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# Exposure to silver nanoparticles does not affect cognitive outcome or hippocampal neurogenesis in adult mice

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ABSTRACT

Due to the unique antimicrobial and many other broad spectrum biotechnological advantages, silver nanoparticles (Ag-NPs) are widely used in biomedical and general applications. However, the current knowledge about the impact of Ag-NPs on the central nervous system is extremely limited. To assess whether Ag-NPs influence spatial cognition and adult hippocampal neurogenesis, male ICR mice received intraperitoneal administration of Ag-NPs (10, 25, and 50 mg/kg body weight) or vehicle every day for 7 days. At the end of this time period, Morris water maze test was performed for the spatial learning and memory. Subsequently, mice were injected with bromodeoxyuridine and sacrificed 1 day or 28 days after the last injection in order to evaluate cell proliferation, survival and differentiation in the hippocampus. Results showed that compared with the control group, both reference memory and working memory were not impaired in Ag-NPs exposed groups. In addition, no differences were observed in hippocampal progenitor proliferation, new born cell survival or differentiation. These data reveal that exposure to Ag-NPs does not affect spatial cognition or hippocampal neurogenesis in mice.


## 1. Introduction

Nanomaterials are defined to be either nano-objects or nano-structured materials (Löestam et al., 2010). Of various nanomaterials, silver nanoparticles (Ag-NPs) are emerging as one of the most commonly used nanomaterials (Chen and Schluesener, 2008). Ag-NPs are now in daily use including bedding, washers, toothpaste, shampoo and fabrics (Wijnhoven et al., 2009). Moreover, the use of Ag-NPs in medical applications is rapidly expanding due to the beneficial physiochemical features they offer (Chaloupka et al., 2010; Wong and Liu, 2010). However, the adverse affects of commercialized Ag-NPs have not been fully examined, especially on the central nervous system (CNS).

It has been reported that Ag-NPs (50–100 nm) may alter the action potential of hippocampal CA1 neurons by depressing voltage-gated sodium current (Liu et al., 2009). In the CNS, voltage-gated sodium current is responsible for modifying the excitability of neuronal cells, neuronal activity and function. Thereby, potential modulation of the current by Ag-NPs would be expected to alter neuronal functions. Furthermore, in vitro studies focused on PC-12 cells, a neuroendocrine cell line with the capability to produce the neurotransmitter dopamine (DA) and contain functional DA metabolism pathways, have shown that Ag-NPs (15 nm) significantly reduce DA and its metabolite (dihydroxyphenylacetic acid) concentrations, and the expression level of genes associated with the dopaminergic system (Hussain et al., 2006; Wang et al., 2009).

Many in vivo studies have demonstrated that Ag-NPs administered systematically may cross the blood brain barrier and accumulate in CNS (Yang et al., 2010; Win-Shwe and Fujimaki, 2011). The long retention of silver in brain has been observed in spite of low concentrations of silver detected in brain compared to other tissues (Lankveld et al., 2010; van der Zande et al., 2012). Further, intraperitoneal, intravenous, intracarotid or subcutaneous administration of Ag-NPs (50–100 nm) induces edema formation, neuronal degeneration, astrocyte swelling as well as myelin damage (Sharma, 2007; Sharma and Sharma, 2007; Sharma et al., 2009a, 2010; Tang et al., 2009). The leakage of Evans blue albumin can be found in several brain regions including hippocampus after Ag-NPs (50 nm) treatment (Sharma, 2007). Importantly, Rahman et al. (2009) have revealed significant alterations in gene expression associated with the toxicity caused

*Abbreviations:* Ag-NPs, silver nanoparticles; BrdU, bromodeoxyuridine; BW, body weight; CNS, central nervous system; DA, dopamine; DLS, dynamic light scattering; GFAP, glial fibrillary acidic protein; ICP-MS, inductively coupled plasma-mass spectrometry; MWM, Morris water maze; NeuN, neuronal nuclear protein; PBS, phosphate-buffered saline; ROS, reactive oxygen species; TEM, transmission electron microscopy

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by Ag-NPs (25 nm) in the caudate, frontal cortex, and hippocampus of mice. It is believed that reactive oxygen species (ROS) and oxidative stress results from an increased generation of ROS or from poor antioxidant defense systems may be responsible for the neurotoxicity of Ag-NPs (Hussain et al., 2006; Rahman et al., 2009; Wang et al., 2009). Oxidative damage has been implicated in age-related decline in cognitive processes and in the pathogenesis of many neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and Huntington's disease (Halliwell, 1992; Cadet and Brannock, 1998; Varadarajan et al., 1999; Ahlemeyer and Krieglstein, 2000). Thus, it can be speculated that Ag-NPs might have deleterious effect on the CNS functions including cognition. However, the effect of Ag-NPs on spatial cognition is a matter of debate. Some studies found that exposure to Ag-NPs induced impairment of reference or working memory (Hritcu et al., 2011; Liu et al., 2012), while other studies showed Ag-NPs had no effects on cognition (Sharma and Sharma, 2007; Sharma et al., 2009b). Although discrepancies exist between these studies using rats, due to the difference of animal species, this impact needs to be further evaluated in mice.

On the other hand, in the adult mammalian hippocampus, cumulative evidence has shown that neurogenesis persists throughout life (Altman and Das, 1965; Gould et al., 1997). Dynamic changes in hippocampal neural precursor cells may contribute to underlying substrates of learning and memory (Gould et al., 1999; Shors et al., 2001; Deng et al., 2010). However, neurogenesis can be influenced by a number of different factors such as environmental enrichment, physical activity, reduced caloric intake, psychosocial stress, aging, and hormones (Gould et al., 1999; Kempermann et al., 2002; Shors et al., 2007; Nagata et al., 2009). To date, little information is available about the impact of exposure to nanomaterials on adult neurogenesis.

The aim of the present study was to assess whether Ag-NPs exposure could result in spatial learning and memory deficits, as well as to assess the effects on cell proliferation, survival and differentiation in the dentate gyrus in mice. These findings will provide an important theoretical basis for evaluating the neurotoxicity underlying impacts of nanomaterials on animals and human.

## 2. Materials and methods

### 2.1. Silver nanoparticles

Noncoated Ag-NPs with a diameter of 25 nm were purchased from Amresco, USA. The suspension of the Ag-NPs was prepared in deionized water, and dispersed by ultrasonic vibration (KQ2200E, Kunshan, China) for 30 min in icy water, followed by stirring on a vortex agitator before every use. The morphologies of Ag-NPs were studied by transmission electron microscopy (TEM, JEM-2000EX, JEOL, Japan), and the size statistical distributions were determined by counting more than one hundred Ag-NPs in TEM photographs. Dynamic light scattering (DLS) and zeta potential measurements were carried out at room temperature using a Zetaplus Analyzer (Zetaplus, Brookhaven, USA).

### 2.2. Animals

Adult male ICR mice, weighing 32–35 g at the beginning of the experiments, were purchased from National Rodent Laboratory Animal Resources (Shanghai Branch, China). All animals were housed in separate cages, with access to standard laboratory food and water ad libitum, and kept in a regulated environment (22–22 °C) under a 12-h:12-h light/dark cycle starting at 7:00 AM. The use of animals in this study was approved by the Institutional Animal Care and Use Committee of the University. All efforts were made to minimize animal suffering and to reduce the number of animals used.

### 2.3. Experimental design

Mice were randomly divided into five groups including control group ($n=10$) which received vehicle (0.9 percent normal saline), and three experimental groups ($n=15$ in each group): 10, 25, and 50 mg/kg body weight (BW) Ag-NPs. The fifth group ($n=10$) received scopolamine (3 mg/kg BW) as a positive control for the behavioral studies. Ag-NPs suspension or the same volume vehicle was administered intraperitoneally once daily in the morning for 7 consecutive days. One day after the last administration, five mice from each experimental group were sacrificed, and the hippocampi were taken out for silver content analysis. At the same time point, Morris water maze (MWM) test was started. 24 h after the behavioral test, mice ($n=5$ in each group/time point) from the control and experimental groups were injected with bromodeoxyuridine (BrdU) and sacrificed 1 day or 28 days after the last BrdU injection.

### 2.4. Determination of tissue silver content

Hippocampi were rapidly separated from brains, weighed, and digested in nitric acid overnight. After adding 1 ml $H_2O_2$, the mixed solutions were heated at about 180 °C until the samples were completely digested. Then, the solutions were heated at 120 °C to remove the remaining nitric acid until the solutions were colorless and clear. At last, the remaining solutions were diluted to 5 ml with 2 percent nitric acid. Inductively coupled plasma-mass spectrometry (ICP-MS, Elan 9000, PE, USA) was used to analyze the silver concentration in the samples. The concentration of silver in the tissue was expressed as μg/g wet weight.

### 2.5. Behavioral testing

The spatial learning and memory ability was assessed using an MWM according to the protocol by Vorhees and Williams (2006). Briefly, before mouse testing, a circular pool (120 cm diameter) was filled to a depth of 50 cm with water at 23 ± 2 °C. A target platform (7 cm diameter) was hidden 1 cm below the water surface in the southeast (fourth) quadrant. In the training task, mice were given 90 s to reach the hidden platform. Four starting positions were used randomly and each mouse was trained with four trials per day. After reaching the platform, the mouse was allowed to remain on it for 20 s. If the mouse failed to find the hidden platform within 90 s, the trial was terminated and the animal was put on the platform for 20 s. The training task was performed at 8:00 AM for consecutive 5 days. Swimming paths were recorded using a computer system with a video camera (AXIS-90, Target/2, Neuroscience). Latency to reach the platform and average swimming velocity were scored on all trials. On the day following the last hidden platform trial, a probe test was done in a single 90 s trial in which the submerged platform was removed from the pool, and the animal released from the quadrant opposite the fourth quadrant. The percentage of time spent in the target quadrant and the number of platform crossing were recorded for each mouse.

Subsequently, a working memory test was performed for 3 consecutive days, which was consisted of five trials per day. Except that the platform location was changed daily, the working memory test was similar, procedurally, to the standard training of the water maze test. The hidden platform remained in the same position across trials on a given test day. The first trial of each session was an informative sample trial in which the mouse was allowed to swim to the platform in its new location and to remain there for 15 s. The latency and distance swum, required to locate the hidden platform, were recorded as above. Spatial working memory was assessed as the mean performance in the second trial of 3 consecutive days.

### 2.6. Bromodeoxyuridine administration and sample collection

After MWM test, mice from the control and experimental groups were intraperitoneally injected with BrdU (Sigma-Aldrich, St. Louis, MO), which can be incorporated into the DNA of dividing cells during S phase, at 100 mg/kg/day for 2 consecutive days. 1 day and 28 days after the last BrdU injection, the animals were anesthetized by a peritoneal injection with 5 μl/g of 7.5 percent chloral hydrate and perfused transcardially with 4 percent paraformaldehyde in phosphate-buffered saline (PBS). Brains were removed from the skulls, and post-fixed overnight at 4 °C in 4 percent paraformaldehyde. Next day, the brains were transferred to a 30 percent sucrose in PBS solution for 48 h at 4 °C. Consecutive coronal sections with a thickness of 40 μm were cut using a cryostat microtome (CM1900, Leica, Germany).

### 2.7. Immunohistochemistry

Biotinylated-BrdU immunostaining was carried out as described previously (Karishma and Herbert, 2002). Briefly, free-floating sections were rinsed repeatedly in PBS (pH 7.4) between steps. The sections were prepared with 50 percent formamide-2 × SSC, at 65 °C for 2 h, denatured by incubating in 2 mol/l hydrochloric acid for 30 min at 37 °C, and then washed for 30 s with boric acid (pH 8.5). Nonspecific binding sites were blocked in blocking solution (PBS in 3 percent bovine serum albumin) containing 1 percent Triton X-100 for 1 h. Mouse monoclonal antibody against BrdU (Chemicon International) was diluted 1:200 in blocking solution containing 0.5 percent Triton X-100 and incubated at 4 °C for