Silver nanoparticle exposure induces rat motor dysfunction through decrease in expression of calcium channel protein in cerebellum

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HIGHLIGHTS

- Silver nanoparticles cause cerebellar ataxia-like symptom in rats.
- Silver nanoparticles induce rat motor dysfunction.
- Silver nanoparticles decrease calcium channel protein expression in rat cerebellum.

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ABSTRACT

Silver nanoparticles (AgNPs) are currently used widely, however, their impact on central nervous system still remains ambiguous and needs to be elucidated. This study is performed to investigate the neurotoxicity of AgNPs and illustrate the potential molecular mechanism. Neonatal Sprague–Dawley (SD) rats are exposed to AgNPs by intranasal instillation for 14 weeks. It is demonstrated that AgNPs exposure causes cerebellar ataxia like symptom in rats, evidenced by dysfunction of motor coordination and impairment of locomotor activity. Observation of cerebellum section reveals that AgNPs can provoke destruction of cerebellum granular layer with concomitant activation of glial cells. AgNPs treatment decreases calcium channel protein (CACNA1A) levels in cerebellum without changing potassium channel protein (KCNA1) levels. The levels of silver in rat cerebellum tissue are correlated with exposure doses. *In vitro* experiments reveal that AgNPs treatment significantly reduces the protein and mRNA levels of CACNA1A in primary cultured cerebellum granule cells (CGCs). These findings suggest that AgNPs-induced rat motor dysfunction is associated with CACNA1A expression decrease, which reveals the underlying molecular mechanism for the neurotoxicity of AgNPs. Possible counteractions may accordingly be suggested to attenuate the unexpected harmful effects in biological applications of AgNPs.

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Abbreviations: AgNPs, silver nanoparticles; SD, Sprague–Dawley; CACNA1A, calcium channel protein; KCNA1, potassium channel protein; CGCs, cerebellum granule cells; BBB, blood–brain barrier; CNS, central nervous system; ICP-MS, inductively coupled plasma mass spectrometry; TEM, transmission electron microscope; H&E, hematoxylin–eosin; IHC, immunohistochemistry; PFA, paraformaldehyde; GFAP, glial fibrillary acidic protein; HRP, horseradish peroxidase; DLS, dynamic light scattering; PDI, polydispersity index.

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1. Introduction

Due to silver nanoparticles' excellent antibacterial activity, they are becoming more and more popular in various fields (Prucek et al., 2011). Despite their wide application, information on their environmental and human health effect is limited. Previous studies revealed that silver nanoparticles (AgNPs) were able to be translocated by blood circulation and distributed throughout the main organs in the form of particles (Tang et al., 2009). Given that AgNPs are capable of permeating the tight blood–brain barrier (BBB) and entering the brain tissue (Tang et al., 2010), the issue on assessing the specific responses of neurons to AgNPs is of great

relevance. Animal studies proved that intravenous injection of AgNPs in Sprague–Dawley (SD) rats caused the functional injury of central nervous system (CNS) by decreasing their locomotor activity (Zhang et al., 2013), showing the potential neurotoxicity of AgNPs.

AgNPs containing nasal drops have been utilized for the treatment of coryza in clinic (Damiani et al., 2011), which introduces a special route for AgNPs exposure. Nasal administration may deliver therapeutic agents preferentially to the brain, which has gained significant interest (Graff and Pollack, 2005). This technique provides a practical, non-invasive method of bypassing the BBB and allows agents to be delivered to the CNS within minutes. The intranasal delivery of AgNPs was demonstrated to induce impairment of hippocampus function in rats (Liu et al., 2012). Clinical case study showed slight sign of cerebellar ataxia in argyria patient using nasal drug administration (Aaseth et al., 1981). Characterized by the impaired coordinate balance, cerebellum ataxia is a non-specific clinical manifestation implying partial dysfunction of the nervous system that coordinates movement, such as the cerebellum (Schmahmann, 2004). Mounting evidences have given some important clues to pathogenic mechanisms in cerebellum ataxia, such as disruption of voltage-gated potassium and calcium channels (Jen et al., 2007; Mori et al., 1991). Our previous study found that AgNPs exposure could attenuate the viability of rat cerebellum granule cells (CGCs) through apoptosis (Yin et al., 2013). Consequently, uncovering *in vivo* neurological deficits induced by AgNPs and understanding the underlying mechanism are of much significance for finding out the solution to their potential harmful effects.

Our objectives in this study were to evaluate neurobehavioral dysfunction in AgNPs exposed rats by nasal administration, examine their neural pathological effects and elucidate the potential molecular mechanism.

2. Materials and methods

2.1. AgNPs characterization

AgNPs coated with citrate (1 mg/mL) was purchased from Sigma–Aldrich Co. LLC (St. Louis, MO, USA). Characterization of AgNPs was performed by morphological observation through transmission electron microscope (TEM, Hitachi H-7500, Japan) as well as particle size distribution and zeta-potential measurement by Malvern Zetasizer Nano ZS (Malvern, UK) at 25 °C. The stock solution was kept at 4 °C and freshly diluted with distilled water for *in vivo* studies or cell culture medium for cell experiments after 30-min sonication when used.

2.2. Animals

Neonatal SD rats with their mother rats were purchased from Peking University Health Science Center. All animals were maintained in accordance with the principles of care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of Peking University. The whole animal room was maintained at 25 °C, <70% of humidity and 12 h/12 h light/dark cycle. Neonatal animals were housed in clear plastic cages with their mother for normal breastfeeding. Twenty eight days later, the weaned animals were transferred to new cages with male and female rats housed in different cages.

2.3. Exposure and sacrifice protocols

The neonatal rats (initially weighting 4–5 g) were set as control, 0.1, 0.2, 0.5 and 1 mg/kg/day of AgNPs exposure groups, respectively. The exposure dose selection in animal studies was based on the preliminary experiments (data not shown) and the guidance on Dose Level Selection for Regulatory General Toxicology Studies for Pharmaceuticals. The number and gender of the rats in each group were listed in Table 1. The total exposure period lasted for 14 consecutive weeks. AgNPs working solutions were administered by intranasal instillation once a day. Equivalent volume of distilled water was used as the vehicle control. The animal body weight was monitored. Rotarod and open field test were performed in the last 2 weeks before exposure termination. The rats were sacrificed by CO₂ and the cerebellum tissues were harvested after 60 mL of cold PBS intracardiac perfusion. The cerebellum were either fixed in 4% paraformaldehyde or frozen at –80 °C for further analysis.

2.4. Rotarod test

The motor coordination of rats was evaluated by rotarod (Panlab Le8500 Harvard Apparatus, Spain). We performed the tests in both the constant speed mode (5, 10, 15 and 20 rpm) and acceleration speed mode (increasing from 4 rpm to 40 rpm during 10 min). The maximum test time of 5 min was set for the constant speed mode. The rats were habituated on the horizontal rotating rod and pre-trained for 3 trials before the formal tests. All the rats were tested 3 trials per day for 7 consecutive days. The apparatus automatically recorded the elapsed time when the rat fell to the base of the apparatus from the spindle.

2.5. Open field test

The open field test was performed for all the rats according to the protocol suggested by Zhang et al. (2013). The dimension of

Table 1
The behavior evaluation for the control and AgNPs exposed rats in open field test.

Behavior parameters	AgNPs concentration (mg/kg/day)				
	0 ^a	0.1 ^b	0.2 ^c	0.5 ^d	1 ^e
Total moving distance (m)	162.5 ± 21.9	132.6 ± 11.1	118.2 ± 13.1 [*]	88.0 ± 7.9 ^{**}	48.8 ± 8.4 ^{**}
Moving velocity (cm/s)	27.9 ± 5.3	25.1 ± 1.9	22.6 ± 2.7	18.1 ± 0.9 [*]	11.1 ± 1.4 ^{**}
The number of entries into center (times)	20.7 ± 3.1	13.5 ± 4.9	9.3 ± 2.5 [*]	4.8 ± 1.1 ^{**}	0.6 ± 0.9 ^{**}
Resting time (s)	82.9 ± 9.4	97.7 ± 2.7	105.1 ± 12.7	127.6 ± 15.6 [*]	172.8 ± 28.1 [*]
Rearing frequency (times)	68.9 ± 20.7	53.5 ± 17.5	45.9 ± 19.7	30.0 ± 10.7 [*]	19.2 ± 3.4 [*]

^a The number of the rats in each group. *n* = 13 (male: 7, female: 6).

^b The number of the rats in each group. *n* = 12 (male: 8, female: 4).

^c The number of the rats in each group. *n* = 14 (male: 9, female: 5).

^d The number of the rats in each group. *n* = 13 (male: 6, female: 7).

^e The number of the rats in each group. *n* = 14 (male: 7, female: 7).

^{*} *p* < 0.05 versus negative control.

^{**} *p* < 0.005 versus negative control.

open field was 100 cm (L) × 100 cm (W) × 40 cm (H), constructed of black PVC plastic. The clear plexiglas cubes were surrounded by two photobeam matrices, one of which measured horizontal activity and the other measured vertical activity (*i.e.*, rearing). These were interfaced with a computer for data collection. Spontaneous horizontal and vertical activities as well as stereotyped movements of rats were measured using a video tracking system (SMART, Panlab Harvard Apparatus, Spain). The computer software defines grid lines that divide the open field into center area (70 cm × 70 cm) and peripheral area (a gallery 30 cm wide). The rat was placed individually into a corner of the open field, and their behavior was continuously recorded for 10 min in the monitoring area. This test was performed once a day for each rat and continued for 7 consecutive days. Endpoints subjected to statistical analysis were moving distance, velocity, entry numbers between the surrounding area and the center area, resting time and rearing frequency.

2.6. Neuropathological examination

To study the neuropathological alterations in AgNPs exposed rat, the cerebellum tissue samples were harvested for hematoxylin–eosin (H&E) staining and immunohistochemistry (IHC) evaluation. According to previously reported protocol (Todorov et al., 2012), the samples were processed by fixation with 4% paraformaldehyde (PFA), dehydration with series of ethanol solution, infiltration and embedding in paraffin. The 5- μ m-thick sections of the cerebellum were cut and subsequently processed with H&E staining. After the morphological screening, three paraffin embedded slides from each group were submitted to immunohistochemistry evaluation. The sections were processed with deparaffinization, dehydration, antigen retrieval and blocking. The primary antibody to glial fibrillary acidic protein (GFAP) (Abcam, UK, 1:1000) was used for overnight incubation. Subsequently, sections were washed and incubated with a secondary horseradish peroxidase (HRP)-labeled antibody (goat anti rabbit IgG, Santa Cruz, USA, 1:5000) for 30 min. The slides were washed and subsequently developed with the substrate (1% DAB and 0.3% H₂O₂, 1:1) for 5 min and counter stained with hematoxylin. The images were photographed by a laser scanning confocal microscope (Leica, TSC SP5, USA).

2.7. Silver levels in rat cerebellum

The total silver content in cerebellum tissue was measured using microwave-assisted digestion method by ICP-MS (Agilent 7500ce, USA). Briefly, approximate 0.1–0.2 g of cerebellum sample from each group ($n=6$ per group, 3 male and 3 female) was digested by the mixture of HNO₃ and H₂O₂ (1:1) at a temperature programming (120 °C hold for 10 min, increase within 10 min to 180 °C and hold for 20 min). Afterwards, the acid solution was evaporated under 120 °C to nearly dry and the residue was redissolved in 3% HNO₃ solution. Silver levels in the samples were quantitatively measured by ICP-MS. The chamber temperature was set at 2 °C and RF power was 1500 W, the flow rate of carrier gas and makeup gas were 1.1 L/min and 0.1 L/min, respectively.

2.8. Cell culture

The primary cultured cerebellum granule cells (CGCs) were prepared according to the protocol reported previously (Yin et al., 2013). Briefly, the cerebellum granule cells dissociated from 7-day-old SD rat pups were plated in 6-well plates (1×10^6 cells/mL) precoated with poly-L-lysine (0.01%, Sigma–Aldrich, USA). The cells were cultured in DMEM-F12 (Hyclone, USA) supplemented with horse serum (5%, GIBCO, USA), fetal bovine serum (5%, GIBCO, USA),

KCl (25 mM, Sigma–Aldrich, USA), L-glutamine (1%, Hyclone, USA) and antibiotics (penicillin 100 U/mL and streptomycin 100 μ g/mL, GIBCO, USA) under the condition of 5% CO₂ at 37 °C. Around 18–22 h later, arabinofuranosylcytosine (10 μ M, Fluca, USA) was added to prevent the proliferation of non-neuronal cells. The cells were continued with 7 days' culture for the formation of synapses, and subsequently submitted to the exposure experiments.

2.9. Cell stimulation

According to the preliminary studies using Alamar Blue assay, the cell stimulation was performed at non-cytotoxic levels of AgNPs (0, 0.01, 0.02, 0.05 and 0.1 μ g/mL) for 24 h. The exposure medium was discarded and the cells were rinsed twice with cold phosphate-buffered saline and harvested for the analysis of protein expression or CACNA1A mRNA levels. Triplicate experiments were performed independently for each assay.

2.10. Ataxia-related protein analysis

Cerebellum tissue samples from different exposure groups ($n=6$ per group, 3 male and 3 female) were homogenized with cold RIPA buffer (Sigma–Aldrich, USA) containing complete protease inhibitor (Roche, EDTA-free Protease Inhibitor Cocktail Tablets, USA). As for the CGCs samples, the cells from different treatments were lysed with RIPA buffer containing protease inhibitor. The protein extraction from the tissues or cell lysates was electrophoresed on 4–20% Mini-PROTEAN Precast Gels (BIO-RAD, USA), transferred to nitrocellulose membrane and blotted with anti-CACNA1A or anti-Kv1.1 potassium channel primary antibodies (Abcam, UK). Goat anti-rabbit IgG HRP-conjugate (Santa Cruz, USA) was used as a secondary antibody. Bands were detected by ECL (PIERCE, USA) and the densitometric analysis of western blots was performed using ImageJ software (<http://rsbweb.nih.gov/ij/>) quantifying mean gray values subtracted from background.

2.11. CACNA1A mRNA analysis

Total RNA from CGCs with different treatments was extracted using TRIzol Reagent (Ambion, USA). After quantitatively analysis of RNA concentration in each sample using NanoDrop 2000 (Thermo Scientific, USA), complementary DNA was synthesized from 2 μ g of total RNA by using reverse transcriptase (Thermo Scientific RevertAid First Strand cDNA Synthesis Kit, USA) following the manufacturer's recommended procedures. PCR primers specific for CACNA1A (5' TTC GTG CAG TCT TTC AAG GC 3', 5' CCG GAA GTT ATT GTG CTC CG 3') and reference gene glyceraldehydes 3-phosphate dehydrogenase (GAPDH) (5' GCC TCG TCT CAT AGA CAA GAT GG 3', 5' TGC TCC TGC TAC TTT AGA CTC CG 3') were added to the reaction (Invitrogen kit, USA) and the samples were amplified in TempCycler (Agilent Technologies, USA) as follows: 95 °C for 2 min, followed by 45 cycles of 95 °C for 15 s, 59 °C for 30 s, and 72 °C for 25 s; and a final cycle of 95 °C for 25 s, 59 °C for 25 s, and 95 °C for 30 s. The comparative Ct method was used to quantify fold changes in target gene transcription (Pfaffl, 2001). The gene transcription levels were measured in triplicate for each control or exposed cell cultures and each exposure was repeated for three times.

2.12. Statistical analysis

The results were analyzed by student's *t*-test or ANOVA test for the evaluation of significant difference in different treatments. A *p* value less than 0.05 was considered as statistically significant, while a *p* value less than 0.005 was considered as highly significant. The dose-response was evaluated by Pearson correlation analysis.

The value of r in the range of 0.8–1.0 indicated a clear positive dose-dependent relationship, while that in the range of -1.0 to -0.8 meant a negative correlation.

3. Results

3.1. AgNPs characterization

Morphology characterization by TEM showed that AgNPs used herein were spherical and mostly uniform in size (around 20 nm in diameter) and the particle size of AgNPs was 23.3 ± 0.9 nm in cell culture medium based on the dynamic light scattering (DLS) measurement (Fig. 1). Similar size was detected for AgNPs dispersed in distilled water (22.1 ± 0.7 nm). The polydispersity index (PDI) value was less than 0.2. Zeta potential values of AgNPs were -17.2 ± 1.2 mV in culture medium and -12.4 ± 2.3 mV in aqueous solution, suggesting the suspensions were stable during the experiment procedures.

3.2. AgNPs induced neuronal behavioral changes

During 14 weeks' exposure of AgNPs, we monitored the body weight of the rats in each group. The results showed that there was no significant difference between different groups in the first 4 weeks. The average body weight gain in AgNPs treatment groups was slightly lower than that in the control during the following 8 weeks' treatment. At the end of the exposure experiment (14 weeks), the average body weights for male rats were 460.6 ± 11.1 g, 379.8 ± 59.6 g, 333.1 ± 16.4 g, 338.7 ± 31.2 g and 318.4 ± 53.9 g for the control, 0.1, 0.2, 0.5 and 1 mg/kg/day AgNPs exposure group, respectively. The average body weights for female rats were 364.7 ± 43.9 g, 290.8 ± 18.2 g, 246.9 ± 16.1 g, 254.0 ± 15.7 g and 233.7 ± 24.8 g for the groups followed by the same order described above. No obvious alterations in food intake or activities were found based on cage observation.

In order to study the potential neurological phenotype of AgNPs, the rotarod test was performed for motor coordination performance of the rats. Two modes including constant speed

mode and acceleration speed mode were tried. The results from constant mode test showed that the duration of rats staying on the rotarod (latency time) was decreased in a dose-related manner, and the increase of the rotate speed amplified the difference between the control and AgNP-treated groups (Fig. 2A). For example, when the speed was set at 20 rpm, the latency time of the control rats was 4.7 ± 0.1 min, while the AgNPs treated rats could barely keep the balance on the rotarod apparatus. Even for the lowest dose of AgNPs exposure (0.1 mg/kg/day), it reduced the latency time of the rats to 1.3 ± 0.5 min. The acceleration speed mode also significantly differentiated the motor coordination performance of the rats with AgNPs treatment from the control (Fig. 2B). Only control rats remained when the rod reached the maximal speed and their latency to fall was 7.5 ± 0.6 min. For AgNPs treated rats, the latency time dramatically decreased with the increase of exposure doses. For instance, the duration of 1 mg/kg/day AgNPs treated rats on rotating rod was only 1.9 ± 0.2 min. These findings revealed that AgNPs were capable of affecting the motor coordination of rats in a dose-dependent manner.

The open field test showed AgNPs treatment obviously decreased the rats' locomotor activity in a dose-related manner compared to that of the control (Fig. 2C). Quantitative analysis shown in Table 1 indicated that AgNPs treated rats were less prone to move around in the new environment. For example, the total moving distance was 162.5 ± 21.9 m and 48.8 ± 8.4 m for the rats in the control and 1 mg/kg/day AgNPs exposed group, respectively. The moving velocity decreased from 27.9 ± 5.3 cm/s in the control to 11.1 ± 1.4 cm/s in the highest dose of exposure group. The endpoints such as total moving distance and rearing frequencies showed the dose-dependent decrease upon AgNPs treatment, while resting time exhibited the opposite change, e.g., the resting time of the rats in the tested area was 82.9 ± 9.4 s, 127.6 ± 15.6 s, and 172.8 ± 28.1 s for 0, 0.5 and 1 mg/kg/day AgNPs exposed groups, respectively. In addition, AgNPs treated rats also preferred to rest in the surrounding field rather than the center area. Comparison of the behavioral performance between male rats and female ones, no significant difference were observed.

Fig. 1. Characterization of AgNPs. (A) Transmission electron microscopy (TEM) image of AgNPs. Scale bar is 100 nm. (B) The particle size distribution of AgNPs dispersed in the culture medium with serum by dynamic light scattering (DLS).

Fig. 2. AgNPs exposure induced ataxia-like behavior phenotype in rats. The motor coordination of SD rats was analyzed by rotarod in constant speed mode (A) and acceleration speed mode (B). The time at which each rat fell off the rod was recorded after pre-training SD rats for 3 trials. All the rats were tested for 3 trials per day for 7 consecutive days. (C) The locomotor activity of SD rats was measured by open field test. The pink color represents the motion trajectory of rats. Animals were monitored for 10 min once a day for 7 consecutive days. The data were presented as mean $\pm$ SD. * $p < 0.05$ and ** $p < 0.005$, versus negative control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. Cerebellum histopathological examination and channel protein analysis

Histopathological examination for the cerebellum section (Fig. 3A) showed the control cerebellar cortex contained the normal morphology of three layers: the outer molecular layer containing stellate and basket cells, the middle Purkinje layer with a single layer of large Purkinje cells and the inner granular layer with numerous granule and Golgi cells. AgNPs treatment (1 mg/kg/day) caused obvious distortions in both Purkinje layer and granular layer. The granular layer was degenerated with loosen and separated structure, and the Purkinje layer was not distinct due to the deficiency of Purkinje cells.

Immunostaining of anti-GFAP for the cerebella cortex (Fig. 3B) indicated that GFAP expression was increased in the glial-rich molecular layer from AgNP-treated group when compared to that of the control, suggesting AgNPs could stimulate astrocytes activation or proliferation. As granule cells have dense nucleus, it may be characterized by compact blue staining in morphology when hematoxylin is used. Obviously, decreased blue color stained granule cells existed in AgNPs treated samples. These histological findings revealed that AgNPs *in vivo* were able to provoke destruction of the cerebellum granular layer with concomitant activation of glial cells.

To investigate the potential molecular mechanism for AgNPs induced neurobehavioral phenotype, some crucial ion channel proteins including calcium channel protein (CACNA1A) and potassium channel protein (KCNA1) in the cerebellum of rats were evaluated. Based on the quantitative analysis of gray levels, AgNPs (1 mg/kg/day) treatment could reduce CACNA1A level to $45.0 \pm 9.2\%$, while KCNA1 showed no difference between the control and AgNPs treated cerebellum samples (Fig. 3C and D),

depicting that AgNPs affected cerebella CACNA1A expression instead of KCNA1 in current treatments.

3.4. Silver accumulation in rat cerebellum

It has been shown that AgNPs were capable of invading the BBB, and then accumulated in different brain regions in the form of particles (Tang et al., 2009). To assess the penetration of AgNPs in rat cerebellum, the silver levels of cerebellum tissue were quantitatively analyzed by ICP-MS. The results demonstrated that silver levels were significantly higher in AgNPs treated groups than that in the control, which increased from $12.2 \pm 3.8 \mu\text{g/g}$ to $32.8 \pm 5.2 \mu\text{g/g}$ when the exposure dose increased from 0.1 to 1 mg/kg/day. These results revealed dose-related accumulation of silver levels existed in AgNPs treated rat cerebellum samples ($r = 0.987$, Fig. 4), which confirmed AgNPs could enter into rat brain and accumulate in the cerebellum tissue after intranasal instillation administration, thus causing potential direct stimulation on neuronal cells.

3.5. Ataxia-related proteins expression in vitro

To make sure if AgNPs exposure could directly induce the alterations of ion channel protein expression observed above, the primary cultured CGCs were used as the cell model for AgNPs exposure and CACNA1A protein expression was investigated by western blot. The results showed AgNPs exposure caused the dose-dependent decrease in CACNA1A expression ($r = -0.912$, Fig. 5A). When normalized by the control, CACNA1A levels in $0.05 \mu\text{g/mL}$ and $0.1 \mu\text{g/mL}$ of AgNPs treatment groups were $37.8 \pm 3.9\%$ and $25.4 \pm 2.0\%$, respectively. The examination on the mRNA level of CACNA1A in CGCs showed it was also inhibited by AgNPs exposure in a dose-related manner ($r = -0.944$, Fig. 5B).

Fig. 3. AgNPs exposure induced histopathological alterations in the rat cerebellum. (A) Pathological observations in cerebellum sections by H&E staining (400 $\times$). The cerebellum section of AgNP-treated group showed a degenerated granular layer with loosen and separated structure, as well as deficiency of Purkinje cells. (B) The cerebellum tissue immunostaining with anti-GFAP antibody (400 $\times$). Scale bar is 25 μ m for A and B. Western blot analysis of (C) voltage-gated calcium ion channel protein (CACNA1A) and (D) voltage-gated potassium channel protein (KCNA1) levels in the cerebellum of rats. The protein was detected using specific antibodies and the levels of corresponding protein were quantified based on gray levels of the band. Triplicate experiments were performed and the data were presented as mean $\pm$ SD. * p < 0.05 versus negative control.

Fig. 4. The dose-related accumulation of silver in the cerebellum of SD rats ($r = 0.987$). Total amount of silver in cerebellum tissue was measured by ICP-MS. The results were presented as mean $\pm$ SD. ** $p < 0.005$ versus negative control.

These findings gave us the hint that AgNPs might be directly involved in the dysfunction of calcium channel protein in granule cells, thus causing neurobehavioral alterations *in vivo*.

4. Discussion

Our findings illustrated that AgNPs-induced rat motor dysfunction was associated with the decrease in expression of calcium channel protein, revealing the potential neurotoxicity of AgNPs. Numerous studies have unequivocally demonstrated that AgNPs are capable of translocating into the blood circulation and accumulating in several organs to cause toxicity, with the deleterious mechanism of oxidative stress or mitochondria damage (Hsin et al., 2008). As for metal nanoparticles that can release metal ions, one important question is whether the toxicity is mediated by the particles or the released ions. Previous study indicates AgNPs may induce size dependent toxicity (Pratsinis et al., 2013). When the particle size is larger than 10 nm, the toxicity of AgNPs is primarily attributed to the dispersed particles rather than the released species due to its limited Ag^+ release (Pratsinis et al., 2013). It is further confirmed by Cronholm et al.

(2013), who showed that AgNPs entered into the cells in the form of particles, through intracellular uptake, with low amount of silver ion release. This is in line with earlier findings that particles with poor solubility can be stable for a long time inside the cell and exert toxicity through reactions on the particle itself (Krug and Wick, 2011). Herein, it could be speculated that AgNPs per se contributed to the toxicity. Similar result was reported for aluminum and copper nanoparticles which exerted biological functions by a nanoparticle-specific mechanism due to the presence of metal-containing nanoparticles (Sharma et al., 2009; Zhang et al., 2011). Moreover, with small size, particles on the nanoscale were able to enter into the brain by disruption of the BBB or be taken up directly into the brain by trans-synaptic transport (Hoet et al., 2004; Oberdorster et al., 2004). It has been shown that AgNPs could induce BBB inflammation and increase permeability in primary rat brain microvessel endothelial cells (Trickler et al., 2010). AgNPs in rats with subcutaneous injection were capable of invading the BBB, and then accumulated in brain regions in the form of nanoparticles, which was evidenced by the presence of AgNPs in neuronal cells (Tang et al., 2009). Besides, *in vivo* studies indicated that AgNPs through the nasal administration had the capacity to induce impairment of hippocampus function, owing to that inhaled nanoparticles could reach the brain through nasopharyngeal system (Liu et al., 2012; Oberdorster et al., 2004). Our current study suggested intranasal instillation could introduce AgNPs into the brain and the occurrence of silver in rat cerebellum was in a dose-related manner, providing an opportunity for the direct stimulation of AgNPs on neuronal cells, thus causing the potential neurotoxicity *in vivo*.

Due to the pivotal role of CNS, the neurotoxicity of AgNPs has now obtained increasing research interest. Some previous studies have reported decreased body weight and locomotor activity in AgNPs exposed adult male rats by tail vein injection (Zhang et al., 2013). It was much of importance to characterize the AgNPs-induced neurobehavioral phenotype and the potential molecular mechanism. Herein, we found both coordination performance and locomotor activity were affected in AgNPs exposed rats though the intranasal instillation. The reduction of motor coordination is ascribed to dysfunction of cerebellum, typically manifesting with

Fig. 5. The influence of AgNPs on the protein and mRNA levels of CACNA1A in CGCs. (A) Dose-related inhibition of CACNA1A protein expression in CGCs treated with AgNPs ($r = -0.912$). The protein was detected using specific antibodies and the levels of CACNA1A protein were quantified based on gray levels of the band. (B) Dose-dependent decrease in CACNA1A mRNA expression of AgNPs treated CGCs ($r = -0.944$). Triplicate experiments were performed and the data were presented as mean $\pm$ SD. * $p < 0.05$ and ** $p < 0.005$, versus negative control.

ataxia (Ferrarin et al., 2005; Schmähmann, 2004). The failure in the proper foot placement in AgNPs exposed rats implied that AgNPs might have potential to induce cerebellar ataxia-like disease. This is in correspondence with the ataxic effect in rats produced by beer consumption (Gallate et al., 2003). A decrease in activity or time spent in the center of the open field indicated the increase in animal anxiety or reduction in their exploration potential (Dulawa et al., 1999). As shown in Table 1, a dramatic reduction in the horizontal and vertical exploratory behavior of rats illustrated that the locomotor activity of animals could be effectively diminished by AgNPs treatment. Consistent with the result from the literature on Mn (Oszlanczi et al., 2010), our data suggested that chronic treatment with AgNPs exerted an increase in the resting time as well as a decrease in the moving distance and moving velocity, revealing the depletion of horizontal activity in the rats. The difference of the rearing frequency between AgNPs-treated rats and the controls demonstrated that AgNPs exerted an inhibitory effect on the vertical exploratory behavior of animals, which was in line with preceding reports on the rats with sedation treatment (Sorensen et al., 2004). Some other indexes such as the number of entries into the center showed the similar down-regulating trend upon AgNPs exposure, indicating AgNPs were able to cause impairment of spontaneous behavior in rats.

Histopathological observation based on H&E staining and IHC showed the destruction of cerebellum granule layer and astrocytes activation in AgNPs-treated rat cerebellum, which provided histopathological evidences on the neurotoxicity of AgNPs. Similar findings were reported that the cerebellum granule cell density was reduced in cerebellar ataxia mice with Ca^{2+} P/Q channelopathies (Ivanov et al., 2004). Voltage-dependent ion channels play important roles in generating the rhythmic membrane potential behavior of neuron and regulating a diversity of cellular processes. As one of the crucial proteins, voltage-gated calcium ion channel (Cav2.1 channel) is involved in neurotransmission at central synapses. Loss-of-function CACNA1A gene mutants often showed early-onset motor dysfunction associated with distinct alterations of Ca^{2+} channel properties, and impaired function of the cerebellar calcium channel Cav2.1 might have a central role in the pathogenesis of certain cases of ataxia (Mori et al., 2000; Wappler et al., 2002). Some types of ataxia, such as spinocerebellar ataxia type 6 were associated with decreased expression or impaired function of CACNA1A protein (Watase et al., 2008). *In vivo* and *in vitro* tests herein indicated the decrease in CACNA1A expression upon AgNPs treatment, showing the inhibition of Cav2.1 channel was involved in AgNPs-mediated cerebellar ataxia-like neurobehavior. Although the dysfunction of voltage-gated potassium channel (Kv1.1 channel) could be associated with the cerebellar ataxia as well (Jen et al., 2007), no alteration was observed in the level of Kv1.1 channel protein upon AgNPs treatment, indicating that voltage-gated potassium channel was not related with AgNPs-induced cerebellar ataxia-like symptom.

AgNPs have been incorporated in a broad range of consumer products and direct contact application on human beings is increasing. The current global annual production is estimated to exceed 400 tons (Pourzahedi and Eckelman, 2015). The wide application of AgNPs contained products will inevitably result in the release of this emerging chemical into the environment, which is likely to cause increasing exposure risks and potential hazards to the environment and human beings. Our study indicated that nasal administration of AgNPs in neonatal rats induced cerebellar ataxia-like neurobehavioral phenotype, which was associated with Cav2.1 channel dysfunction. This finding offered the solid toxicological evidences for the risk assessment on the potential biological effects from AgNPs application. In view of rescuing the neurotoxicity of AgNPs, effective counteraction is highly recommended.

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