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Combined enrichment of neuromelanin granules and synaptosomes from human *substantia nigra pars compacta* tissue for proteomic analysis

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Abstract

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Conflict of interest statement
There was no conflict of interest.

Appendix A. Supplementary data
Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2013.07.015.

This article gives a detailed description of a protocol using density gradient centrifugation for the enrichment of neuromelanin granules and synaptosomes from low amounts (≥0.15 g) of human *substantia nigra pars compacta* tissue. This has a great advantage compared to already existing methods as it allows for the first time (i) a combined enrichment of neuromelanin granules and synaptosomes and (ii) just minimal amounts of tissue necessary to enable donor specific analysis. Individual specimens were classified as control or diseased according to clinical evaluation and neuropathological examination. For the enrichment of synaptosomes and neuromelanin granules from the same tissue sample density gradient centrifugations using Percoll® and Iodixanol were performed. The purity of resulting fractions was checked by transmission electron microscopy. We were able to establish a reproducible and easy to handle protocol combining two different density gradient centrifugations: using an Iodixanol gradient neuromelanin granules were enriched and in parallel, from the same sample, a fraction of synaptosomes with high purity using a Percoll® gradient was obtained. Our subfractionation strategy will enable a subsequent in depth proteomic characterization of neurodegenerative processes in the *substantia nigra pars compacta* in patients with Parkinson's disease and dementia with Lewy bodies compared to appropriate controls.

Keywords

Neuromelanin; Synaptosomes; Density gradient; *Substantia nigra pars compacta*; Parkinson's disease

Technical brief

Neuromelanin (NM) is a black-brownish pigment, which can be found in brains of humans and some mammals like primates, cows and horses [1]. Furthermore, it was described that the pigment accumulates in dopaminergic neurons during aging [2]. Until now the function of NM granules in neurons is not exactly known. Indeed there are indications for an involvement of NM granules in cell iron homeostasis and neuronal protection from toxic agents, like products of the dopamine synthesis [3-5]. NM is of special interest because in Parkinson's disease pallor of the *substantia nigra pars compacta* (SN) can be seen, due to the depletion of dopaminergic neurons containing NM. It is obvious that the characteristic symptoms of the disease are a consequence of an impaired neuronal signal transmission. Therefore, the analysis of NM granules and purified synaptic terminals of neurons (synaptosomes) are of special concern. Additionally, a typical phenomenon of synucleinopathies like Parkinson's disease or dementia with Lewy bodies (DLB) is the formation of Lewy bodies or — neurites mainly consisting of aggregated alpha-synuclein.

For the enrichment of NM granules [6] or synaptosomes individually [7,8] we tested different published protocols. One of the protocols was established several years ago by our group allowing the enrichment of NM granules after tissue disruption via low-speed centrifugation with sucrose and additional Percoll® gradients [6]. However, this protocol could not be applied to low tissue amounts under 0.5 g making donor specific analysis nearly impossible. Additionally, in our hands the protocol was incompatible with the combined purification of NM granules.

Consequently we changed the protocols and established a new and very efficient two-step protocol for the combined enrichment of both NM granules and synaptosomes from low tissue amounts (Fig. 1).

Tissues were provided by the German Brain Bank in Würzburg, by the Brain Bank of the Netherlands in Amsterdam and by the Brazilian Brain Bank in São Paulo. The use of *post mortem* human brain tissue was approved by the Ethics Committee of the University Clinics of Würzburg (file number 78/99), the Ethics Committee of the University of São Paulo (file number 361/10) and the Brazilian national health ministry (file number 16380). Due to current restrictions tissues prepared in São Paulo were only used for the preparation of NM granules. All subjects died from natural death, their brains were dissected as described [9] and stored at −80 °C.

Prior to purification of NM granules and synaptosomes 0.15 to 0.35 g of SN tissue was washed in homogenization buffer (0.32 M Sucrose, 1 mM EDTA, 0.25 mM DTT, 5 mM Tris, pH 7.4, 1 tablet protease inhibitor complete mini EDTA-free (Roche Pharma AG, Mannheim, Germany) per 10 ml). Then the tissue was transferred to a Dounce homogenizer (7 ml Wheaton, VWR International S.A.S, West Chester, USA) and was homogenized with 10 strokes of the tight fitting pestle in 4 ml homogenization buffer (s. Fig. 1). Remaining tissue sticking to the pestle was rinsed off with 1 ml of homogenization buffer and homogenized with additional 5 strokes. The lysate was transferred to a 15 ml tube (BD Biosciences, Erembodegem, Belgium) and centrifuged for 20 min at 1000 ×g and 4 °C in a Multifuge (Heraeus X3R with TX-750 swinging bucket rotor, Thermo Scientific, Rockford, USA) to pre-fractionate the lysate to a synaptosome-rich supernatant and a NM-granule rich pellet. The supernatant was transferred to a fresh 15 ml tube and the pellet was dissolved in 2 ml resuspension buffer (0.25 mM Sucrose, 1 mM EDTA, 10 mM Tris–HCl, pH 7.4). The protein concentration of each fraction was measured by Bradford tests (Bio-Rad Protein Assay, Bio-Rad Laboratories, Inc., Hercules, USA) and diluted, if necessary in resuspension buffer to 1–3 μg/μl. For ultracentrifugation polyallomer tubes (Herolab/Beranek Laborgeräte, Weinheim, Germany) were used. The supernatant was layered on top of a discontinuous Percoll® (GE Healthcare) gradient (3%, 10%, 15%, 23% in gradient buffer (0.32 M sucrose, 1 mM EDTA, 0.25 mM DTT, 5 mM Tris, pH 7.4)) and separated by centrifugation at 31,000 $\times g_{av}$ and 4 °C for 5 min at full speed according to Dunkley and co-workers [8] in a Sorvall WX-100 ultracentrifuge with a Surespin 630 swinging bucket rotor, 17 ml buckets (Thermo Scientific, Rockford, USA) and acceleration and deceleration levels of nine and three. The pellet was dissolved in 500 μl EDTA buffer (gradient buffer without DTT) and the interphases were collected in 1.5 ml tubes and diluted twofold with EDTA buffer. The fractions were centrifuged for 30 min at 13,000 ×g and 4 °C in a table top centrifuge (Eppendorf 5415R, Eppendorf). The supernatants were discarded and the sediments were once more washed with EDTA buffer. Finally, the sediments were resuspended in appropriate buffers for further analysis. The NM-rich pellet was adjusted to 20% Iodixanol (OptiPrep™, Axis-Shield, Norway) with 50% Iodixanol working solution (50% Iodixanol, 0.042 mM sucrose, 1 mM EDTA, 10 mM Tris, pH 7.4) and layered on top of a discontinuous Iodixanol gradient (26%, 31%, 36%, 50%). Fractionation was done by ultracentrifugation for 3 h at 81,000 $\times g_{av}$ and 4 °C with acceleration and deceleration levels

of nine and three. The resulting pellet was resuspended in 500 μl isolation buffer (10 mM Hepes, 1 mM EDTA, 10% (w/v) sucrose, 100 mM KCl, pH 7.4, 1 tablet protease inhibitor per 10 ml) and the interphases were collected and diluted twofold with isolation buffer. After 5 min centrifugation with 13,000 ×g at 4 °C in a table top centrifuge sediments were washed again with isolation buffer.

To check the purity of both fractions, the collected samples were analyzed by transmission electron microscopy. Hereto, sediments were incubated overnight in 2.5% glutaraldehyde (in phosphate buffered saline). After washing with phosphate buffered saline the samples were fixed with Dalton fixing solution according to Coulter [10] and embedded in Epon 812 (SERVA Electrophoresis GmbH, Heidelberg, Germany) [11]. The samples were dehydrated and counterstained by incubating them for 15 min in 50% ethanol and overnight with 70% ethanol, 1% uranyl acetate and 1% phosphotungstic acid. Afterwards, the samples were completely dehydrated with an ascending ethanol series (80–100%) and transferred to Epoxypropane. Samples were stepwise brought into Epon 812 by incubation with a series of Epoxypropane Epon 812 mixtures with increasing amounts of Epon 812. Samples in Epon 812 with hardener (18% Dodecenylsuccinic anhydride, 33% of 4,7-Methanoisobenzofuran-1,3-dione and 1.5% of 2-4-6-Tris(dimethylaminomethyl)phenol) were cast in stencils. After two days of polymerization at 60 °C blocks were sliced in 70 nm sections using a diamond knife containing ultramicrotome. These sections were mounted on formvar coated 75 mesh copper grids. The samples were analyzed using a transmission electron microscope (Philips-420, Philips, Hamburg, Germany) with a digital camera (Gatan, Inc., Pleasanton, USA).

The transmission electron microscopy demonstrated that NM granules were enriched in two fractions of the Iodixanol gradient with different purities. In the 20% to 26% interphase NM granules were co-isolated with membranes (Fig. 2A), whereas in the interphase between 26% and 31% Iodixanol NM granules of adequate purity were found (Fig. 2B). After establishing the protocol with control cases, we were also able to transfer it successfully to the SN tissue of specimens suffering from dementia with Lewy bodies (DLB) for the enrichment of NM granules (Fig. 2C) indicating its applicability to tissue showing substantial loss of neurons.

As expected and described by Dunkley and co-workers [8], synaptosomes were found in two interphases of the Percoll® gradient. In the interphase 10% to 15% Percoll® synaptosomes were enriched together with membranes and myelin but in interphase 15% to 23% Percoll® highly pure synaptosomes were enriched (Fig. 2D–F).

Additionally, NM granules from fraction 26/31% Iodixanol generated in Germany or Brazil were analyzed by LC–MS/MS. Hereto, proteins were solubilized by heating the granules in LDS-buffer (26.5 mM Tris–HCl, 35.2 mM Tris base, 0.5% (w/v) Lithium dodecyl sulfate, 2.5% (w/v) glycerol, 0.13 mM EDTA) at 95 °C for 10 min followed by sonication for 15 min (RK31, BANDELIN electronic, Berlin, Germany). Then the granules were incubated again for 10 min at 95 °C and centrifuged and supernatants were transferred to fresh tubes. The protein concentrations were determined by amino acid analysis (see Supplementary material) and 10 μg protein of each sample were reduced by adding 2 M DTT and heating

the samples for 10 min at 95 °C. For clean up reasons samples were separated on a 12% polyacrylamide Bis-Tris gel with MOPS buffer (50 mM 3-(N-Morpholino)-propanesulfonic acid, 50 mM Tris, 0.1% (v/v) SDS, 0.8 mM EDTA, pH 7.2) in a mini-gel chamber (XCell-SureLock™ MiniCell, Invitrogen, Life Technologies™ Corporation, Carlsbad, USA). After a protein separation of 0.5 cm the separation was stopped and after an additional Coomassie stain gel pieces were excised, destained and subsequently used for an in-gel digest with 0.15 µg trypsin (Serva Electrophoresis GmbH, Heidelberg, Germany) in 50 mM ammonium bicarbonate at 37 °C overnight. The peptide concentration was determined by amino acid analysis (see Supplementary material) and 200 ng peptides were used for nanoLC-MS/MS analysis using an Ultimate 3000 RSLCnano HPLC-system online-coupled to an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, Germany). After loading the samples on a trap column (Acclaim®PepMap 100 C18, 300 µm × 5 mm, particle size: 5 µm, pore size: 100 Å; Thermo Fisher Scientific) and washing for 10 min with 0.1% trifluoroacetic acid, the peptides were separated on an analytical C18 column (Acclaim®PepMap RSLC C18, 75 µm × 50 cm, particle size: 2 µm, pore size: 100 Å; Thermo Fisher Scientific) using the following solvents: 0.1% formic acid (A); 84% acetonitrile, 0.1% formic acid (B); and HPLC-gradient: 5% to 10% B in 5 min, 10–30% B in 45 min, 30% to 95% B within 1 min and held at 95% B for another 4 min before the system was equilibrated for the next analysis. The column oven temperature was set to 60 °C. The LC was coupled to the mass spectrometer using a nano-electrospray ion source (Thermo Fisher Scientific) and distal coated Silica Tips (PicoTip Emitter SilicaTip FS360-20-10-D-20, New Objective, Woburn, USA). MS spectra were scanned between 300 and 2000 m/z at a resolution of 60,000 for m/z = 400. The 20 most intensive ions (charge > 1) were selected for MS/MS-fragmentation in the ion trap. Fragments were generated by collision-induced dissociation on isolated ions with normalized collision energy of 35%. Afterwards these ions were set on a dynamic exclusion list for 30 s to prevent fragmentation of previously analyzed ions. Raw-files were transformed into MGF files using ProteomeDiscover 1.3 (Thermo Fisher Scientific) software with default parameters for database searches with the Mascot search algorithm (Matrix Science, London, UK, version 2.2.0) using a peptide mass tolerance of 5 ppm and a fragment mass tolerance of 0.4 Da. For the protein identification the enzyme settings were set on “trypsin” and up to one possible missed cleavage site was considered for the database searches, as well as oxidation of methionine as a variable modification. All data were searched against the human UniProt/Swissprot decoy database (20121025; 1,076,020 sequences; 381,978,470 residues) using a decoy strategy to reduce false positive identifications. The results of the Mascot searches were assigned as true until the first decoy entry and proteins were accepted with Mascot scores higher than 25 and a minimal assignment of two peptides to the protein. The results of the samples generated in Germany or Brazil were compared resulting in an identification of 960 and 989 proteins, respectively. An overlap of 749 proteins was determined. Table 1 (Supplementary material) shows 278 proteins, which were identified in both samples with sequence coverage of at least 15% in one of the cases. Twenty of these 278 proteins were already described [6,12] to be NM related and another 32 proteins represent isoforms or known interaction partners of the published proteins [6,12].

In summary, we established a robust protocol allowing the reproducible parallel enrichment of NM granules and synaptosomes from limited amounts of human SN tissue of one individual, which is very advantageous as these tissues are rare and valuable. It allows for processing of small tissue amounts down to 0.15 g making for the first time a donor-specific subsequent analysis possible. Using Percoll® and Iodixanol instead of sucrose as density media grants relatively low centrifugation times and forces without osmolarity changes in the gradient ensuring optimized conditions for the organelle-purification under protective conditions. The established protocol is compatible with subsequent mass spectrometric analysis and will be used for future comparative proteomic studies in order to get a deeper insight in molecular mechanisms leading to neurodegeneration in Parkinson's disease and dementia with Lewy bodies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

$\mathbf{g_{av}}$	average g-force in the ultracentrifugation tube
NM	neuromelanin
SN	substantia nigra pars compacta

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Biological significance Key features of Parkinson's disease are the degeneration of dopaminergic neurons in the *substantia nigra pars compacta*, an associated loss of the brain pigment neuromelanin and a resulting impairment of the neuronal network. The accumulation of iron binding neuromelanin granules is age- and disease-dependent and disease specific alterations could affect the neuronal iron homeostasis leading to oxidative stress induced cell death. The focus of the described method is the analysis of neuromelanin granules as well as axonal cell-endings of nerve cells (synaptosomes) of individual donors (control and diseased). It is the basis for the identification of disease-relevant changes in the iron homeostasis and the generation of new insight into altered protein compositions or regulations which might lead to disturbed communications between nerve cells resulting in pathogenic processes.

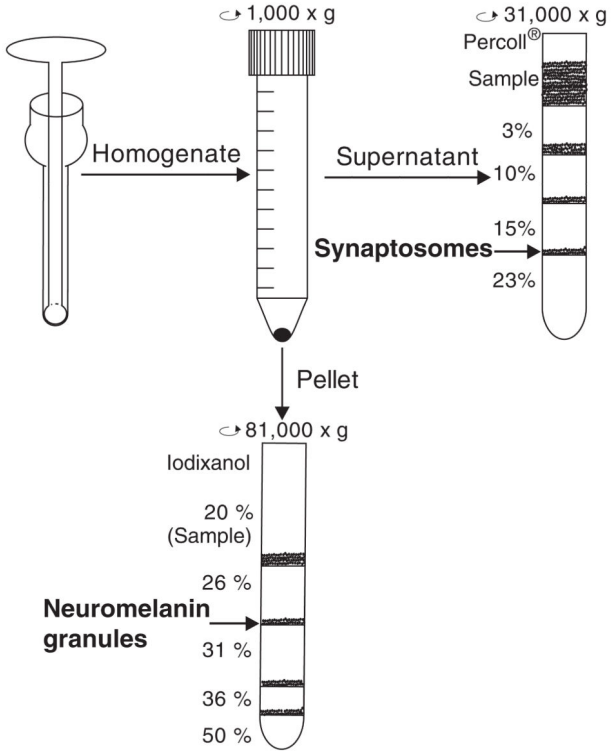

Fig. 1.

Schematic workflow of the combined NM granule and synaptosomal purification from human SN. After tissue disruption using a Dounce homogenizer the lysate was separated by low speed centrifugation. The supernatant was layered on top of a Percoll® gradient and the sediment was resuspended and added to the top of an Iodixanol gradient. After ultracentrifugation NM granules and synaptosomes accumulated at the indicated interphases.

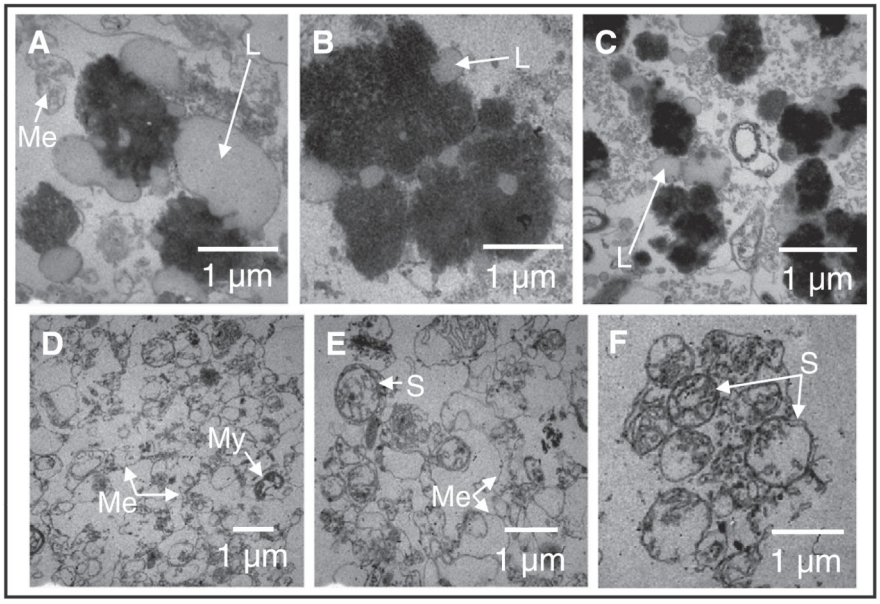

Fig. 2.

Transmission electron microscopy of NM granules and synaptosomes enriched from human *substantia nigra pars compacta* (SN). Transmission electron microscopy images of enriched NM granules from a control case (A, B) and a dementia with Lewy bodies case (C). In A the NM granules containing not only lipid droplets (L) that were enriched at the interphase of 20% to 26% Iodixanol, but also membranes (Me) were present. In B, NM granules of adequate purity were enriched at the interphase of 26% to 31% Iodixanol. The method enabled also the enrichment of NM granules from dementia with Lewy bodies cases (C). In D, 3% to 10% Percoll® interphase containing membranes (Me) and Myelin (My) can be seen. E shows an interphase of 10% to 15% Percoll® with enriched synaptosomes (S) and membrane (Me) impurities. In F, pure synaptosomes (S) enriched at the interphase of 15% to 23% Percoll® are shown. A, B: magnification factor (×) 60,000; C: × 34,000; D: × 18,000; E, F: × 23,000.