

Inhibition of Sirtuin 2 exerts neuroprotection in aging rats with increased neonatal iron intake

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Abstract

Impaired iron homeostasis may cause damage to dopaminergic neurons and is critically involved in the pathogenesis of Parkinson's disease. At present, very little is understood about the effect of neonatal iron intake on behavior in aging animals. Therefore, we hypothesized that increased neonatal iron intake would result in significant behavior abnormalities and striatal dopamine depletion during aging, and Sirtuin 2 contributes to the age-related neurotoxicity. In the present study, we observed that neonatal iron intake (120 µg/g per day) during postnatal days 10–17 resulted in significant behavior abnormalities and striatal dopamine depletion in aging rats. Furthermore, after AK-7 (a selective Sirtuin 2 inhibitor) was injected into the substantia nigra at postnatal 540 days and 570 days (5 µg/side per day), striatal dopamine depletion was significantly diminished and behavior abnormality was improved in aging rats with neonatal iron intake. Experimental findings suggest that increased neonatal iron intake may result in Parkinson's disease-like neurochemical and behavioral deficits with aging, and inhibition of Sirtuin 2 expression may be a neuroprotective measure in Parkinson's disease.

Key Words: nerve regeneration; Parkinson's disease; iron homeostasis disruption; aging; dopamine; corpus striatum; neurotoxicity; Sirtuin; AK-7; NSFC grants; neural regeneration

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Introduction

Parkinson's disease is one of the most prevalent age-related neurodegenerative diseases. Its main clinical features are resting tremor, rigidity, bradykinesia, and abnormal postural reflexes. Increasing evidence indicates that the causes of Parkinson's disease are multifactorial, including aging, genetic predisposition, exposure to environmental toxins, immune/inflammatory factors, and innate characteristics of the nigrostriatal dopaminergic system in the brain (Olanow and Tatton, 1999; Kidd, 2000; Gao et al., 2003; Wang et al., 2005a, b, 2007a, b, 2011; Connolly and Lang, 2014). Among multiple factors suspected to play a role in Parkinson's disease, aging is a major risk factor for idiopathic Parkinson's disease (Dillin and Kelly, 2007; Yankner et al., 2008; Gureviciene et al., 2009; Hindle, 2010). Epidemiological studies show that Parkinson's disease affects approximately 1–2% of the population over the age of 65 years, with incidence and prevalence further increasing with advancing age (von Campenhausen et al., 2005; Yankner et al., 2008; Hindle, 2010).

Sirtuins are NAD⁺-dependent protein deacetylases that regulate a variety of cellular functions (Saunders and Verdin,

2007; Milne and Denu, 2008; Outeiro et al., 2008; Finkel et al., 2009; Kalle et al., 2010). Sirtuin 2 is mainly distributed in the brain (Dillin and Kelly, 2007; Gan and Mucke, 2008; Finkel et al., 2009) and has been shown to be associated with the aging process and age-related neurodegeneration (Longo and Kennedy, 2006; Dillin and Kelly, 2007; Gan and Mucke, 2008; de Oliveira et al., 2010). AK-7 is a cell- and brain-permeable selective Sirtuin 2 inhibitor (Taylor et al., 2011) and was used to investigate the effects of Sirtuin 2 inhibition on striatal dopamine depletion and behavioral abnormalities in aging rats with increased neonatal iron intake.

Iron is an essential trace metal. It plays an important role in electron transfer, oxygen transport, neurotransmitter synthesis, and myelin production in the central nervous system (Stankiewicz et al., 2007; Xiong et al., 2012). However, impaired iron homeostasis may be harmful to neurons, especially dopaminergic neurons (Stankiewicz et al., 2007; Snyder and Connor, 2009; Lee and Andersen, 2010). Iron dyshomeostasis is associated with the etiopathogenesis of Parkinson's disease (Barnham and Bush, 2008; Rhodes and Ritz, 2008; Bolognin et al., 2009; Snyder and Connor, 2009).

Insufficient iron content can lead to iron-deficient anemia (Anand et al., 2014), and severe iron deficiency early in life can result in impaired brain development (Lozoff and Georgieff, 2006; Radlowski and Johnson, 2013). Children who are not breast-fed or who are partially breast-fed should be given an iron-fortified formula. For these reasons, it is of interest to investigate the long-ranging effects of neonatal iron intake in adulthood and senescence. Kaur et al. (2007) reported that elevated neonatal iron intake in mice contributed to age-related neurodegeneration similar to Parkinson's disease. However, little is known about the effect of neonatal iron treatment on motor behavior in aging animals. Thus, we hypothesized that increased neonatal dietary iron may result in behavior abnormalities and striatal dopamine depletion during aging, and Sirtuin 2 may be involved in the age-related neurotoxicity.

Materials and Methods

Iron intake and Sirtuin 2 inhibitor intervention

All animals were provided from Sino-British SIPPR/BK Lab Animal, Shanghai, China. All experiments were performed according to the Guide for *the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the Guideline for Animal Experimentation of Shanghai Jiao Tong University School of Medicine (China). Seventy male and female, specific-pathogen free, Sprague-Dawley rat pups were maintained in a temperature-controlled (21–22°C) room with a 12-hour light/dark cycle (lights on: 06:00–18:00). Ambient humidity was set between 30% and 70%. Sprague-Dawley rat pups were fed either saline vehicle ($n = 20$) or carbonyl iron ($n = 50$) daily by oral gavage from postnatal days 10 to 17. Previous studies (Kaur et al., 2007) demonstrated that increased murine neonatal iron intake (120 µg/g per day) resulted in Parkinson's disease-like neurodegeneration during aging, so the rat pups in this study were fed an increased iron diet (120 µg/g per day; Sigma-Aldrich, St. Louis, MO, USA). Rats were assigned to young ($n = 20$, 10 non-fed rats and 10 high iron-fed rats) and aging ($n = 50$, 10 non-fed rats and 40 high iron-fed rats) groups. The rats in the young and aging groups were aged to 170 days and 615 days, respectively, and behavior tests were conducted on the rats. The rats were then sacrificed for further experiments.

At the age of 540 and 570 days, respectively, 20 aging rats received intranigral injections of a selective Sirtuin 2 inhibitor, 3-(1-azepanylsulfonyl)-N-(3-bromophenyl) benzamide (AK-7) (Sigma-Aldrich) in both hemispheres, 1 µg/side per day ($n = 10$) or 5 µg/side per day ($n = 10$), respectively. The aging rats were anesthetized with ketamine and xylazine (60 mg/kg and 3 mg/kg, respectively; Sigma-Aldrich) *via* intramuscular injection and were positioned in a stereotaxic apparatus (Narishige Scientific Instrument Lab, Tokyo, Japan). Then, AK-7 (2 or 10 µg, respectively) was dissolved in DMSO (4 µL) or vehicle (4 µL of DMSO), respectively, and was injected into the substantia nigra at a flow rate of 1 µL/min using a 10-µL microsyringe (Hamilton, Bonaduz, Switzerland), with 2 µL volume of intranigral injection per hemisphere. The following coordinates were used: anterior-

or-posterior –5.4 mm, medial-lateral ±2.1 mm, dorsal-ventral –7.8 mm (Manfredsson et al., 2009; Klein et al., 2010). The needle was left in place for 5 minutes to avoid reflux along the injection track prior to being withdrawn.

Behavior tests

Rotarod performance test and open field test were conducted to evaluate rat behaviors during the light period (Graham and Sidhu, 2010). The basic requirements for the rotarod test consisted of a power source, a roller, and four separators dividing the roller into equal-sized compartments (IITC Life Science, Woodland Hills, CA, USA). Following training, the rats were tested three times at rotarod speeds of 5, 10, and 15 rotations per minute (r/min), respectively. The latency time to fall was recorded for each test. For locomotor activity, each rat was placed into an open field chamber made of wood covered with impermeable Formica. The chamber had a white floor (100 cm × 100 cm) divided into 25 squares (20 cm × 20 cm) and 50-cm-high walls. Before testing, each animal was placed in the center of the open field and habituated for 10 minutes. Rat motor behavior was recorded for 30 minutes. The following parameters were evaluated: (1) number of crossings: entering of another square with all four paws; (2) number of rearings: rearing with and without wall contact (standing only on hind legs).

High-pressure liquid chromatography-ECD analysis of dopamine content

High-pressure liquid chromatography-ECD was used to assay neurotransmitter content in the rat striata. Briefly, rat striata were dissected on ice and weighed. The striata were then homogenized (10%, w/v) through sonication in ice-cold homogenization buffer containing perchloric acid (0.1 mol/L). 3,4-Dihydroxybenzylamine was used as the internal standard. Obtained samples were centrifuged at 25,000 × *g* for 10 minutes at 4°C and the supernatants were collected. Dopamine and serotonin (5-hydroxytryptamine) content were detected by high-pressure liquid chromatography (Eicom, Kyoto, Japan) with an electrochemical detector, equipped with a column of 5 µm spherical C18 particles. The mobile phase was composed of 0.1 mol/L phosphate buffer (pH 2.6) containing 2.5% methanol, 0.2 mmol/L octane sulfonic acid, and 4.5% acetonitrile. Dopamine content was expressed as ng/g equivalent striatal tissue. The percentage of the detected concentrations of dopamine and serotonin to baseline levels was defined as contents of dopamine and serotonin in the striata of aging rats.

Statistical analysis

The GraphPad Prism 5.0 (GraphPad software, San Diego, CA, USA) program was used for statistical analyses. Data were expressed as the mean ± SEM. Differences were determined using the two-tailed Student's *t*-test for comparison between two groups and an analysis of variance and Bonferroni *post hoc* test for comparison between more than two groups. Normality of sample distribution and homogeneity of variances were tested before each analysis of variance. Values of $P < 0.05$ were considered statistically significant.

Results

Increased neonatal iron intake resulted in age-related behavior abnormalities and striatal dopamine depletion in rats

The rotarod performance test and open field test were performed to evaluate the effect of neonatal iron intake on motor behavior in young and aging rats. As shown in **Figure 1**, neonatal iron intake had no impact on behavior changes in young rats compared with the vehicle-treated rats. However, significant decreases in latency and the number of crossings and rearings were observed in aging rats with neonatal intake of the same dose of iron compared with the vehicle-treated rats ($P < 0.01$; **Figure 1**). In agreement with the behavioral tests, neonatal iron intake did not result in significant striatal dopamine depletion in young rats compared with the vehicle-treated rats (**Figure 2A**). However, significantly decreased striatal dopamine content was observed in aging rats with neonatal iron intake compared with the vehicle-treated rats ($P < 0.01$; **Figure 2A**). No significant change in striatal serotonin level was observed in aging rats with neonatal iron intake compared with vehicle-treated rats ($P > 0.05$; **Figure 2B**).

AK-7 was neuroprotective in aged rats with increased neonatal iron intake

As shown in **Figure 3**, although intranigral injection of AK-7 did not significantly change behavior abnormalities in aging rats with increased neonatal iron intake compared with vehicle-treated rats at a dose of 1 $\mu\text{g}/\text{side}$ per day, behavior abnormalities in aging rats with increased neonatal iron intake at 5 $\mu\text{g}/\text{side}$ per day were significantly improved ($P < 0.01$). In agreement with behavior tests, neurochemical analysis results also showed that AK-7 administration significantly diminished striatal dopamine depletion in aging rats with increased neonatal iron intake compared with vehicle-treated rats ($P < 0.05$: 1 $\mu\text{g}/\text{side}$ per day, $P < 0.01$: 5 $\mu\text{g}/\text{side}$ per day; **Figure 4**).

Discussion

Many studies have shown that aging is one of the strongest risk factors for idiopathic Parkinson's disease (Dillin and Kelly, 2007; Yankner et al., 2008; Gureviciene et al., 2009; Hindle, 2010; Lu et al., 2013). Parkinson's disease is rarely seen before 50 years of age and its incidence and prevalence increase with aging. Aging people gradually manifest pathological features of Parkinson's disease, such as Lewy bodies, striatal dopamine reduction, and motor signs similar to those observed in Parkinson's disease (Guang et al., 2012; Yu et al., 2012). In mice with elevated neonatal dietary iron feeding, Kaur et al. (2007) observed increased substantia nigra iron content at 3 months of age, as well as increased markers of oxidative stress and reduced striatal dopamine content at 12, 16, and 24 months, but not at 2 months, when compared with vehicle-treated animals. They also observed significantly decreased tyrosine hydroxylase-immunoreactive neurons in iron-fed mice compared with vehicle-treated mice at 24 months of age, but not at 2, 12, or 16 months. In the present study, we observed that elevated neonatal iron (120 $\mu\text{g}/\text{g}$ per day) resulted in significant behav-

ior abnormalities and striatal dopamine depletion in aging rats, while there was no change in young rats. No significant change in striatal serotonin content was observed in aging rats with the same amount of neonatal iron supplementation. These data support and extend previous findings showing that increased neonatal iron supplementation, given enough time, might cause some features of Parkinson's disease. In addition, striatal serotonin content was not significantly affected in these aging rats, showing the selective neurotoxicity. Our results indicate that increased neonatal iron intake has long-lasting effects and could potentially represent a novel risk factor for age-related dopaminergic neurodegeneration. The potential toxic effects of elevated dietary iron in early life, as revealed by our study, should be taken into consideration and certainly warrant further studies in humans, especially as they impact neurological function in aging individuals. It is of interest to develop effective therapeutic strategies to attenuate age-related dopaminergic neurotoxicity as a consequence of increased neonatal iron exposure.

Sirtuins are a family of seven distinct NAD⁺-dependent deacetylase enzymes with homology to the yeast SIR2 (Outeiro et al., 2008; Finkel et al., 2009; Harting and Knöll, 2010). Growing evidence indicates that sirtuins participate in the regulation of a variety of biological activities (Saunders and Verdin, 2007; Milne and Denu, 2008; Outeiro et al., 2008; Finkel et al., 2009; Kalle et al., 2010). Recent studies have suggested a role for sirtuins during the aging process and age-related neurodegeneration (Longo and Kennedy, 2006; Dillin and Kelly, 2007; Gan and Mucke, 2008; de Oliveira et al., 2010). Among the seven sirtuins, Sirtuin 2 is strongly expressed in the brain (Dillin and Kelly, 2007; Southwood et al., 2007; Gan and Mucke, 2008; Pandithage et al., 2008; Finkel et al., 2009; Maxwell et al., 2011). Sirtuin 2 has been shown to be expressed in the cytoplasm, and not the nucleus, of neurons and oligodendrocytes (Li et al., 2007; Southwood et al., 2007; Werner et al., 2007; Pandithage et al., 2008), and Sirtuin 2 overexpression decreases survival of healthy neurons (Pfister et al., 2008). Sirtuin 2 inhibition was identified as a promising approach for treating Huntington's disease (Luthi-Carter et al., 2010; Taylor et al., 2011; Chopra et al., 2012). Outeiro et al. (2007) reported that Sirtuin 2 inhibition ameliorates α -synuclein-induced toxicity in three different models (*in vitro* and *in vivo*) relevant to Parkinson's disease. AK-7 is a brain-penetrating, selective, Sirtuin 2 inhibitor (Taylor et al., 2011), which has been shown to attenuate mutant Huntingtin fragment-induced neurodegeneration (Taylor et al., 2011). AK-7 treatment results in decreased brain atrophy, extended survival, and improved motor behavior in two genetic mouse models of Huntington's disease (Chopra et al., 2012). Based on its properties and results from previous studies (Taylor et al., 2011; Chopra et al., 2012), AK-7 was used as a Sirtuin 2 inhibitor in the present study. The results showed that selective Sirtuin 2 inhibition significantly diminished striatal dopamine depletion and improved behavior abnormality in aging rats with increased neonatal iron intake, suggesting the potential dopaminergic neuroprotection of AK-7 in Parkinson's disease.

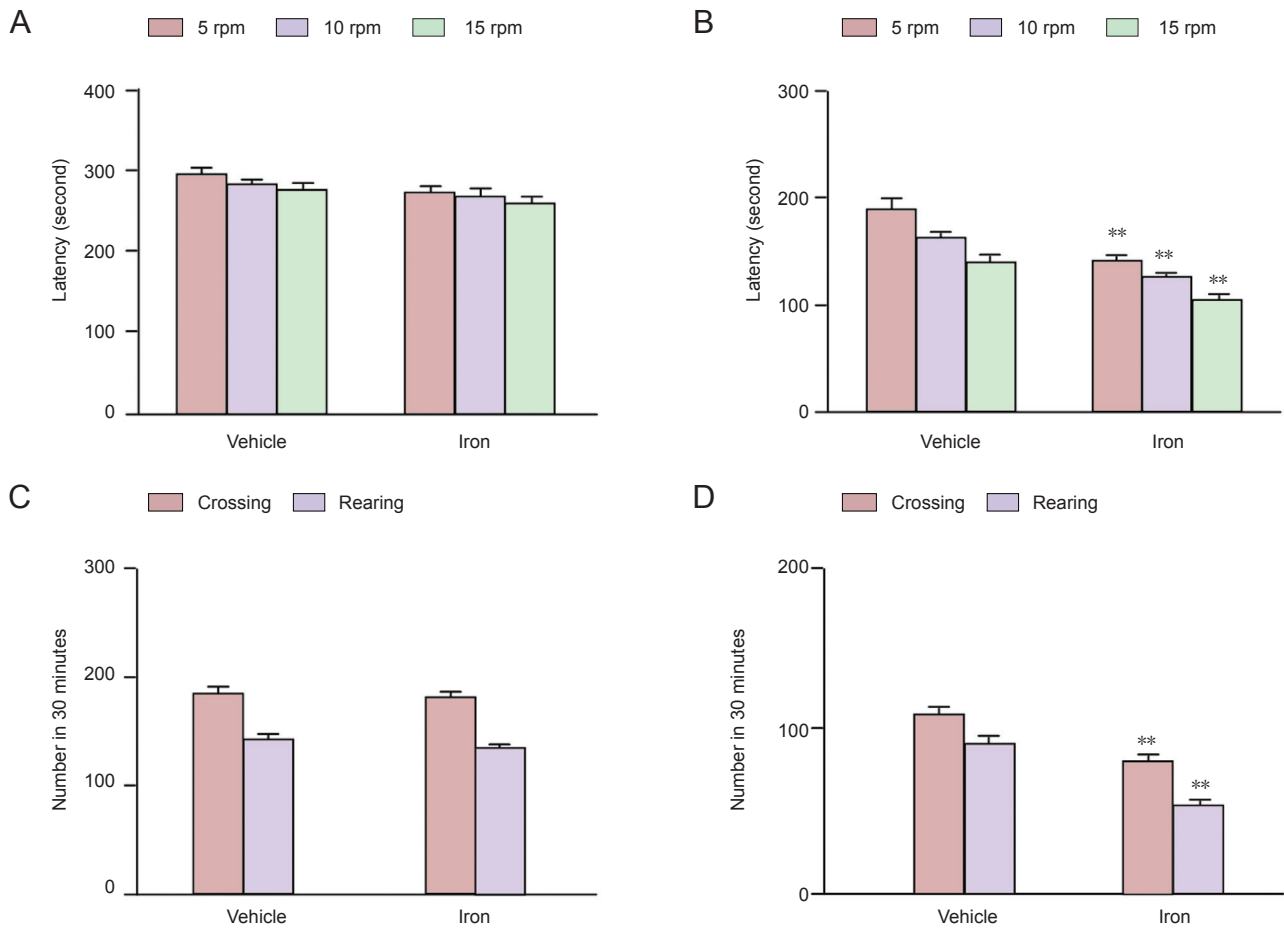


Figure 1 Increased neonatal iron intake resulted in age-related behavior abnormalities.

(A, B) Rotarod test in young (170 days old) and aging (615 days old) rats, respectively; (C, D) open field test in young (170 days old) and aging (615 days old) rats, respectively. Data are expressed as the mean \pm SEM. (A, C) There were 20 young rats: 10 rats with neonatal iron intake and 10 vehicle-treated rats (B, D) and 20 aging rats: 10 rats with neonatal iron intake and 10 vehicle-treated rats. Differences were determined using the two-tailed Student's *t*-test for comparison between two groups and an analysis of variance and Bonferroni *post hoc* test for comparison between more than two groups. ** $P < 0.01$, vs. aging rats treated with vehicle. rpm: Rotation/min.

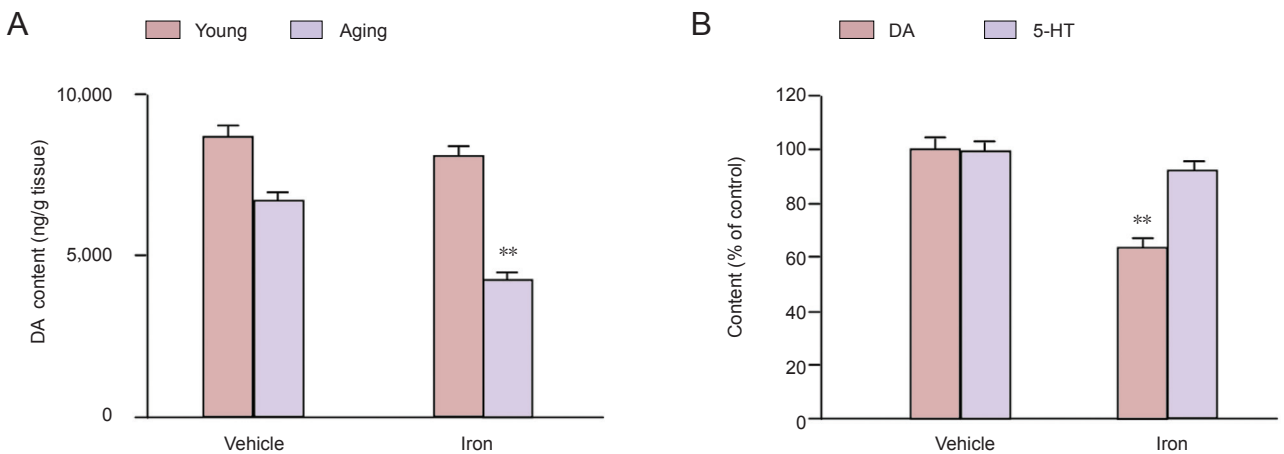


Figure 2 Increased neonatal iron intake in rat pups resulted in age-related striatal dopamine (DA) depletion in rats.

(A) DA content. (B) DA and serotonin (5-HT) content in aging rats. Data are expressed as the mean \pm SEM. (A) There were 20 young rats (10 rats with neonatal iron intake and 10 vehicle-treated rats) and 20 aging rats (10 rats with neonatal iron intake and 10 vehicle-treated rats). (B) There were 20 aging rats: 10 rats with neonatal iron intake and 10 vehicle-treated rats. Differences were determined using the two-tailed Student's *t*-test for comparison between two groups and an analysis of variance and Bonferroni *post hoc* test for comparison between more than two groups. ** $P < 0.01$, vs. aging rats treated with vehicle.

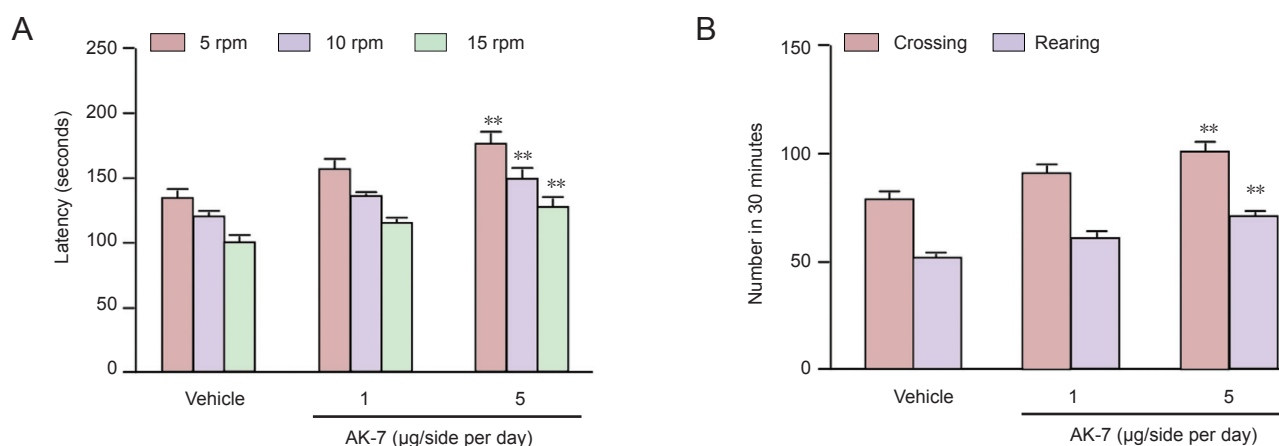


Figure 3 Effect of AK-7 treatment on motor behavior of aging rats with increased neonatal iron intake in rotarod test (A) and open field test (B). Data are expressed as the mean \pm SEM. Ten aging rats received neonatal iron, and 20 aging rats received both neonatal iron and AK-7 (a selective Sirtuin 2 inhibitor) administration at postnatal days 540 and 570. Among the 20 aging rats, 10 rats were injected with 1 μ g AK-7/side per day, and the remaining 10 rats were injected with 5 μ g AK-7/side per day. Differences were determined using the two-tailed Student's *t*-test for comparison between two groups and an analysis of variance and Bonferroni *post hoc* test for comparison between more than two groups. Normality of sample distribution and homogeneity of variances were tested before each analysis of variance. ** $P < 0.01$, vs. aging rats treated with vehicle. rpm: Rotation/min.

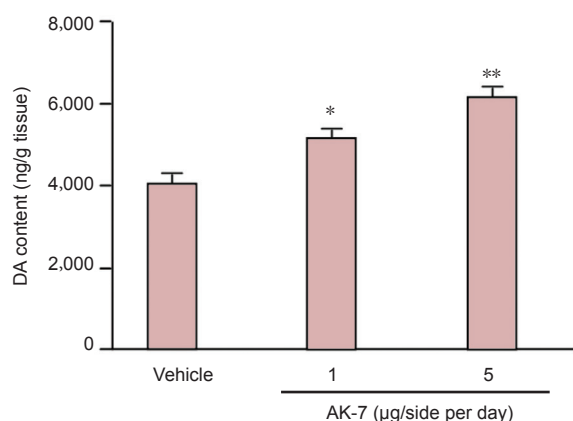


Figure 4 Effect of AK-7 treatment on striatal dopamine (DA) content of aging rats with increased neonatal iron intake.

Data are expressed as the mean \pm SEM. There were 10 vehicle-treated aging rats without neonatal iron, and 20 aging rats receiving both neonatal iron and AK-7 (a selective Sirtuin 2 inhibitor) administration at postnatal days 540 and 570. Among the 20 aging rats, 10 rats were injected with 1 μ g AK-7/side per day, and the remaining 10 rats were injected with 5 μ g AK-7/side per day. Differences were determined using the two-tailed Student's *t*-test for comparison between two groups and an analysis of variance and Bonferroni *post hoc* test for comparison between more than two groups. Normality of sample distribution and homogeneity of variances were tested before each analysis of variance. * $P < 0.05$, ** $P < 0.01$, vs. aging rats treated with vehicle. rpm: Rotation/min.

In summary, the results from our study suggest that increased neonatal iron intake may result in Parkinson's disease-like neurochemical and behavioral deficits with aging, and AK-7 may be neuroprotective in Parkinson's disease. We will further investigate this age-related neurotoxicity and its underlying mechanisms through other detection methods in our future research. Further studies will bring us a greater understanding of the potential role of Sirtuin 2 in the aging process and Parkinson's disease, as well as the development of effective therapeutic strategies to slow the progression of aging and Parkinson's disease neurodegeneration (Lavu et al., 2008; Outeiro et al., 2008; Han, 2009; Donmez and Outeiro, 2013).

Author contributions: Wang XJ and Liu ZG designed the study and wrote the paper. Wang XJ, Wang MH, Yang L, Bai J, Yan ZQ and Zhang YH performed the experiments and data analysis. All authors approved the final version of the manuscript.

Conflicts of interest: None declared.

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