***L*-type Calcium Channels are Involved in Iron-induced Neurotoxicity in Primary Cultured Ventral Mesencephalon Neurons of Rats**

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Abstract In the present study, we investigated the mechanisms underlying the mediation of iron transport by *L*-type Ca^{2+} channels (LTCCs) in primary cultured ventral mesencephalon (VM) neurons from rats. We found that co-treatment with 100 $\mu\text{mol/L}$ FeSO_4 and MPP^+ (1-methyl-4-phenylpyridinium) significantly increased the production of intracellular reactive oxygen species, decreased the mitochondrial transmembrane potential and increased the caspase-3 activation compared to MPP^+ treatment alone. Co-treatment with 500 $\mu\text{mol/L}$ CaCl_2 further aggravated the FeSO_4 -induced neurotoxicity in MPP^+ -treated VM neurons. Co-treatment with 10 $\mu\text{mol/L}$ isradipine, an LTCC blocker, alleviated the neurotoxicity induced by co-application of FeSO_4 and $\text{FeSO}_4/\text{CaCl}_2$. Further studies indicated that MPP^+ treatment accelerated the iron influx into VM neurons. In addition, FeSO_4 treatment significantly increased the intracellular Ca^{2+} concentration. These effects were blocked by isradipine. These results suggest that elevated extracellular Ca^{2+} aggravates iron-induced neurotoxicity. LTCCs mediate iron transport in dopaminergic neurons and this, in turn, results in elevated intracellular Ca^{2+} and further aggravates iron-induced neurotoxicity.

Keywords *L*-type Ca^{2+} channels · Iron overload · Parkinson's disease · Isradipine · Dopamine neuron

Introduction

Parkinson's disease (PD) is a common neurodegenerative disease. It is characterized by a selective loss of dopamine (DA) neurons in the substantia nigra pars compacta (SNpc), and results in DA exhaustion in the striatum. Many factors have been implicated in the pathological process of PD [1–6]. Recently, growing evidence has indicated that iron accumulation plays a key role in the pathogenesis of DA neuron degeneration [7–10]. Iron is an important co-factor in maintaining cellular functions. It is extensively involved in metabolic processes [11, 12]. When the dynamic balance of iron is interrupted, it leads to mitochondrial DNA damage [13, 14], mitochondrial dysfunction, lipid peroxidation, and DNA breakage [15]. Excessive deposition of iron in the brain and the consequent oxidative stress are considered to play key roles in the pathogenesis of PD [16]. However, the precise mechanisms underlying iron-selective accumulation in the SN and iron-mediated neuronal toxicity remain unclear.

There is evidence that *L*-type Ca^{2+} channels (LTCCs) are involved in the DA neuron degeneration [17–20]. Epidemiological studies have shown that the risk of PD is significantly decreased by dihydropyridines, LTCC blockers, in patients with hypertension [21, 22]. It has been reported that LTCCs may mediate iron entry into cardiomyocytes under high-iron conditions [23]. In addition to cardiomyocytes, iron may compete with Ca^{2+} for entry into nerve growth factor-treated PC12 cells and mouse N-2a cells *via* LTCCs [24]. Our previous study also demonstrated that the LTCC blocker nifedipine attenuates iron aggregation in the SN of iron-overloaded rats [25]. However, the mechanisms underlying LTCC-mediated iron accumulation in the SN are not fully understood.

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It is well known that excessive iron reacts with hydrogen peroxide to produce hydroxyl radicals by the Fenton reaction, thus aggravating oxidative stress. Recently, studies have indicated that iron overload increases the level of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$), thereby aggravating the iron-mediated neurotoxicity. In addition, inhibition of Ca^{2+} signals prevents the mitochondrial fragmentation and neuronal death induced in hippocampal neurons by iron-overload [26]. However, the effects of extracellular Ca^{2+} on iron-overload-induced neurotoxicity remain unclear. In the present study, we investigated the mechanisms underlying LTCC-mediated iron transport and the effects of Ca^{2+} on iron-induced neuronal toxicity in primary cultured ventral mesencephalon (VM) neurons from rats.

Materials and Methods

Materials

All the reagents were from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated. Dulbecco's modified Eagle's medium Nutrient Mixture-F12 (DMEM/F12) and B27 were from Gibco (Grand Island, NY, USA).

Culture of VM Primary Neurons

All procedures were carried out in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Animal Ethics Committee of Qingdao University. Primary cultures of VM neurons were prepared from the mesencephalon of embryonic Wistar rats (14 days) as previously reported, with some modifications [27, 28]. Briefly, the abdomen of female rats was cleaned with 70% ethanol, and then the abdominal wall and the uterine horns were opened. Each embryo was collected from the uterine horns and the amniotic membranes were removed. For dissection, the embryos were placed in a Petri dish containing pre-cooled DMEM/F12 under a stereomicroscope and the VM was isolated using ultra-fine forceps. Cells were suspended in serum-free DMEM/F12 supplemented with 2% B27, and then seeded onto poly-D-lysine-coated culture plates. Neurons were cultured for 7 days before use. Neuron purity was approximately 90% based on immunofluorescence staining with the specific neuron marker microtubule-associated protein 2.

Assessment of Mitochondrial Transmembrane Potential ($\Delta\Psi_m$)

After culture for 7 days, changes in the $\Delta\Psi_m$ of VM neurons were measured with rhodamine123, using flow

cytometry as described previously [29, 30]. The uptake of rhodamine123 into mitochondria is an indicator of the $\Delta\Psi_m$. The neurons were pre-incubated with 5 $\mu\text{mol/L}$ 1-methyl-4-phenylpyridinium (MPP^+) for 24 h, and then treated with FeSO_4 and/or CaCl_2 for 3 h. The neurons were divided into control, MPP^+ treatment, $\text{MPP}^+/\text{CaCl}_2$ treatment, $\text{MPP}^+/\text{CaCl}_2/\text{isradipine}$ treatment, $\text{MPP}^+/\text{FeSO}_4$ treatment, $\text{MPP}^+/\text{FeSO}_4/\text{isradipine}$ treatment, $\text{MPP}^+/\text{CaCl}_2/\text{FeSO}_4$ treatment, and $\text{MPP}^+/\text{CaCl}_2/\text{FeSO}_4/\text{isradipine}$ treatment groups (500 $\mu\text{mol/L}$ CaCl_2 , 100 $\mu\text{mol/L}$ FeSO_4 , 10 $\mu\text{mol/L}$ isradipine). After different treatments, the neurons were incubated in HEPES-buffered saline (HBS) containing rhodamine123 in a final concentration of 5 $\mu\text{g/mL}$ for 30 min at 37 °C. After washing twice with HBS, the fluorescence intensity was recorded at 488 nm excitation and 525 nm emission. Results were presented as a Fluorescence 1-Histogram, setting the gated regions M1 and M2 as a marker to measure the changing levels of fluorescence intensity with Cellquest software.

Reactive Oxygen Species (ROS) Assay

Intracellular ROS in the VM neurons were measured using $\text{H}_2\text{DCF-DA}$ (2,7-dichlorodihydrofluorescein) as previously described [30]. The fluorescence emitted by $\text{H}_2\text{DCF-DA}$ reflects intracellular ROS generation. After the different treatments, the neurons were incubated in HBS containing $\text{H}_2\text{DCF-DA}$ in a final concentration of 5 $\mu\text{mol/L}$ for 30 min at 37°C. After washing twice with HBS, fluorescent intensity was recorded at 488 nm excitation and 525 nm emission. The results were presented as for the $\Delta\Psi_m$ assay.

Western Blot Analysis

The VM neurons were seeded in six-well plates and treated as described above. After washing with PBS, the cells were digested on culture plates with RIPA lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% Nonidet, 0.5% deoxycholate, 1 mmol/L EDTA, and 1 mmol/L PMSF) with 1% phenylmethylsulfonyl fluoride on ice for 30 min. Then, the samples were centrifuged at 12,000 rpm for 20 min at 4 °C. Protein concentration was determined with a bicinchoninic acid kit. The lysates containing 40 μg of protein sample were separated on 12% sodium dodecyl sulfate polyacrylamide gel and transferred to polyvinylidene difluoride membranes with a diameter of 0.22 μm . The membranes were blocked with 5% non-fat milk at room temperature for 2 h. Blots were probed with rabbit anti-mouse cleaved caspase-3 (Cell Signaling, Boston, MA, USA, 1:1000) and rabbit anti-mouse β -actin primary antibody (Bioss, Boston, MA, USA, 1:10000) overnight at 4 °C. After washing with TBST, the membranes were incubated with goat anti-rabbit and goat anti-mouse

secondary antibodies conjugated with horseradish peroxidase (Bioss, Boston, MA, USA, 1:10,000) for 1 h at room temperature. The antigen-antibody complexes were visualized with enhanced chemiluminescence reagent and then analyzed by scanning densitometry on a Tanon Image System (Shanghai, China).

Calcein Loading of Cells and Iron Influx Assay

Calcein-AM is a membrane-permeable, non-fluorescent molecule that becomes fluorescent upon intracellular cleavage by cytoplasmic esterases to calcein (which is membrane impermeable). It is pH-independent and is rapidly quenched by divalent metals and reversed easily by their chelators. Ferrous iron influx into VM neurons was determined by the quenching of calcein fluorescence as described previously [31]. The VM neurons were divided into 4 groups. Control: VM neurons were cultured in serum-free DMEM/F12 supplemented with 2% B27 for 24 h; MPP⁺: VM neurons were cultured in serum-free DMEM/F12 supplemented with 2% B27 with 5 $\mu\text{mol/L}$ MPP⁺ for 24 h; MPP⁺ with isradipine or Bayk8644: MPP⁺-treated cells underwent the same procedures except that isradipine or 10 $\mu\text{mol/L}$ Bayk8644 was included in the perfusion fluid. The cells were incubated with calcein-AM at a final concentration of 1 $\mu\text{mol/L}$ in HBS for 30 min at 37 °C. Excess calcein on the cell surface was washed 3 times with HBS. Calcein fluorescence was recorded at 488 nm excitation and 525 nm emission. Fluorescence intensity was measured every 3 min for the next 30 min during perfusion with 100 $\mu\text{mol/L}$ ferrous iron (FeSO_4 in ascorbic acid solution, 1:44 molar ratio, pH 6.0). The mean fluorescence intensity of 30–35 cells in 4 separate fields was monitored at 200 $\times$ magnification and processed with Leica Application Suite X (Leica, Mannheim, Germany).

Determination of Intracellular Ca^{2+} Concentration

Fluo-3 AM is a fluorescent probe used to assess the $[\text{Ca}^{2+}]_i$. Fluo-3 AM can penetrate the cell membrane and it is cleaved by esterase to form Fluo-3, which binds to Ca^{2+} and produces strong fluorescence. The excitation wavelength is 488 nm and emission is 525 nm–530 nm. VM neurons were divided into 6 groups. Control: VM neurons were cultured in serum-free DMEM/F12 supplemented with 2% B27 for 24 h; MPP⁺: VM neurons were cultured in serum-free DMEM/F12 supplemented with 2% B27 with 5 $\mu\text{mol/L}$ MPP⁺ for 24 h; MPP⁺/FeSO₄ treatment: MPP⁺-treated cells underwent the same procedures except that 100 $\mu\text{mol/L}$ FeSO₄ was included in the perfusion fluid; MPP⁺/CaCl₂ treatment: MPP⁺-treated cells underwent the same procedures except that 2.5 mmol/L CaCl₂ was included in the perfusion fluid; MPP⁺/FeSO₄/CaCl₂

treatment: MPP⁺-treated cells underwent the same procedures except that 100 $\mu\text{mol/L}$ FeSO₄ and 2.5 mmol/L CaCl₂ were included in the perfusion fluid; and MPP⁺/FeSO₄/CaCl₂/isradipine treatment: MPP⁺-treated cells underwent the same procedures except that 100 $\mu\text{mol/L}$ FeSO₄, 2.5 mmol/L CaCl₂, and 10 $\mu\text{mol/L}$ isradipine were included in the perfusion fluid. The cells were incubated with Fluo-3 AM at a final concentration of 5 $\mu\text{mol/L}$ in HBS for 30 min at 37 °C and then perfused with Krebs–Henseliet buffer (K–H buffer). Fluo-3 AM fluorescence was recorded at 488 nm excitation and 525 nm emission. Fluorescence intensity was measured every 2 min for the next 20 min during perfusion with K–H buffer. The mean fluorescence intensity of 30–35 cells in 4 separate fields was monitored at 200 $\times$ magnification and processed with Leica Application Suite X (Leica, Mannheim, Germany) [32].

Statistical Analysis

The results are expressed as the mean $\pm$ SEM. Data were analyzed using Prism Graphpad 5.0 software (Graphpad Software, San Diego, CA, USA). One-way analysis of variance (ANOVA) followed by the Newman-Keuls test was used to compare the differences between the means. For iron influx and $[\text{Ca}^{2+}]_i$ imaging experiments, regular two-way ANOVA followed by Bonferroni *post hoc* comparison of the means was performed. $P < 0.05$ was considered to be significant.

Results

Isradipine Prevents FeSO₄-induced $\Delta\Psi_m$ Reduction in VM Neurons

$\Delta\Psi_m$ is a key index of mitochondrial function, which is closely related to oxidative stress and apoptosis. By flow cytometry, we assessed the $\Delta\Psi_m$ changes in VM neurons after FeSO₄ and/or CaCl₂ treatment. The $\Delta\Psi_m$ in the MPP⁺ and MPP⁺/CaCl₂ groups were slightly lower than that in the control group, however the difference was not statistically significant (Fig. 1). The $\Delta\Psi_m$ in the MPP⁺/FeSO₄ group was decreased by 26% compared to the MPP⁺ group, and the difference was statistically significant ($P < 0.001$). Co-treatment with isradipine alleviated the FeSO₄-induced decrease of $\Delta\Psi_m$ in VM neurons ($P < 0.01$). The $\Delta\Psi_m$ in the MPP⁺/FeSO₄/CaCl₂ group was decreased by 11% compared to the MPP⁺/FeSO₄ group ($P < 0.01$). Treatment with isradipine alleviated the neurotoxicity induced by co-treatment with FeSO₄ and CaCl₂ ($P < 0.001$).

Fig. 1 Isradipine prevents FeSO₄-induced $\Delta\Psi_m$ reduction in VM neurons. **A** Representative fluorometric assays of the $\Delta\Psi_m$ in different groups of VM neurons. **B** Summary of the changes in

$\Delta\Psi_m$ in different treatment groups (mean $\pm$ SEM of 6 independent experiments; ** P < 0.01, *** P < 0.001).

Isradipine Prevents FeSO₄-induced ROS Production in VM Neurons

We further measured the changes of ROS production in VM neurons using the fluorescence dye H₂DCF-DA. The fluorescence value of the control was set to 100%. Co-application of MPP⁺ with FeSO₄ significantly increased the intracellular ROS production (Fig. 2). The intracellular ROS production in the MPP⁺/FeSO₄ group was increased by 80% compared to the MPP⁺ group (P < 0.001). Co-treatment with isradipine inhibited FeSO₄-induced ROS production (P < 0.05). The ROS production in the MPP⁺/FeSO₄/CaCl₂ group was increased by 28% compared to the

MPP⁺/FeSO₄ group (P < 0.05). Isradipine markedly inhibited the ROS production induced by FeSO₄ and CaCl₂ co-treatment (P < 0.001).

Isradipine Inhibits FeSO₄-induced Caspase-3 Activation in VM Neurons

Caspase-3 is a key protein in the process of apoptosis. We further assessed the caspase-3 activity in the different treatment groups. The expression of cleaved caspase-3 in the MPP⁺/FeSO₄ group was up-regulated by 202% compared to the MPP⁺ group (P < 0.001). This increase was alleviated by isradipine (P < 0.05 versus the MPP⁺/FeSO₄

Fig. 2 Isradipine prevents FeSO_4 -induced ROS production in VM neurons. **A** Representative fluorometric assays of ROS in different groups. **B** Statistical analysis of the ROS production in different treatment groups. Fluorescence values of the control were set to 100% (mean $\pm$ SEM of 6 independent experiments; * $P < 0.05$, *** $P < 0.001$).

group). The cleaved caspase-3 expression in the MPP⁺/FeSO₄/CaCl₂ group was up-regulated by 59% compared to the MPP⁺/FeSO₄ group ($P < 0.01$). The up-regulation of cleaved caspase-3 induced by MPP⁺/FeSO₄/CaCl₂ co-treatment was alleviated by isradipine ($P < 0.001$ versus MPP⁺/FeSO₄/CaCl₂ group) (Fig. 3).

Isradipine Inhibits MPP⁺-induced Iron Influx in VM Neurons

Iron influx into VM neurons was determined by the quenching of calcein fluorescence. Time-dependent intracellular fluorescence quenching occurred with 100 $\mu\text{mol/L}$ ferrous iron perfusion (control), indicating increased intracellular iron levels. After treatment with 5 $\mu\text{mol/L}$ MPP⁺ for 24 h, the quenching was more rapid than in the control. The quenching was further accelerated when neurons were perfused with 10 $\mu\text{mol/L}$ Bayk8644, a Ca²⁺ channel agonist, compared with the MPP⁺ group, indicating a further iron influx. Fluorescence quenching in the MPP⁺ group was inhibited by both 1 $\mu\text{mol/L}$ and 10 $\mu\text{mol/L}$

isradipine, indicating that iron influx was blocked by isradipine (Fig. 4).

Intracellular Ca²⁺ Levels Increase with Iron Treatment

We did not find a significant difference in the $[\text{Ca}^{2+}]_i$ between the MPP⁺ group and MPP⁺/CaCl₂ (2.5 mmol/L) co-perfusion groups ($P > 0.05$ vs control). However, the $[\text{Ca}^{2+}]_i$ significantly increased with FeSO₄ perfusion in K-H buffer. A marked increase in $[\text{Ca}^{2+}]_i$ occurred when 2.5 mmol/L CaCl₂ was perfused in K-H buffer compared to the MPP⁺/FeSO₄ co-application group. Bath application of isradipine inhibited iron-induced elevation of $[\text{Ca}^{2+}]_i$ (Fig. 5).

Discussion

In the present study, we found that isradipine, a specific antagonist of LTCCs, prevented the iron-induced reduction in $\Delta\Psi_m$ and inhibited iron-induced ROS production and

Fig. 3 Isradipine inhibits FeSO₄-induced caspase-3 activation in VM neurons. **A** Original bands showing the expression of cleaved caspase-3 in VM neurons. β-actin was used as a loading control. **B** Statistical

summary of the expression of cleaved caspase-3 in the different treatment groups (ratio of cleaved caspase-3 to β-actin from 6 independent experiments; **P* < 0.05, ***P* < 0.01, ****P* < 0.001).

Fig. 4 Time-course of calcein fluorescence in VM neurons. The mean fluorescence intensity was calculated from 30–35 separate cells from 4 separate fields at each time point (mean ± SEM of 6 independent experiments; ****P* < 0.001, MPP⁺ group versus control;

#*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001, Bayk8644 group vs MPP⁺ group; ^*P* < 0.05, ^^*P* < 0.01, ^^*P* < 0.001, 10 μmol/L isradipine group vs MPP⁺ group; &*P* < 0.05, 1 μmol/L isradipine group vs MPP⁺ group)

caspase-3 activation in VM neurons. These findings suggest that LTCCs are involved in iron-induced DA neuron degeneration in the pathogenesis of PD.

Iron overload in the SN plays a key role in the etiology and pathogenesis of PD [33]. However, the precise

mechanisms underlying such an overload remain unknown. The uptake of iron by neurons is divided into a transferrin-dependent pathway and a non-transferrin-bound iron (NTBI) pathway [34]. Studies have revealed that iron enters DA neurons in the SN of PD patients mainly via the

Fig. 5 Time-course of fluo-3 AM fluorescence in VM neurons. The mean fluorescence intensity was calculated from 30 to 35 separate cells from 4 separate fields at each time point (mean $\pm$ SEM of 6 independent experiments; * P < 0.05, ** P < 0.01, *** P < 0.001 vs MPP⁺ group; # P < 0.05, ### P < 0.001 vs MPP⁺/FeSO₄ group; ^^^ P < 0.001 vs MPP⁺/FeSO₄/CaCl₂ group)

NTBI-dependent pathway [35]. Therefore, NTBI pathways, including divalent metal transporter 1 (DMT1) and voltage-gated Ca²⁺ channel-mediated iron transport, have attracted more attention. Previous studies have confirmed that the expression of DMT1 protein is up-regulated in the pathogenesis of PD and is involved in the uptake of iron by DA neurons in the SN [36, 37]. Other studies have shown that iron can enter neurons through LTCCs, and this is associated with the pathology and progression of neurodegenerative diseases [38, 39]. LTCCs may also provide an alternative route for iron import in neuronal cells [24]. However, there is no direct evidence for LTCC-mediated iron transport in DA neurons in the pathogenesis of PD. In this study, we found that activation or blockade of LTCCs altered the intracellular iron content in MPP⁺-treated VM neurons, which implied that LTCCs might mediate iron influx. These results indicate that LTCCs can contribute to iron accumulation in the SN. It has been shown that dihydropyridines such as nitrendipine and nimodipine block LTCC-mediated iron transport in the micromolar range [24, 40]. Indeed, we found that both 1 and 10 μ mol/L isradipine protect cells against iron-induced toxicity. Previous studies have also shown that isradipine in the nanomolar range inhibits the LTCCs in DA neurons [41, 42]. We further tested the effects of 200 nmol/L isradipine on the iron transport in MPP⁺-treated VM neurons. However, we did not find significant inhibition of iron influx by 200 nmol/L isradipine perfusion. It has been reported that 1 μ mol/L isradipine is a saturating concentration that is known to inhibit Cav1 channels, but not to disrupt the gating of other plasma membrane channels [41]. Both Cav1.2 and Cav1.3 Ca²⁺ channels are functionally expressed in DA neurons. It is still unclear which type of Ca²⁺ channel mediates iron transport in DA neurons. Further studies are needed to determine the sub-type selectivity of LTCC-mediated iron transport.

In addition, we found that the intensity of Ca²⁺ fluorescence in VM neurons was enhanced by iron stimulation, which suggested that iron promotes Ca²⁺ release and/or Ca²⁺ influx in VM neurons. It has been

reported that iron overload might delay LTCC inactivation and cause more Ca²⁺ influx in cardiomyocytes [40]. Therefore, we hypothesized that the iron-induced Ca²⁺ influx might further aggravate the iron-induced neurotoxicity in DA neurons.

Consistent with our hypothesis, we also found that the neurons reacted more to the stress of iron and Ca²⁺ co-application, which suggests that an increase in extracellular Ca²⁺ aggravates the iron-induced neurotoxicity by decreasing $\Delta\Psi$ m and aggravating intracellular oxidative stress. Ca²⁺ is an important second messenger and Ca²⁺ overload may lead to cell death. Our study focused on the effects of Ca²⁺ on iron-induced neuronal toxicity, and attempted to minimize the effect of Ca²⁺ itself on cell damage. So a relatively low Ca²⁺ concentration was chosen, based on preliminary studies. As expected, 500 μ mol/L Ca²⁺ itself did not induce significant changes in ROS, $\Delta\Psi$ m, and caspase-3 activation; however, it markedly increased the iron-induced toxicity. It has been shown that iron overload increases [Ca²⁺]_i and affects the calcineurin-dependent regulation of nuclear factor in the activated T-cell signaling pathway during cardiomyopathy [43]. Recently, Lee *et al.* demonstrated that iron overload results in the elevation of [Ca²⁺]_i. Chelation of Ca²⁺ by BAPTA attenuates the mitochondrial fragmentation and neuron death induced by iron overload [26, 44]. It is known that iron homeostasis is vital for cellular metabolism and important for maintaining cellular functions. Elevated cellular iron may induce hydroxyl radical production by the Fenton reaction, which in turn leads to mitochondrial dysfunction, oxidative stress, caspase-3 activation, and ultimately apoptosis in the pathogenesis of PD. Previous studies have shown that ROS production causes the release of Ca²⁺ by activating redox-sensitive Ca²⁺ channels [45, 46]. Munoz *et al.* [47] also showed that iron overload increases intracellular ROS levels and stimulates RyR-mediated Ca²⁺ release from the endoplasmic reticulum in primary hippocampal neurons. Lee *et al.* [26] confirmed that iron overload induces Drp1 (Ser637)-dependent mitochondrial damage and neuronal death via Ca²⁺ signaling, including Ca²⁺/calmodulin and

Ca^{2+} /calpain-calcineurin signaling. Taken together, all these studies support the concept that an interaction of iron and Ca^{2+} may aggravate the damage to neurons.

In conclusion, our studies indicate that LTCCs mediate iron influx in DA neurons. An elevation of extracellular Ca^{2+} concentration aggravates iron-induced neurotoxicity. The LTCC antagonist isradipine might protect neurons against iron-induced neurodegeneration.

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Conflict of interest The authors declare that they have no conflict of interest.

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