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Systemic and Behavioral Effects of Intranasal Administration of Silver Nanoparticles

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Abstract

Use of silver nanoparticles (AgNPs) for their antimicrobial properties is widespread. Much of the previous work on the toxicity of AgNPs has been conducted *in vitro* or following oral or intravenous administration *in vivo*. Intranasal (IN) instillation of AgNPs mimics inhalation exposure and allows further exploration of the toxicity of these particles via respiratory tract exposure. The present study involved 1) single-dose exposures to assess tissue distribution and toxicity and 2) repeated exposures to assess behavioral effects of IN AgNP exposure (nominally uncoated 25 nm AgNP). AgNP deposition was localized in the liver, gut-associated lymphoid tissue, and brain. Decrease cellularity in spleen follicles was observed in treated mice, along with changes in cell number and populations in the spleen. The splenic GSH:GSSG ratio was also reduced following AgNP exposure. Expression of the oxidative stress-responsive gene *Hmox1* was elevated in the hippocampus, but not cortex of treated mice, as was the level of HMOX1 protein. Mice receiving 7 days of IN exposure to 50 mg/kg AgNPs exhibited similar learning- and memory-related behaviors to control mice, except that treated mice spent significantly less time in the target quadrant of the Morris Water Maze during the acquisition phase probe trial. These findings indicate systemic distribution and toxicity following IN administration of AgNPs.

Keywords

silver nanoparticles; neurobehavioral effects; autometallography; intranasal instillation; Morris water maze

1. Introduction

Use of silver nanoparticles (AgNPs) in consumer goods has become increasingly prevalent due to their antimicrobial properties. Textiles, aerosol sprays, water purification systems, and coatings on medical implants and instruments may contain AgNPs (Seltenrich 2013; Chen and Schluesener 2008; U.S. EPA 2012). Despite their prevalence, little is known about the risks of inhalation exposure for manufacturing employees or consumers. It is estimated that between 2.8 and 20 tons of nanosilver are produced each year in the United States (Hendren et al. 2011). Lee et al. (2012) demonstrated that employees are exposed to these particles during production.

Previous research with AgNPs has illustrated their ability to cause damage to various types of tissue throughout the body. Oral administration of AgNPs was shown to cause hepatotoxicity and systemic inflammation (Park et al., 2010). Intraperitoneal (IP) injection of AgNPs led to changes in oxidative stress-mediated gene expression in the brain (Rahman et al. 2009). Sung et al. (2008) found decreased pulmonary function and lung inflammation in rats exposed by inhalation to AgNPs. We previously showed the ability of AgNPs to distribute throughout the body following intranasal (IN) exposure, including to the brain, spleen, lungs, and kidneys (Genter et al. 2012). Administration of particles via the IN route mimics exposure via inhalation through the nose, the most likely exposure route for manufacturing workers.

The present study was performed to expand the range of IN doses of AgNPs from our previous study and to explore additional endpoints. These additional endpoints included evaluation of additional tissues (liver and gut-associated lymphoid tissues), as well as more extensive evaluation of the responses of the spleen and brain. Our previous work and that of others suggest that silver nanoparticles can cause oxidative stress (Genter et al. 2012; Rahman et al. 2009). Therefore, changes in cell number and populations and glutathione status were evaluated in the spleen, as well as brain regions for oxidative stress markers, were examined. Increased expression of the oxidative stress responsive gene heme oxygenase (*Hmox1*; (Keyse and Tyrell 1989)), suggesting particle-induced oxidative stress in the hippocampus of treated mice, also led to exploration of the effects of IN AgNP administration on learning and memory.

2. Materials and Methods

2.1 Reagents

The silver nanoparticles under investigation in this study were nominally 25 nm uncoated particles supplied by Dr. Syed Ali (National Center for Toxicological Research, Jefferson, AR) and originally obtained from NovaCentrix (Austin, TX, USA).

2.2 Transmission electron microscopy (TEM)

Nanoparticles were subjected to TEM in the Advanced Materials Characterization Center at the University of Cincinnati. AgNPs were suspended in ethanol and deposited on a TEM grid and dried before analysis. An FEI CM-20 microscope operated at 200 Kv was used to observe particle diameter and distribution. Mean AgNP particle diameter was found to be 39.97 ± 17.98 nm (Figure 1). These particles were previously characterized by Rahman et al., (2009), with DLS results showing the particles 118 nm in DI water and at 1090 nm in PBS. These authors also showed a zeta potential value of -34.6 mV.

2.3 Study Design

The present study was conducted in two parts:

2.3.1)—Single-dose IN exposures (10–500 mg/kg) were conducted at doses of 10–500 mg/kg, and the mice euthanized 7d later. These doses were selected to examine the effects of doses lower than those in our previous publication (Genter et al., 2012), while keeping the highest dose previously used as an internal control. No behavioral analysis was performed on these mice.

2.3.2)—A repeat-dose study was performed (50 mg/kg/d for 7 d, followed by a 7d wait period) for neurobehavioral assessment.

2.4 Mouse treatments

Male C57BL/6 mice (Charles River Laboratories, Wilmington, MA for behavior studies, Jackson Laboratory, Bar Harbor, ME for all other studies) were acclimated for one week following arrival. Mice were housed two to four per cage and provided water and pelleted chow *ad libitum*. Housing was under consistent environmental conditions (50% humidity, 12

hr light-dark cycle, $22 \pm 1^\circ\text{C}$). Particles were suspended in sterile ultrapure water, sonicated for 10 seconds, and vortexed immediately prior to instillation. The AgNP dosing suspension (50 – 62.5 mg/mL, depending on the body weight of the mice), or sterile water for control, was delivered in a volume of 10 μL per nostril via sterile pipette tip, as previously described (Genter et al. 2012). Mice were cared for in accordance with federal animal care guidelines. This research was approved by the University of Cincinnati Institutional Animal Care and Use Committee and was performed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility.

2.5 Evaluation of endpoints after single-dose treatments

Histology and autometallography (AMG): At necropsy, tissues were collected and put into formalin (e.g. brain, kidney, liver, spleen, and gut-associated lymphoid tissue (GALT)) for histological analysis. Tissues were then paraffin-embedded, sectioned at 5 microns, and subjected to hematoxylin and eosin (H&E) staining or AMG. AMG is an established method for the detection of metal colloid aggregates in histological sections (Danscher and Stoltenberg 2006) and was previously used for localization of AgNP deposition in our laboratory (Genter et al. 2012). Briefly, deparaffinized sections were incubated at 30°C for 90 minutes in a solution containing 1.2% carboxymethylcellulose (w/v) (Sigma-Aldrich, St. Louis, MO), citrate buffer solution (1.2 M citric acid + 0.8 M sodium citrate, pH 3.7), 8.6% hydroquinone (w/v), and 0.12% silver lactate (w/v) (Acros Chemicals, Brunswick, NJ) in the dark. Following a wash step, slides were incubated in a 5% sodium thiosulfate solution at RT for 5 min. Sections were washed, counterstained with nuclear fast red, and coverslipped with permanent mounting medium following dehydration. Slides were photographed on a Nikon Eclipse E400 microscope and images captured using a Nikon D80 digital camera. Images were optimized using the Auto Correct function in Microsoft Office Picture Manager software.

2.6 Spleen Processing and Flow Cytometry

Spleens were processed for cell differentiation and flow cytometry as previously described (Kruisbeek 2001; Motz et al. 2008). Spleens were collected at necropsy, stored in DMEM with 10% FBS, and then homogenized by pressing through a 100 μm filter. Following a low-speed spin, the pellet was incubated with Gentra Systems RBC Lysis Solution (Qiagen, Valencia, CA). Total cell number was evaluated from this solution. Cells were subjected to a low-speed spin and then a fraction was aliquoted for cytospin analysis. Remaining cells were incubated with antibodies to CD3e (T-cell marker), B220 (B-cell marker), NkP46 (NK cell marker), and F4/80 (myeloid cell marker). Cells were fixed and analyzed by flow cytometry.

2.7 Glutathione Assay

GSH and GSSG levels in spleen homogenate were fluorometrically following *o*-phthalaldehyde derivatization as previously described (Senft et al. 2000). Protein concentrations were determined with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA).

2.8 Hmox1 qPCR

Heme oxygenase, encoded by *Hmox1*, is very sensitive to induction in response to oxidative stress, and endpoint that is suggested by previous findings (Keyse and Tyrell 1989; Rahman et al., 2009; Genter et al., 2012). Brain slices were microdissected on ice into individual regions, including cortex and hippocampus at necropsy. Samples were stored at -80°C until RNA isolation. RNA was isolated via Tri Reagent (Molecular Research Center, Cincinnati, OH) according to manufacturer's instructions. cDNA was synthesized by the Verso cDNA kit (Thermo Scientific, Pittsburgh, PA) according to kit instructions. qPCR was performed using SYBR Green 2 $\times$ RT-PCR master mix (Bio-Rad, Hercules, CA) and primers for *Hmox1* or 18S rRNA (Integrated DNA Technologies, Coralville, IA). Reactions were performed in duplicate, and gene expression values were determined by the difference in *Hmox1* expression relative to 18S rRNA via the $2^{-\text{ct}}$ method (Livak and Schmittgen 2001). Primers:

Hmox1

F5'-CCTCACTGGCAGGAAATCATC

R5'-CTCGTGGAGACGTTTACATA

18S rRNA

F5'-CGGCTACCACATCCAAGGAA

R5'-GCTGGAATTACCGCGGCT

2.9 Western Blot

Protein concentration of brain homogenates was determined by Bradford Assay after microdissection to enrich for specific brain regions. Samples were subjected to SDS-PAGE (12% acrylamide) and transferred to nitrocellulose. Blocking was performed with LI-COR Odyssey Blocking Buffer (LI-COR Biosystems, Lincoln, NE). The blot was incubated for 2 hr or overnight with the primary antibodies (goat anti-HMOX1, 1:500, Santa Cruz Biotechnology, Dallas, TX and mouse anti- β -actin, 1:10,000, Sigma-Aldrich), followed by washes with PBS-T and secondary antibody incubation (1 hour, 1:10,000, IRDye 680RD donkey anti-mouse and IRDye 800CW donkey anti-goat, LI-COR Biosystems). Following washes, the blot was imaged on the LI-COR Odyssey instrument.

2.10 ICP-MS

Each sample was transferred to a pre-cleaned 35mL Teflon digestion vessel, 0.5 mL ultra-pure nitric acid and 2.5 mL hydrofluoric acid (HF) were added, and the vessels were vented for 5 minutes in hood prior to being sealed. The samples were dissolved using microwave assisted digestion (CEM MARS) as follows: 1600 W at 100% at temperature ramp to 200°C in 25 minutes, 40 minute hold, and 20 minute cool down. Stock solutions of silver were mixed for 1 minute then sonicated for 20 minute prior to aliquoting and digestion. Water and acid blanks were included within the digestion, and were negative for Ag. A dilution of 100uL AgNP stock solution in mq H_2O (final total volume 15mL) was mixed and sonicated (20 min) and several aliquots were digested to determine the stock AgNP concentration. Following digestion samples were transferred to 15 mL conical tubes, internal standard

(indium) was added, and samples were diluted to 15 mL total volume with mq H₂O. Analysis occurred within two days of sample preparation. Standards were prepared from Ag⁺ standard obtained from Agilent and contained 0.5 mL of HNO₃ and 2.5 mL of HF and indium as internal standard. Standards included a blank (0 ppb), 1, 3, 5, 10, and 25 ppb Ag⁺. Analysis of the silver content by ICP-MS was conducted essentially as previously described (Li et al., 2014). The Method Detection Limit (MDL) for this analysis was 3.69ppm and the critical concentration was 1.845ppb.

2.11

Neurobehavioral studies (7 consecutive days of dosing at 50 mg/kg, followed by 7 days of recovery)

2.11.1 Novel Object Recognition—Novel object recognition was performed as previously described, with minor modifications as described (Hautman et al. 2014). Mice (n= 13 controls, 14 treated; some mice were lost to fighting-related injuries) were habituated to the Anybox chamber (Stoelting Company, Wood Dale, IL) for 10 minutes at least 1 hour prior to the beginning of the experiment. Mice were first allowed to explore two identical objects for 30 cumulative seconds or 10 minutes, whichever came first. After at least one hour of rest, they were re-introduced to the chamber, containing two objects, one with which they were already familiar, and one novel object for the same time period. Analysis was done on time spent with each object during familiarization phase compared to retention phase. If there was no preference between the identical objects during familiarization and preference for the novel object during retention, the animal was thought to have intact memory. Tracking was performed with AnyMaze software (Stoelting Company).

2.11.2 Morris Water Maze—The Morris Water Maze test for spatial learning and memory was administered as previously described, with some minor changes listed below (Hautman et al. 2014). Mice (n=17 per treatment group) received 1 d of cued training at the beginning of the Morris maze test. During cued training, the 10 cm hidden platform was marked with an orange ball on a rod above the platform, and animals were given 6 trials of up to 90 sec to learn that reaching the platform led to their removal from the maze with a fixed start and fixed platform position. Trials for the whole study were conducted in a rotational fashion, with each animal receiving at least a 10 min inter-test interval.

Two phases (acquisition and reversal) made up the hidden platform portion of the test, with four 90-second trials per day for 5 d for mice to find and learn the location of the hidden platform. Mice that did not find the platform in 90 sec were placed on it for 5 sec after the trial to facilitate learning the location of the platform. On the sixth day, animals were given one 60-sec probe trial with no platform present. The Morris Water Maze was 122 cm in diameter with room temperature water. The platform for the acquisition trials was 10 cm in diameter and for the reversal trials was 7 cm in diameter. All data was collected with Anymaze tracking software. After reversal, mice were given 5 days of cued-random trials with 2 trials per day with the start and platform positions randomized, the platform marked as during cued training, and curtains closed around the maze to block wall cues as a control procedure for non-specific performance effects.

3. Results and Discussion

3.1 Systemic Effects of AgNPs (single-dose study)

Mice were exposed once to varying doses of AgNPs by IN instillation and sacrificed 7 days later. No differences in body weight were noted in the treated mice, compared to controls. Additionally, IN exposure to AgNPs had no significant effect on normalized organ weights (including heart, thymus, lungs, liver, kidney, and spleen). Complete blood counts were determined for exposed animals, and no significant changes were observed among any of the AgNP treated groups compared to controls (not shown).

Samples of organ tissue were collected and autometallography (AMG) was performed to evaluate the accumulation of silver. AMG is an established technique used for enhancing metals present in tissue and has been previously used to localize the deposition of silver nanoparticles (Genter et al. 2012; Danscher and Stoltenberg, 2006). AgNPs were detected in the liver, GALT (Figure 2), choroid plexus (Figure 3), and lateral ventricles (Figure 4) of treated mice (note black AMG reaction product, indicating location of deposited AgNP). The deposition of silver appears to increase in a dose-dependent manner from 10 to 500 mg/kg AgNPs in the choroid plexus (Figure 3).

AgNP could theoretically access the brain by movement along the olfactory nerve, by paracellular movement between nasal epithelial cells, or through the bloodstream. A study demonstrating this point with another agent showed movement of a modified distemper virus to various brain subregions, including the choroid plexus, as well as to distant organs, following IN administration (Rudd et al., 2006), indicating transmission to the nervous system, lymphatics, and bloodstream. The presence of silver in liver, GALT, and brain indicates the ability of AgNPs to distribute systemically following IN instillation, and likely via multiple routes. A one-time exposure to AgNPs led to a dose-dependent increase in the expression of *Hmox1* in the hippocampus, but not the cortex, 7 d later (Figures 5A, B). Expression of HMOX1 protein was also increased after exposure to 50 mg/kg AgNPs (5C). Silver was detected in whole brain homogenate by ICP-MS following a one-time 500 mg/kg AgNP dose and 7 d of treatment with 50 mg/kg AgNP (brains collected after completion of behavioral assessments) (5D). Deposition of AgNPs in the lumen of the portal vein points to distribution via the bloodstream, while their presence in GALT is more likely indicative of exposure via the intestine or lymphatic system.

It is unavoidable to have some fraction, albeit unknown in this case, swallowed when dosing by the IN route. While no data are available on this topic for IN-administered AgNP, Fattinger et al. (2000) estimated the relative contributions of nasal vs. gastrointestinal absorption of IN administered cocaine in human volunteers and estimated that the fraction of the administered dose absorbed into the systemic circulation following IN administration was 19%. In terms of absorption of materials from the nose to the brain, Westin et al. (2006) showed that the brain concentration of morphine in rats treated either IN or IV was the same 5 and 15 min following the respective route of administration, but the brain/plasma morphine AUC ratios were ~3 and 0.1 after IN and IV dosing, respectively, showing an “early distribution advantage” of morphine to the brain via the IN route. While food packaging materials containing nanosilver are in commercial use (e.g. to preserve cheeses;

Incoronato et al. 2011), there are some studies showing effects of oral administration of AgNP on the gut microbiota and immune responses (e.g. Williams et al. 2014; Han et al. 2014). Environmental microbe populations may also be adversely affected by discharge of silver nanomaterials (e.g. Pradhan et al. 2011; Kumar et al. 2014), though *Bacillus* sp. have been shown to be adaptive to nanosilver (Gunawan et al. 2013).

3.2 Effects of AgNPs on the Spleen

In our previously published study, IN AgNP administration led to accumulation of AgNPs in the spleen, deposition of hemosiderin in the red pulp, and depletion of the red pulp (Genter et al. 2012). These changes prompted further exploration into the effects of IN AgNP on the spleen. Treatment with 50 mg/kg (single treatment) of AgNP led to loss of cellularity in the spleen follicles, as observed by H&E staining (Supplemental Figure 1). Total spleen cell number and percentage of each population of cell subtypes were evaluated across a range of doses (0–500 mg/kg) (Figure 6). Total cell number was relatively unchanged among treated animals and was not significantly different from controls (6A). The percent of monocytes and macrophages was increased from control in all treated groups, but only the 25 and 50 mg/kg doses of AgNPs were statistically significant (6B). The neutrophil population was very similar among treated and control groups (6C). The total percentage of lymphocytes was decreased in animals treated with AgNPs, with a significant decrease observed following treatment with 25 and 50 mg/kg AgNPs (6D). The individual lymphocyte populations were also affected by AgNP exposure. The percent of NK cells in the whole spleen was significantly different than control for doses of 25, 50, 100, and 500 mg/kg AgNPs (6E). Treatment with 10 mg/kg AgNPs led to a significant decrease in the percent of B cells in the spleen (6F). However, this decrease tapered off with increasing concentrations of AgNPs. The T cell population was decreased at all doses but was only statistically significant at 25 mg/kg AgNPs (6G).

To further characterize the changes in the spleen following AgNP exposure, the ratio of reduced glutathione to oxidized glutathione was measured. Mice treated with AgNPs at 50 and 500 mg/kg had a significantly reduced GSH:GSSG ratio compared to control (Table 1). Decreased glutathione levels are associated with decreased immune function, specifically for lymphocytes (Hamilos et al. 1989; Dröge and Breitkreutz 2000; Morris et al. 2013). Kurihara et al. (2013) found supplementation with oral cystine and theanine, precursors of glutathione prevented inflammation and weakened immunity (including lowered NK cell activity) following intense exercise in healthy volunteers. A study by Wang et al. (2013) found a high fat diet, known to generate a lowered immune response, to cause a decreased GSH:GSSG ratio and an increase in the production of proinflammatory cytokines within the spleen. The inability of lymphocytes to respond normally in conditions of low glutathione indicate the decreased GSH:GSSG ratio observed in AgNP treated animals could lead to a decreased immune response. Additionally, little to no change was observed in cell numbers in the spleen following AgNP treatment; however the number of lymphocytes was generally decreased. Specifically, the T-cell and NK-cell populations were reduced, with the NK-cell population being significantly reduced. A decrease in T- and NK-cells also points toward reduced immunity in AgNP-treated animals. Further studies based on these results would allow assessment of changes in immune function in NP treated animals.

3.3 Neurobehavioral Effects of IN AgNPs (repeat-dose study)

The single-dose study showed an increase in the expression of *Hmox1*, a gene that is induced in response to oxidative stress, as well as increased levels of HMOX1 protein in the hippocampus, but not the cortex, 7 days after a single treatment (Figure 5). Therefore, the effects of AgNP exposure on learning- and memory-related behaviors were assessed.

Novel object recognition provides a measure of short-term memory (Clark and Zola 2000). Mice dosed intranasally with 50 mg/kg AgNPs for 7 d showed no difference in preference for the novel object, compared to controls (Supplemental Figure 2).

The Morris Water Maze is a measure of spatial learning and reference memory (Vorhees and Williams 2006). No differences were found on day-1 cued training (not shown). No significant difference in cumulative distance traveled to the hidden platform was observed during the acquisition or reversal phases of testing among animals treated with 50 mg/kg AgNPs for 7 days, compared to control (Figure 7, panels A & C). On both acquisition and reversal, there were significant effects of day, indicating that mice learned over days, although the learning curves were more pronounced during reversal than acquisition. However, treated mice spent significantly less time in the target quadrant of the maze during the probe trial following the acquisition phase (Figure 7B). As these changes were only observed during the probe trial, and not during the learning phase, these findings suggest that AgNP treatment leads to a reduction in spatial reference memory, but not spatial learning. Treated animals did not spend significantly less time in the target zone during the reversal probe trial (Figure 7D), nor did they spend significantly more time in the non-target quadrant (Supplemental Figure 3). On cued random trials conducted after reversal, no treatment effects were found (Supplemental Figure 4).

Our research into the disposition and toxic effects of the AgNP under study in this manuscript was prompted by the work of Rahman et al. (2009) who showed altered expression of genes related to oxidative stress and inflammatory responses in multiple brain regions using a gene expression plate assay, with no follow up on changes in protein or behavioral endpoints. The present study used uncoated AgNP from the same batch as Rahman et al. (2009), which were re-characterized by TEM to verify the size, as they had been stored for some time; neither DLS nor zeta potential measurements were re-characterized. We innovated on the prior study by administering the particles by the IN route, with the intent of more closely modeling inhalation exposure to the particles. In terms of the doses used in the present study, they represent a range that is not inconsistent with potential workplace exposures. Given that the current workplace exposure limit for silver dust and fumes is 100 $\mu\text{g}/\text{m}^3$, and the average adult working male breathes 16.8 m^3 of air per day, this would yield a daily exposure of 1680 micrograms, or 1.68 mg of silver/day (American Conference of Governmental Industrial Hygienists, 2001; iQ Power Tools, 2014). Our low and high doses in this study were 10 and 500 mg/kg, so for a 25 g mouse, the daily exposure was 0.25 mg at the low dose, and for the high dose, 12.5 mg, the former of which is well within the range of a potential acceptable workplace exposure, and the latter about 7.5 times higher than an acceptable 8-hr workplace exposure level.

In other work, smaller silver nanoparticles (5 nm) were found to cause weight loss and locomotor activity when repeatedly administered via tail vein injection (Zhang et al. 2013). However, no effect on body weight or swim speed was observed among mice that received IN instillation of AgNPs in the present study. A previous study by Liu et al. (2013) found no effect in the Morris Water Maze or on neurogenesis in the hippocampus following 7 days of IP injection of AgNPs in mice, despite the presence of Ag in the hippocampus, determined by ICP-MS.

While the behavioral effects of AgNP exposure were modest in the present study, the effects on gene and protein expression and spatial reference memory indicate some changes taking place in the hippocampus. The increased expression of *Hmox1* could be indicative of a compensatory mechanism that prevents further damage to memory systems in the hippocampus. Whether the modest changes in behavior would be observed in humans with significant exposure to silver nanomaterials remain to be determined.

4. Conclusions

The systemic distribution of AgNPs following IN instillation indicates the broad potential for toxicity of nanoparticles. The observed decreases in NK- and T-cell populations taken together with the decrease in the GSH:GSSG ratio point toward a potential lowered immune response following AgNP exposure. In addition, changes in gene expression in the hippocampus and spatial reference memory indicate the need for further investigation into the neurotoxicity of AgNPs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Intranasally-administered silver nanoparticles (AgNP) are deposited in the brain.
- Intranasally-administered AgNP caused induction of *Hmox1*, in the hippocampus.
- Neurobehavioral studies in mice treated with AgNP showed minor neurotoxic effects.
- Intranasal AgNP reduced spleen cell populations and GSH:GSSG ratio.

Figure 1. Representative TEM images of the particles used in these studies. Approximately 200 particles were sized, with a mean value of 39.97 ± 17.98 nm in diameter. Bar=20 nm.

Figure 2. Autometallography showing silver deposition in peripheral tissues 7d after IN administration of single doses of AgNP. Black staining is indicative of the presence of AgNP aggregates. Top panel: central vein of liver from a mouse treated with 10 mg/kg; bottom panel: gut associated lymphoid tissue from a mouse treated with 500 mg/kg. No black depositions were found in tissues from vehicle-treated controls in these tissues.

Figure 3.

Autometallography (AMG) of the choroid plexus in control (A–D). Panel A is from a control mouse, and treated B–D are from treated mice (B, 10 mg/kg AgNPs; C, 50 mg/kg AgNPs; D, 500 mg/kg AgNPs) mice. All samples were collected 7d after a single treatment. Black staining indicates the location of AgNP deposition.

Figure 4. Autometallography (AMG) of lateral ventricles of mice treated with a single 50 mg/kg dose of AgNPs (20 $\times$). Upper panel: Black staining (arrows) is indicative of AgNP deposition. Lower panel (left) No black AMG reaction product was detected in vehicle-treated control mice; (right) enlarged image of ventricle of AgNP-treated mouse. (200 $\times$).

Figure 5.

Gene and protein expression of heme oxygenase 1. (A) Levels of *Hmox1* gene expression in cortex by qPCR; (B) Levels of *Hmox1* in hippocampus by qPCR; (C) Representative Western blot of HMOX1 protein levels in hippocampus (Lane 1, Control; Lane 2, 50 mg/kg AgNP) and average intensity of HMOX1 Western bands normalized to β -actin levels from hippocampal tissue; (D) Concentration of silver in brain tissue determined by ICP-MS. ($n=3$ or $4 \pm$ SEM for qPCR results, *, significantly different from control ($p<0.05$) by one-way ANOVA with Tukey post-test. $n=3 \pm$ SD for Western results. $n=4 \pm$ SD for ICP-MS results.)

Figure 6.

Spleen cell populations determined by cell differentials and flow cytometry in control and AgNP treated animals (10–500 mg/kg). (A) Total cell number, followed by % of each cell type in whole spleen: (B) Monocytes and macrophages, (C) Neutrophils, (D) Lymphocytes, (E) NK Cells, (F) B Cells, (G) T Cells. ($n=3 \pm$ SEM for each condition, *, significantly different from control ($p < 0.05$) by one-way ANOVA with Dunnett post-test.)

Figure 7. Morris Water Maze results. (A) Cumulative distance traveled to reach the platform per day during acquisition phase; (B) Time spent in target zone during acquisition probe trial (* different from control). $F(1,20) = 4.33$, $P < 0.05$; in addition, treated mice spent significantly more time in the non-goal zones (all $P < 0.05$), but there were no differences in swim speed; (C) Cumulative distance traveled per day to reach the platform during reversal phase; (D) Time spent in target zone during reversal probe trial ($n=17/\text{group}$).

Table 1

Reduced glutathione (GSH), oxidized glutathione (GSSG) and ratio of GSH/GSSG in spleens from mice treated with a single dose of AgNP (n=4/group)

Treatment	GSH (nmol/mg)	GSSG (nmol/mg)	GSH/GSSG
Vehicle	3.00 (0.23) *	0.51 (0.04)	5.85 (0.16)
50 mg/kg	1.60 (0.16) **	0.42 (0.03)	3.95 (0.14) **
500 mg/kg	2.36 (0.27)	0.58 (0.06)	4.07 (0.09) **

*
mean $\pm$ SEM

**
significantly different from control p<0.05