Apoferitin improves motor deficits in MPTP-treated mice by regulating brain iron metabolism and ferroptosis

Li-Mei Song, Zhi-Xin Xiao, Na Zhang, Xiao-Qi Yu, Wei Cui, Jun-Xia Xie, Hua-Min Xu

jxiaxie@public.qd.sd.cn (J.-X.X.)
huamin102@163.com (H.-M.X.)

Highlights
Apoferitin improved MPTP-induced motor deficits.
Apoferitin rescued dopaminergic neurodegeneration in the SN of MPTP-treated mice.
Apoferitin inhibited MPTP-induced iron aggregation.
Apoferitin prevented MPTP-induced ferroptosis by regulation of ACSL4 and FSP1.

Song et al., iScience 24, 102431 May 21, 2021 © 2021 The Author(s).
https://doi.org/10.1016/j.isci.2021.102431
Apoferritin improves motor deficits in MPTP-treated mice by regulating brain iron metabolism and ferroptosis

Li-Mei Song,1 Zhi-Xin Xiao,1 Na Zhang,1,2 Xiao-Qi Yu,1,2 Wei Cui,1 Jun-Xia Xie,2,* and Hua-Min Xu1,2,3,*

SUMMARY
Iron deposition is one of the key factors in the etiology of Parkinson’s disease (PD). Iron-free-apoferritin has the ability to store iron by combining with a ferric hydroxide-phosphate compound to form ferritin. In this study, we investigated the role of apoferritin in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD mice models and elucidated the possible underlying mechanisms. Results showed that apoferritin remarkably improved MPTP-induced motor deficits by rescuing dopaminergic neurodegeneration in the substantia nigra. Apoferritin inhibited MPTP-induced iron aggregation by down-regulating iron importer divalent metal transporter 1 (DMT1). Meanwhile, we also showed that apoferritin prevented MPTP-induced ferroptosis effectively by inhibiting the up-regulation of long-chain acyl-CoA synthetase 4 (ACSL4) and the down-regulation of ferroptosis suppressor protein 1 (FSP1). These results indicate that apoferritin exerts a neuroprotective effect against MPTP by inhibiting iron aggregation and modulating ferroptosis. This provides a promising therapeutic target for the treatment of PD.

INTRODUCTION
Parkinson’s disease (PD) is a neurodegenerative disease that is common in middle-aged and elderly people. The pathological feature of PD is the damage of dopamine (DA) neurons in the substantia nigra pars compacta (SNpc), resulting in the progressive loss of motor functions. However, the etiology and pathogenesis are currently not fully understood. Increasing evidence has confirmed that iron content in the substantia nigra (SN) of patients with PD increased significantly compared with the control (Gerlach et al., 2006; Chen et al., 2019; Bergslund et al., 2019; Hirsch, 2009; Dexter et al., 1989; Hopes et al., 2016). Excessive labile iron in the SN resulted in oxidative stress and increased the production of reactive oxygen species (ROS) by the Fenton reaction (Wypijewska et al., 2010; Dixon and Stockwell, 2014; Weinreb et al., 2013). Iron is also an important participant in ferroptosis, which is a newly discovered iron-dependent cell death and has been found in patients with PD (Mahoney-Sánchez et al., 2021; Bellinger et al., 2011; Vallerga et al., 2020). It has also been reported that ferroptosis is related to the pathogenic changes observed in PD models, including elevated iron deposit in the SN, consumption of glutathione (GSH), lipid peroxidation, increased production of ROS, and oxidation of DA (Wypijewska et al., 2010; Dixon et al., 2012). Therefore, it is important to find therapeutic target to prevent iron deposition and iron-dependent ferroptosis in PD.

Studies have confirmed that the cellular mechanism leading to iron accumulation in the SN of PD might be related to abnormal brain iron metabolism (Hentze et al., 2010; Wang and Pantopoulos, 2011; Zecca et al., 2001, 2004). Iron homeostasis is maintained by interactions between iron transporters and iron storage protein. Impaired iron transport or altered iron storage could disrupt the balance of iron homeostasis. Transferrin (Tf)-transferrin receptor (TfR) and the divalent metal transporter 1 (DMT1)-mediated non-transferrin binding iron (NTBI) are two major pathways responsible for iron uptake (Moos and Morgan, 2000; Kielmannowicz et al., 2005). A large number of experiments have confirmed that the up-regulation of iron importer DMT1 might be involved in nigral iron accumulation and the degeneration of DA neurons in PD (Salazar et al., 2008; Saadat et al., 2015; Hirsch, 2009). Iron storage protein ferritin was known to play an important role in maintaining iron homeostasis. Ferritin is composed of 24 subunits of two types: H-ferritin (FTH) and L-ferritin (FTL) (Arosio et al., 1978; Harrison and Arosio, 1996). FTH has ferrous oxidase activity and can oxidize ferrous iron into ferric iron. FTL has a nucleation site to promote the formation of iron core and
complete iron storage. Evidence has shown that the load of ferritin is significantly increased in PD models (Kaur et al., 2007; Goto et al., 1996). It has been reported that the extracellular ferritin interacts with the cell through the specific binding of FTH to transferrin receptor 1 (TR1) (Fan et al., 2012; Daniels et al., 2006). The discovery of extracellular ferritin indicated that ferritin might be an important factor in the regulation of brain iron homeostasis.

Apoferritin is an iron-free form of ferritin, which has been used as a non-toxic nanomaterial in clinical treatments such as drug delivery, in vivo imaging, and photothermal therapy (Truffi et al., 2016; Srinivasan et al., 2014; Dominguez-Vera et al., 2010). Owing to the iron-binding function, apoferritin might play a role in chelating excess iron to protect DA neurons against PD. In this study, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was used to induce PD mice models. We explored the effects of apoferritin on MPTP-induced motor deficits and elucidated the possible underlying mechanisms. Our results showed that apoferritin could remarkably improve the MPTP-induced motor deficits and inhibit nigral iron aggregation and ferroptosis. This provides new discoveries and possibilities for the prevention and treatment of PD.

RESULTS

Apoferritin pretreatment rescued the weight loss and improved motor deficits induced by MPTP

In this study, we investigated the potential therapeutic effects of apoferritin in MPTP-induced PD mice model. The experimental paradigm is shown in Figure 1A. Results showed that the body weight of MPTP-treated mice decreased significantly compared with the control. And pretreatment with 10 or 15 mg/kg apoferritin had a significant recovery on the weight loss induced by MPTP. There was no difference between the apoferritin group and the control group (Figures 1B and 1C).

Then, we tested the effect of apoferritin on motor ability of MPTP-treated mice using the open-field test and pole-climbing test. Our results showed that MPTP mice exhibited a significantly reduced capacity for pole-climbing, including a significantly longer climb time and longer time to turn their heads. Pretreatment with 10 or 15 mg/kg apoferritin improved the pole-climbing ability (Figures 1D and 1E). There is no significant difference between 10 mg/kg apoferritin and 15 mg/kg apoferritin pretreatment. Therefore, 10 mg/kg apoferritin pretreatment was used in the following experiments. Results of open-field test showed that compared with the healthy control group, the total distance in MPTP-treated mice decreased significantly, whereas the total distance in the mice with apoferritin pretreatment was significantly increased compared with the MPTP group (Figures 1F and 1G).

Apoferritin effectively inhibited MPTP-induced degeneration of DA neurons

To further verify whether apoferritin pretreatment has a neuroprotective effect on DA neurons in the SN of MPTP-induced PD mice, tyrosine hydroxylase (TH) staining and western blots were used to evaluate the survival of DA neurons and the level of TH protein in the SN. As shown in Figures 2A and 2B, TH-positive neurons in the SN of MPTP-treated mice were significantly decreased compared with the healthy control. Conversely, pretreatment with apoferritin could effectively inhibit MPTP-induced loss of TH-positive neurons. Furthermore, we also detected the protein level of TH in the SN of mice in different groups. Results showed that the protein level of TH in the SN of MPTP-treated mice was significantly decreased compared with the control. Consistent with the effect on rescuing the loss of TH-positive neurons caused by MPTP, pretreatment with apoferritin could significantly attenuate the decrease in TH protein level in the SN of MPTP-treated mice (Figure 2C). Thus, these results suggested that apoferritin could protect DA neurons against MPTP neurotoxicity.

Apoferritin decreased the number of Iba-1-positive microglia in the SN of MPTP-induced mice

The expression of Iba-1 is a hallmark of microglia in the CNS. In this study, immunofluorescence was used to stain Iba-1-positive microglia. Results showed that MPTP treatment induced a robust increase in the number of Iba-1-positive microglia in the SN as detected by Iba-1 staining, compared with the control. The number of Iba-1-positive microglia markedly decreased after 10 mg/kg apoferritin pretreatment (Figures 3A and 3B). This suggested that the effect of apoferritin on the number of Iba-1-positive microglia in the SN might also contribute to its neuroprotection against MPTP.
Apoferritin decreased iron content in the SN and regulated iron transporters

Increased iron content in the SN of PD has been indicated in patients with PD and PD animal models. Apoferritin is an iron-free ferritin and might exert its neuroprotective effect in PD by chelating excess iron. Therefore, in this study, we investigated the effect of apoferritin on the number of iron-positive cells and the expression of iron transporters in MPTP-induced PD models. Results showed that the number of iron-positive cells in the SN as measured by iron staining was higher in MPTP-treated mice compared with the control. On the contrary, pretreatment with apoferritin suppressed MPTP-induced increase in the number of iron-positive cells in the SN of MPTP-induced PD mice (Figures 4A and 4B). This indicated that the protection of apoferritin might be related to reducing iron accumulation in the SN of MPTP-induced PD mice.

Consistently, altered expressions of iron-related proteins have been reported to be involved in the nigral iron accumulation in PD. We further investigated whether the effect of apoferritin on iron levels was related to the regulation on the expression of iron-related proteins. Therefore, the expressions of iron importer DMT1 and TfR1 were detected by western blot analysis. Results showed that MPTP-induced PD mice expressed higher levels of DMT1 in the SN than the control mice, and pretreatment with apoferritin drastically inhibited the up-regulation of DMT1 induced by MPTP (Figure 4C). However, the expression of TfR1 was not significantly altered by MPTP or apoferritin treatment (Figure 4D). These results suggest that apoferritin might exert its neuroprotective effect in PD by regulating iron transporters and thus reducing iron accumulation in the SN.
decreased after MPTP treatment, and apoferritin restored the expression of TfR1 in MPTP-treated mice (Figure 4D). Thus, the effect of apoferritin on suppression of nigral iron accumulation in MPTP-induced PD mice might be associated with regulating the abnormal expression of iron import protein DMT1.

Apoferritin protected DA neurons against MPTP by inhibiting ferroptosis
Ferroptosis is an iron-dependent cell death. Studies have confirmed that ferroptosis is involved in the pathology of PD. To further investigate whether apoferritin could inhibit ferroptosis, we conducted the experiments to detect the expressions of proteins related to ferroptosis including glutathione peroxidase 4 (GPX4), long-chain acyl-CoA synthetase 4 (ACSL4), and ferroptosis suppressor protein 1 (FSP1). Results showed that the expression of GPX4 decreased significantly in the SN of MPTP-treated PD mice. However, pretreatment with apoferritin did not restore MPTP-induced decrease in the expression of GPX4 as shown in the Figure 5A. Further study showed that MPTP and pretreatment with apoferritin did not affect the levels of GSH/oxidized glutathione (GSH/GSSG) (Figure 5B). In addition, results showed that the expression of ACSL4 increased, whereas the expression of FSP1 decreased significantly in the SN of MPTP-treated PD mice. Apoferritin pretreatment suppressed MPTP-induced increase in the expression of ACSL4 and decrease in the expression of FSP1 (Figures 5C and 5D). These results indicated that the neuroprotective effect of apoferritin might be associated with the regulation of ferroptosis by affecting the expression of ACSL4 and FSP1.
Evidence suggests that iron deposition in the SN is involved in the pathogenesis of PD (Hirsch et al., 1991; Dexter et al., 1987; Riederer et al., 1989). Treatment strategies that inhibit iron accumulation and iron-dependent cell damage might halt the progression of PD. Apoferritin is an iron-free ferritin that has the function of storing iron. Here, we investigated the neuroprotective effect and the possible underlying mechanisms of apoferritin against MPTP toxicity. We presented evidence supporting that apoferritin can effectively improve motor deficits and protect against the degeneration of DA neurons of MPTP-induced PD mice. More importantly, we found that suppression of nigral iron accumulation and regulation of iron transporter DMT1 might contribute to the neuroprotection of apoferritin against MPTP toxicity. Furthermore, we showed here that apoferritin could inhibit MPTP-induced ferroptosis by up-regulating FSP1 and down-regulating ACSL4.

MPTP is a by-product in the synthesis of 1-methyl-4-phenyl-4-propionoxypiperidine (MPPP) (Ziering and Lee, 1947). MPTP itself is not toxic, but after entering the brain, it is converted into toxic 1-methyl-4-phenylpyridiniumion (MPP+) under the action of monoamine oxidase B. MPP+ is specifically transported to dopaminergic neurons of the SN by DA transporter and then induces the occurrence of PD by inhibiting mitochondrial complex I (Langston et al., 1984; Chiba et al., 1984; Castagnoli et al., 1985; Lee et al., 2011). Our results demonstrated that apoferritin can prevent MPTP-induced weight loss and improve motor deficits. And there was no significant difference in the protection against MPTP-induced motor deficits between intragastric gavage and intravenous injection of apoferritin (Figure S1). In addition, apoferritin protected against MPTP-induced degeneration of DA neurons, including restoring TH-positive neurons and increasing the expression of TH protein of the SN in MPTP-induced

**Figure 3. Apoferritin suppressed MPTP-induced increase of Iba1-positive microglia in the SN**

(A) MPTP treatment caused a significant increase in the number of Iba1-positive microglia in the SN, which was suppressed by apoferritin. Scale bars, 200 µm (top) and 50 µm (below).

(B) Statistical analysis.

*"p < 0.01, compared with the control. *p < 0.05, compared with the MPTP. Data were expressed as mean ± SEM (n = 6 in each group).
PD mice. Further results showed that apoferritin decreased the numbers of Iba-1-positive microglia in the SN of MPTP-induced mice. These results indicate that apoferritin has a protective effect against MPTP-induced neurotoxicity.

In vivo and in vitro results also showed that the expression of L-ferritin in the SN of mice and primary cultured ventral mesencephalon (VM) neurons increased significantly after apoferritin treatment compared with the control. This indicated that apoferritin might exert its effect by entering neurons and capturing intracellular iron (Figure S2).

Studies have confirmed that MPTP induced iron deposition in the SN of mice (Shi et al., 2019; Li et al., 2020; Goto et al., 1996; Temlett et al., 1994). In this study, we also observed the increased iron-positive cells in the SN of MPTP-induced PD mice. And we further proved that apoferritin pretreatment could inhibit MPTP-induced increase of iron-positive cells. DMT1 and TfR1 are two major iron import proteins, responsible for non-transferrin-bound iron import and transferrin-bound iron import, respectively. Our previous study has shown that MPTP induced iron accumulation and up-regulation of DMT1, whereas iron chelator

**Figure 4. Apoferritin suppressed MPTP-induced iron accumulation in the SN via regulating iron-transport proteins**

(A) Perls’ iron staining revealed that MPTP treatment resulted in the increase in iron-positive cells in the SN, which could be suppressed by apoferritin. Scale bars, 200 µm (top) and 50 µm (below).

(B) Statistical analysis of iron positive cells as shown in (A).

(C) DMT1 was up-regulated in the SN of MPTP-induced mice. Apoferritin suppressed MPTP-induced up-regulation of DMT1.

(D) The expression of TfR1 decreased in the SN of MPTP-induced mice. Apoferritin suppressed MPTP-induced decrease in the expression of TfR1.

**p < 0.01, ***p < 0.001, compared with the control. #p < 0.05, compared with the MPTP. Data were expressed as mean ± SEM (n = 5–7 in each group).**
deferoxamine (DFO) abolished these effects (Zhang et al., 2009). In this study, our results showed that up-regulation of DMT1 in the MPTP-induced PD model was inhibited by apoferritin. This indicated that the iron-suppressing effect of apoferritin might be achieved through the suppression of DMT1, but not TfR1. Up-regulation of DMT1 might increase iron uptake by neurons, which in turn leads to lipid peroxidation and ROS production by the Fenton reaction (Wypijewska et al., 2010; Aguirre et al., 2012; Dixon and Stockwell, 2014; De Farias et al., 2016). Ferroptosis is an iron-dependent and regulated process of cell death, which is characterized by the accumulation of ROS and lipid peroxidation products. It has been reported that ferroptosis was involved in the neuropathology of MPTP neurotoxicity (Do Van et al., 2016). Previous studies have shown that ferroptosis inhibitors ferrostatin-1 (Fer-1) and liproxstatin-1 can effectively prevent the loss of DA neurons in the SN of PD (Do Van et al., 2016; Dixon et al., 2012), whereas the iron chelator DFO can...
effectively inhibit the occurrence of ferroptosis (Cheng et al., 2019). Therefore, apoferritin might protect DA neurons against MPTP by modulating iron homeostasis and iron-dependent ferroptosis.

Glutathione peroxidase 4 (GPX4) is one of the regulator of ferroptosis (Yang et al., 2014) and is essential for maintaining the redox balance of cells (Matsushita et al., 2013). In this study, we investigated the effect of apoferritin on the expression of GPX4 in MPTP-induced PD mice. Results showed that the expression of GPX4 decreased compared with the control. However, apoferritin did not restore MPTP-induced decrease in the expression of GPX4. To further confirm the possible mechanisms underlying the effect of apoferritin on ferroptosis in MPTP-induced PD mice model, we further detected other proteins related to ferroptosis. Recent studies showed that the balance of redox states was regulated by FSP1-NAD (P) H-CoQ10 axis (Bersuker et al., 2019; Doll et al., 2019), which is in parallel with the typical GPX4 pathway to suppress lipid peroxidation and ferroptosis (Doll et al., 2019). As a novel CoQ10 plasma membrane oxidoreductase, FSP1 protects cells from ferroptosis by reduced form of CoQ10, which is a potent antioxidant to prevent lipid peroxidation. Our results showed that MPTP induced decreased FSP1 in the SN, which can be inhibited by apoferritin pretreatment. This indicated that decreased FSP1 might cause CoQ10 to be in an oxidized state and further aggravated lipid peroxidation, leading to the occurrence of ferroptosis and ultimately the degeneration of DA neurons in PD. However, apoferritin can effectively inhibit ferroptosis by up-regulating FSP1, thereby inhibiting the production of lipid peroxidation to exert its neuroprotective effects against MPTP.

In addition, we also detected the expression of ACSL4, a member of the long-chain family of acyl-CoA synthetase proteins, which have recently been shown to play an important role in ferroptosis (Doll et al., 2017). ACSL4 catalyzes arachidonic acid and adrenaline to produce coenzyme A derivatives, which is a process associated with ferroptosis. Free iron or iron-containing lipoxigenase enzymes are responsible for oxidizing membrane polyunsaturated fatty acids (PUFAs), potentially leading to the formation of lipid ROS. It has been reported that ACSL4 was required for the activation or incorporation of PUFAs into membrane phospholipids, which is important in ferroptosis. Inhibiting the expression of ACSL4 prevents lipid peroxidation in ferroptosis and related cell death (Doll et al., 2017). Our results showed that MPTP induced up-regulation of ACSL4, which might aggravate lipid peroxidation, whereas apoferritin can effectively inhibit ferroptosis by inhibiting the expression of ACSL4. The results together with previous studies suggest that ferroptosis is probably an important cell death pathway of DA neurons in MPTP-induced PD mice models, and apoferritin might be a potential drug candidate to pharmacologically modulate the ferroptosis via regulation of ACSL4 and FSP1.

In conclusion, our study demonstrates the effect of apoferritin in MPTP-induced PD animal models and elucidates the possible mechanisms underlying its neuroprotection on DA neurons, which is summarized in Figure 6. Our study supports the pathophysiological significance of ferroptosis in the pathogenic process of MPTP-induced PD animal models. Suppression of iron accumulation and iron-dependent ferroptosis in the brain contributes to the neuroprotection of apoferritin against MPTP. This provides a promising therapeutic direction for the clinical prevention and treatment of PD.

Limitations of the study
The mechanisms underlying the effect of apoferritin on MPTP-induced ferroptosis still need to be elucidated. In addition, the transport of apoferritin in the brain is not experimentally demonstrated.

Resource availability
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Hua-Min Xu (huamin102@163.com).

Materials availability
This study did not generate new unique reagents.

Data and code availability
Raw data will be shared upon receipt of a reasonable request.
**METHODS**

All methods can be found in the accompanying transparent Methods supplemental file.

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.102431.

**ACKNOWLEDGMENTS**

This work was supported by grants from the National Natural Science Foundation of China (31871202, 31771124), the Department of Science and Technology of Shandong Province (ZR2019MC057), Excellent Innovative Team of Shandong Province (2020KJK007), and Taishan Scholars Construction Project, Shandong.

**AUTHOR CONTRIBUTION**

H.-M.X. conceived the project, designed the experiments, and supervised the project. L.-M.S., Z.-X.X., N.Z., X.-Q.Y., and W.C. performed the experiments. L.-M.S. analyzed the data, prepared the figures, and wrote the manuscript. J.-X.X. revised the manuscript. All authors read and approved the final manuscript.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

Received: January 23, 2021  
Revised: March 7, 2021  
Accepted: April 12, 2021  
Published: May 21, 2021

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Supplemental information

Apoferitin improves motor deficits in MPTP-treated mice by regulating brain iron metabolism and ferroptosis

Li-Mei Song, Zhi-Xin Xiao, Na Zhang, Xiao-Qi Yu, Wei Cui, Jun-Xia Xie, and Hua-Min Xu
Supplementary Figure 1. Intragastric Gavage or Intravenous Injection of Apoferritin Improved Motor Deficits of MPTP-treated Mice, Related to Figure 1

MPTP resulted in significant motor deficits, which were inhibited by apoferritin given by intragastric gavage (IG) or intravenous (IV) injection. A. Turnaround time; B. Pole-climbing time. **P < 0.01, compared with the control; #P < 0.05, ##P < 0.01, compared with the MPTP. Data were expressed as mean ± SEM (n = 5–11 in each group).
Supplementary Figure 2. The Expression of Ferritin after Apoferritin Treatment in vivo and in vitro, Related to Figure 4
The expression of L-ferritin increased significantly after apoferritin treatment, compared with the control.

A. The expression of L-ferritin in C57BL/6J mice treated with 10 mg/kg apoferritin;
B. The expression of L-ferritin in the primary VM neurons treated with 50 ug/mL apoferritin;
C. The expression of H-ferritin in C57BL/6J mice treated with 10 mg/kg apoferritin;
D. The expression of H-ferritin in the primary VM neurons treated with 50 ug/mL apoferritin.

*P < 0.05, **P < 0.001, compared with the control. Data was expressed as mean ± SEM (n = 6–12 in each group).
Supplementary Table 1. Key Antibodies Used in the Experiments, Related to Figure 2-5

| REAGENT      | WORKING DILUTION | SOURCE          | IDENTIFIER          |
|--------------|------------------|-----------------|---------------------|
| TH           | 1:3000/1:1000    | United States   | Cat# AB152; RRID: AB_390204 |
|              |                  | Millipore       |                     |
| GPX4         | 1:10000          | United States   | Cat# ab125066; RRID: AB_10973901 |
|              |                  | Abcam           |                     |
| FSP1         | 1:1000           | United States   | Cat# 07-2274; RRID: AB_10807552 |
|              |                  | Millipore       |                     |
| ACSL4        | 1:1000           | United States   | Cat# sc-365230; RRID: AB_10843105 |
|              |                  | Santa Cruz      |                     |
| β-actin      | 1:10000          | China           | Cat#bs0061R; RRID: AB_0855480 |
|              |                  | Bios            |                     |
| DMT1         | 1:1000           | United States   | Cat# TA324527; RRID: AB_2758340 |
|              |                  | OriGene         |                     |
| TfR1         | 1:1000           | United States   | Cat#ab84036; RRID: AB_10673794 |
|              |                  | Abcam           |                     |
| Iba-1        | 1:200            | United States   | Cat# 17198; RRID: AB_2820254 |
|              |                  | CST             |                     |
| H-ferritin   | 1:1000           | United States   | Cat#ab183781        |
|              |                  | Abcam           |                     |
| L-ferritin   | 1:1000           | United States   | Cat#ab69090; RRID: AB_1523609 |
| Goat Anti-Rabbit IgG-Alexa Fluor 488 | 1:500 | China Absin | Cat#abs20025 |
| Goat Anti-Rabbit IgG-Alexa Fluor 594 | 1:500 | China Absin | Cat#abs20021 |
| Goat Anti-Rabbit IgG | 1:10000 | China Absin | Cat#abs20011 |
| Goat Anti-Mouse IgG | 1:10000 | China Absin | Cat#abs20012 |
Transparent Methods
Method details
Chemicals
Apo ferritin from horse spleen, MPTP and tyrosine hydroxylase (TH) were purchased from Sigma (St. Louis, MO, USA). The primary antibodies of glutathione peroxidase 4 (GPX4), transferrin receptor 1 (TfR1), H-ferritin and L-ferritin were from Abcam (Cambridge, MA, USA). The primary antibody of ferroptosis suppressor protein 1 (FSP1) was from MilliporeSigma (Billerica, MA, USA). The primary antibody of long-chain acyl-CoA synthetase 4 (ACSL4) was from Santa Cruz Biotechnology (CA, USA). The primary antibody of DMT1 was from OriGene Technology (Maryland, USA). The monoclonal β-actin antibody was from Bioss (Beijing, China). The goat anti-rabbit IgG labeled with HRP and goat anti-mouse IgG labeled with HRP were from absin (Shanghai, China). ECL ultrasensitive chemiluminescence kit was from MilliporeSigma (Billerica, MA, USA). Other biological reagents and materials are from local commercial sources.

Primary Cultured Ventral Mesencephalon (VM) Neurons
Primary cultures of VM neurons were obtained from embryonic 14-day Sprague–Dawley rat. Briefly, VM was dissected from the embryonic rat brain under the dissection microscope and then mechanically dissociated with a pipette until the tissue was dispersed. After centrifugation, cells were suspended in DMEM/F12 supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin and seeded on poly-d-lysine-coated 12-well culture plates. Cells were grown in a humidified atmosphere of 5% CO₂ at 37°C for 18 h, and then the culture medium was changed to serum free DMEM/F12 supplemented with 2% B27. Cells were grown for a further 4 days before use. For experiments, VM neurons were treated with 50 μg/ml Apoferritin for 24 h.

Animal Treatments
The C57BL/6J male mice used in this study were all from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). The mice were housed one animal per cage with food pellets and water available ad libitum. The room was maintained at a constant temperature and humidity on a 12 h light/dark cycle. The mice were adapted to the laboratory environment for 1 week before the experiments. The Animal Ethics and Experimentation Committee of the Qingdao University approved the use of animals for this study.
MPTP is diluted with normal saline (NS) to 6 mg/ml, and the injection volume is 30 mg/kg; Apoferritin is diluted with NS to 2 mg/ml stock solution. The working concentration is 10 mg/kg or 15 mg/kg. Male C57BL/6J mice aged 9-10 weeks were randomly divided into four groups: control group (normal saline group), MPTP group, apoferritin+MPTP group, and apoferritin group. Pre-protection was given for 3 days before the experiments (Mice in the control group and MPTP group were given NS by intragastric gavage; Mice in apoferritin+MPTP group and apoferritin group were given apoferritin by intragastric gavage (IG) or intravenous (IV)
injection). On the 4th day after apoferritin treatment, MPTP was given intraperitoneally for 5 consecutive days to induce PD models (control group and apoferritin group intraperitoneally injected NS: MPTP group and apoferritin+MPTP group intraperitoneally injected MPTP).

**Open-field Test**

The open-field test was used to assess the general behavior and locomotor activity. Mice were gently placed in the center of a dedicated black box (40 cm×40 cm×40 cm). Locomotor behavior was video-recorded for 10 min by a computer for automatic analysis and the total movement distance of each animal was recorded.

**Pole-climbing Test**

A self-made straight wooden pole with a diameter of 1.2 cm and a height of 50 cm was used to do the pole-climbing test. A small wooden ball is placed on the top of the pole and wrapped with gauze to prevent mice from slipping. Mice were habituated to the pole on the day before testing. And then the animals were recorded via digital video on the next day. The amounts of time for the mouse to turn towards the ground (time to orient down) and to reach the ground (time to descend) were recorded. The average scores for each mouse were determined based on five tests.

**Perls’ Iron Staining**

After the mice were perfused with NS and 4% paraformaldehyde (PFA). The brains were taken and fixed in 4% PFA for 4-6 hours, and then immersed in 20% and 30% sucrose for sugar precipitation. Brain blocks containing the SN were sectioned coronally at 20 µm on a freezing microtome and stored at -20°C. Perls' staining was utilized to detect the presence of iron in brain sections by a complex hydrated ferric ferrocyanide substance as described previously (Jiang et al., 2010). Sections were fixed with 4% PFA for 5 minutes and washed with ddH2O for 30 seconds (not overtime). Sections were immersed for in a ready-to-use iron staining solution (2% HCL-potassium ferrocyanide), followed by three washes with PBS. Negative control sections were prepared in which the HCl and potassium ferrocyanide solutions were omitted. The sections were then immersed in 99% methanol and 1% hydrogen peroxide for 20 min to eliminate endogenous peroxidase activity. The DAB reaction product was observed under an Olympus microscope and the images were captured by a video camera (OLYMPUS, Japan) at a final magnification of 200×.

**Immunofluorescence**

For immunofluorescence, the sections of SN were stained with TH antibody. After three washes with 0.01% phosphate-buffered saline (PBS, pH 7.4) for 10 min, sections were incubated in 5% donkey serum-PBS for 1 h at room temperature and then incubated overnight with the primary antibody of TH (1:1000) or lba-1(1:200). After washing, secondary antibody of Alexa Fluor ® 488 donkey anti-rabbit IgG was applied to sections for 2 h at room temperature.
Nuclei were stained with DAPI at room temperature for 10 min in the dark and washed twice with PBS. Then sections were mounted with 70% glycerin and examined using digital pathology section system (OLYMPUS, Japan). The same anatomical landmarks were used to select three sections through the SN from each mouse to count TH-positive cells at a final magnification of 400×. Values represent the mean TH-positive cells from each section.

Western Blotting
SN tissues were dissected from the brain of mice and lysed with lysis buffer on ice for 30 min. The harvested lysates were centrifuged at 12,000×g for 20 min at 4 °C, and the supernatants were used for analysis. Protein concentration was determined by a BCA protein assay kit (CW BIO China). Proteins with 5 × loading buffer (Beyotime) were incubated at 100°C for 5 min. The total 20 µg protein was separated by 8-12% SDS polyacrylamide gels and the protein in the gel was transferred to a PVDF membrane (Millipore, MA, USA). After overnight blocking with TBST containing 5% non-fat milk or BSA for 2 hours at room temperature, membranes were incubated overnight at 4 °C with the primary antibodies against TH (1:3000), DMT1 (1:800), TIR1 (1:1000), H-ferritin (1:1000), L-ferritin (1:1000), ACISL4 (1:1000), GPX4 (1:10000), FSP1 (1:1000), β-actin (1:10000). Goat anti-rabbit or goat anti-mouse IgG labeled with HRP (Santa Cruz Biotechnology, Texas, USA) was used at 1:10,000 and incubated with the membranes for 1 h at room temperature. Cross-reactivity was visualized using ECL western blotting detection reagents (Millipore, USA) and then was analyzed through scanning densitometry by a UVP BioDoc-It Imaging System (UVP, Upland, USA).

GSH/GSSG Ratio Detection
The total reduced GSH and GSSG level were detected by GSH/GSSG assay kit (Abcam, USA) according to the manufacturer’s instructions. 400 µl 0.5% NP-40 (Solarbio, China) was added to 20 mg tissue to grind the tissue thoroughly, and centrifuged at 4°C, 12000 rpm for 15 min. Extract the supernatant into a new Ep tube, then add 1 volume of TCA (Abcam, USA) to 5 volume of the sample and vortex to mix. After incubation on ice for 5-10 min and centrifugation at 4°C, 12000 g for 5 min. The supernatant was used to detect the level of GSH/GSSG by GSH/GSSG assay kit. The signal was read by a fluorescence microplate reader at Ex/Em = 490/520 nm.

Statistical Analysis
The results were analyzed by GraphPad Prism 6.0 statistical software and data was expressed as mean ± SEM. One-way analysis of variance (ANOVA) followed by Turkey’s test was used to compare the differences between means. P<0.05 was considered to be statistically significant.

Supplemental References
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