# Silver Nanoparticles Decrease Body Weight and Locomotor Activity in Adult Male Rats

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Considering the next industrial revolution, nanotechnology is estimated to have significant economic impact and is anticipated to comprise a more than one trillion dollar global market by 2015.[1] Silver nanoparticles (Ag-NPs) are one of the most commonly used nanomaterials in a variety of commercial applications. Given the fact that Ag-NPs exhibit strong antimicrobial and anti-inflammatory properties, they have also become widely commercially available for wound dressings, contraceptive devices, and surgical instruments.[2] Moreover, these nanomaterials are currently being used in food packaging to prevent mold and bacterial growth during storage, processing, and transportation.[3] The large scale use of Ag-NPs raises safety concerns due to the considerable potential for high exposure to humans and the environment and the lack of sufficient information about their corresponding health risks. In vitro cell culture models have demonstrated that Ag-NPs exposure can produce toxicity.[4–8] In vivo rodent studies have further described Ag-NP-induced toxicity after oral, injection and inhalation exposures.[8–10] Given that Ag-NPs easily cross the blood–brain barrier[11] and are measurable in the rodent brain after oral or intravenous administration,[8,12,13] their potential to alter central nervous function is clear. However, studies of central nervous system functional alterations resulting from Ag-NPs treatment in a mammalian model have not been described to the best of our knowledge. As noted by Simko and Mattsson,[14] an adequate risk assessment of potential neurological effects in response to nanoparticle exposure is not available due to a lack of knowledge. Here, we evaluated the acute effects of Ag-NPs on body weight and locomotor activity of adult male Sprague-Dawley rats following intravenous injection. As this seems to be the first report of neurobehavioral responses after Ag-NP treatment in a rodent model, this study begins to fill the substantial data gap regarding the potential neurotoxicity of Ag-NPs. These results suggest that behavior can be altered with high doses of Ag-NPs in rodents and should help to guide future studies of the health risks associated with this new technology.

Physical and chemical characterization of the nanomaterials used in nanotoxicological studies is critical.[15] Size, surface charge, shape, aggregations, stability, and purities of nanomaterials are important parameters that may contribute to toxicological responses. For certain endpoints, toxicity appears related to Ag-NP size as lower concentrations of smaller particles produce more cytotoxic responses.[4] Further, in a zebrafish model, it appears that it was the release of Ag ions from Ag-NPs that was critical in neurodevelopmental toxicity.[16] Therefore, the widely used uncoated Ag-NPs were selected for use in this preliminary study. Ag-NPs were characterized using transmission electron microscopy (TEM) and dynamic light scattering (DLS) techniques for determination of size, and the zeta-potential as a measure of particle charge.

The size of the Ag nanoparticles used here was reported by the manufacturer as 5 nm. Using TEM, the primary size of Ag-NP in water solution was validated (**Table 1**). Average size of Ag-NPs was 7.2 ± 3.3 nm (mean ± SD). The TEM images in **Figure 1** show the sphere morphology and distribution of the various nanoparticle sizes. DLS was used to characterize the behavior of Ag-NPs in an aqueous environment as exposure condition. Therefore, the nanostructure size and zeta potential in 0.9% NaCl solution were measured using a Zetasizer (Malvern Instruments, Inc., Westborough, MA). The hydrodynamic size and surface charge characteristics of Ag-NPs are summarized in Table 1. Zetasizer analysis indicated that the average hydrodynamic size of Ag-NPs in 0.9% NaCl was 60.5 ± 2.4 (mean ± SD). The sample of Ag-NPs in

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**Table 1.** Physical-chemical properties of Ag-NPs.

| Parameter | Value |
|---|---|
| Primary Size [nm][a)] | 7.2 ± 3.3 |
| Hydrodynamic Size [nm][a,b)] | 60.5± 2.4 |
| Shape | Spherical |
| Zeta Potential [mV][b)] | –9.4 ± 0.5 |
| Ag [%] | 95.5% |
| Fe [%] | 2.6% |
| S [%] | 1.0% |
| Others [%] | 0.9% |

[a)]Diameter of Ag NPs was expressed as mean± SD; [b)]Characterization of Ag NPs in vehicle.

0.9% NaCl with a size of 60.5 ± 2.4 nm by Zetasizer analysis was measured as 7.2 ± 3.3 nm by TEM. This discrepancy is due to the differences in the weighted averages determined by these two techniques, and also the differences in the physical properties measured. These challenges for physical characterizations of Ag NPs have been recently reported, and the approaches of measurement as well as dispersion status greatly affected the reported size and size distribution.[17] TEM directly measures the primary size of the nanoparticles based on the projected area; while DLS measures the hydrodynamic diameter of the nanoparticles based on the translational diffusion area of the particle being measured. Therefore, TEM is sensitive to the size of primary particles, whereas DLS is sensitive to the presence of small quantities of large particles or aggregates. The salt in 0.9% NaCl solution may also contribute to agglomeration potential, and produce an increase in agglomeration size. The size of the Ag-NPs used in this study was relatively uniform, although there were small amounts of aggregations in the nanoparticle solution. This aggregation occurs in many other nanomaterials and likely cannot be avoided if the nanomaterial surface lacks coating or functionalization.

Beyond shape and size, it is important to fully understand the surface chemistry of the nanoparticles.[18,19] The surface charge (zeta potential) value was –9.4 ± 0.5 as determined by DLS (Table 1). The biological properties of the nanomaterials may be altered in biological systems due to the surface protein binding.[20] It has been found that the surface charge of some nanoparticles affected blood–brain barrier integrity and permeability.[21] Indications of inflammatory responses and increased blood–brain barrier permeability after Ag-NPs exposure in an in vitro model provide evidence that exposure in an in vivo model might produce significant neurotoxicity.[4] Purity is also critical in nanotoxicological studies. The STEM image (Figure 1C) of an Ag cluster showed it contained several Ag-NPs. The overlay of Ag EDS map onto the corresponding STEM image demonstrated that the brightest nanoparticles were indeed Ag-NPs. The Ag-NPs were further studied by EDS elemental analysis (Figure 1D–G, Table 1) which showed that the overall purity of the Ag-NPs was above 95.5%. The large Cu peaks in the spectra were due to the Cu grid. There was a small amount of Fe (2.6%) and S (1.0%) impurity in the samples.

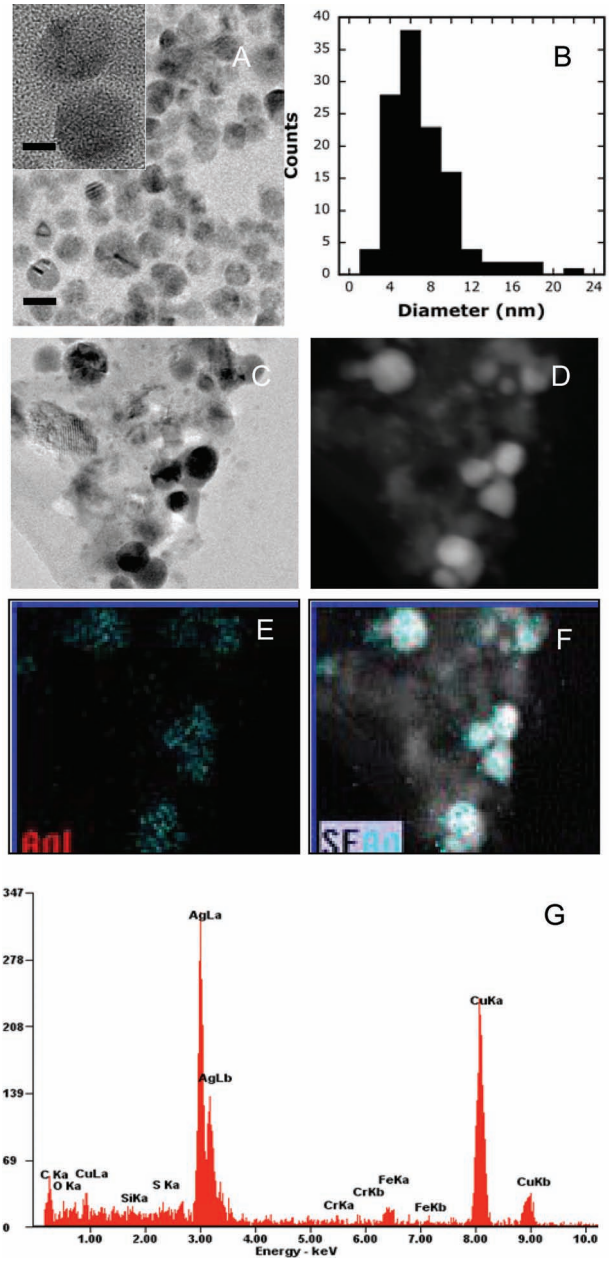


**Figure 1.** TEM and STEM characterization of silver nanoparticles (Ag-NPs). (A) Nanoparticles were deposited on formvar carbon coated Cu grids and dried for TEM imaging. Images were analyzed in a high resolution mode with an acceleration voltage 100 kV (Bar = 10 nm). Insert image of a high magnification images of representative particles (scale bar = 2 nm). (B) Nanoparticle size distribution was analyzed by Image J software (N > 100). (C) TEM image of the STEM image. The contrast is reversed in TEM images; Ag-NPs appear darker. (D) HAAD STEM image of an Ag cluster containing several Ag-NPs. (E) EDS map of Ag L-α line. (F) Overlay of Ag EDS map onto the corresponding STEM image showing the brightest particles are indeed Ag-NPs. (G) EDS analysis showed Ag element and other small amount of elements. The large Cu peaks are due to the Cu grid.

To investigate potential functional neurotoxicity, the initial experiment measured locomotor activity and body weight of male Sprague-Dawley rats (postnatal day 49 or 50) after three consecutive daily injections with saline (n = 4) or

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**Table 2.** Body weight of rats treated with Ag-NPs.

| Group | Body Weight [g][a] | | | Percent change in body weight from day 1[a] | |
|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 2 | Day 3 |
| Initial Experiment | | | | | |
| Control [n = 4] | 230.5 ± 11.9 | 236.8 ± 11.6 | 243.9 ± 11.9 | 2.79 ± 0.53 | 5.86 ± 0.33 |
| 45 mg/kg [n = 4] | 261.9 ± 18.1 | 245.4 ± 20.6 | 234.9 ± 33.1 | −6.54 ± 1.64[b] | −8.44 ± 4.23[b] |
| Subsequent Experiment | | | | | |
| Control [n = 12] | 365.7 ± 9.0 | 369.7 ± 8.8 | 371.3 ± 9.0 | 1.13 ± 0.32 | 2.05 ± 0.26 |
| 5 mg/kg [n = 6] | 333.2 ± 12.3 | 326.6 ± 13.6 | 331.0 ± 13.4 | −2.37 ± 1.05[b] | −0.67 ± 1.35 |
| 10 mg/kg [n = 5] | 390.9 ± 16.8 | 366.0 ± 22.4 | 361.4 ± 24.6 | −5.32 ± 1.59[b] | −6.57 ± 2.66[b] |

[a]Body weights are expressed as mean± SEM; [b]Significantly different from same-experiment control group.

45 mg/kg Ag-NPs (n = 4). One rat treated with 45 mg/kg/day Ag-NPs was found dead on day 3 prior to the third injection. A second rat treated with 45 mg/kg/day Ag-NPs was found dead on the day after the third injection (i.e., day 4). Analysis of percent body weight change indicated a significant interaction of treatment x day ($F(1, 5) = 9.836$, $p < 0.026$). The 45 mg/kg/day Ag-NPs group lost weight on days 2–3 and the percent change was significantly different from the control group on both days ($p < 0.008$ for both comparisons to control) (see **Table 2**).

A technical error caused the loss of activity and rearing data on day 1 for 4 rats (two saline control and two 45 mg/kg/day Ag-NPs). Analysis of total activity indicated a significant effect of day ($F(2, 7) = 15.35$, $p < 0.003$). Total activity on day 1 was significantly more than on days 2 or 3 ($p < 0.003$ for both). Despite a lack of statistical significance, it was clear that the activity decrease on days 2 and 3 was much more for the 45 mg/kg/day Ag-NPs group than the control group (**Figure 2,** top left). Analysis of rearing frequency indicated a significant effect of treatment ($F(1, 7) = 11.10$, $p < 0.015$) (Figure 2, top right). Rats treated with 45 mg/kg/day Ag-NPs reared less frequently on all days than did the saline-treated controls (overall mean±SEM for 45 mg/kg/day Ag-NPs and control groups, respectively: 5.9 ± 1.3 and 45.9 ± 11.8).

Because of the substantial effects of 45 mg/kg/day on behavior, body weight, and mortality (see above), the subsequent experiment assessed the effects of a single injection of saline (n = 12) 5 (n = 6) or 10 (n = 6) mg/kg Ag-NPs. Here, rats

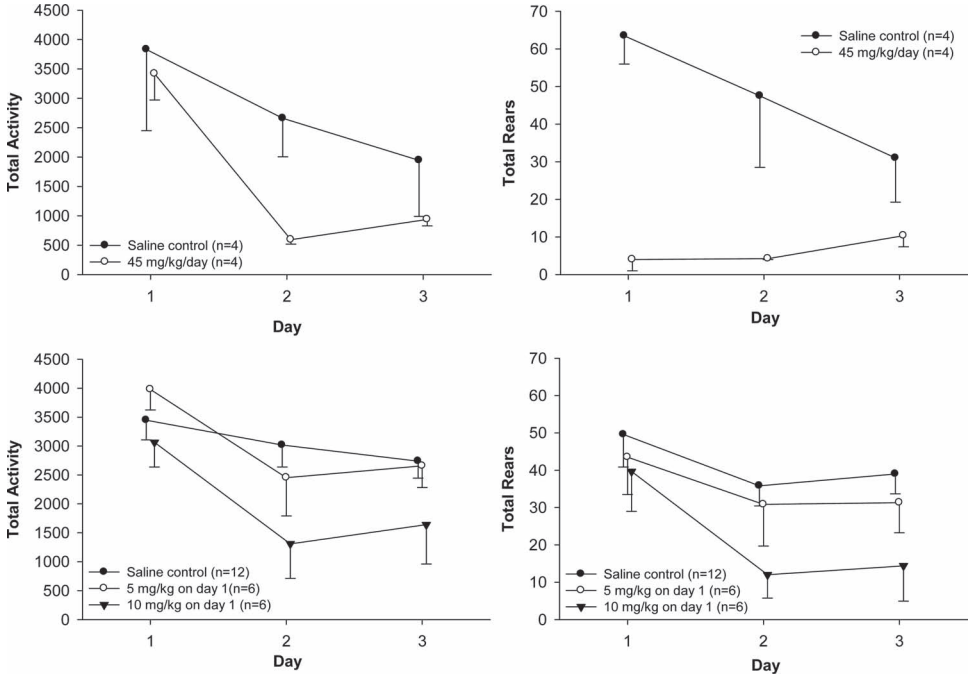


**Figure 2.** Open field horizontal activity (left) and rearing frequency (right) in adult male rats treated with 45 mg/kg/day Ag-NPs (top) or either 5 or 10 mg/kg Ag-NPs for a single day (bottom). Points are slightly offset from the *x* axis for clarity. Despite the statistical results, it is clear that the 45 mg/kg/day Ag-NPs group was less active on day 2 than the control group. Rearing frequency was significantly less on all days for rats treated with 45 mg/kg/day Ag-NPs (top right). Although there were no statistically significant effects of 5 or 10 mg/kg Ag-NPs treatment, activity and rearing frequencies were less on days 2 and 3 relative to controls.

  

were aged postnatal day 62–77. One rat treated with 10 mg/kg Ag-NPs was found dead on the morning of day 2. Analysis of percent body weight change indicated a significant interaction of treatment x day ($F(2, 20) = 6.218$, $p < 0.008$). Both Ag-NPs treated groups lost weight on day 2 and the percent of body weight change was significantly different from the control group ($p < 0.019$ for both comparisons). On day 3, only the percent body weight change for the 10 mg/kg group was significantly different from control ($p < 0.001$) (see Table 2).

Analysis of total activity indicated a significant effect of day ($F(2, 38) = 9.45$, $p < 0.001$). Activity on day 1 was significantly more than on days 2 and 3 ($p < 0.002$ for both comparisons) (Figure 2, bottom left). Analysis of rearing frequency indicated a significant effect of day ($F(2, 38) = 5.312$, $p < 0.009$). Rearing frequency on day 1 was significantly higher than on days 2 and 3 ($p < 0.026$ for both comparisons) (Figure 2, bottom right).

Body weight proved to be quite sensitive to Ag-NPs treatment. All Ag-NPs groups (5 and 10 mg/kg, and 45 mg/kg/day) lost weight after the first injection and a single treatment with 10 mg/kg or 3 consecutive days with 45 mg/kg Ag-NPs was sufficient to decrease body weight further on day 3. While it is unfortunate that body weight measurements did not continue beyond day 3 in the current study, others have reported significantly decreased body weight in rats intravenously injected with 20 and 40 mg/kg Ag-NPs,[10] but not after chronic oral treatment at doses up to 1000 mg/kg/day or acute inhalation exposure at high doses.[8,22] Given the Ag-NPs-induced decrease in body weight, it is difficult to know if the activity alterations are due entirely to central nervous system toxicity. Future studies should measure food consumption and continue measurements for a longer duration. In addition, the dissolution studies of Ag nanoparticles could identify if the Ag ion or Ag-NPs contribute to the body weight and locomotor activity responses.

Locomotor activity of adult male rats appeared sensitive to 45 mg/kg/day Ag-NPs treatment, but not 5 or 10 mg/kg Ag-NPs. Total activity typically decreases over sessions due to habituation as described in previous study.[23] However, the decrease from day 1 was more substantial for those rats treated with 45 mg/kg/day Ag-NPs than for controls (83% vs 31% decrease on day 2 relative to day 1 for 45 mg/kg/day Ag-NPs-treated and control groups, respectively). Further, rearing frequency was significantly decreased on all three days by 45 mg/kg/day Ag-NPs treatment. A similar dose-related decrease in horizontal activity occurred in the subsequent study in which the control group exhibited a 13% decrease in activity from day 1 to 2 while the 5 and 10 mg/kg Ag-NPs groups exhibited activity decreases of 38% and 57%, respectively. Given that the half-life of an iv injection of 1 mg/kg Ag-NPs (8 nm size) in adult male Sprague-Dawley rats is approximately 99 h,[24] it is likely that there was some accumulation in rats treated daily with 45 mg/kg Ag-NPs which may have contributed to the increased mortality of this dose. Further, the small group sizes here may have limited the statistical power and, while the effects appear reasonable, further studies are needed to confirm these activity effects. It should be noted that the maximum dose used in this study may be higher than the practical use of silver NPs product in the market. A recent study demonstrated there are significant changes in clinical chemistry and hematological parameters after the rats intravenously repeatedly with up to 40 mg/kg silver nanoparticles (15–40 nm in size).[25] However, the lack of a substantial effect on locomotor activity in the 5 and 10 mg/kg groups demonstrates that there is still much to be learned about the toxicity of these doses.

In summary, Ag-NP size was characterized using transmission electron microscopy and dynamic light scattering technique. The surface charge of Ag-NPs in solution was measured by Zeta sizer. Ag-NPs were characterized as 7.3 nm diameter with sphere morphology and a negative charge in solution. The time- and dose-dependent activity and body weight alterations suggest that the nervous system may be a target of Ag-NPs, however, this remains to be thoroughly investigated. In any case, these results raise concerns for the wide application of silver nanomaterials.

## Experimental Section

*Silver Nanoparticles*: Ag-NPs (5 nm) were obtained from Nanocentrix Inc. (Austin, TX). The stock solution was mechanically mixed and sonicated for 5 min in an ultrasonic water bath to ensure a uniform suspension prior to each experiment. The Ag-NP suspension was then diluted with 0.9% sterile NaCl solution to produce the needed concentrations.

*Nanoparticle Characterization Using Transmission Electron Microscopy (TEM)*: TEM characterization was carried out to obtain the primary size, size distribution and morphology of the Ag-NPs using JEOL JEM-2100 TEM (JEOL USA Inc., Peabody, MA) equipped with a CCD camera. The acceleration voltage was 100 kV. Nanoparticles were measured after samples were homogeneously dispersed in water, and 5 μL of the suspension was deposited on a formvar/carbon-coated TEM grid, dried, and evacuated before analysis. The mean and standard deviation (SD) of the particle size was calculated from the measurement of over 100 nanoparticles in random fields of view. Images show the general morphology (shape) of the nanoparticles. The size distribution of the nanoparticles was analyzed using Image J software for over 100 random nanoparticles.

*Nanoparticle Characterization Using Scanning Transmission Electron Microscopy (STEM) with EDX*: For scanning transmission electron microscopy (STEM), a field emission transmission electron microscope, JEOL JEM-2100F, equipped with a Genesis XM2 X-ray energy dispersive spectroscopy (EDS) analyzer (EDAX Inc., Mahwah, NJ) was utilized. The probe size of 1.5 nm at 100 keV was utilized to collect high angle annular dark (HAAD) STEM images which yield qualitative information (contrast based on atomic masses) on the STEM images, and accompanying EDS elemental maps determined the chemical nature of the components on the images. For Ag-NPs, we utilized Ag Lα line for the elemental mappings. Example of such analyses are shown in Figure 1 (TEM images) and Figure 2 (STEM analysis with EDS spectrum). The latter shows STEM images, elemental map overlays of Ag and the corresponding TEM image.

*Nanoparticle Characterization Using Dynamic Light Scattering*: Dynamic light scattering, for characterization of size and zeta potential of the nanoparticles in solution, was performed on a Zetasizer Nano ZS instrument (Malvern Instruments, Inc., Westborough, MA). Samples were measured after dilution of the Ag-NP

  

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stock solution to 50 μg/mL suspensions in water or 0.9% NaCl solution. The dilution was vortexed and sonicated for 5 min to provide a homogenous dispersion. For the size measurement, 1 mL of the diluted dispersion Ag-NPs was transferred to a 1 $cm^2$ cuvette for dynamic size measurement; for zeta potential measurement, a Malvern zeta potential cell was washed three times with ultrapure water followed by transferring 850 μLof diluted dispersion Ag-NPs to this cell to measure the zeta potential. The concentration of the samples and experimental methods were optimized to assure the quality of the data. 10, 30, and 60 nm NIST standard gold nanoparticles were used in the validation of the instrument. Both size and zeta potential were measured at least three times. The data were calculated as the average size or zeta potential of Ag-NPs (Table 1).

*Animals and Ag-NP Treatment*: Adult male Sprague-Dawley rats (n = 32) were obtained from the National Center for Toxicological Research (NCTR) Breeding Colony at weaning (postnatal day 21). Rats were randomly assigned to treatment groups, although treatment groups were not mixed within cages. The rats were group housed in standard polycarbonate cages in a room with a 12/12 h light/dark cycle (7 am to 7 pm) at 22 ± 1 °C (mean ± SEM), 45–55% humidity and ad lib access to food and water. Daily body weight was collected prior to tail vein injection with saline (1 mL/kg) or 5, 10, or 45 mg/kg Ag-NPs (or prior to activity measurement for those subjects injected on a single day). Immediately after injection, locomotor activity and rearing frequency were measured. The initial experiment examined 3 consecutive daily injections of 45 mg/kg Ag-NPs. Selection of this dose was based on a previous study indicating that iv injections every fifth day of up to 40 mg/kg Ag-NPs (15–40 nm size) in adult Wistar rats did not affect body weight until after several injections.[10] All animal procedures followed the "Guide for the care and use of laboratory animals"[26] and were approved in advance by the NCTR Institutional Animal Care and Use Committee.

*Locomotor Activity Measurement*: Immediately after saline or Ag-NP injection (or after body weight measurement for those subjects injected on a single day), each rat was assessed for 60 min using one of four open field apparatus previously described in detail.[27] The clear Plexiglas cubes (each 40 cm × 40 cm × 40 cm) 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 using the SR-LAB program (San Diego Instruments, San Diego, CA). Each rat was placed into the same apparatus for 60 min on each of three consecutive days. The software recorded and defined activity as horizontal and vertical (rearing). Endpoints subjected to statistical analysis were total horizontal activity/session and frequency of rearing/session for each of the three test days.

*Statistical Analyses*: Because cages were randomly assigned to treatment, the average group body weights were not similar. Thus, body weight analyses were conducted on the percent body weight change from day 1 since that weight was measured prior to any treatment. The three consecutive days of 45 mg/kg Ag-NPs treatment caused substantial body weight decrements and two deaths; therefore, data from this portion were analyzed separately from the subsequent study which assessed the effects of a single injection of 5 and 10 mg/kg Ag-NPs. For both portions, the percent body weight change for days 2 and 3 was analyzed via repeated measures ANOVA with factors of treatment and day. Two repeated measures ANOVA analyzed locomotor activity data, each with factors of treatment and day: one for total activity and one for frequency of rearing. Pairwise comparisons of significant effects were done using the Holm-Sidak method.

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