The zinc ionophore clioquinol reduces Parkinson's disease patient-derived brain extracts-induced neurodegeneration

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Abstract

Parkinson's Disease (PD) is characterized by intracellular α -synuclein-rich protein aggregates, named Lewy Bodies (LB), and by the progressive loss of dopaminergic neurons in the substantia nigra. Several heavy metals, including zinc (Zn), have been suggested to play a role in PD progression, although the exact role of Zn in neurodegeneration remains to be fully elucidated. To address this gap, we investigated the effects of Zn modulation on the progression of degeneration in mice injected with PD patient-derived LB-extracts carrying toxic α -synuclein aggregates. Zn modulation was achieved using either a clioquinol-enriched diet, a Zn ionophore that redistributes cellular Zn, or a Zn-enriched diet that increases Zn levels. Clioquinol treatment significantly prevented dopaminergic neurodegeneration and reduced α -synuclein-associated pathology in LB-injected mice, while no differences were observed with Zn supplementation. Biochemical analyses further demonstrate that the expression levels of vesicle-specific Zn transporter ZnT3 in the striatum of LB-injected mice treated with clioquinol were decreased, suggesting an intracellular redistribution of Zn. Additionally, we found that clioquinol modulates the autophagy-lysosomal pathway by enhancing lysosomal redistribution within the neuronal compartments. Collectively, we found that in vivo pharmacological chelation of Zn, by dampening Zn-mediated cytotoxicity, can result in an overall attenuation of PD-linked lysosomal alterations and dopaminergic neurodegeneration. The results support zinc chelation as a disease-modifying strategy for treating PD.

Background

Parkinson's Disease (PD) is the second most common neurodegenerative disease. Clinically, PD is characterized by motor dysfunction, including bradykinesia, rigidity, postural instability, and rest tremor [1]. PD pathology is described by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the presence of α -synuclein (α -syn)-positive intracytoplasmic inclusions called Lewy bodies (LB) in the brain. In physiological conditions, α -syn can be either unfolded or in a membrane-bound conformation in the brain. In pathological conditions, it is now well established that α -syn can misfold and aggregate into fibrillar species with a propensity to progressively and sequentially spread from affected to unaffected central nervous system (CNS) regions. Accumulation of pathological α -syn has been shown to disrupt vital cellular functions, including endoplasmic reticulum stress response, induction of reactive oxygen species by the mitochondria, and lysosomal dysfunction [2]. Understanding α -syn misfolding, its implication in the cell, and aggregate formation are essential to grasp PD induction and neurodegeneration mechanisms.

Several heavy metals such as copper, iron, and zinc (Zn) are thought to play a role in the progression and development of PD [3–6]. Among these metals, Zn role in neurodegeneration remains to be elucidated. After iron, Zn is the second most predominant metal in the body [7]. Zn plays many cellular functions, including as a second messenger and a catalyzer for enzymatic reactions. In the cell, Zn concentrations are tightly regulated. Every modification in Zn levels could induce changes in cellular mechanisms, notably the autophagy-lysosomal pathway (ALP) through the mTOR-dependent pathway [8, 9]. To this end, the free Zn levels in the cell are very low in the cytoplasm. To avoid free Zn, it is either coupled to

other proteins, such as metallothioneins, or sequestered in organelles, such as the lysosome. This free and sequestered Zn regulation is mainly accomplished by Zn transporters [10]. Dysregulation of Zn transporter levels has been observed in certain neurodegenerative diseases, notably in PD and Alzheimer's Disease (AD) [11, 12]. Zn transporters are dysregulated in the case of sporadic PD patients and experimental PD models, thus suggesting a Zn dyshomeostasis in the pathological process [13]. This phenotype is also observed in familial PD cases, particularly with *ATP13A2/PARK9* mutations [14–17]. *ATP13A2* deficiency causes Zn dyshomeostasis by reducing intralysosomal Zn concentrations, impairing lysosomal function [14, 16]. Conversely, *ATP13A2* overexpression induces a decrease in α -syn toxicity via an increase in α -syn exosomal release from the cell [17, 18]. Moreover, α -syn has been shown to bind Zn, positing that Zn binding could modify α -syn secondary conformations [19].

In an attempt to correct the excess levels in certain metals, chelators have been used in models of PD and AD to evaluate their ability to reduce protein aggregation and/or improve cellular functions [20–22]. Clioquinol (ClQ), or 5-chloro-7-iodo-8-hydroxyquinone, is a lipophilic metal ionophore that affects zinc, copper and iron homeostasis, and that has previously been used in studies on neurodegenerative diseases, particularly *in vivo* models of PD, AD and Huntington's Disease [23–27]. ClQ may directly have a neuroprotective effect on aggregate formation and neuronal loss in transgenic rodent models of neurodegeneration, suggesting a potential therapeutic strategy. However, its impact on α -syn seeding-based rodent models remains to be investigated. However, mechanistic explanations of ClQ effects are unclear despite direct chelation effect and ALP enhancement have, notably, been proposed as playing a key role in the clearance of α -synuclein, the major pathological trigger in PD [27].

Here, we aimed to clarify the role of Zn and the modulation of its quantities in a clinically relevant model of PD related to α -syn-induced dopaminergic neurodegeneration. We used a mouse model based on the intracerebral injection of PD patient-derived LB in the SN previously shown to promote progressive nigrostriatal degeneration, α -syn pathology and ALP impairment in wild-type mice [13, 28, 29]. Following the model induction, mice were fed for four months with diets featuring either ClQ, to deplete Zn, or supplemented in Zn, to increase Zn levels. We show that *in vivo* chronic ClQ exposure can prevent LB-triggered dopaminergic neurodegeneration and reduce α -syn pathology in the SNpc. Both effects may be related to *in vitro*-demonstrated enhanced α -syn degradation and the ALP activation in neurons. These results highlight Zn chelation potential as a disease-modifying strategy for treating PD.

Methods

Mice Stereotaxic injections and diet

Wild-type C57BL/6J mice (3 months old) received 2 μ l of either LB fractions (n = 30) or sucrose (n = 30) by stereotactic delivery to the region immediately above the right substantia nigra (SNpc, coordinates from Bregma: AP=-2.9, L = + 1.3, DV=-4.5) at a flow rate of 0.4 μ l/min and the pipette was left in place for 5 min after injection to avoid leakage as previously described. One week after surgery, mice were divided into three different groups, depending on the received diet, until the end of the experiment: clioquinol

supplemented diet (30mg/kg/day), Zn sulfate supplemented diet (180mg/kg/day), or a standard diet. Mice were perfused four months after injection using 0.9% NaCl followed by 4% paraformaldehyde. Brains were then post-fixed for 24 hours in 4% paraformaldehyde at 4°C, cryoprotected in gradient 20% sucrose in PBS before being frozen by immersion in a cold isopentane bath (-60°C) and stored immediately at -80°C until sectioning for histochemical analysis.

Purification of Lewy bodies from human PD Brains

The samples were obtained from brains collected in a Brain Donation Program of the Brain Bank “GIE NeuroCEB” run by a consortium of Patients Associations: ARSEP (association for research on multiple sclerosis), CSC (cerebellar ataxias), France Alzheimer, and France Parkinson. According to the French Bioethical Laws, the patients themselves or their next of kin signed the consent. The Brain Bank GIE NeuroCEB (Bioresource Research Impact Factor number BB-0033-00011) has been declared at the Ministry of Higher Education and Research and has received approval to distribute samples (agreement AC-2013-1887). Human SNpc was dissected from fresh frozen postmortem midbrain samples from 5 patients with sporadic PD exhibiting conspicuous nigral LB pathology on neuropathological examination (mean age at death: 75 ± 2.75 years; frozen post-mortem interval: 31.8 ± 7.45 h; GIE Neuro-CEB BB-0033-00011). Tissue was homogenized in 9 vol (w/v) ice-cold MSE buffer (10 mM MOPS/KOH, pH 7.4, 1M sucrose, 1mM EGTA, and 1mM EDTA) with protease inhibitor cocktail (Complete Mini; Boehringer Mannheim) with 12 strokes of a motor-driven glass/Teflon homogenizer. A sucrose step gradient was prepared for LB purification by overlaying 2.2 M with 1.4 M and 1.2 M sucrose in volume ratios of 3.5:8:8 (v/v). The homogenate was layered on the gradient and centrifuged at 160,000 x g for 3 h using a SW32.1 rotor (Beckman). Twenty-six fractions of 1500 µl were collected from each gradient from top (fraction 1) to bottom (fraction 26) and analyzed for the presence of α -synuclein aggregates by filter retardation assay, as previously described [29]. Further characterization of LB fractions was performed by immunofluorescence, α -synuclein ELISA quantification, and electron microscopy as previously described [29]. For stereotactic inoculations, LB-containing fractions from PD patients were mixed together in the same proportion (PD#1, fractions 19 and 20; PD#2, fractions 19 and 20; PD#3, fraction 22; PD#4, fractions 17, 18 and 19; PD#5, fractions 20, 21 and 23). LB fractions were adjusted to ~24 pg α -synuclein per microliter of injected samples, as measured by a specific enzyme-linked immunosorbent assay (ELISA) kit against human α -synuclein (Invitrogen, #KHB0061) according to the manufacturer’s instructions. In all cases, samples were bath-sonicated for 5 min prior to *in vivo* injections.

Histological Analysis

Neurodegeneration. To assess the effect of LB injections on dopaminergic neurons, tyrosine hydroxylase (TH) immunohistochemistry was performed on substantia nigra pars compacta (SNpc) as previously described [13]. Serial sections (1 to 6 slices) corresponding to the whole SNpc were incubated with rabbit monoclonal TH antibody (Abcam, EP1532Y, 1:5000) for one night at room temperature (RT) and revealed the next day by an anti-mouse peroxidase EnVision secondary antibody, followed by 3,3'-diaminobenzidine (DAB) visualization. Free-floating sections were mounted on gelatin-coated slides, counterstained with 0.1% cresyl violet solution, dehydrated and cover-slipped. TH-positive neurons of the

SNpc were counted by stereology blind to the experimental condition using a Leica DM6000B microscope coupled with the Mercator software (Explora Nova, France). The SNpc was delineated for each slide, and probes for stereological counting were applied to the map obtained. Each TH-positive cell with the neuron included in the probe was counted. The total number of TH-positive neurons in the SNpc per hemisphere was then assessed using the optical fractionator method.

α -synuclein pathology. Synuclein burden has been assessed with a mouse monoclonal antibody raised against murine α -syn (BD Biosciences, Syn1, 1:1,000) immunostaining, as previously reported [13]. Briefly, selected sections of 1 rostrocaudal level of SN were specifically identified and incubated in the same well to directly compare immunostaining intensity. For pretreatment with PK, sections were incubated first with PK at 10 μ g/ml in PBS before long sequential washes in distilled water and then in PBS. Sections were incubated overnight at room temperature with the aforementioned antibody. The following day, the revelation was performed with anti-species peroxidase EnVision system (DAKO) followed by DAB incubation. Sections were then mounted on gelatinized slides, dehydrated, and cover-slipped until further analysis. Sections were analyzed by optical density (OD). The slides were scanned using Epson expression 10000XL high-resolution scanner. Images were analyzed using ImageJ open-source software (version 1.53) to compare mean grey levels.

Biochemical Analysis

Total protein extraction. Tissue patches collected on 300 μ m-thick cryostat-cut sections of SN and striatum were extracted on ice using 100 μ l of RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1.0% Triton X-100, 0.5% Na-deoxycholate, 0.1% sodium dodecyl sulfate) with a protease and phosphatase inhibitor cocktail as previously described [13, 30]. Lysates were incubated for 20 minutes and then centrifugated at 14,000 rpm for 15 minutes at 4°C. Supernatants were collected, and the total amount of protein in the lysates was assessed by Bicinchoninic Acid (BCA) assay before storage at -80°C.

Based on total protein concentrations from the BCA assays, aliquots of tissue lysates corresponding to known amounts of total protein per sample were prepared for each animal in Laemmli buffer (Tris-HCl 25mM pH = 6.8, Glycerol 7.5%, SDS 1%, DTT 250mM, and Bromophenol Blue 0.05%) for immunoblotting experiments.

Immunoblotting. Western blots were run in all conditions using 10 μ g of protein separated by SDS-PAGE and transferred to nitrocellulose membranes, as previously described [13, 30]. Incubation of the primary antibodies was performed overnight at 4°C with LC3-IIB (Novus Biologicals, 1:1000), p62 (Progen, 1:1000), ZnT3 (Abcam, 1:1000), ZnT4 (Invitrogen, 1:1000), ZIP8 (Merck, 1:1000), and SOD1 (R&D Systems, 1:2000). Anti- β -actin (1:10000, Sigma) was used to control equal loading. Appropriate secondary antibodies coupled to peroxidase were revealed using a Super Signal West Chemiluminescent kit (Immobilon Western, Chemiluminescent HRP substrate, Millipore). Chemiluminescence images were acquired using the ChemiDoc + XRS system measurement (BioRad). Signals per lane were quantified using ImageJ (version 1.53). A ratio (protein of interest normalized to β -Actin protein levels, then to Control values) of the signal on loading per animal was performed and used in statistical analyses.

Immunofluorescent Labeling

Double-immunofluorescent labeling was performed on nigral sections to localize lysosomes in dopaminergic neurons. Sections were permeabilized and blocked using normal goat serum diluted in PBS 1X-Saponine 0.2% for one hour before being incubated overnight in primary antibodies rabbit anti-TH (1:1000, ab237961, Abcam) and rat anti-LAMP2 (1:1000, ab25339, Abcam). The next day, sections were incubated in secondary antibodies sequentially: first with goat anti-rabbit Alexa 488 secondary antibodies, then with goat anti-rat Alexa 568 secondary antibodies. After washing, sections were incubated with 10 μ M of Hoechst staining for 8 min to color the nuclei. Sections were mounted on non-gelatinized slides and cover-slipped using fluorescent mounting media without DAPI (Vector Labs). Images were acquired using a Zeiss SP5 confocal microscope (x63). Analysis of images was done using ImageJ (version 1.53), with a macro designed to delineate individual cells, delineate the nucleus of each cell and localize the perinuclear and cytosolic LAMP2 puncta within the cell.

Synchrotron radiation X-ray fluorescence (SR-XRF) microscopy elemental mapping of brain tissue cryosections

The synchrotron experiments were carried out at Diamond Light Source, Harwell Science and Innovation Campus (Didcot, UK) with a 3 GeV energy of the storage ring and 300 mA currents with top-up injection mode. All SR-XRF microscopy investigations reported herein were carried out on the microfocus spectroscopy beamline (I18) [31]. The micro X-ray fluorescence (μ -XRF) elemental mapping was acquired at room temperature with an incident X-ray energy set to 12 keV using an Si(111) monochromator, resulting in an X-ray photon flux of 2.4×10^{11} ph/s in the final focused beam. The SN of each animal were collected from free-floating sections and mounted onto an X-ray transparent metal-free 4 μ m thickness Ultralene R foil (SPEXCert Prep, Metuchen, NJ, U.S.A.) secured to a customized Polyetheretherketone (PEEK) holder ensuring contamination-free samples and reduced X-ray scattering contribution. The samples were affixed to a magnetic plate that connects to the sample stage. Two 4-element 1mm thick sensor Si Drifts Vortex ME4 energy dispersive detectors (Hitachi Hi-Technologies Science America) resulting in an effective detection area of 2.9 cm², equipped with Xspress-3 processing electronics, were used to collect the X-ray fluorescent signal. They were positioned at 90° to the incident X-ray focused beam to minimize scattered background signal. The sample-detector distance was fixed (80 mm). The sample was held at 45° to the incident X-ray beam and rastered in front of the beam while the X-ray fluorescence spectra were collected. An area of 500 μ m x 500 μ m within the SN was mapped for each sample with a step-size that matched the focused beam size (5 μ m x 5 μ m) and a dwell time of 1 s per pixel due to low concentration element. The raw data consist of full energy dispersive spectra for each sample pixel exposed to the beam. A thin-film XRF reference material (AXO Dresden GmbH) was measured to calibrate experimental parameters. This was followed by elemental quantification through the open-source software PyMCA [32] in which both the reference material and the sample are modelled in terms of main composition, density and thickness. The fluorescence spectrum obtained from each pixel was fitted, the elemental concentration (μ g/g dry weight or ppm) maps were generated, and an average elemental concentration of the SN regions was obtained.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 9.1 (GraphPad Software, Inc., San Diego, CA). For all experiments, comparisons among means were performed by using two-way ANOVA. In all analyses, statistical significance was set at $p < 0.05$. The debate about the need to move beyond p value is raging. Data must now be analyzed further with estimation graphics [33] that emphasized the effect size. Therefore, all data appear as estimation graphics called 'Gardner–Altman plots': on the left of each graph, data are presented as a scatter plot showing the observed values and above defined descriptive statistics (mean $\pm$ standard deviation). Contrast graphs using the difference axis to display an effect size on the bottom of each graph, here mean difference. Horizontally aligned with the mean of the test group, the mean difference is indicated by the black circle. The black vertical line illustrates the mean difference's 95% confidence interval (CI). Given the observed data, the colored curve indicates the resampled distribution of the effect size.

Results

To explore the *in vivo* effect of Zn chelation in an experimental PD mouse model, we used the widely-used membrane-permeable (and safe) Zn chelator named clioquinol [34, 35]. We injected low doses (pg/ μ l) of α -syn containing LB extracts purified from the SNpc of PD brains into the SN of C57Bl/6J WT mice. These LB extracts were previously shown to promote progressive dopaminergic degeneration and α -syn pathology in wild-type mice after 4 months [13, 29]. Following the injection of LB or vehicle, we used different Zn-related diets for the remaining 4 months of the experiment. To determine whether Zn modulation influenced the viability of ventral midbrain dopaminergic neurons, we examined the total number of TH-positive cells in the SN by stereology (Fig. 1A) (**Table 1** features all raw data). First, we observed a 30% decrease in the ipsilateral to contralateral TH staining ratio in LB-injected mice compared to Vehicle mice (Fig. 1B-C), according to our previous studies [13, 28, 29, 36]. However, we did not observe the same neurodegeneration magnitude in LB-injected mice with altered diets. Stereological counts of SNpc TH-positive neurons in mice treated with ClQ (blue groups) revealed that ClQ reduced significantly dopaminergic cell loss to 15% (Fig. 1B), instead of the $\sim 35\%$ loss typically observed in this model. On the contrary, no significant differences were observed in LB-injected mice with Zn supplementation (green groups) (Fig. 1C). Still, the number of TH-positive neurons remained similar to the LB-injected mice. This result suggests a significant neuroprotective effect of ClQ on LB-induced dopaminergic neuron loss, which is not observed with a Zn-supplemented diet. However, no changes in striatal TH were observed in any of the groups (**Supp.** Figure 1A-C). To evaluate the impact of Zn modulation on α -syn pathology in mice, we quantified the levels of phosphorylated α -syn at Ser129, but with no visible alterations, which in turn, we chose a refined detection using total monomeric α -syn and proteinase K (PK)-resistant α -syn in the SN, i.e., a proxy of aggregated α -syn (Fig. 2A). No significant differences were observed between groups when quantifying total α -syn levels without PK treatment (Fig. 2B). The expression of monomeric α -syn also remained unchanged when quantifying using immunoblotting (**Supp.** Figure 2). However, we observed that untreated LB-injected mice had significantly increased content in PK-resistant α -syn

(Fig. 2C). The amount of PK-resistant α -syn staining in ClQ-treated groups demonstrated a trend to decrease in LB-injected mice compared to untreated mice ($p = 0.099$), an effect confirmed by the effect size distribution. This alteration in PK-resistant staining was not observed in Zn supplemented LB-injected mice ($p = 0.2983$), indicating the beneficial effect of ClQ treatment compared to Zn supplementation in this mouse model.

Several Zn transporters regulate Zn homeostasis and are crucial for proper cellular functions to avoid Zn-associated toxicity [10]. Thus, we next explored the expression levels of Zn transporters associated with synaptic vesicles and the lysosome and Zn-binding proteins (Fig. 3). First, the only Zn transporter present at the membrane in synaptic vesicles is ZnT3, responsible for Zn import. Measuring striatal ZnT3 expression levels, we observed a significant decrease in ZnT3 levels in ClQ-treated LB-injected mice, but not in any other examined groups (Fig. 3A). This data might indicate a reorganization of Zn concentrations away from the synapse, leading to less Zn released at the synapse [11]. Second, we assessed the levels of ZnT4 and ZIP8, two lysosomal transporters responsible for the import and export of Zn from the cytosol to the lysosome, respectively. In the striatum, the levels of ZnT4 remained generally unchanged, with a trend towards a decrease of ZnT4 in untreated LB-injected mice (Fig. 3B). However, ZIP8 levels were significantly increased in LB-injected mice having received a Zn diet, suggesting an increase in cytosolic zinc and a reduction of intralysosomal Zn in LB-treated mice with Zn impairing lysosomal function by disrupting the ionic balance in lysosomes (Fig. 3C). This latter result would potentially explain the mechanism-based toxicity in LB + Zn-treated mice. Third, superoxide dismutase 1 (SOD1) is a Zn-binding protein in the cytosol, responsible for regulating mitochondrial oxidative stress, that is dysregulated in multiple neurological disorders [37], and notably PD [38]. We thus investigated whether SOD1 expression was altered in our experimental conditions. In vehicle and LB-injected mice, SOD1 showed no changes in expression in the striatum of mice treated with either ClQ or zinc supplementation (Fig. 3D). This result could indicate that this protein is not directly implicated in Zn binding in our model, at least at the striatal level. Altogether, these striatal transporters and Zn-binding proteins showed minor alterations, except ZnT3 in ClQ-treated LB-injected mice and ZIP8 in zinc supplemented LB mice. We then measured heavy metals levels in the SN of the four experimental groups. First, considering the sensitivity of the synchrotron microprobe ($< \mu\text{g/g}$ sensitivity) and that ClQ intrinsically contains an iodine atom, we could potentially detect the iodine in the mapped tissues section. The I-L_{β} ($E = 4.22 \text{ keV}$) X-ray fluorescent line does not overlap other element in a spectral region with low background counts, yet no iodine peak could be detected. Surprisingly, we observed an increase in Zn levels only in control mice receiving ClQ while control mice supplemented with Zn showed decreased Zn concentration. However, no alteration in Zn levels was observed in LB-injected mice, independently of any treatment (**Supp. Figure 3A**). Alterations in metal concentrations were also seen in the case of calcium and iron (**Supp. Figure 3B-C**), however no changes were observed for copper, manganese or sulfur (**Supp. Figure 3D-F**). These changes in metal concentrations, in particular Zn, calcium and iron indicate that ClQ and Zn supplementation induce metal-specific content variations within the cell.

Given the implication of Zn in the ALP, we next determined whether CIQ or zinc supplementation diets altered the general function of ALP (Fig. 4, **Supp.** Figure 4). We first measured the expression of autophagy-related proteins, including LC3-II and p62, in the SN of vehicle and LB-injected mice. LC3-II is present at the autophagosome membrane and binds to p62, a cargo protein that brings misfolded proteins for their degradation. Immunoblot results showed no significant difference in the steady-state levels of LC3-II in LB-injected mice having received both CIQ and Zn diets (**Supp.** Figure 3A). Furthermore, p62 levels were increased only in LB-injected mice having received a zinc diet, suggesting impaired autophagy flux in zinc supplementation condition (**Supp.** Figure 3B). Second, we examined whether a change in cellular localization of lysosomes could be observed, as an index of the lysosomal function [39]. Functional lysosomes are preferentially located in the perinuclear region, whereas dysfunctional lysosomes are further from the nucleus [40, 41]. To assess lysosomal distribution, we used co-immunofluorescence to determine the distribution of the lysosome-specific marker LAMP2 in TH-positive neurons (Fig. 4A). We found that in untreated LB-injected mice, the percentage of perinuclear LAMP2 puncta in TH-positive neurons was significantly reduced, indicating lysosomal impairment [40, 41] (Fig. 4B). However, in LB mice treated with CIQ, the number of perinuclear lysosomal puncta was significantly increased compared to untreated LB-injected mice. The restoration of the percentage of perinuclear fractions of LAMP2 was not observed in LB-injected mice treated with Zn. Taken together, this result suggests that one of the neuroprotective effects of CIQ is the restoration of proper lysosomal function.

Discussion

CIQ treatment in LB-injected mice protected the dopaminergic neurons in the SN associated with a reduction in of α -syn PK-resistant staining. In addition, CIQ treatment modified Zn synaptic transmission and modulated the lysosomal function in LB-injected treated mice. Both changes would independently or synergistically participate in rescuing SN dopaminergic neurons, proving its potential neuroprotective effects (Fig. 5). Conversely, Zn supplementation neither induced nor enhanced dopaminergic neurodegeneration. Nonetheless, Zn supplementation might impair ALP, as p62 expression is increased and disrupts the ionic balance between lysosomal and cytosolic Zn in neurons by increasing lysosomal ZIP8 expression. These latter results might potentially explain the toxicity in LB + Zn-treated mice (Fig. 5).

Compared to iron and copper, very few studies have considered the role of Zn in PD. In particular, evidence that zinc concentrations were increased in the hair of PD patients [42], in the SN of 6-hydroxydopamine-induced rats [43], and in human parkinsonian SN when compared to tissue from age-matched controls [44], thus demonstrating the importance of Zn in pathology. In addition, excess of Zn in the diet has been shown to induce a loss of dopaminergic neurons, the accumulation of α -syn, and responsiveness to levodopa in a rat model [45]. This ability to induce PD-like pathology implicated Zn directly in the development of pathology.

With the implication of multiple metals in the progression of PD, we determined whether the concentrations of these heavy metals were altered in the SN of LB-injected non-human primates. A

synchrotron X-ray fluorescence-based micro-analysis allowed a precise measure of SN metal concentrations and showed that Zn levels were the only dysregulated metal concentrations of all metals measured. This increase in nigral zinc was identified as a good predictor for neurodegeneration in an artificial network used in machine-learning [13]. With this in mind, our study based on Zn modulation was a direct follow-up to this demonstration and aimed at further understanding these zinc variations. However, we observed very little changes in this metal when measuring Zn concentrations using synchrotron X-ray fluorescence in our mice. The differences seen between this study and the previous experimental study could be due to timing differences. During the last study, variations in zinc occurred after 24 months in non-human primates. The present 4 months survival time could well be not sufficient to observe such the development of changes in zinc concentrations. A study using the tau knockout mouse model also reports an unaltered Zn level in the SN by CIQ treatment [25]. In the present work, the perfusion and fixation of the brains could have contributed to alterations in the detection of metal levels, as previous studies have demonstrated that the 4% paraformaldehyde post-fixation method no doubt alters metal concentrations [46, 47]. It would thus be interesting to assess metal concentrations in animals having not been perfused. In addition, Zn concentrations are very tightly regulated within the brain, given its importance and toxicity. First, variations could appear in other Zn-rich organs such as the liver or kidney before reaching the brain. Given these constantly changing dynamics, it could be interesting to observe the Zn concentrations in these organs in addition to brain zinc in the future.

The prion-like properties of α -syn and the Braak's staging hypothesis cannot thoroughly explain what we observe in synucleinopathy patients [48, 49]. One other hypothesis relies on the potential cellular vulnerability of dopaminergic neurons, which makes these neurons more susceptible to degeneration [50]. Among the discussed hypotheses, one depends on the increased metabolism of oxidized dopamine via neuromelanin production in nigral dopaminergic neurons [51]. Neuromelanin can stock heavy metals, particularly iron and Zn, which are increased in LB-injected non-human primates [13, 52]. When inducing the non-physiological expression of neuromelanin in rats, neuromelanin chelates the metals present within the SN, indicating its role in metal homeostasis [53]. Therefore, the dysregulation of metals in the SN, potentially in neuromelanin-positive neurons, could explain a part of the vulnerability observed in synucleinopathies.

Studies have also demonstrated that oxidative stress, as is observed in PD, could lead to the release of Zn by metal-binding proteins. Increasing cytosolic Zn would activate the ERK1/2 pathway, in turn inducing an inhibition of mTOR and thus activating autophagy [54–56]. This same Zn release allows for its binding to the metal transcription factor 1 (MTF1), leading to the transcription of the *ATG* genes, involved in ALP activation [57]. With these several roles in autophagy, Zn dyshomeostasis could be implicated in the dysregulation of this pathway in neurodegenerative diseases such as PD. In animal models of PD, variations of Zn concentrations have been measured, mostly using zinc-binding fluorescent probes which are not the most reliable measuring technique *in vivo* [58–60]. Zn dyshomeostasis patterns have been variable from one species to another, with no Zn variations being observed in A53T- α -syn transgenic rats compared to mice and non-human primates injected with patient-derived fractions [13].

Several studies have tried to understand the role of ClQ within the cell and how it could have beneficial effects in neurodegeneration and cancer. With the use of *in vivo* and *in vitro* experiments, multiple potential roles of ClQ have emerged, in particular in transgenic rodent models of neurodegenerative diseases [23–26]. Until now, it was unknown whether ClQ would be effective in an animal model based on α -synuclein pathology seeding. One of the first identifications of the role of ClQ was its potential role in ALP. ClQ is thought to be implicated in the ALP through its Zn ionophore abilities, particularly in cases of oxidative stress in cultured astrocytes [61]. Given its ability to activate autophagy through the Akt/mTOR pathway [27], we thought that the neuroprotective effects of ClQ could be related to this ability in our PD mouse model. For this reason, we analyzed the effect of ClQ and Zn-supplemented diets on our LB-injected mice. ClQ has also previously shown an ability to increase lysosomal zinc, restoring lysosomal pH and correct autophagy function [62–64]. Even though this lysosomal targeting carries the potential to activate autophagy, lysosomal dysfunction occurring after LB-injection could be compensated by lysosomal Zn [62]. We looked at the lysosomal function in LB-injected mice with ClQ and Zn-treated mice. In LB-injected mice, we noticed that lysosomal function is impaired by the mislocalization of these organelles within the cell. When the lysosomes are functional, they are predominantly located around the nucleus, and their increased distance to the nucleus demonstrates their inactivity [39–41]. Our study showed that when LB mice were treated with ClQ, the number of lysosomes located close to the nucleus was increased compared to untreated LB-injected mice. This result determines one of the potential mechanisms of neuroprotection of ClQ in our PD mouse model. Further experiments should focus on the alterations in autophagy, potentially through the mTOR/Akt pathway, TFEB activation, and the verification of other zinc transporters or proteins that are implicated in neurons.

Second, ClQ has other potential targets in the synaptic transmission. That ClQ is a zinc ionophore indicates its ability to redistribute the zinc contained within the cell depending on cellular requirements [34]. Zinc has demonstrated increased importance at the synapse, and transmission has been implicated in correct brain function [7, 11, 12]. The decrease in ZnT3 expression observed in our LB-injected mice treated with ClQ, hypothesizes that this phenomenon is related to the trafficking of cytosolic zinc towards synaptic vesicles. ZnT3 decrease could indicate a lower zinc concentration at the synapse, leading to potential neuroprotection. ClQ could play a protective role in LB-injected mice through postsynaptic zinc signaling combined with increasing lysosomal function.

Conclusion

Altogether, our study suggests that *in vivo* pharmacological chelation of Zn, by dampening Zn-mediated cytotoxicity, would result in an overall attenuation of PD-linked lysosomal alterations and dopaminergic neurodegeneration (Fig. 5) and may thus carry therapeutic potential. Clinical trial phases based on iron chelation with deferiprone (FAIR-PARK-I_NCT00943748) in PD patients are currently ongoing (Fair Park II), after having shown promise in certain pre-clinical models of PD [20, 65, 66]. With this in mind, it is essential to note that heavy metal chelation as a whole, if not carefully dosed and extensively studied in pre-clinical conditions, could have detrimental effects of human patients given the widespread effect of these molecules. Therefore, this ClQ approach will require both rodent and non-human primate studies over long-term periods of time to ensure their proper clinical potential in PD patients over time.

Abbreviations

AD
Alzheimer's disease
ALP
Autophagy-Lysosomal Pathway
CIQ
clioquinol
CNS
central nervous system
ELISA
enzyme-linked immunosorbent assay
PD
Parkinson's disease
 α -syn
 α -synuclein
LB
Lewy bodies
LC3
MAP1LC3B, microtubule-associated protein 1 light chain 3 β
MTF1
metal transcription factor 1
PK
proteinase K
SNpc
substantia nigra *pars compacta*
SOD1
superoxide dismutase 1
TH
tyrosine hydroxylase
Zinc
Zn

Declarations

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fellowship (France). The human samples were obtained from the Brain Bank GIE NeuroCEB (BRIF number 0033-00011), funded by France Alzheimer, France Parkinson, ARSEP, and Connaître les Syndromes Cérébelleux, to which we express our gratitude.

AUTHORS' CONTRIBUTIONS

BD isolated LB fractions. MT performed the surgeries. MT and ED performed the animal termination. MT performed histologic and immunohistochemical analysis of the data. MT and MLT performed the biochemistry experiments. MT, SB, and BD performed the synchrotron analysis. MT prepared the figures. MT, EB and BD wrote the paper with input from all authors. BD, and EB conceptualized the study and designed experiments. EB and BD secured funding.

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AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during this study are included in Table 1 of supplementary data, further inquiries can be directed to the corresponding author.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Animals. Experiments were performed following the European Union directive of September 22, 2010 (2010/63/EU) on the protection of animals used for scientific purposes. The Institutional Animal Care and Ethical Committee of Bordeaux University (CE50, France) approved experiments accepted by the ministry under references APAFIS#17592-2018111914281699.

Human tissues. The samples were obtained from brains collected in a Brain Donation Program of the Brain Bank "GIE NeuroCEB" run by a consortium of Patients Associations: ARSEP (association for research on multiple sclerosis), CSC (cerebellar ataxias), France Alzheimer, and France Parkinson. The consent documents were signed by the patients themselves or their next of kin in their name, in accordance with the French Bioethical Laws. The Brain Bank GIE NeuroCEB (Bioresource Research Impact Factor number BB-0033-00011) has been declared at the Ministry of Higher Education and Research and has received approval to distribute samples (agreement AC-2013-1887).

CONSENT FOR PUBLICATION

Not applicable.

COMPETING INTERESTS

E.B. owns equity stake in Motac Holding Ltd. and receives consultancy payments from Motac Neuroscience Ltd. All other authors declare no competing interests.

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Figures

Figure 1

Determining the effect of zinc modulation on neurodegeneration in the substantia nigra of Vehicle and LB-injected mice. Illustrative images (**A**) and quantifications (**B-C**) of TH staining in the right SN in mice injected with Vehicle or LB fractions that received one of three diets: (i) Standard diet (black color framework), (ii) Clioquinol-supplemented diet (blue color framework), or (iii) Zinc supplemented diet (green color framework). Comparisons were made using two-way ANOVA followed by Tukey's post-hoc analysis, $n=5$ per group. * $p<0.05$ vs Veh; # $p<0.05$ vs LB. Scale bar = $500\mu\text{m}$.

Figure 2

Evaluation of the effect of zinc modulation on α -synuclein accumulation in the substantia nigra of LB-injected mice. (A) Illustrative images of untreated or proteinase-K (PK) exposed α -synuclein staining in the substantia nigra of Vehicle and LB-injected mice treated with Clioquinol or Zinc. **(B)** Quantification of α -synuclein staining in the substantia nigra of in Vehicle and LB-injected mice treated with Clioquinol or Zinc. **(C)** Quantification of PK resistant α -synuclein in Vehicle and LB-injected mice treated with Clioquinol or Zinc. Comparisons were made using two-way ANOVA followed by Tukey's post-hoc analysis, n=5 per group. * p<0.05 vs Veh and Veh ClQ. Scale bar =20 μ m.

Figure 3

Zinc Transporters are altered in mice with modulated zinc diets. Representative immunoblots and histograms of ZnT3 (A), ZnT4 (B), ZIP8 (C) and SOD1 (D) expressions in the striatum. Scatter plots represent the mean protein expression normalized by β -Actin levels in Vehicle and LB mice treated with clioquinol or zinc. Comparisons were made using two-way ANOVA followed by Tukey's post-hoc analysis, n=4-5 per group. * p<0.05 vs Veh.

Figure 4

Clioquinol and Zinc induce autophagy-lysosomal pathway changes in the brain of LB-injected mice. (A-B) Representative images of TH and LAMP2 immunofluorescence in the SN **(A)** and quantification **(B)** to evaluate the distance between lysosomes and the nucleus. LAMP2-positive lysosomes were segmented, and the distance to the border of the nucleus was calculated for each lysosome. Comparisons were made using two-way ANOVA followed by Tukey's post-hoc analysis, $n=12-20$ neurons per group. * $p<0.05$ vs LB CIQ (LAMP2). Scale bars= 20µm (left) and 10µm (right).

Figure 5

Schematic representation of zinc alterations in neurons of Vehicle and LB-injected mice and proposed therapeutic action of clioquinol in experimental PD. Expression of transporters ZnT4, ZIP8 and ZnT3 are altered and provoke a change in distribution of Zn (orange dots) within the cell, whether in the soma or at the synaptic terminals in LB-injected mice treated with Clioquinol or Zn supplementation.

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