Nano-imaging trace elements at organelle levels in substantia nigra overexpressing α-synuclein to model Parkinson’s disease

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Sub-cellular trace element quantifications of nano-heterogeneities in brain tissues offer unprecedented ways to explore at elemental level the interplay between cellular compartments in neurodegenerative pathologies. We designed a quasi-correlative method for analytical nanoimaging of the substantia nigra, based on transmission electron microscopy and synchrotron X-ray fluorescence. It combines ultrastructural identifications of cellular compartments and trace element nanoimaging near detection limits, for increased signal-to-noise ratios. Elemental composition of different organelles is compared to cytoplasmic and nuclear compartments in dopaminergic neurons of rat substantia nigra. They exhibit 150–460 ppm of Fe, with P/Zn/Fe-rich nucleoli in a P/S-depleted nuclear matrix and Ca-rich rough endoplasmic reticula. Cytoplasm analysis displays sub-micron Fe/S-rich granules, including lipofuscin. Following AAV-mediated overexpression of α-synuclein protein associated with Parkinson’s disease, these granules shift towards higher Fe concentrations. This effect advocates for metal (Fe) dyshomeostasis in discrete cytoplasmic regions, illustrating the use of this method to explore neuronal dysfunction in brain diseases.
Several neurodegenerative diseases of the central nervous system, including Parkinson’s disease, lead to perturbations in the tissue content and distribution of specific metals. In particular, a lot of attention was paid to the level and distribution of iron, which normally contributes to essential cellular functions, including mitochondrial respiration, via its capability to transfer electrons. In vulnerable populations of neurons however, iron dysregulation can have detrimental effects such as the production of reactive oxygen species via the Fenton reaction. Hence, it is critical for neurons and glial cells to tightly control metal metabolism. In the case of iron, this is in large part mediated through interactions with iron-binding proteins and molecules such as transferrin, ferritin, specific transporters, heme, or neuromelanin, which determine iron distribution in cellular compartments and organelles. Genetic defects affecting iron metabolism cause brain diseases, including neurodegeneration with brain iron accumulation\(^1\).\(^{1}\) In more common neurodegenerative diseases such as Parkinson’s and Alzheimer’s diseases, the pathology is also associated with iron overload\(^2\)–\(^4\). As several central mechanisms involved in Parkinson’s disease, such as mitochondrial dysfunction, are tightly coupled with metal dyshomeostasis\(^6\), it is important to explore perturbations in metal distribution in neurons and glia, with the aim to identify potential causal mechanisms in neurodegeneration\(^6\).

Parkinson’s disease is a complex neurodegenerative motor disorder characterized by the degeneration of nigral dopaminergic neurons and by the accumulation and aggregation of the alpha-synuclein protein (\(\alpha\)-syn), leading to the formation of Lewy bodies in vulnerable brain regions. Mitochondrial dysfunction, and in particular complex I deficiency, has been identified as a pathophysiological mechanism underlying Parkinson’s disease\(^7\). Genetic associations have shown that the overabundance of \(\alpha\)-syn can cause both familial as well as sporadic forms of the disease\(^8\)–\(^10\). The \(\alpha\)-syn protein can moderately bind Fe(II) and Fe(III) and displays ferrireductase activity\(^1,11,12\). Furthermore, interaction with Fe(III) contributes to \(\alpha\)-syn oligomer formation\(^13\). However, the role of this protein in metal dyshomeostasis remains unclear. Abnormal levels of iron and copper have been reported in cases of Parkinson’s disease, mainly observed in the substantia nigra, a region of the ventral midbrain which is selectively vulnerable to the underlying pathology\(^14\)–\(^17\). These histopathological manifestations of neurodegenerative processes may reflect metal dyshomeostasis linked to mitochondrial and lysosomal dysfunctions\(^18\), ultimately contributing to cell death via mechanisms such as ferroptosis\(^19\)–\(^21\). In brain tissue affected by Parkinson’s disease, little is known about the cellular compartments that contribute to metal dyshomeostasis, although higher Fe concentrations have been mainly observed in the soluble fraction extracted from the substantia nigra\(^22\).

Developing direct imaging of metals at the subcellular level, in particular that of organelles in the neurons which are vulnerable to disease, would be a major step toward understanding the pathogenic mechanisms involved\(^23\). Among the analytical approaches developed to investigate physiological heterogeneities of trace elements in brains\(^24,25\), synchrotron radiation-induced X-ray fluorescence (XRF hereafter) is part of the few imaging approaches\(^26,27\), the only non-destructive multi-elemental method of high sensitivity. XRF has been used to analyze Fe traces in brains affected by Parkinson’s disease\(^28\), showing that this element is heterogeneously distributed in brain tissues and accumulates in both the neuronal cells and the extra-neuronal substantia nigra tissue of Parkinson’s patients. It reaches levels three times higher than in non-affected subjects\(^29,30\). XRF also showed that in the human brain, Fe is abundantly present in neuromelanin and increases with age\(^31\).

The highest spatial resolution elemental imaging applied to date to neuronal cultures revealed heterogeneous intracellular Fe distributions\(^33,34\). Neuronal cells show Fe-enriched perinuclear “puncta” (size < 100 nm), corresponding to granules similar to siderosomes. When neuronal cell culture models are exposed to Fe-supplemented media and induced to overexpress \(\alpha\)-syn, the Fe-enriched puncta imaged by XRF appear to be enlarged, which indicates perturbed iron homeostasis\(^35,36\). Aside from the Fe puncta, XRF maps of neuronal cells typically display blurred regions of elemental contrasts in the nuclear and cytoplasmic compartments. Both low elemental count-rates as well as low contrasts at the minimal detection limits of various elements, prevented from extracting robust areas for the elemental maps.

Although challenging, imaging multiple trace elements at organelle level by XRF\(^37\)–\(^39\) can be tackled and reach the required sensitivity and spatial resolution at third-generation synchrotrons that develop high brilliance hard X-ray nanoprobes exploiting efficient nanofocusing optics and compact ultra-sensitive multiple-element detector systems. Cryo-preparations of neuron cultures were successfully implemented\(^40,41\), and are considered to be the optimal way to preserve the cells in a close-to-native state. In the case of brain tissues and biopsies, alternative approaches have to be considered when targeting a specific region implying the preservation of a large volume and vitrification at an excessive depth\(^40,41\). Furthermore, coupling to an ultrastructural microscopy method becomes mandatory to unambiguously identify neuronal organelles. This prompted the development of an analytical method based on a transmission electron microscopy (TEM)-nano-XRF imaging correlation, which must be carried out without heavy metal staining usual in preparations for ultrastructural analysis, to avoid elemental contamination impeding XRF analysis. Preparations must also be compliant with the general principles of trace element quantification\(^42\). In practice, using extremely brilliant nano-beams (>10\(^11\) ph/s in a 30 nm spot) forces a trade-off between the exposure time for imaging trace elements and the sample radiation damage. Likewise, increasing the sample thickness up to organelle sizes (500–1000 nm) is a trade-off between enhancing the count rates and preserving subcellular spatial resolution.

Here, we introduce an analytical method to measure trace elements in inferred subcellular structures inside neuronal cell bodies located in the substantia nigra tissue. The method is based on quasi-correlative nano-imaging using XRF applied to a 500-nm-thick section of the rat ventral midbrain, and TEM to reveal ultrastructure in an adjacent 80-nm-thin section (Fig. 1). The first survey step of the method consists of (i) displaying histological structures, both in XRF and TEM images, to identify fiducial marks as distant as possible from the region of interest to align these images, and (ii) measuring the elemental content of the cytoplasmic and nuclear compartments. In the second zoom-in step, the elemental composition of the sub-compartments delimited in the ultrastructure TEM images, including organelles, is investigated. This method was applied to several neurons using sections of substantia nigra, revealing elemental compositions specific to different organelle types compared with the nuclear compartment. Next, taking advantage of the highest signal-to-noise ratio (SNR) reached by finely delineating the cytoplasmic regions, we determined changes in the elemental distribution at the highest resolution (down to 20 nm pixel size) following local overexpression of the \(\alpha\)-syn protein in the substantia nigra of wild-type rats. In these animals, the accumulation of the human \(\alpha\)-syn protein, which is associated to Parkinson’s disease, leads to the selective degeneration of nigral dopaminergic neurons\(^43\). Although the pathology induced in the rat midbrain does not replicate changes observed in the human substantia nigra, such as the formation of Lewy bodies as well as the age-dependent deposition of neuromelanin, this animal model likely mimics the early pathogenic process downstream of \(\alpha\)-syn.
accumulation associated with Parkinson’s disease. In nigral dopaminergic neurons exposed to the α-syn-induced pathology, nano-imaging resolves the presence of Fe-enriched multi-pixels granules in the cytoplasm. Although better statistics would enhance differences between neurons exposed or not to this pathological condition, the measured Fe and S content of these granules revealed a local shift toward more heterogeneous and higher Fe concentrations in the granules of diseased neurons. Our proposed analytical method provides a tool to assess trace metal dyshomeostasis at organelle levels in an animal model of Parkinson’s disease.

Results

Nano-XRF visualization of neurons in the substantia nigra. Several histological structures including neuronal cell bodies were revealed in the substantia nigra by nano-XRF. An unstained 500-nm-thick section containing a portion of the substantia nigra pars compacta was placed on a thin, ultrapure Si$_3$N$_4$ window, and introduced in the nano-XRF setup of the ID16A beamline at the European Synchrotron Radiation Facility (ESRF) (see Supplementary Fig. 1). Low resolution and fast (low count-rate) XRF maps of the section were first recorded by Scanning X-ray Microscopy at 17 keV (see coarse-scan mode in “Methods”). They showed features, such as blood vessels, as well as folds and scratches on the section that were also seen in optical microscopy, on the same section or in an adjacent section, stained with toluidine blue. This first mapping was used to locate neurons in the XRF maps of substantia nigra (Fig. 2a).

This step was followed by a higher resolution scan of neuronal bodies using a 25–50 nm step size (see fine-scan mode in “Methods”). The scanning procedure was optimized to adjust the dose just below our observed sample breakdown threshold (ca. $4 \times 10^7$ photons/s nm$^2$) (Fig. 2b). As expected, the sum spectrum of all pixels measured in the neuronal cell body shows major contributions from the Si-rich membrane used to support the brain section, and from the Cl-rich EPON™ resin (Fig. 2c). For each spectrum, the Fe and Zn peaks, as well as the P, S, and Ca overlapping ones, were deconvoluted using a fitting procedure with the freely available PyMca software (“Methods”).

To obtain elemental maps, the procedure was optimized to define background levels and applied to a set of ~50,000 spectra covering the entire region of a neuronal cell body. This was used to extract the raw count values for each of the elemental $K_x$ and $K_b$ lines identified in the spectrum.

Maps of P and S revealed several cellular features, recognizable in the adjacent optical images of the substantia nigra. In particular, these maps showed contrasted structures in the neuropil and the glial cells and neuronal cell bodies, in which the nuclear and cytoplasmatic compartments could be distinguished (Fig. 2a, b). The Fe map mainly showed enriched structures in the basal lamina of the blood vessels, but also a few hot spots in the neurons that are not observed in the stained section.

Trace elements in the cytoplasm and nuclei of neurons. Elemental patterns of large cellular compartments in neuronal cell bodies in the substantia nigra were compiled from nano-XRF imaging. Distinct elemental compositions were measured for the cytoplasmatic, nuclear, and nucleolar compartments. The elemental profiles measured across the neuronal cell body showed P enrichments and S depletions in the cytoplasmatic and nuclear regions, compared with the extracellular region mainly composed of the neuropil (Fig. 3a). Levels of both P and S were lower in the nuclear compartment compared with the cytoplasm, whereas the perinuclear region was characterized by a P-rich contour (Fig. 3a, b). Based on these observations, the P and S raw fluorescence counts were used to delineate the boundaries of the cytoplasmatic and nuclear compartments. Of note, elemental...
imaging of the nucleoli revealed granular structures highly enriched in Fe and P (Fig. 3c).

Next, we compared the elemental composition of these cellular compartments relative to the nuclear area, which was defined as the main part of the nucleus excluding the nucleolus and P-rich perinuclear regions. The P and S contrasts of low SNR per pixel are displayed by an average over all the pixels in a given compartment, $n_{pixels}$. Elemental contrasts were calculated from the average counts per pixel of the XRF sum spectra of a given compartment. Although the SNR increases, the corresponding minimum detection values decrease so the sensitivity of the elemental contrasts is improved. The relative count rate $\Delta_{i,n}$ of compartment $i$ with respect to the nuclear compartment $n$ and the corresponding uncertainty are defined in “Methods.”

![Fig. 2 Visible and nano-XRF images of substantia nigra.](image)

**Fig. 2 Visible and nano-XRF images of substantia nigra.** a Optical micrograph of a 500-nm-thick toluidine blue-stained section used to locate large-size neurons in the rat substantia nigra. Neurons (*), glial cells (g), and blood vessels (v) can be identified. b Nano-XRF image of the neuron (*) and glial cells (g) observed in the dashed frame shown in a recorded on a 500-nm-thick adjacent section. This image is extracted from nano-XRF mapping and represents a stack of the P/Fe/S $K_{\alpha}$ distributions color-coded as red/green/blue, respectively. Acquisition time: 50 ms; pixel size: $50 \times 50\, \text{nm}^2$; X-ray excitation energy: 17 keV; X-ray fluorescence collection: 12-element Silicon Drift Detector.

![Fig. 3 Nano-XRF of nuclear and cytoplasmic compartments in neuron bodies.](image)

**Fig. 3 Nano-XRF of nuclear and cytoplasmic compartments in neuron bodies.** a Nano-XRF stack of a neuron body (control non-injected condition) showing the P and S maps color-coded as red and blue, respectively. Dotted white lines delimit the nucleus (nu), the nucleolus (n), the P-rich contour of the nucleus (p), the cytoplasm (cy), and the neuropil (np). b Raw count XRF profiles (counts/pixel) of P (in red) and S (in blue). Crosses show the P and S raw counts per pixel measured along the 1-pixel-wide dashed line shown in a. The P and S counts shown as continuous lines are obtained by averaging values along a 1-pixel-wide horizontal line in the 80-pixel-wide rectangular box displayed in a. c Nano-XRF stack of a second neuronal body (control condition) showing P and Fe maps color-coded as red and green, respectively. The average $\Delta$ for Si, present in the Si$_3$N$_4$ substrate, is used as an internal control. Scale bars: 4 $\mu$m. The XRF acquisitions were carried out at 17 keV with a 6-element detector (Fig. 3a, b) or a 12-element detector (Fig. 3c, d) on two neurons in the rat substantia nigra overexpressing human $\alpha$-syn and two in the control. d Average of the relative count rates ($\Delta$) calculated for Si, P, S, Ca, Fe, and Zn in the cytoplasm ($76.5 \pm 19\, \mu$m$^2$ average scanned surface $\pm$ SD per neuron, for four neurons) and two nucleoli ($5.5 \pm 0.8\, \mu$m$^2$) with respect to a reference value measured for each element in the nuclear compartment ($24 \pm 3\, \mu$m$^2$). Error bars represent SD values.
Given the different sections, cells, and experimental acquisition setups, average contrast values and their standard deviations $\Delta c_{\text{org}} \pm \sigma$ were calculated only for relative uncertainties $\sigma c_{\alpha}/\Delta c_{\text{org}} < 30\%$ and at least triplicate measurements.

Doing so, the nuclei appeared to be markedly enriched in P (+66 ± 16%), S (+22.4 ± 6%), Ca (+53 ± 10%), Fe (+65 ± 10%), and Zn (+47 ± 9%) (Fig. 3d). Similarly, the cytoplasm was enriched in P (+41.5 ± 12%), S (+19.7 ± 4%) and Ca (+97 ± 7%) (Fig. 3d).

Testing the significance of lower Fe and Zn contrasts, and of different high P, S, and Ca contrasts between the α-syn and the control non-injected conditions (Table 1) would have required statistics that are out of reach of the present study. The corresponding elemental areal masses in ng/cm$^2$ (and concentrations in ppm) of the nuclei, cytoplasm in several neuronal cell bodies of the substantia nigra are in the ranges of 73–155, 47–107, 1–2.5, and 7–23 ng/cm$^2$ for P, S, Ca, and Fe, respectively (Table 1).

Trace element contrasts of organelles. The characteristic elemental contrasts in the soma of neurons were further refined using XRF and TEM quasi-correlative microscopy. Neuronal cell bodies were imaged by TEM in sections adjacent to the ones analyzed by XRF. Ultrastructure revealed nuclear and cytoplasmic compartments, as well as specific organelles, such as mitochondria, lipofuscin granules, and the rough endoplasmic reticulum (RER) (Fig. 4a, b, d). Based on TEM mosaic images, specific masks were generated for each type of subcellular compartment (nucleus, cytoplasm) and organelles (mitochondria, lipofuscin granules, RER) (Fig. 4c, e), and applied to the corresponding nano-XRF maps (with an uncertainty of 1 pixel) (Fig. 4f and "Methods").

For the alignment of TEM images and nano-XRF maps (with an uncertainty of 1 pixel) (Fig. 4g). The elemental composition was determined by comparison to reference values measured in the nuclear compartment (Fig. 4g). The values of the elemental contrasts confirmed the elemental enrichments in Ca, P > S for the cytoplasm (Fig. 4g).

Note that the nuclear membrane (defined from the TEM contrast) extends to the nuclear and perinuclear cytoplasmic P-rich region. Nevertheless, the unavoidable uncertainty if one uses only the P/S contrasts to delineate the nucleus/cytoplasm boundary, does not change the average cytoplasmic contrast. Compared with the cytoplasm, lipofuscin granules displayed higher levels of Ca and Fe, whereas the region containing the RER was particularly enriched in P and Ca (Fig. 4g). Mitochondria were enriched in Ca and to a lesser extent in P and S, without noticeable contrast compared with the cytoplasm (Fig. 4g). The Zn chemical contrast was remarkably flat for the different subcellular compartments, except for the nuclei (Figs. 4g and 3d).

This method reveals the compartment- and organelle-specific distribution of metals within neuronal cells in the brain tissue. However, it is important to consider that the retention of elements imaged in situ depends on the method used for sample preparation, which is based here on methanol for freeze substitution before resin embedding. Indeed, metal retention in presence of solvents depends on local interactions with cellular components which may vary across the different compartments.

### α-syn overexpression alters Fe/S in cytoplasmic granules.

Following overexpression of the α-syn protein associated to Parkinson’s disease, we analyzed Fe and S contrasts in nigral dopaminergic neurons to explore possible pathological alterations in iron and sulfur distributions. Nano-XRF imaging revealed the presence of sub-micrometer granules enriched in Fe or S in the neuronal cytoplasm (Fig. 5a, b and Supplementary Fig. 2). These granules were systematically defined by thresholding the Fe and S counts at two standard deviations above the corresponding mean background of counts in the cytoplasm, for both the control non-injected hemisphere, and the AAV-α-syn-injected one. The areal masses compiled from the sum spectrum of all the granules per

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**Table 1 Elemental areal masses** $am$, concentrations $c$, and uncertainties $\epsilon$ of Fe, Ca, S, and P, calculated for compartments (nucleus, nucleolus, cytosol) in a neuron body of the substantia nigra in the α-syn and control non-injected conditions (CTRL), using an AXO XRF standard. All areal masses, concentrations, and corresponding errors, largely dominated by the AXO standard uncertainties, were compiled by semi-quantitative analysis (see “Methods”).

|       | Nucleus | Nucleolus | Cytoplasm | FeS-G |
|-------|---------|-----------|-----------|-------|
|       | $am$ ng/cm$^2$ | $c$ ppm (%) | $\epsilon$ | $am$ ng/cm$^2$ | $c$ ppm (%) | $\epsilon$ | $am$ ng/cm$^2$ | $c$ ppm (%) | $\epsilon$ |
| **CTRL** | | | | | | | | | |
| N 1 Fe | 10 | 200.9 | 14.0 | 14.3 | 286.3 | 13.3 | 2.5 | 50.3 | 13.7 |
| Ca | 1.2 | 24.5 | 16.7 | 1.5 | 29.4 | 13.3 | 66.6 | 1332.7 | 16.9 |
| S | 59.2 | 1183.8 | 16.9 | 61.8 | 1235.7 | 16.8 | 112.8 | 2256.3 | 20.7 |
| P | 79 | 1579.4 | 20.6 | 97.9 | 1958.9 | 20.7 | 86 | 20.7 |
| N 2 Fe | 9.8 | 196.9 | 13.3 | 19.6 | 391.8 | 13.3 | 7.6 | 152.5 | 13.5 |
| Ca | 1.2 | 24.6 | 16.7 | 1.9 | 38.3 | 13.5 | 2.5 | 49.2 | 13.6 |
| S | 60.7 | 1213.1 | 16.8 | 78.9 | 1577.1 | 16.9 | 72.3 | 1447 | 16.9 |
| P | 78 | 1559.5 | 20.6 | 155.1 | 3102.4 | 20.7 | 100.9 | 2018.7 | 20.7 |
| **α-syn** | | | | | | | | | |
| N 1 Fe | 8.9 | 178.6 | 13.5 | 14.3 | 285.6 | 13.3 | 8.5 | 169.1 | 13.5 |
| Ca | 1.1 | 22 | 18.2 | 1.9 | 37.6 | 15.8 | 2.2 | 43.2 | 13.7 |
| S | 52.9 | 1058 | 16.8 | 58.2 | 1164 | 16.8 | 60.7 | 1214.7 | 16.9 |
| P | 72.8 | 1455.7 | 20.7 | 106.6 | 2131.8 | 20.7 | 97.4 | 1948.9 | 20.7 |
| N 2 Fe | 8.7 | 173.4 | 13.8 | 13.6 | 272.5 | 13.2 | 8.8 | 175.4 | 13.5 |
| Ca | 1.1 | 21.2 | 9.1 | 1.7 | 34.4 | 11.8 | 1.9 | 38.9 | 13.6 |
| S | 52.9 | 1058 | 16.8 | 69 | 1380.7 | 17.0 | 62.7 | 1254.1 | 16.9 |
| P | 74.6 | 1492.1 | 20.6 | 141.6 | 2831.9 | 20.7 | 117.4 | 2347.6 | 20.7 |
A detector on two neurons in the rat substantia nigra. The error bars represent SD values. Si is used as an internal control. The XRF acquisition was carried out at 17 keV with a 6-element S-rich granules did not co-localize with any recognizable organelle surrounding a S-rich core (Fig. 5g, bottom). Correlative analysis of signal was often located at the periphery of the largest granules, appearing to be highly inhomogeneous within the granules (see Fig. 5c). No grain size effects were registered for our 500-nm-thick sections, since comparable low spreads of the data for Fe/S mass ratios were obtained for both homogeneous and inhomogeneous granules of ca. Ø 200 nm average size. In the non-injected control hemisphere, these granules were found to contain mainly sulfur in the control condition (in the ranges of 98–108 and 10–11 ng/cm² for S and Fe, respectively, for two neurons shown in Table 1) and to be more enriched in iron in the AAV-α-syn-injected condition (66–72 and 22–23 ng/cm² for S and Fe, respectively, for two neurons shown in Table 1). The distribution and relative fractions of the Fe and S areal masses of each granule versus its size were further analyzed using the Fiji Analyze Particle plugin. In the AAV-α-syn-injected hemisphere, the granules were found to have on average a larger size (mean and SD area: 0.15 and 0.27 µm² for 63 granules) than in the control neurons (mean and SD area: 0.08 and 0.1 µm² for 82 granules) (Fig. 5c). No grain size effects were registered for our 500-nm-thick sections, since comparable low spreads of the data for Fe/S mass ratios were obtained for both homogeneous and inhomogeneous granules of ca. Ø 200 nm average size.

In the non-injected control hemisphere, these granules were found to be rather homogeneous in composition, with a relatively constant Fe/S mass ratio, showing mainly S enrichment (Fig. 5c, d (bottom)), while in the AAV-α-syn-injected hemisphere, a large fraction of the granules were characterized by high Fe content and high Fe/S ratios (Fig. 5c, f (top)). High Fe enrichment was mainly attributed to the granules with sizes ≥ 0.07 µm² (Fig. 5c) and appeared to be highly inhomogeneous within the granules (see α/mean Fig. 5d (middle)).

The XRF maps recorded in the very fine-scan mode (“Methods”) showed that in the α-syn condition, the intense Fe signal was often located at the periphery of the largest granules, surrounding a S-rich core (Fig. 5g, bottom). Correlative analysis of XRF with adjacent TEM images showed that in most cases, the Fe/S-rich granules did not co-localize with any recognizable organelle structures in the neuronal cytoplasm. However, some of the largest Fe/S-rich granules were found to co-localize with electron-dense organelles, assigned to lipofuscin granules (Fig. 5g, h).

Discussion
This method quantifies trace elements in identified compartments of neurons of brain tissues. Clear-cut contrasts and higher levels of P and S were obtained in the cytoplasmic compartment as compared with the nucleus, unlike what has been reported either in cell cultures or in vivo. Although the cause of these differences in P and S levels needs to be further explored, it is also worth noting that in the present study, thin sections (500 nm) were probed (see Fig. 3d and Table 1). Hence, the enhanced trace contrasts and subsequent trace quantifications are obtained from uniform matter, either of nuclear or cytoplasmic origin, whereas imaging thicker sections, it is likely that elemental composition reflects a mix of various subcellular structures.

The nucleolar compartment of nigral neurons, even in non-diseased conditions, displayed clear evidence for the abundant presence of P, S, Ca, but also of Zn and Fe. The presence of Fe in the nucleoli has so far been imaged by XRF in plants and mammalian cells, and by other techniques in aged or diseased brains. Fe levels in nucleoli of dopaminergic neurons were quantified in the present work (see Table 1). As the substantia nigra is a brain region of notoriously high Fe content, the possibility that the presence of Fe in the nucleoli might be an in vivo property of dopaminergic neurons calls for further investigations. Besides, the weak overlaps between the Fe and P, Ca, Zn distributions within the nucleolus question the role of metals in close contact with nucleic acids.
The method also displays RERs as particularly enriched in P and Ca, consistent with the role of this organelle in Ca storage, processing of nucleic acids and phospholipid synthesis. Moreover, similar P and Ca enrichments and expected higher Fe levels were observed in lipofuscin granules\textsuperscript{51}. High Fe levels in lipofuscin may indicate a role similar to that of the neuromelanin pigment in human dopaminergic neurons, which binds Fe and inactivates toxic Fe cations\textsuperscript{52}. By establishing trace metal compositions of the neuronal ultrastructure down to specified organelles, our correlation method provides a tool to further explore metal dyshomeostasis in brain tissue.

The method also showed the existence of Fe-rich granules (diameter < 600 nm) throughout the cytoplasm in the substantia nigra, previously observed only in cell cultures\textsuperscript{34,36}. Subsets of granules of different Fe/S ratios and sizes, which vary with AAV-\(\alpha\)-syn injections, were singled out owing to the better SNR and spatial resolution in uniform and thin preparations. The Fe and S contents of these granules and in particular the shift toward higher Fe concentrations in diseased neurons may indicate that perturbations of iron homeostasis in dopaminergic neurons may play a role downstream of the accumulation of the human \(\alpha\)-syn protein following overexpression in the rat midbrain. However, these findings will need to be confirmed in samples of human origin. Remarkably, similar changes in the Fe/S ratio were previously measured at much lower resolution (1 \(\mu\)m) using electron probe microanalysis in both neuronal cells and the neuropil previously measured at much lower resolution (1 \(\mu\)m) using electron probe microanalysis in both neuronal cells and the neuropil.

It is therefore important to explore how \(\alpha\)-syn accumulation may induce these effects, prompting further research on the role of iron–sulfur clusters in neurodegeneration\textsuperscript{34}. Partial co-localization with lipofuscin indicates that Fe-rich granules are likely linked to the deposition of lysosomal material, similar to the co-localization of Fe with neuromelanin observed in human samples. However, most of these Fe/S-rich granules were not as yet matched to any recognizable subcellular structure. Given their small size, the granules observed in the 500-nm-thick XRF sections may not always extend to the adjacent 80-nm-thick TEM sections. Further analysis in thinner XRF sections is therefore warranted, provided these sections can withstand radiation damage. X-ray spectroscopic studies of Fe-speciation and complexation such as those performed in soft X-rays on iron in amyloid plaque cores from Alzheimer’s disease subjects would help the identification of these nanostructures and have to be further investigated\textsuperscript{55–57}. Similarly, the interpretation of the elemental levels of the mitochondria, close to the cytoplasmic levels, should also benefit from section thinning, if tolerant of higher X-ray doses. Our method quantifies unique multi-elemental nano-heterogeneities at the organelle levels in normal and diseased states, to unfold their contribution to degenerative mechanisms in neurons vulnerable to Parkinson’s disease.

In conclusion, we introduce an analytical method to measure trace elemental contents down to organelle levels in dopaminergic neurons within substantia nigra. It is based on the quasi-correlative nano-imaging of 500-nm-thick XRF sections and 80-nm-thick adjacent TEM sections of chemically embedded tissues that compensates for the difficulty in targeting specific brain regions and an inability to rapidly vitrify large depths of brain tissue when using only cryo-fixation. To record the highest XRF count rates at the highest spatial resolution, sections of organelle-size thicknesses (ca. 500 nm) were prepared and irradiated up to the damage limits using the highest beam fluences typically withstood by much sturdier thicker sections (>20 \(\mu\)m)\textsuperscript{22}. Image processing was carried out by exacting extraction of the low SNR per pixel for each cell (>\(10^4\) XRF spectra/cell) of the highest fluorescence...
signal currently attainable but still close to the limit of detection of our setup. This yielded improved contour maps of the cytoplas- 
and nuclear compartments, for which elemental average counts per pixel and total loads were compiled. XRF of K-line 
using hard X-rays has high trace sensitivity and is the only 
method to detect few ppb. It complements the scope of com- 
positional analyses of organic structures and speciation of minor 
metal contents linked to them, reachable by soft X-ray correlative 
microscopy, with a comparable 20–30 nm resolution in 100–200 
mm thin tissue sections of brains. Hence, different types of 
compartments, organelles, or subsets of granules were imaged 
from which pathological perturbations were discriminated in 
several neurons.

This method offers considerable opportunities for exploring 
cellular ultrastructure and function in brain tissues, subject to 
cautious cytological interpretations. Both the organelles 
that showed elemental levels very close to the cytoplasm, e.g., 
the numerous TEM-identified mitochondria in the neurons, and 
conversely the granules with strong elemental contrasts that do 
not match any recognizable TEM- ultrastructure, call for further 
investigations. Testing new sample preparations (varying thick- 
ness of the fixing solution) would better immobilize 
metals or other bio-essential elements (P, S, K, and Ca), 
tolerate higher X-rays radiation doses while enhancing TEM contrasts is a 
prerequisite to increasing the detection contrasts and making 
them more accurate. Such improvements will be complemented 
by the next generation of X-ray multi-element detectors coupled 
with ultra-fast pulse processors allowing the use of a largest 
fraction of the available intensity of synchrotron beams without 
saturating. Implementing correlative nano-imaging of XRF 
with other contrasts is also paramount to constrain biological inter- 
pretations. Coupling with X-ray microtomography or phase contrast imaging at the highest spatial resolutions (<20 nm) was shown to 
be very efficient to reveal subtle elemental differences in single 
cells, anticipating a finer detection of nano-XRF contrasts in brain 
tissues. The development of new techniques for super-resolution near-field optical imaging (resolution < 100 nm), or 
other contrasts yielding statistical cytological or biochemical dis- 
criminants is a contribution of major importance for diagnosing 
and exploring the causes of neurodegenerative diseases.

Methods

Animal model of Parkinson’s disease. Three 8-weeks old adult female Sprague- 
Dawley rats weighing ~200 g were housed in standard 12 h light/dark cycles, with 
ad libium access to water and food. Anesthetized animals were unilaterally 
injected in the substantia nigra (right hemisphere) with 2 μl of a suspension of the 
AAV2/6-pgk-α-syn-prepro vector encoding wild-type human α-syn protein. 
The concentration of transducing units (TU) in the vector suspension (8.35 × 10^9 
TU/ml) was determined by real-time polymerase chain reaction on total DNA 
derived from HEK293T cells, 48 h after injection with AAV2/6. The injected 
vector dose was set at 1.5 × 10^7 TU. The vector was injected using a standard 
neurosurgical guide. For each injection, 0.5 µl of the vector was injected 
side of the sample to collect the fluorescence signals. Nanopositioning is performed 
by a piezo-driven short-range hexapod stage regulated with the metrology of twelve 
capacitive sensors. All scanning uses “on-the-fly” acquisition with the sample 
translated at constant speed in the horizontal direction. Low resolution and fast 
scan steps were recorded with a scan size of 400 × 400 μm (dwell time 100 ms, flu- 
scence count rate reproducing the scan step). Neuron maps were recorded 
with 50 × 50 μm^2 fixed scan steps, called fine-scan mode, and details 
with fine-scan steps fixed at 25 × 25 μm, very fine-scan mode (dwell time 50 ms, 
3 × 10^8 photons/s in both cases). The summed spectra from the multi-element 
detectors were fitted using the PyMca software.

Statistics and reproducibility. The relative count rate $\Delta_{\alpha}$ of a compartment “i” 
with respect to the nuclear compartment “n” and its uncertainty $\sigma_{\Delta_{\alpha}}$ are defined as:

$$
\Delta_{\alpha} = \frac{(S_\alpha/N_\alpha) - (S_n/N_n)}{(S_n/N_n)}, \quad S_\alpha = \frac{N_{\text{org}}}{N_n}, \quad 1.
$$

$$
\sigma_{\Delta_{\alpha}} = \Delta_{\alpha} \times \sqrt{\left[ \frac{\sigma_{S_\alpha}}{S_\alpha} \right]^2 + \left[ \frac{\sigma_{N_n}}{N_n} \right]^2}, \quad 2.
$$

Where $S_\alpha$, $\sigma_\alpha$ and $N_n$ designate the number of counts, their uncertainties, and the 
number of pixels, respectively, in the compartments. $S_n$ and $\sigma_n$ are the output of the 
PyMca fitting procedure. As a measure of the fluorescence count rate reproduc- 
ability, the uncertainty of each pixel, subject to Poisson statistics, is the square root 
of the number of counts. The number $N_n$ of pixels in a given compartment thus 
becomes an important parameter in order to evaluate a $\sigma_{\Delta_{\alpha}}$ value with good 
precision. The organelle/nucleus ratios are typically 100 times less precise than 
those of cytoplasm/nucleus as the organelle areas are typically ≥0.25 μm^2 (10^6 
pixels at 50 nm steps) while the cytoplasm and nucleus have a typical area ≥ 25 μm^2 
(or 10^4 pixels at 50 nm steps).

Finally, to qualify the reproducibility of the animal/neuron sampling, SD values 
were calculated and used throughout.

Semi-quantitative analyses of elemental areal masses. Elemental areal masses am 
and their uncertainties, $\sigma_m$, were compiled by semi-quantitative analysis, 
comparing the ratio of counts in the $K_{\alpha}$ line of the considered element X over 
that of Si ($S_{X}/S_{\text{Si}}$) to that of the Fe $K_{\alpha}$ line (in grey in the Table 1) over that 
of the Si for the AXO Dresden GmbH RF200-52371 thin multilayer standard 
(100% SiO2(AO,XO)). Mounting a 500-nm-thick section of substantia nigra on a 
500-nm-thick ultrapure SiN window has the advantage of providing an internal 
Si standard whose buffer calibration is then used for the contrast detection.

The level of Si in the tissue is indeed negligible with respect to the level of Si measured 
in the window. The calibration used the average sum spectra per pixel of the
AXO standard, corresponding to 1054 pixels and a total integration time of 105 s. The applied semi-quantitative analysis correction of \( \alpha_{\text{AXO}} \) and \( \varepsilon_{\text{AXO}} \) estimated from the Fe Kc and Si Kc fluorescence lines of samples and from the AXO standard is presented below:

\[
\alpha_{\text{x}} = \frac{\alpha_{\text{AXO}} \cdot \frac{f_{\text{Fe,AXO}}}{f_{\text{Fe}}} \cdot \frac{t_{\text{Fe}}}{t_{\text{AXO}}} \cdot \frac{\rho}{\rho_{\text{AXO}}}}{1}
\]

\[
\varepsilon_{\text{x}} = \frac{\varepsilon_{\text{AXO}} \cdot \frac{f_{\text{Fe,AXO}}}{f_{\text{Fe}}} \cdot \frac{t_{\text{Fe}}}{t_{\text{AXO}}} \cdot \frac{\rho}{\rho_{\text{AXO}}}}{1}
\]

where \( \alpha \) and \( \varepsilon \) are the thicknesses (cm\(^{-1}\)) of the Si\(_N\) sample holder membranes of the X sample and AXO standard and \( t \) is the total fundamental parameter correction due to the \( \varepsilon \) fluorescence cross-section (cm\(^2\)/g), \( \delta \) detector efficiency, and \( A \) atomic mass, respectively, of the specific element. The conversion from ng/cm\(^2\) of the areal masses \( am \) to ppm for the \( c \) concentration is obtained as follows:

\[
c = \frac{am}{t \cdot \rho}
\]

where \( t \) is the section thickness, and a value of 1 g/cm\(^3\) was assigned to the density of the chemically fixed brain slices.

**Reporting summary** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability** The datasets generated during and/or analysed during the current study are available from the corresponding author upon reasonable request.

Received: 16 November 2019; Accepted: 18 June 2020; Published online: 09 July 2020.

**References**

1. Rouault, T. A. Iron metabolism in the CNS: implications for neurodegenerative diseases. *Nat. Rev. Neurosci.* **14**, 551–564 (2013).
2. Bush, A. I. The metal theory of Alzheimer’s disease. *J. Alzheimers Dis.* **33** (Suppl 1), S277–281 (2013).
3. Ayton, S., Lei, P. & Bush, A. I. Metallostat in Alzheimer’s disease. *Free Radic. Bio. Med.* **62**, 76–89 (2015).
4. Sian, H., Mandel, S., Youdim, M. B. H. & Riederer, P. The relevance of iron in the pathogenesis of Parkinson’s disease. *J. Neurochem.* **118**, 939–957 (2011).
5. Richardson, D. R. et al. Mitochondrial iron trafficking and the integration of iron metabolism between the mitochondrion and cytosol. *Proc. Natl Acad. Sci. USA* **107**, 10775–10782 (2010).
6. Ndayisaba, A., Kaindlstorfer, C. & Wenning, G. K. Iron in neurodegeneration—cause or consequence? *Front. Neurosci.* **13**, 180 (2019).
7. Schapira, A. H. V. et al. Mitochondrial complex I deficiency in Parkinson’s disease. *Lancet* **333**, 1269 (1989).
8. Ibáñez, P. et al. Causal relation between alpha-synuclein gene duplication and Parkinson’s disease. *Lancet Neurol.* **14**, 965–974 (2015).
9. Pihlstrøm, L. et al. A comprehensive analysis of SNCA-related genetic risk in Parkinson’s disease. *Sci. Rep.* **6**, 36009 (2016).
10. Davies, K. M. et al. Copper pathology in vulnerable brain regions in Parkinson’s disease. *Neurobiol. Aging* **35**, 858–866 (2014).
11. Muñoz, Y., Carrasco, C. M., Campos, J. D., Aguirre, P. & Núñez, M. T. Parkinson’s disease: the mitochondria-iron link. *Parkinsons Dis.* **2016**, 7049108 (2016).
12. Micsik, A. et al. Ferroptosis: a regulated cell death nexus linking metabolism, redox biology, and disease. *Cell* **171**, 273–285 (2017).
13. Belaidi, A. A. & Bush, A. I. Iron neurochemistry in Alzheimer’s disease and Parkinson’s disease: targets for therapeutics. *J. Neurochem.* **139**, 179–197 (2016).
14. Guiney, S. J., Adlard, P. A., Bush, A. I., Finkelstein, D. I. & Ayton, S. Ferroptosis and cell death mechanisms in Parkinson’s disease. *Neurochem. Int.* **104**, 34–48 (2017).
15. Genoud, S. et al. Subcellular compartmentalisation of copper, iron, manganese, and zinc in the Parkinson’s disease brain. *Metallomics* **9**, 1447–1455 (2017).
16. New, E. J., Wimmer, V. C. & Hare, D. J. Promises and pitfalls of metal imaging in biology. *Cell Chem. Biol.* **25**, 7–18 (2018).
17. Fahrni, C. J. Biological applications of X-ray fluorescence microscopy: exploring the subcellular topography and speciation of transition metals. *Curr. Opin. Chem. Biol.* **11**, 121–127 (2007).
18. Collingwood, J. F. & Adams, F. X-ray microscopy for detection of metals in the brain. *In Metals in the Brain: Measurement and Imaging* (ed White, A. R.) 7–32 (Springer, New York, 2017), https://doi.org/10.1007/978-1-4939-6918-0_2.
19. Szczerskowska-Boruchowska, M. et al. Topographic and quantitative microanalysis of human central nervous system tissue using synchrotron radiation. *X-Ray Spectrom.* **41**, 3–11 (2002).
20. Carboni, E. et al. Imaging of neuronal tissues by x-ray diffraction and x-ray fluorescence microscopy: evaluation of contrast and biomarkers for neurodegenerative diseases. *Biomed. Opt. Express* **8**, 4331–4347 (2017).
21. Hackett, M. J. et al. Elemental characterisation of the pyramidal neuron layer within the rat and mouse hippocampus. *Metalomics* **11**, 151–165 (2019).
22. Bohic, S. et al. Intracellular chemical imaging of the developmental phases of human neurormelanin using synchrotron X-ray microspectroscopy. *Anal. Chem.* **80**, 9557–9566 (2008).
23. Hütt, D. et al. X-ray fluorescence analysis of iron and manganese distribution in primary dopaminergic neurons. *J. Neurochem.* **124**, 250–261 (2013).
24. Kohal, R. A., Jin, Q., Lai, H.-J. & Kiedrowski, L. Visualizing metal content and intracellular and intracellular distribution in primary hippocampal neurons with synchrotron X-ray fluorescence. *PLoS One* **11**, e0159582 (2016).
25. Κοσιό, Κ. et al. Combined use of hard X-ray phase contrast imaging and X-ray fluorescence microscopy for sub-cellular metal quantification. *J. Struct. Biol.* **177**, 239–247 (2012).
26. Ortega, R. et al. α-Synuclein over-expression induces increased iron accumulation and redistribution in iron-exposed neurons. *Mol. Neurobiol.* **53**, 1925–1934 (2016).
27. Kashiv, Y. et al. Imaging trace element distributions in single organelles and subcellular features. *Sci. Rep.* **6**, 21437 (2016).
28. Deng, J. et al. X-ray pt'hographic and fluorescence microscopy of frozen-hydrated cells using continuous scanning. *Sci. Rep.* **7**, 445 (2017).
29. Carmona, A. et al. SLC30A10 mutation involved in Parkinsonism results in manganese accumulation within nanovesicles of the Golgi apparatus. *ACS Chem. Neurosci.* **10**, 599–609 (2019).
30. Korogod, N., Petersen, C. C. & Knott, G. W. Ultrastructural analysis of adult mouse neocortex comparing aldehyde perfusion with csox fixation. *eLife* **4**, e05793 (2015).
31. Ohno, N., Terada, N., Saitoh, Y. & Ohno, S. Overview on recent applications of in vivo cryoetchine in neurosciences. *In Vivo Cryoetchine in Biomedical Research and Application for Biomaging of Living Animal Organs* (eds Ohno, S., Ohno, N. & Terada, N.) 179–183 (Springer, Japan, 2016), https://doi.org/10.1007/978-4-431-55723-4_34.
32. Simionovici, A. & Chevallier, P. Micro-XRF with synchrotron radiation. *in Exploring the Subcellular Topography and Speciation of Transition Metals* (eds Ohno, S., Ohno, N. & Terada, N.) 179–183 (Springer, Japan, 2016), https://doi.org/10.1007/978-4-431-55723-4_34.
33. Guenther, M. N. et al. Nigrostriatal overabundance of α-synuclein leads to decreased vesicle density and deficits in dopamine release that correlate with reduced motor activity. *Acta Neuropathol.* **123**, 653–669 (2012).
44. Silva, J. C. da et al. Efficient concentration of high-energy x-rays for diffraction-limited imaging resolution. Optica 4, 492–495 (2017).

45. Solé, V. A., Papillon, E., Cotte, M., Waller, P. H. & Susini, J. A multiplatform code for the analysis of energy-dispersive X-ray fluorescence spectra. Spectrosc. Acta B 62, 63–68 (2007).

46. Schindelin, J. et al. Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682 (2012).

47. Koschuttner, H. et al. Plant cell nucleolus as a hot spot for iron. J. Biol. Chem. 286, 27863–27866 (2011).

48. Robinson, I. et al. Nuclear incorporation of iron during the eukaryotic cell cycle. J. Synchrotron Rad. 23, 1490–1497 (2016).

49. Yumoto, S., Kakimi, S. & Ishikawa, A. Colocalization of aluminum and iron in nuclei of nerve cells in brains of patients with Alzheimer’s disease. J. Alzheimers Dis. 65, 1267–1281 (2018).

50. Reintir, A., Morawski, M., Seeger, J., Arendt, T. & Reintir, T. Iron concentrations in neurons and glial cells with estimates on ferritin concentrations. BMC Neurosci. 20, 25 (2019).

51. Hön, A. & Grune, T. Lipofuscin: formation, effects and role of macroautophagy. Redox Biol. 1, 140–144 (2013).

52. Double, K. L. et al. The comparative biology of neuromelanin and lipofuscin in the human brain. Cell. Mol. Life Sci. 65, 1669–1682 (2008).

53. Oakley, A. E. et al. Individual dopaminergic neurons show raised iron levels in Parkinson disease. Neurology 68, 1820–1825 (2007).

54. Isaya, G. Mitochondrial iron-sulfur cluster dysfunction in neurodegenerative disease. Front. Pharmacol. 12, 29 (2014).

55. Zhang, P. et al. Electron tomography of degenerating neurons in mice with abnormal regulation of iron metabolism. J. Struct. Biol. 150, 144–153 (2005).

56. Everett, J. et al. Nanoscale synchrotron X-ray spectiation of iron and calcium compounds in amyloid plaque cores from Alzheimer’s disease patients. Nano Res. 10, 11782–11796 (2018).

57. Lernyse, F. et al. Emerging approaches to investigate the influence of transition metals in the proteinopathies. Cell 8, 1231 (2019).

58. James, S. A. et al. Quantitative comparison of preparation methodologies for x-ray fluorescence microscopy of brain tissue. Anal. Bioanal. Chem. 401, 853–864 (2011).

59. Piaugè, E., Lindh, U. & Roomans, G. M. Comparative study of freeze-substitution techniques for x-ray microanalysis of biological tissue. Microsc. Res. Tech. 28, 254–258 (1994).

60. da Cunha, M. M. L. et al. Overview of chemical imaging methods to address biological questions. Micron 84, 23–36 (2016).

61. Gao, R. et al. Cortical column and whole-brain imaging with molecular contrast and nanoscale resolution. Science 363, eaau3302 (2019).

62. Duonchet, J., Bensadoun, J.-C., Schneider, B. L. & Aebischer, P. Targeted overexpression of the parkin substrate Pael-R in the nigrostriatal system of adult rats to model Parkinson’s disease. Neurobiol. Dis. 35, 32–41 (2009).

63. Bobela, W., Nazeru, S., Knott, G., Aebischer, P. & Schneider, B. L. Modulating the catalytic activity of AMPK has neuroprotective effects against α-synuclein toxicity. Mol. Neurodegener. 12, 80 (2017).

64. Villar, F. et al. Nanopositioning for the ESRF ID16A nano-imaging beamline. Synchrotron Radiat. News 31, 9–14 (2018).

65. Brunetti, A., Sanchez del Rio, M., Golosio, B., Simionovici, A. & Somogyi, A. A library for X-ray–matter interaction cross sections for X-ray fluorescence applications. Spectrosc. Acta B 59, 1725–1731 (2004).

Acknowledgements

The nano-XRF experiments were supported by the ESRF in the frame of the proposals MD900 and MD970. B.L.S. and Ph.C. were supported by the Swiss National Science Foundation, Grant No 31003A_135696. A.S. and L.L. were supported by the French National Research Agency in the framework of the Investissements d’Avenir program (ANR-15-IDEX-02).

Author contributions

A.S. and B.L.S. designed the research project; G.K., Ph.C., B.L.S. prepared samples and did the TEM work; L.L., A.S., Ph.C., S.B., P.C., and B.L.S. performed the XRF data acquisition; L.L. and A.S. performed the XRF data analyses; B.L.S., L.L., A.S., P.C., and S.B. wrote the paper.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s42003-020-1084-0.

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