granules marks an important step toward understanding iron metabolism of the human SN and will no doubt give rise to new hypotheses on iron-driven degeneration of the SN in PD.

Acknowledgment—We are grateful to Siglinde Schenk for assistance at the transmission electron microscope.

* This work was supported by the BrainNet Europe II and the Bundesministerium für Bildung und Forschung (BMBF) projects “Human Brain Proteome Project”/“Functional Genomics of Parkinson’s disease” and BMBF Grants 031U102F and 01 GS 08143. This research was completed within “The National Parkinson Foundation Center of Excellence Research Laboratories”, Miami, FL, the “Verein zur Durchführung des 16th International Congress on Parkinson’s disease and Allied Disorders e.V.”, and the Clinic and Polyclinic for Psychiatry and Psychotherapy, University of Würzburg (to P. R.).

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