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# NOX4 as a critical effector mediating neuroinflammatory cytokines, myeloperoxidase and osteopontin, specifically in astrocytes in the hippocampus in Parkinson's disease

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ABSTRACT

Oxidative stress and mitochondrial dysfunction have been believed to play an important role in the pathogenesis of aging and neurodegenerative diseases, including Parkinson's disease (PD). The excess of reactive oxygen species (ROS) increases with age and causes a redox imbalance, which contributes to the neurotoxicity of PD. Accumulating evidence suggests that NADPH oxidase (NOX)-derived ROS, especially NOX4, belong to the NOX family and is one of the major isoforms expressed in the central nervous system (CNS), associated with the progression of PD. We have previously shown that NOX4 activation regulates ferroptosis via astrocytic mitochondrial dysfunction. We have previously shown that activation of NOX4 regulates ferroptosis through mitochondrial dysfunction in astrocytes. However, it remains unclear why an increase in NOX4 in neurodegenerative diseases leads to astrocyte cell death by certain mediators. Therefore, this study was designed to evaluate how NOX4 in the hippocampus is involved in PD by comparing an MPTP-induced PD mouse model compared to human PD patients. We could detect that the hippocampus was dominantly associated with elevated levels of NOX4 and α-synuclein during PD and the neuroinflammatory cytokines, myeloperoxidase (MPO) and osteopontin (OPN), were upregulated particularly in astrocytes. Intriguingly, NOX4 suggested a direct intercorrelation with MPO and OPN in the hippocampus. Upregulation of MPO and OPN induces mitochondrial dysfunction by suppressing five protein complexes in the mitochondrial electron transport system (ETC) and increases the level of 4-HNE leading to ferroptosis in human astrocytes. Overall, our findings indicate that the elevation of NOX4 cooperated with the MPO and OPN inflammatory cytokines through mitochondrial aberration in hippocampal astrocytes during PD.

## 1. Introduction

Parkinson's disease (PD), a progressive neurodegenerative disease, is currently considered the most common neurodegenerative disorder following Alzheimer's disease (AD) [1,2]. Nowadays, it is generally accepted that mitochondrial dysfunction [3,4], oxidative stress [5,6], chronic inflammation [7], aberrant protein folding [8], and abnormal protein aggregation [9] are the main causes contributing to the initiation and progression of PD [10]. Moreover, evidence has been developed for oxidative stress and mitochondrial dysfunction, which play a significant role in neuronal loss under physiological conditions in PD, evidently disrupt several biological processes in the central nervous system (CNS), eventually leading to cell death [10–13].

Typically, PD is characterized by a range of motor symptoms [14,15]. Nevertheless, there is increasing evidence for non-motor symptoms such as cognitive impairment, sleeping disturbances and psychiatric symptoms including depression and anxiety, which have gained increasing attention due to their major impact on the quality of a patient's life and their onset before the typical motor symptoms [14,16–18]. In addition, the involvement of hippocampal dysfunction in non-motor symptoms of PD has gained increasing interest because depression, and cognitive impairment are commonly seen in PD patients [19–21].

The two primary sources of CNS endogenous reactive oxygen species (ROS) are mitochondria and the NADPH-oxidized (NOX) pathway [22,23]. Mitochondrial ROS (mtROS) are mainly produced by ROS in

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electron transfer processes [24–26]. The NOXs family containing seven members (NOX1-5, DUOX1, DUOX2), is specifically expressed on the cell membrane of different cell types [27–29]. It catalyzes the electron transport process from NADPH and ultimately converts it to ROS [29]. Among the ROS, the superoxide anion radical ($O^{\bullet -2}$) is a crucial redox signaling molecule prominently generated by the NOX enzyme family and the mitochondrial electron transport chain [30]. Growing evidence suggests that NOX activity and superoxide levels of astrocytes increase with ageing and that astrocytes play a significant role in PD progression [31]. Astrocytes constitute the majority of cells responsible for regulating homeostatic cells in the brain and maintaining CNS-related physiological properties, such as maintaining the blood-brain barrier (BBB), producing trophic factors, controlling neuronal synaptic activity, regulating extracellular ion balance, transferring lactate and glutamate to neurons and processing cell metabolites [32].

Particularly, NOX4 is a member of the NOX family of ROS-generating NADPH oxidases and these enzymes transport electrons from cytoplasmic NADPH across cell and organelle membranes to generate superoxide ($O_2^{\bullet -}$) and other downstream ROS. NOX4 has been found in the brains of PD patients [33–38]. Our previous study also revealed that elevated levels of NOX4 in AD induce robust ROS production in astrocytes, impair the mitochondrial metabolism and modulate astrocyte ferroptosis [39]. Unlike NOX1, 2, or 3, NOX4 is present in all neurons, astrocytes, and microglia [40], and is located in the mitochondrial membrane [41,42]. Due to these features, NOX4 is expected to play a crucial role in mitochondrial ROS production. According to recent research and our results, NOX4 was found converting oxygen to ROS in both neurons and astrocytes in the central nervous system (CNS) [30,39]. Neuronal NOX4 is cellularly autotoxicity under ischemia or hypoxia [43,44]. NOX4 likely plays a pivotal role in the progression of pathologies such as PD and AD, and neuronal-targeted NOX4 knockdown was sufficient to reduce neurotoxicity and prevent cognitive decline, suggesting a direct and causal role for neuronal NOX4 [44]. In addition, NOX4 is mainly expressed in astrocytes [45]. During stress conditions in the brain, high levels of ROS contribute to the dysfunction of astrocytes that consequently induce neurotoxicity [35]. According to many reports on NOX4, it is not considered as leading different roles in neurons or astrocytes, but elevated NOX4 levels cause cell death of neurons and astrocytes by severely reducing their complementary or sometimes independent functions [39]. Therefore, we expected that the NOX4 expression in a neurodegenerative disease, such as PD, would increase symptom severity by regulating abnormally robust mtROS in astrocytes. Moreover, for this pathology to become clinically problematic, there is the possibility of overacceleration in a specific area of the brain by overexpression of NOX4, a pathological feature that is not simply a function of NOX4 itself but is controlled through intermediation with other proteins.

In this study, since the connections between NOX4 and biological processes believed to contribute to programmed cell death in PD progression remain unknown, 1-methyl-4-phenyl-1,2,3,6-tetrahydrophyridine (MPTP) was administered chronically to induce PD in a PD mouse model for comparison with tissues of human PD patients. We demonstrate that NOX4 is dominantly expressed in the hippocampus of an MPTP-induced PD mice model and in PD patients with accumulated α-synuclein, which has been identified as an important neurological hallmark of PD. We discovered significant increases in myeloperoxidase (MPO) and osteopontin (OPN) in the hippocampus through the proteome array analysis of PD mice. The results were similar to those in the PD patients' hippocampus. Our study provides evidence for specific astrocyte death being mediated by increased MPO and OPN from NOX4 overexpression in the pathogenesis of PD.

## 2. Materials and methods

### 2.1. Animal study

C57BL/6J mice (n = 24; 7–8 weeks old male, 18–25 g) obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and NOX4 knockout mice (n = 12; 7–8 weeks old male, 18–25 g) from the Jackson laboratory (Jackson Laboratory, Farmington, CT, USA) were used for the experiments. All experiments were conducted according to the principles established by the Institutional Animal Care and Use Committee (IACUC) of Soonchunhyang University (protocol #: SCH22-0005). All mice were raised in an environment at room temperature (22 ± 2 °C) with 60% humidity on a 12-h light: dark cycle (light cycle: dark cycle from 07:00 to 19:00) and were provided with available food (2018S; Harlan, USA) and distilled water.

### 2.2. Experimental procedures

Experimental procedures were conducted as follows (Supplementary 1). Mice were used in this study after a week of acclimatization and had no diseases, pathogens or genetic defects. The mice were divided into two groups: a control group (vehicle, 0.9% normal saline) and an MPTP group (MPTP-HCl 30 mg/kg free base), and body weight, food intake volume and water consumption were measured daily for 7 weeks. MPTP hydrochloride was obtained from Selleckchem (S4732, Selleckchem, USA). C57BL/6J mice (n = 12 per group) and NOX4 knockout mice (n = 6 per group) received intraperitoneal (i.p.) injections of freshly diluted MPTP-HCl (30 mg/kg free base) dissolved in 0.9% (w/v) NaCl. MPTP was administered once a day for 30 consecutive days, while the control group received vehicle injections (0.9% NaCl, i.p.) at the time of MPTP administration. After behavioral evaluation, all animals were sacrificed for further study.

### 2.3. Behavioral test

Prior to administration, all mice were trained on a rotarod (B.S Technolab, Daejeon, Korea) at 4 rpm for 120 s. As part of the protocol, mice were placed on the rod and tested sequentially at 10, 20, 30 and 40 rpm for 120 s at each speed and the latency to fall was recorded. The animals were tested three times at each speed, with a 5-min break between each trial. The average of three trials was used for further analysis. The time of testing the post-MPTP intoxication was observed 1 day and 7 days after the last injection.

### 2.4. Tissue preparation and dissection of brain regions in mice and human

All animals were sacrificed under deep anesthesia with Urethane (1 g/kg; Sigma-Aldrich, St. Louis, MO, USA) and transcardially perfused with 0.9% NaCl and/or followed by 4% PFA for paraffin embedding and isolated through the hippocampus for tissue lysate. The brain sectioning starts with consecutive sections every 4 μm by coronal sections, which are collected at the critical section according to the mouse brain atlas at bregma (+1.18 ~ +0.74 mm) for the striatum, (−1.46 ~ −2.06 mm) for the hippocampus, (−2.80 ~ −3.28 mm) for the substantia nigra and (−5.80 ~ −6.24 mm) for the cerebellum [46]. Human brain tissues were donated from The Netherlands Brain Bank, a total of six paraffin-embedded hippocampal brains of PD patients, and paraffin-embedded normal adult hippocampal brain tissue slides were obtained from Novus Biologicals (NBP2-77762, Centennial, CO, USA). For the cerebellum, normal adult and PD patient tissues were obtained from Novus Biologicals (NBP2-50617 and NBP2-77999, Centennial, CO, USA), respectively. Hippocampal and cerebellar brain tissues, including a normal adult and a PD patient, were analyzed by immunohistochemistry studies.

### 2.5. Antibodies

The following antibodies were used: polyclonal goat anti-NOX4 (N-15) antibody (sc-21860, Santa Cruz Biotechnology, Dallas, TX, USA), polyclonal rabbit anti-tyrosine hydroxylase (TH) antibody (ab3517, Abcam, Cambridge, UK), monoclonal mouse anti-alpha synuclein (211) antibody (sc-12767, Santa Cruz Biotechnology, Dallas, TX, USA), polyclonal rabbit anti-GFAP antibody (ab27642, Abcam, Cambridge, UK, polyclonal rabbit anti-NeuN antibody (ab104225, Abcam, Cambridge, UK), polyclonal goat anti-osteopontin (OPN) antibody (AF808, R&D system), polyclonal goat anti-myeloperoxidase (MPO) antibody (AF3667-3D, R&D system), monoclonal mouse anti-GFAP (#3670, Cell signaling technology, Danvers, MA, USA), Alexa Fluor® 594 donkey anti-goat IgG (H + L) (A11058, Life Technologies, Eugene, OR, USA), Alexa Fluor® 546 donkey anti-goat IgG (H + L) (A11056, Life Technologies, Eugene, OR, USA), Alexa Fluor™ Plus 488 donkey anti-goat IgG (H + L) (A32814, Life Technologies, Rockford, IL, USA), Alexa Fluor™ Plus 488 donkey anti-rabbit IgG (H + L) (A32790, Life Technologies, Rockford, IL, USA), Alexa Fluor™ 488 donkey anti-mouse IgG (H + L) (A21202, Life Technologies, Eugene, OR, USA), polyclonal donkey anti-rabbit IgG H&L Texas red (TR) (ab6800, Abcam, Cambridge, UK), Biotinylated rabbit anti-goat IgG (H + L) (BA-5000, Vector Laboratories, Burlingame, CA, USA), Biotinylated goat anti-rabbit IgG (H + L) (BA-1000, Vector Laboratories, Burlingame, CA, USA), Biotinylated goat anti-mouse IgG (H + L) (BA-9200, Vector Laboratories, Burlingame, CA, USA), Peroxidase labeled goat anti-rabbit IgG (H + L) (PI-1000, Vector Laboratories, Burlingame, CA, USA), Peroxidase labeled horse anti-goat IgG (H + L) (PI-9500, Vector Laboratories, Burlingame, CA, USA). Peroxidase labeled horse anti-mouse IgG (H + L) (7076P2, Cell Signaling Technology, Danvers, MA, USA).

### 2.6. Immunohistochemistry and immunofluorescence analysis

For immunofluorescence analysis, the sections of paraffin-embedded tissues were dewaxed and rehydrated. Citrate buffer (C9999, Sigma-Aldrich, St. Louis, MO, USA) was used for antigen retrieval and sections were blocked in CAS-Block™ Histochemical Reagent (008126, Thermo Fisher Scientific, Waltham, MA, USA). The primary antibodies were then performed overnight at 4 °C and the secondary antibodies were treated for 2 h at room temperature. Vectashield® Antifade Mounting Medium with DAPI (H-1200, Vector Laboratories, Burlingame, CA, USA) was used for nuclear staining. For immunohistochemistry, sections were blocked with peroxidase blocking solution (0.3% $H_2O_2$, 4104–4405, Daejung Chemicals and Metals, Gyeonggi-do, Korea) for 15 min and then were blocked in CAS-Block™ Histochemical Reagent. The primary antibodies were incubated overnight at 4 °C. After performing the biotinylated secondary antibody, sections were incubated in ABC-Peroxidase solution (PK-6100, Vector Laboratories, Burlingame, CA, USA) for detection for 1 h at room temperature and stained with DAB solution (D5637-5G, Sigma- Aldrich, St. Louis, MO, USA) until a brown stain appears. Sections were mounted on Eukitt® Quick-hardening mounting medium (03898, Sigma- Aldrich, St. Louis, MO, USA) after dehydration. For α-synuclein immunohistochemistry, sections were subjected to a specific antigen retrieval method [47] which were submersed in 10 mM EDTA Buffer pH 8.0 (17385-0401, Junsei Chemical, Koshigaya-shi, Saitama, Japan) for 2 min at 95–100 °C and then submersed in 90% formic acid (25010-0350, Junsei Chemical, Japan) for 10 min at room temperature. The sections were then subjected to a standard immunohistochemistry protocol.

Stained brain sections were analyzed on an OLYMPUS BX53F (Olympus Corporation, Tokyo, Japan) with an OLYMPUS U-HGLGPS (Olympus Corporation, Tokyo, Japan). Stained brain sections were quantified by ImageJ software v1.52t (Bethesda, MD, USA). To ensure objectivity, all measurements were performed under conditions blinded by two observers per experiment under identical conditions. For human astrocytes immunofluorescence, cells were fixed in 1% formalin-methanol solution and were blocked in 5% BSA. Primary antibodies (1:100), which are monoclonal mouse anti-GFAP and polyclonal rabbit anti-4-HNE, were used and incubated overnight at 4 °C. Cells were treated with secondary antibodies for 2 h at room temperature and Fluoroshield™ with DAPI (F6057, Sigma- Aldrich, St. Louis, MO, USA) was used for nuclear staining. Stained cells were analyzed with an immunofluorescence microscope (Thermo Fisher Scientific, Invitrogen™ EVOS™ M700 Imaging System, USA).

### 2.7. Immunoblot

All proteins were quantified using the BCA assay kit (21071, iNtRON Biotechnology, Gyeonggi-do, Korea). Proteins were electrophoresed on Novex 4%–12%, Tris-Glycine, 1.0 mm, Mini Protein Gel, 15-well (XP04205, Thermo Fisher Scientific, Carlsbad, CA, USA) and transferred to a PVDF membrane (PR04574, Merck Millipore, Carrgtwohil, Cork, Ireland). Membranes were blocked in 5% (w/v) bovine serum albumin (BSA) (SM-BOV, GeneAll Biotechnology, Songpa-gu, Seoul, Korea) in TBS-T (TR2007, Biosesang, Gyeonggi-do, Korea) for 1 h at room temperature. Membranes were incubated with primary antibodies including polyclonal goat anti-NOX4, polyclonal rabbit anti-GFAP, polyclonal rabbit anti-NeuN, polyclonal goat anti-OPN, polyclonal goat anti-MPO, polyclonal rabbit anti-4-HNE, monoclonal mouse anti-OXPHOS complexes and polyclonal rabbit anti-GAPDH, diluted in 1% (w/v) BSA in TBS-T overnight at 4 °C followed by horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000) diluted in TBS-T for 2 h at room temperature. The results were detected with ECL western blotting detection reagents (RPN2232, GE Healthcare, Buckinghamshire, UK) and exposed with the Chemi-luminescence Bioimaging Instrument (CELLGENTEK, Daejeon-si, Korea).

### 2.8. Proteome arrays

Hippocampal protein lysate from control and MPTP- groups (100 μg) was used for Proteome Profiler™ Mouse XL Cytokine Array (ARY028, R&D systems, Minneapolis, MN, USA) to analyze 111 soluble proteins that include cytokines, chemokines, and growth factors. The protein lysate samples were incubated on nitrocellulose membranes each containing 111 different capture antibodies printed in duplicate overnight at 4 °C. The detection antibody, a cocktail of biotinylated antibodies was used to incubate the membranes for 2 h at room temperature and then incubated with streptavidin conjugated to horseradish-peroxidase (HRP) for 30 min at room temperature. Membranes were detected with immunoreactive spots with Chemi Reagent Mix and exposed to X-ray film for 1–10 min. The results were analyzed using image software (HLImage$^{++}$ Version 25.0.0r, https://www.wvision.com/QuickSpots.html, Western Vision Software, Salt Lake City, UT, USA).

### 2.9. Human astrocytes

Human astrocytes were obtained from N7805100 (Thermo Fisher Scientific, Waltham, MA, USA) and #1800 (ScienCell Research Laboratories, Carlsbad, CA, USA). Human astrocytes are for research use only and are derived from human brain tissue of normal human cells. Gibco™ Astrocyte Medium containing Dulbecco's Modified Eagle Medium (DMEM) with N-2 Supplement and 10% (vol/vol) One Shot™ Fetal Bovine Serum (FBS) was added with 100 units/ml penicillin and 100 mg/ml streptomycin (A1261301, Thermo Fisher Scientific, Waltham, MA, USA) for culturing human astrocytes. To overexpress human myeloperoxidase (MPO) and osteopontin (OPN), human astrocytes were seeded and transfected using Lipofectamine® LTX and Plus™ reagent (life technologies, Carlsbad, CA, USA) with pCMV6-AC-GFP constructs against human MPO (NM_000250, PS100010, Origene, Rockville, MD, USA) or pCMV6-AC-GFP constructs against human OPN (SPP1) (NM_001040058, PS100010, Origene, Rockville, MD 20850, USA) or pCMV6-AC-GFP (PS100010, Origene).

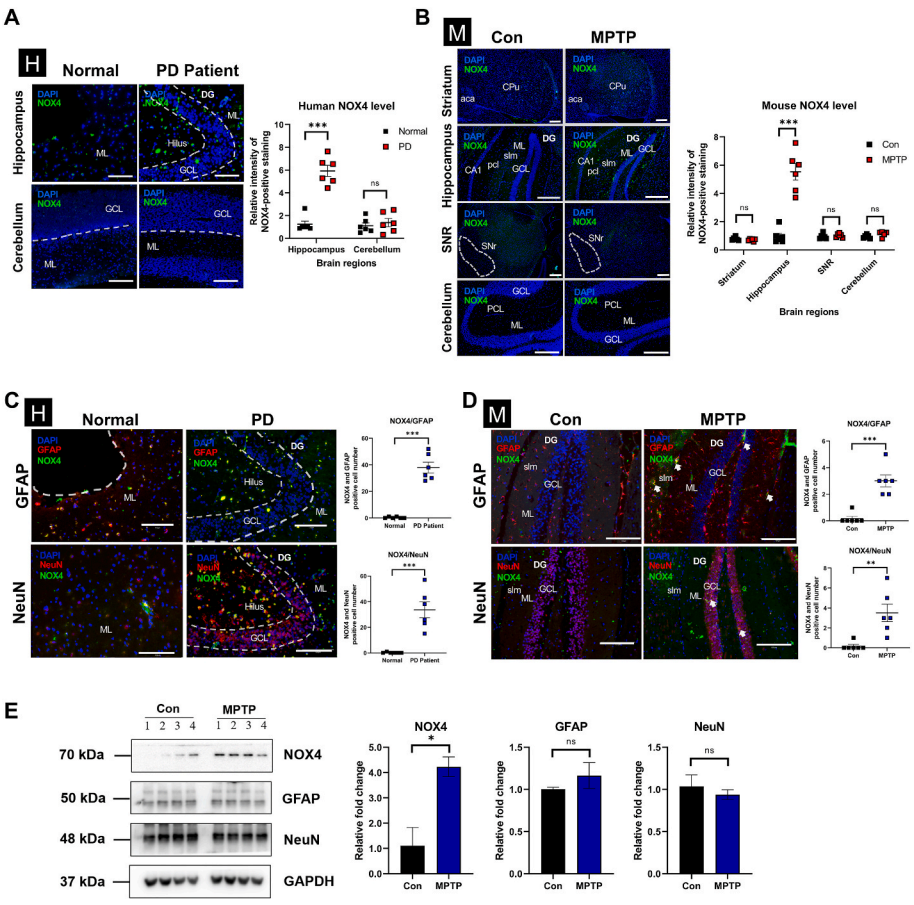


**Fig. 1. NOX4 is significantly upregulated in astrocytes of the hippocampus in PD models.** (A, B) Images and quantification of NOX4 expression in different regions of the human brain (hippocampus and cerebellum) and the PD mice model (striatum, hippocampus, substantia nigra, cerebellum). (C, D) Representative double-immunofluorescence images and quantification of PD patients and PD mouse models for NOX4 using Alexa flour 488 (green) in combination with the astrocyte marker GFAP (Texas red) or with the neuron-specific marker NeuN (Texas red). Cell nuclei were stained with DAPI (blue) for visualization. Scale bar: 125 and 300 μm (E) Immunoblot analysis and quantification of NOX4, GFAP, and NeuN levels from hippocampal lysates of a MPTP-induced PD mouse model compared to control. GAPDH was used as a loading control. Values are means ± standard error of the mean (SEM). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (one-way ANOVA test or Student's t-test) vs control, aca: anterior commissure, anterior part, CA1: field CA1 hippocampus; Cpu: Caudate putamen (striatum); DG: Dentate gyrus; GCL: granular layer; ML: molecular layer; PBP: parabrachial pigmented nucleus of the VTA; pcl: pyramidal cell layer; PCL: purkinje cell layer; slm: stratum lacunosum-molecular; SNc: substantia nigra pars compacta; SNr: substantia nigra pars reticulate; VTA: ventral tegmental area. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

### 2.10. 3D images and morphological cell death

Human astrocytes were seeded in a cell culture imaging dish (81156, Martinsried, IBIDI, Germany). Cells were transduced with pCMV6-AC-GFP constructs against human MPO (NM_000250, PS100010, Origene, Rockville, MD, USA) or pCMV6-AC-GFP constructs against human OPN (SPP1) (NM_001040058, PS100010, Origene, Rockville, MD 20850, USA) or pCMV6-AC-GFP (PS100010, Origene). Cells were incubated for 24 h or 48 h 3D images were analyzed by 3D Cell Explorer (NANOLIFE, Ecublens, Switzerland) and images were represented from a total of 100 cells in six individual images per group. For cell morphology, the percentage of morphological cell death quantification was counted for 100 cells in 12 individual images per group.

### 2.11. Mitochondrial ROS (mtROS) production

MitoSOX™ Red mitochondrial superoxide indicator (M36008, Invitrogen) was used to detect superoxide in the mitochondria of living cells according to the manufacturer's instructions. Human astrocyte cells were plated in 6-well plates and transduced with pCMV6-AC-GFP constructs against human MPO (NM_000250, PS100010, Origene, Rockville, MD, USA) or pCMV6-AC-GFP constructs against human OPN (SPP1) (NM_001040058, PS100010, Origene, Rockville, MD, USA).) or pCMV6-AC-GFP vector (PS100010, Origene). Then, the cells were incubated for 48 h and washed with PBS. Trypsin was treated and cells were resuspended in OPTI-MEM® (31985-070, Gibco, Green Island, NY, USA). Data was obtained using the Guava® Muse® Cell Analyzer (Luminex Corporation, Austin, TX, USA) with Muse® Software V.1.9.0.2.

### 2.12. Statistical analysis

All data is represented as mean ± standard error of the mean (SEM). In analyzing all statistical tests, a two-tailed Student's *t*-test was applied to compare the two groups using a statistical software package (GraphPad Prism version 8.0, GraphPad Software Inc. (San Diego, CA, USA)). For the comparison of multiple groups, the analysis of variance (ANOVA) (with post hoc comparisons using Duncan's multiple range test) was used by the SPSS (IBM Corp., ver. 22) statistical software package and P values (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$) were considered statistically significant.

## 3. Results

### 3.1. Chronic MPTP treatment leads to loss of dopaminergic neurons and motor function in mice

The repeated MPTP administration schedule for constructing the PD mouse model is depicted in Supplementary Fig. S1A. To confirm the neuronal degeneration effect in MPTP-treated mice, Nissl staining and tyrosine hydroxylase (TH) immunohistochemistry, which are commonly used to detect dopaminergic neurons were evaluated in the substantia nigra and striatum (Supplementary S2). There were no differences in physiological phenotypes between the groups in all experimental periods (Supplementary S3).

We examined hippocampal α-synuclein levels, which have been established to characterize PD pathology, in PD patients and in the chronically MPTP-treated mice. An accumulated abnormality of α-synuclein was found in both chronically MPTP-injected mice and PD patients in the hippocampus (Supplementary S4A), indicating that the mice model of chronic MPTP-induced PD had similar effects on

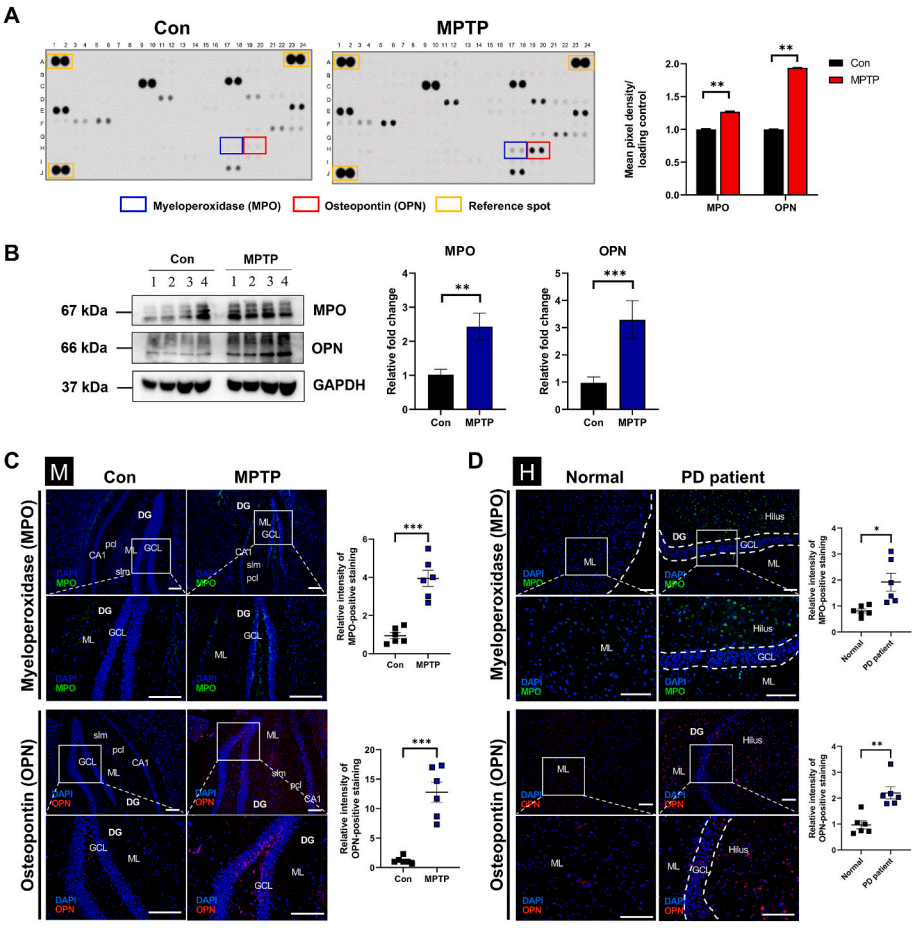


**Fig. 2. Myeloperoxidase (MPO) and Osteopontin (OPN) are dominantly elevated in hippocampus in PD models.** (A) Representative images and quantification of the Proteome Profiler Mouse XL Cytokines Array immunoblot analysis of 111 different cytokines, chemokines, growth factors, or extracellular signaling molecules of the MPTP hippocampus and control groups. (B) Representative immunoblot analysis and quantification of OPN and MPO levels in MPTP groups compared to control groups. For immunoblots, GAPDH was used as a loading control. Data are representative of three independent experiments. (C, D) Immunofluorescence staining and quantification of MPO and OPN, labeled with Alexa Fluor 546-conjugated antibody (red) in the hippocampal brain of MPTP mice and a PD patient. Scale bar: 300 and 125 μm. Cell nuclei were stained using DAPI (blue) for visualization. Scale bar: 300 μm and 125 μm. DG: Dentate gyrus; ML: molecular layer; GCL: granule cell layer. Data are mean ± standard error of the mean (SEM); *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs control by Student's two-tailed *t*-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

α-synuclein as observed in PD patients.

As expected, our experimental results showed that the MPTP-induced PD mice model significantly declined in rotarod performance (Supplementary S4B). Rotarod retention time decreased at 30 and 40 rpm in chronic MPTP-treated mice compared to that in control mice. MPTP intoxication leads to dopaminergic depletion in the striatum and substantia nigra which is consistent with the observed deficits of motor function in the MPTP-treated group. These results confirmed that MPTP treatment effectively destroyed dopaminergic neurons in the substantia nigra, resulting in a reduction of DA secretion in the striatum. This then leads to a lack of triggering of the movements evaluated with the rotarod assay.

### 3.2. *NOX4 is increased in the hippocampus of PD models*

In our previous studies, NOX4 was found to be predominant in the hippocampus of AD patients [39]. We concluded that the regulation of NOX4 in the hippocampus has a critical impact on dementia symptoms in non-Alzheimer's brain degenerative diseases [39]. Notably, the cell type in the hippocampus that expresses NOX4 can be a significant indicator for the function of NOX4.

To examine the role of NOX4 during PD, we analyzed NOX4 levels in the brain of PD patients. Compared to healthy human tissue, NOX4 was upregulated in PD patients and prominently expressed in the hippocampus of PD patients but not in the cerebellum compared to normal human tissue (Fig. 1A). To support the results obtained in human tissues, the mice NOX4 levels were observed in the tissues of mice chronically MPTP-treated mice in the four main brain regions which are the striatum, hippocampus, substantia nigra and cerebellum. NOX4 was only significantly increased in the hippocampus of MPTP-induced PD mice (Fig. 1B) which is remarkably consistent with human PD patients. These results indicate that NOX4 is highly expressed specifically in the hippocampus in PD models.

In addition, this study was carried out to visualize NOX4 expression in neuroinflammatory responses in hippocampus in PD models. NOX4 protein levels and NOX4 double immunofluorescence staining were investigated with several brain cells merged with GFAP, NeuN, and Iba-1 as markers of astrocytes, neurons, and microglia cells, respectively. NOX4 was primarily expressed in neurons and astrocytes (Fig. 1C and D), but was less defined in the microglia (Supplementary S5) in the hippocampus in PD models. These results were confirmed by Western Blot that showing that NOX4 was enhanced in the hippocampus brain lysate of the MPTP mice model (Fig. 1E), and the quantified NOX4 levels were significantly increased in the MPTP-treated group compared to those in the control group.

### 3.3. *MPO and OPN are elevated in PD models*

It is important to understand how NOX4 expression in astrocytes, commonly identified in PD models, contributes to the regulation of neuroinflammation in the hippocampus in PD according to the results depicted in Fig. 1. The effects of cytokine or chemokine production in hippocampal MPTP-induced PD mice were examined using the proteome profiler mouse XL cytokines array containing 111 soluble proteins, including cytokines, chemokine and growth factors immunoblot analysis, compared to control. Interestingly, we found significantly increased expression of MPO and OPN among 111 cytokines in hippocampal lysates in MPTP-induced PD mice compared to that in normal control mice (Fig. 2A). Changes in the ratio of cytokines in normal mice and 111 inflammatory cytokines in hippocampal lysates of MPTP-induced PD

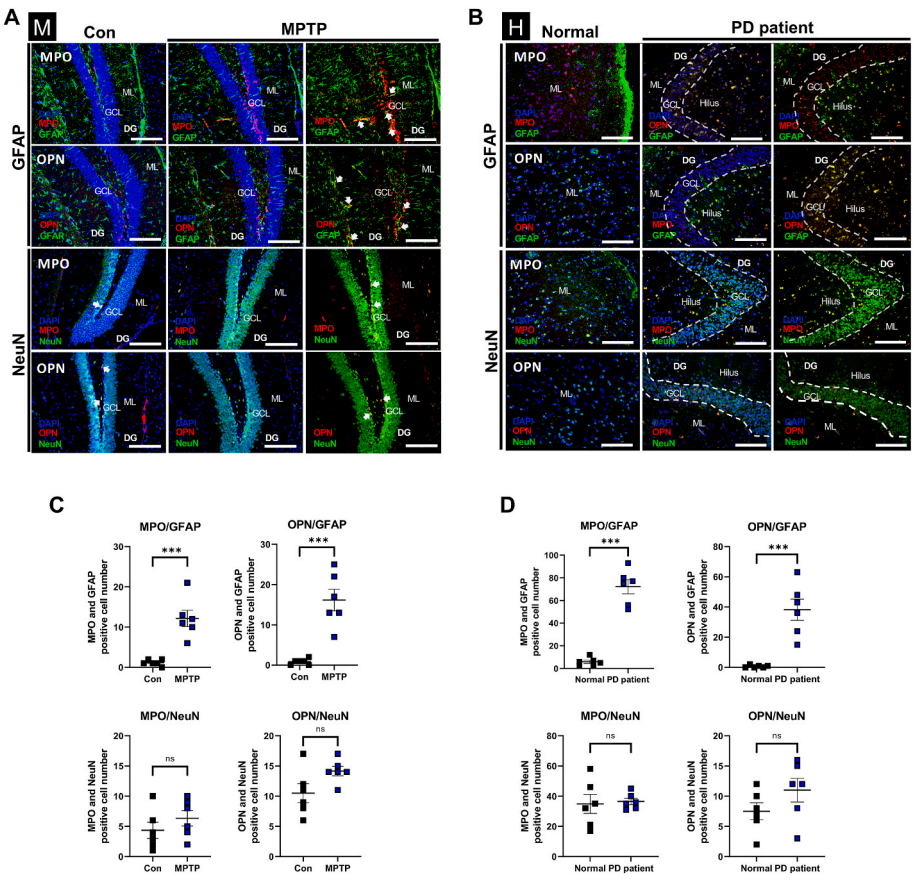


**Fig. 3. Myeloperoxidase (MPO) and Osteopontin (OPN) are highly increased in hippocampal astrocytes in PD models.** (A, B) Representative visualization by immunofluorescence of OPN or MPO (red) expression on astrocyte markers (GFAP) and neuronal markers (NeuN) (green) in PD mice and humans. Cell nuclei were stained using DAPI (blue) for visualization. (C, D) Quantification of MPO and OPN in the hippocampal brain of MPTP mice and PD patients co-localized with GFAP and NeuN. Scale bar: 125 μm. DG: Dentate gyrus; ML: molecular layer; GCL: granule cell layer. Data are mean ± standard error of the mean (SEM); *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs. control by Student's two-tailed *t*-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

mice are shown in Supplementary S6. These results were again confirmed by Western Blot analysis of hippocampal lysates using anti-MPO and anti-OPN antibodies (Fig. 2B). MPO is a peroxidase type that converts $H_2O_2$ to hypochlorous acid (HOCl). The NADPH complex is vital to generate superoxide anion ($O_2^{\bullet-}$) from oxygen, and the brain has a large oxygen consumption which correlates with oxidative stress [50,51]. MPO activity occurs in several types of tissue injuries and in the pathogenesis of neurodegenerative diseases, including AD [52,53] and PD [54]. OPN, an O-glycosylated phosphoprotein, is a multifunctional protein synthesized in multiple tissues and secreted into body fluids [55]. OPN can be a regulator in various physiological and pathological processes such as inflammation process, ischemia, osteoclast function, immune responses, cell viability processes, and cancer [55]. In the CNS, OPN expression is upregulated during injury or inflammation processes [56]. Studies reported that OPN is also associated with AD [57–59] and PD [60–62].

We found that MPO and OPN had higher expression in the hippocampal dentate gyrus (DG) in MPTP-induced PD mice, which is consistent with the results achieved in PD patients (Fig. 2C and D). As a result, GFAP-positive astrocytes were dominantly merged with MPO and OPN in PD models (Fig. 3). During PD pathogenesis, a significant increase in the level of neuroinflammatory cytokines (MPO and OPN) was observed in astrocytes of the hippocampus region.

### 3.4. NOX4 associates MPO and OPN expressions in the hippocampal astrocytes

We introduced NOX4 knockout mice to elucidate the relationship between MPO and OPN that NOX4 specifically increases in astrocytes. MPO and OPN were assessed by measuring the expression in neuronal and astrocytic cells in NOX4 knockout mice compared to that in normal control mice (Supplementary S7A and B). We found that the absence of NOX4 was not accompanied by elevated levels of MPO and OPN in hippocampal neurons and astrocytes (Supplementary S7). There were no differences in any cell type in the hippocampal DG of NOX4 knockout mice compared to control mice. Moreover, the expression of MPO and OPN were evaluated on hippocampal astrocytes in NOX4 knockout mice after chronic MPTP administration (Fig. 4). Repeatedly MPTP administered NOX4 knockout mice did not show upregulated MPO or OPN during PD which was in contrast to the MPTP-treated mice (Fig. 4). This result suggests that NOX4 is directly interrelated with MPO and OPN, and it is strongly suggests that MPO and OPN expressions are upregulated in PD progression in a NOX4-dependent manner.

### 3.5. MPO and OPN promote impairment of the mitochondrial metabolism and induce lipid peroxidation in human astrocytes

NOX4 and the two cytokines were closely related, especially in hippocampal astrocytes. Therefore, it is considered very important to reveal a functional change in astrocytes depending on the increase in the expression of these two cytokines. To elucidate how human astrocytic cells are affected by MPO and OPN evaluation, we transfected human astrocytes with a vector containing GFP-tagged MPO or OPN. Through live cell imaging, we observed the transfected cells as black arrows with control and overexpression of MPO or OPN using GFP tracking (black arrows, Fig. 5A). Next, 3D imaging of the living cells showed morphological changes where the shape of the cells showed shrinkage and blebbing compared to the control (white arrows, Fig. 5B). Furthermore, cells showing morphological apoptosis were counted and compared to controls (Fig. 5C). We demonstrated that overexpression of MPO and OPN resulted in significant induction of morphological cell death.

To understand the importance of elevated MPO and OPN for the mitochondrial metabolism, it is essential to assess the levels of five protein complexes in the mitochondrial electron transport chain (ETC),

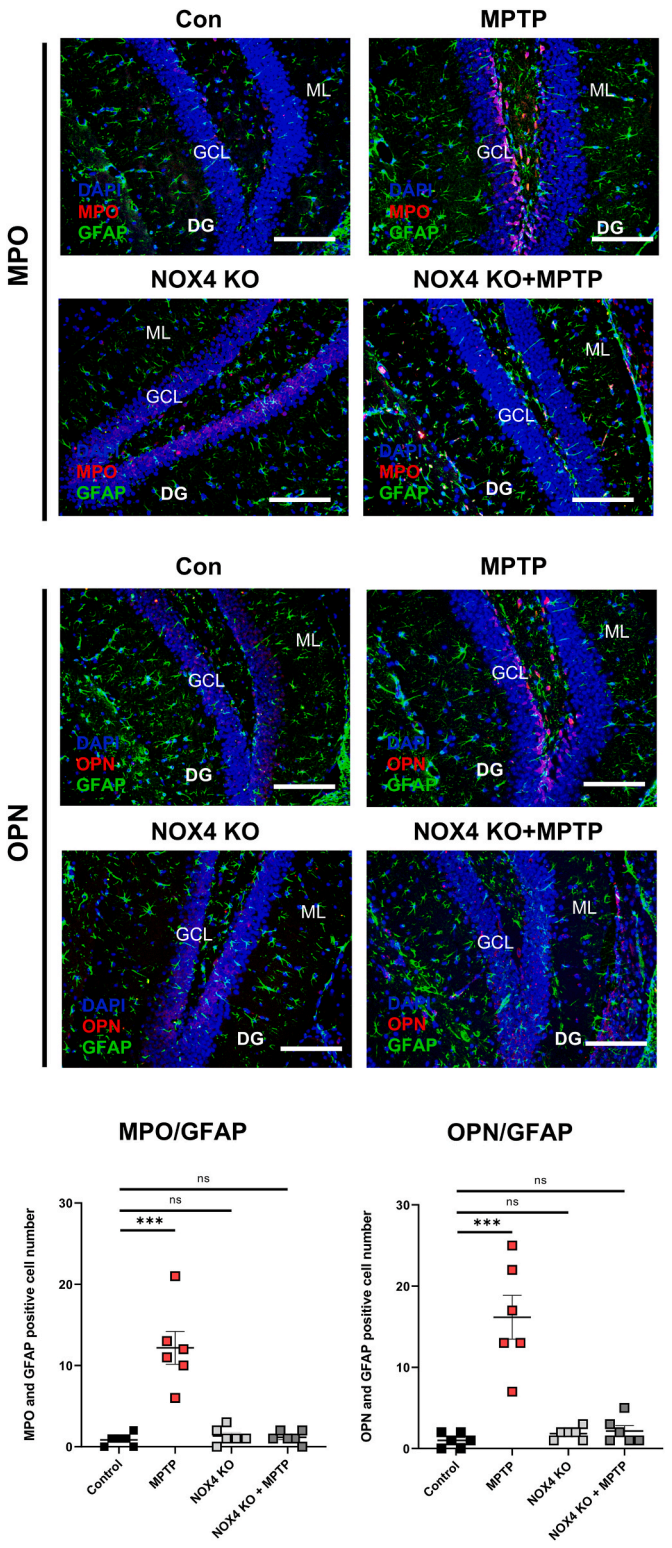


**Fig. 4. Myeloperoxidase (MPO) and Osteopontin (OPN) expressions mediated by NOX4 in astrocytes in PD models.** Representative images of immunofluorescence staining and quantification for OPN or MPO (red) expressing on astrocyte markers (GFAP) (green) in the hippocampus of control, MPTP, NOX4 knockout and NOX4 knockout mice with MPTP administration. Cell nuclei were stained using DAPI (blue) for visualization. Scale bar: 125 μm. DG: Dentate gyrus; ML: molecular layer; GCL: granule cell layer. Data are mean ± standard error of the mean (SEM); *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs. control by Student's two-tailed *t*-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

including Complex I–V: which are the B8 subunit of ubiquinone oxidoreductase (Complex I; NDUFB8), succinate dehydrogenase complex iron sulfur subunit B (Complex II; SDHB), ubiquinol-cytochrome c reductase core protein 2 (Complex III; UQCRC2), mitochondrially encoded cytochrome *c* oxidase I (Complex IV; MTCO1), ATP synthase F1 subunit alpha (Complex V; ATP5F1A) (Fig. 6A). We detected significantly reduced Complex' I–V after MPO and OPN overexpression, and overexpression of MPO and OPN after transfection was confirmed by Western Blot. These results show that mitochondrial activity was suppressed by MPO and OPN overexpression, leading to a causal impairment of mitochondrial function in astrocytes.

4-hydroxy-2-nonenal (4-HNE) is a major end-product of lipid peroxidation and well-known to propagate oxidative damage and contribute to cell death [63–65]. In particular, the accumulation of lipid peroxidation is a fundamental reason for promoting cell death [66]. In addition to 4-HNE, various makers for lipid peroxidation exist [39,66], but several studies have judged the measured value of 4-HNE as evidence of lipid peroxidation [39,67–69]. 4-HNE was shown to react with proteins and form stable adducts that can be used as markers of oxidative stress-induced cellular damage [70]. Thus, 4-HNE levels were measured using immunofluorescence and Western Blot to assess the degree of lipid peroxidation that induces cell death. Transfected cells with MPO or OPN showed a significantly increased level of 4-HNE protein in human astrocytes compared to that in controls (Fig. 6B). In addition, immunofluorescence staining revealed that upregulation of MPO and OPN increased 4-HNE expression with co-localization of the GFAP-positive astrocyte marker (Fig. 6B). When we observed the level of 4-HNE by immunoblot analysis, 4-HNE was also significantly increased by MPO and OPN overexpression (Fig. 6C). For subsequent analyses of mitochondrial dysfunction, we examined whether the mitochondrial metabolism was impaired by elevated MPO and OPN in human astrocytes. We tried to elucidate whether ROS are produced to activate lipid peroxidation, in line with our previous study. To that end, human astrocytic cells with MPO or OPN overexpression were evaluated with MitoSOX Red superoxide indicators that selectively target mitochondria. We found a significantly increased production of mtROS in MPO-overexpressing human astrocytes while in astrocytic cells with elevated OPN, only a negligible change in mtROS was observed when compared to that of the control (Fig. 6D). These results suggest that the pathway of oxidative stress-induced lipid peroxidation is only upregulated through mitochondrial dysfunction only in overexpressed MPO human astrocytes. However, according to the results, the pathway of OPN overexpression in astrocytes inducing apoptosis seems to be different from that of MPO.

## 4. Discussion

We previously reported that the elevation of NOX4 induces mitochondrial dysfunction and oxidative stress-induced lipid peroxidation [39]. We concluded that NOX4 promotes the ferroptosis of astrocytes in the brains of AD patients, APP/PS1 mice models, and human astrocyte cell lines [39]. In particular, we suggested that NOX4 could be an upstream molecular target in impairing the mitochondrial oxygen metabolism via inhibition of mitochondrial respiration and ATP production by reducing five protein complexes in the mitochondrial electron transport chain (ETC) in astrocytes during AD [39]. An increase in NOX4 was observed in the brain cortex of APP/PS1 mice and AD patients. The increased NOX4 resulted in decreased mitochondrial respiration, increased mtROS, and ferroptotic cell death of astrocytes due to lipid peroxidation. However, we did not investigate which mediators causing the death of astrocytes the increase in NOX4 contributes to.

In the present study, we expected that this mechanism would not be a pathological mechanism that could only be attributed to AD but also found in other neurodegenerative diseases such as PD. Thus, we tried to apply these hypotheses directly to PD. Therefore, we designed the experiment almost precisely as the previous one, except that NOX4 does

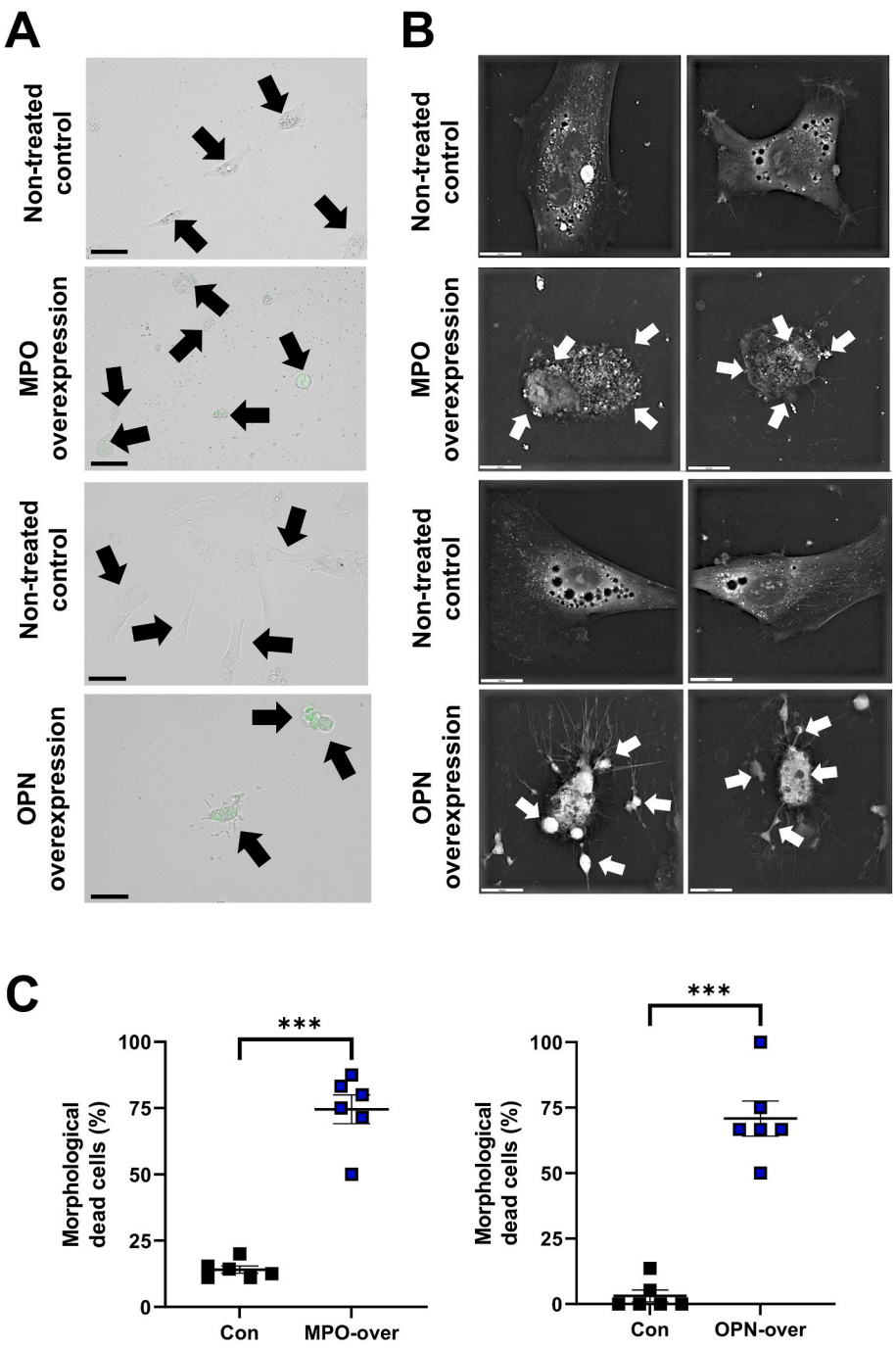


**Fig. 5. Upregulation of Myeloperoxidase (MPO) and Osteopontin (OPN) induces cell death in human astrocytes.** (A) Representative images of human astrocytes transfected with MPO- or OPN-GFP-tagged plasmids. (B) Representative 3D images of control and MPO and OPN overexpression in human astrocytes. (C) Quantification of the morphological dead cells (%) in control compared to MPO and OPN overexpressing human astrocytes. Scale bars: 20 μm. Data are mean ± standard error of the mean (SEM); *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs. control by Student's two-tailed *t*-test.

not cause astrocyte death directly but rather through certain mediators. We also speculated that increased NOX4 would only be observed only in the hippocampus in PD. In this study, we found novel intermediators under NOX4 modulation, namely MPO and OPN. The discovery of NOX4 upregulation in the hippocampus of PD is supports the hypothesis that NOX4 might be pivotal for oxidative stress in neurodegenerative disease progression. Furthermore, we believe that NOX4 is an essential effector mediating MPO and OPN in astrocytes, particularly in the hippocampus in PD. Since several studies have proven that increased MPO and OPN promote neurodegenerative diseases [71–75], increased NOX4 expression in astrocytes is expected to correlate with MPO or OPN increases in AD, resulting in astrocytic death; however, further validation studies are needed.

Although some studies have revealed the function of NOX4 in the hippocampus using PD animal models or cell system [71,76,77], our study is the first to focus on molecular pathological mechanisms using PD patient samples and human astrocytes compared with a PD animal model. Many research results on the production of ROS in astrocytes in neurodegenerative diseases have recently been published [78–81]. Therefore, elucidating the molecular pathological mechanisms underlying the relationship between NOX4 expression and ROS in the dementia process may be the building block to also strengthen our understanding of neurodegenerative diseases other than PD. In other words, it is essential to find a modulator that is involved in the pathological mechanism underlying astrocyte death by NOX4 upregulation. By profiling specific target cytokines that participate in neuroinflammation by proteome array, we found that MPO and OPN show characteristically significant expression differences in the hippocampus

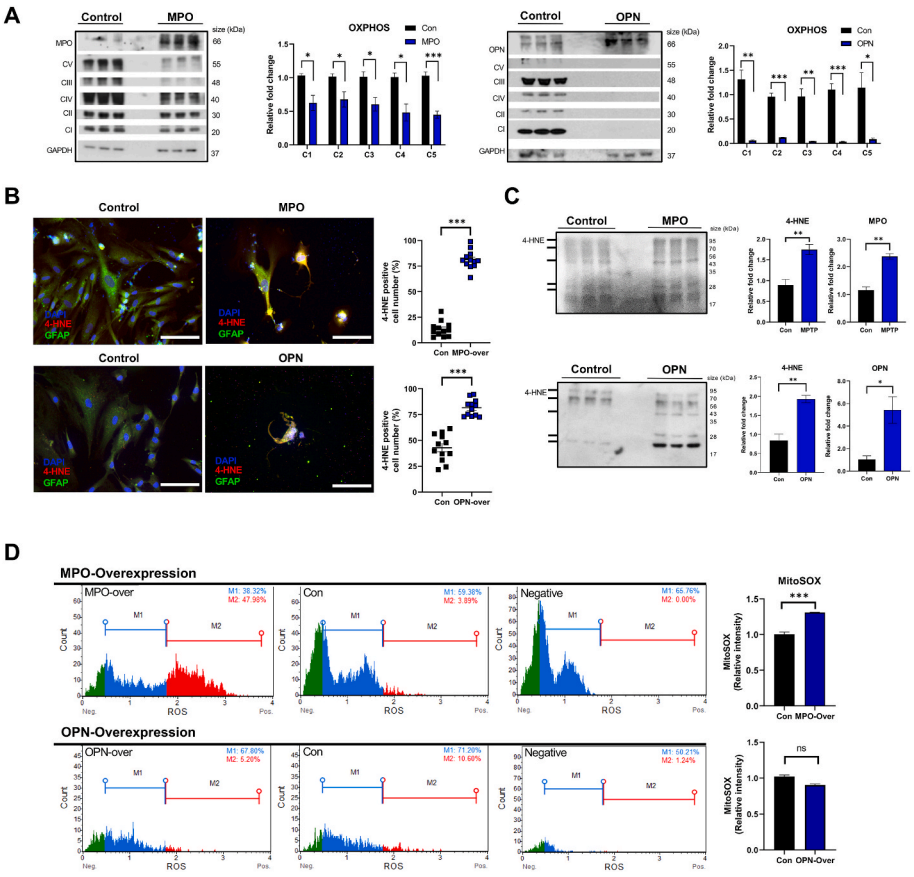


**Fig. 6. Elevated Myeloperoxidase (MPO) and Osteopontin (OPN) promote mitochondrial dysfunction and increase lipid peroxidation in human astrocytes.** (A) Images and quantification of immunoblot analysis for five mitochondrial ETC protein levels, including Complex I (CI (NDUFB8)), Complex II (CII (SDHB)), Complex III (CIII (UQCRC2)), Complex IV (CIV (MTCO1)), and Complex V (CV (ATP5F1A)) in control and overexpressing MPO and OPN human astrocytes. (B) Representative images of immunofluorescence for 4-HNE expression (red) with astrocyte marker (GFAP) (green) and percentage of 4-HNE-positive cells on overexpression of MPO and OPN in human astrocytes compared to control. DAPI staining is shown in blue. Scale bar; 125 μm. (C) Immunoblot analysis images and quantification of 4-HNE protein levels of MPO and OPN overexpressing human astrocytes. (D) mtROS levels and quantification using MitoSOX staining in human astrocytes of control (Con) and MPO and OPN overexpression. Data are mean ± standard error of the mean (SEM); *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs. control by Student's two-tailed *t*-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

of MPTP-induced PD mice. Remarkably, both these proteins were increased in PD mice and in PD patients and had clearly co-localized positive labelling on astrocytes in both PD patients and PD mice. Interestingly, several reports have demonstrated a robust correlation between the elevation of MPO and OPN in neurodegenerative disease [71,72,74,75,82].

MPO has been detected occasionally in microglia [71], neurons in the AD brain [75], and in the astrocytes of the brains of PD patients [72,74,82]. MPO is an oxidant-generating enzyme that is not present in the normally aged brains and reacts with $H_2O_2$ to oxidize chloride, producing the potent oxidant hypochlorous acid [52,53]. Phospholipid peroxidation and neuronal dysfunction have been found to contribute to AD caused by an aberrant human MPO expression in astrocytes [74]. As a result, MPO expression correlated with increased levels of the lipid peroxidation product 4-HNE. Similar to the results of other researchers, our results also showed a consistent effect of increased expression of MPO in astrocytes in a PD model that led to apoptosis by downregulating the mitochondrial metabolism, increasing ROS production and lipid peroxidation. Features of ferroptosis, such as iron dysregulation, lipid peroxidation and inflammation are considered key pathological circumstances of cognitive dysfunction [83]. In particular, the abundant diffusible outcomes of lipid peroxidation chemically reactive aldehydes, including malondialdehyde (MDA), acrolein, 4-hydroxy-2-hexenal and 4-HNE are speculated [84,85]. Reactive aldehydes derived from lipid peroxidation show biological activity [86]. 4-HNE, which is an electrophilic lipid peroxidation product, also exhibits various cytotoxic effects of lipid peroxidation, such as glutathione deficiency, dysfunction of structural proteins, reduction of enzyme activity and induction of cell death [84,87]. Although many studies have investigated the relationship between NOX4 and MPO [88–91], no study has clarified the pathological PD process through the relationship between NOX4 and MPO in PD models. Most studies on NOX4 and MPO have described a relationship between endothelial cell damage and vascular fibrosis in various organs. Compared to our results, if astrocyte apoptosis is induced in the hippocampus of PD patients, damage to the BBB will also be severe, and one would expect that the smooth supply of glucose that must continuously flow from cerebral blood vessels to neurons via astrocytes could not be sustained. A number of relationships between NOX4 and MPO of cerebrovascular cells, and the BBB require further research; however, considering the results of several reports [92–95], the outcomes of augmenting astrocytic ROS and lipid peroxidation in the hippocampus as a result of increasing MPO by NOX4 increases are not expected to cause severe damage the BBB, which plays an essential role in brain homeostasis.

OPN, a glycosylated phosphoprotein, is downregulated in the remaining dopaminergic neurons in PD [96] and is a multifunctional molecule that acts both an immobilized extracellular matrix protein and a secreted-free cytokine in the body fluids [97]. It has also been suggested that OPN may induce self-injury of inflammatory activity in neurodegenerative diseases including multiple sclerosis, PD and AD [98]. In MPTP treatment, neuronal cells, TH-positive fibers, and microglial activation cells of the substantia nigra and striatum were reported to have less damage in OPN knockout mice compared to wild-type mice [60]. OPN has often been described as an inflammatory promoter, but it has also been reported as an oxidative stress inhibitor during microglial activation [99,100]. OPN function has shown decreased mitochondrial activity in PD patients and high OPN expression has been detected in autopsy brains of AD patients and mice models in hippocampal neurons [101]. In this study, we newly demonstrated that a PD-induced increase in NOX4 in the hippocampus specifically increased OPN expression in astrocytes. There is abundant research focused on OPN in microglial cells and neurons in neurodegenerative diseases; however, no previous study has yet clearly shown results that OPN expression in hippocampal astrocytes contributes to the

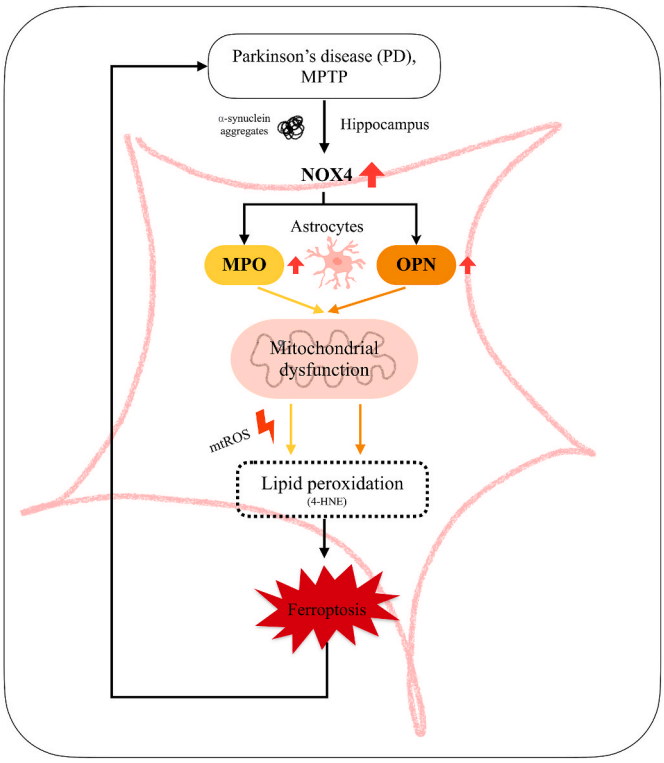


**Fig. 7. Schematic diagram of a possible pathway contributing to PD in the astrocytic hippocampus.** The red arrows indicate growth. The yellow arrows follow the MPO path, and the orange arrows follow the OPN path. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

pathogenesis of PD. In our study, mitochondrial dysfunction was clearly detected by increased OPN, but ROS generation by OPN was very insignificant; thus, the mechanisms of astrocyte death caused by increased expression of MPO and OPN are likely different. Therefore, further studies on the astrocyte death mode by increasing MPO and OPN are needed. Nevertheless, it should be noted that OPN must be an essential modulator of PD following NOX4 expression in hippocampal astrocytes.

In summary, mitochondrial dysfunction induced by NOX4 upregulation and ultimately astrocyte ferroptosis mediated by the neuroinflammatory intermediators, that are MPO and OPN in the hippocampus, contributes to the PD progression. The rapid decrease in mitochondrial function by MPO and OPN elevation and the production of mtROS or ferroptotic cell death through an increase in lipid peroxidation is a novel finding. The increase in MPO and OPN expression by triggering the NOX4 induction in the hippocampus in neurodegenerative diseases is expected to be a common mechanism causing the pathological death of astrocytes. NOX4-induced increases in MPO and OPN were both observed to induce astrocytic death characteristically in the hippocampus of PD models. Nevertheless, based on our results, we believe that they may trigger cell death by mechanisms independent of each other in PD.

In conclusion, our study demonstrates that mitochondrial dysfunction induced by NOX4 upregulation and ultimately astrocyte ferroptosis mediated by the neuroinflammatory intermediators that are MPO and OPN in the hippocampus contribute to PD progression (Fig. 7).

## Author contributions

N.B., and S.S.Y. conceived the study. N.B. contributes to all experiments. S.Y., and C.Y.K. contributes to performed analysis for IH and IF images. N.B., and J.S.M. participated in cell analysis. N.B., and S.S.Y. participated in data acquisition and interpretation of the results. N.B., and S.S.Y. wrote the article. S.S.Y. supervised the entire project. All authors read, revised, and approved the final manuscript.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2023.102698.

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