**Silver nanoparticles at sublethal concentrations disrupt cytoskeleton and neurite dynamics
in cultured adult neural stem cells.**

Robert J. Cooper¹ and Nadja Spitzer^{1*}

*corresponding author

¹ Department of Biological Sciences

Marshall University

One John Marshall Dr.

Huntington, WV, 25755, USA

spitzern@marshall.edu

Tel: 304-696-7147

Abstract

Silver nanoparticles (AgNPs) have potent antimicrobial properties at concentrations far below those that cause cytotoxic and genotoxic effects in eukaryotic cells. This property has resulted in the widespread use of AgNPs in consumer products, leading to environmental exposures at sub-lethal levels through ingestion and inhalation. Although the toxicity of AgNPs has been well characterized, effects of environmentally relevant exposures have not been extensively investigated in spite of studies that suggest accumulation of silver in tissues, including brain. To assess the sublethal effects of AgNPs on neural cell function, we used cultured SVZ-NSCs, a model of neurogenesis and neural cells. Throughout life, neural stem cells (NSCs) in the subventricular zone (SVZ) of the lateral ventricles proliferate and migrate via the rostral migratory stream to the olfactory bulb. Once there, they complete differentiation into neurons and glia and integrate into existing circuits. This process of neurogenesis is tightly regulated, and is considered a part of healthy brain function. We found that 1.0 μ g/mL AgNP exposure in cultured differentiating NSCs induced the formation of f-actin inclusions, indicating a disruption of actin function. These inclusions did not co-localize with AgNPs, and therefore do not represent sequestered nanoparticles. Further, AgNP exposure led to a reduction in neurite extension and branching in live cells, cytoskeleton-mediated processes vital to neurogenesis. We conclude that AgNPs at sublethal concentrations disrupt actin dynamics in SVZ-NSCs, and that an associated disruption in neurogenesis may contribute to documented deficits in brain function following AgNP exposure.

Keywords: subventricular zone; neurogenesis; differentiation; emerging environmental contaminant; f-actin

1. Introduction

The adult mammalian brain contains pools of adult neural stem cells (NSCs) that proliferate and differentiate into mature neurons and glia throughout life (Altman and Das, 1965, Eriksson et al. , 1998, Gage, 2000). Adult NSCs are found in several areas of the brain, including two well-characterized niches, the subventricular zone of the lateral ventricles (SVZ) and the subgranular zone of the dentate gyrus in the hippocampus (reviewed in (Curtis et al. , 2012)). In the SVZ, progenitor cells proliferate, giving rise to rapidly amplifying precursor cells that begin differentiation while migrating along the rostral migratory stream towards the olfactory bulb. There, they complete differentiation and integrate into existing circuits, primarily as GABAergic interneurons (Doetsch et al. , 1997, Gerard et al. , 1994, Luskin, 1993, Scheffler et al. , 2005). Adult neurogenesis continues throughout life and is a part of healthy adult brain function, and is implicated in memory, learning, and endogenous brain repair (Snyder et al. , 2001, van Praag et al. , 1999). In culture, undifferentiated adult-SVZ-derived NSCs can be maintained as neurospheres (Reynolds and Weiss, 1992, Wachs et al. , 2003) consisting primarily of progenitor cells (Doetsch et al. , 2002). When provided an extracellular matrix and subjected to growth factor withdrawal, cells attach, migrate, and differentiate into cells with mature neural phenotypes. These cells therefore offer a useful *in vitro* model of neurogenesis and neural cells in general. In addition, because adult neural stem cells recapitulate many of the processes that are active during development (Ge et al. , 2008), findings with this model system can also shed light on neurodevelopmental effects of environmental contaminants.

Silver nanoparticles (AgNPs) are 1-100nm long on at least one dimension (Kruszewski et al. , 2011) and, due to their unique structure, small size, and high surface area, have novel chemical and physical properties that differ from other forms of silver (Garcia-Reyero et al. , 2014, Tang et al. , 2008). In particular, AgNPs have potent antimicrobial effects (Pal et al. , 2007, Sondi and Salopek-Sondi, 2004) that have led to their widespread use in industrial and commercial products ranging from medical dressings, clothing, and toys to household appliances. The toxicity of high concentrations of AgNPs on vertebrate systems has been well studied (Arora et al. , 2008, AshaRani et al. , 2009b, de Lima et al. , 2012, Li et al. , 2014). AgNPs cross and disrupt biological membranes, breaking down or bypassing the blood brain barrier (Tang, Xiong, 2008), and they accumulate in tissues including the lungs, kidneys, and brain (Lee et al. , 2013, Sharma

et al. , 2010, Tang et al. , 2009). In cultured cells, toxic levels of AgNPs damage the cytoskeleton (Asharani et al. , 2010, Schrand et al. , 2008, Xu et al. , 2013) and DNA (Ahamed et al. , 2008), and inducing oxidative stress, apoptosis, and necrosis (Arora, Jain, 2008, AshaRani, Low Kah Mun, 2009b, Haase et al. , 2012, Kim et al. , 2009).

To date, most of the toxicity assays have used concentrations of AgNPs in excess of those thought to be relevant to environmental exposure. However, given that AgNPs persist in biological environments (Dobias and Bernier-Latmani, 2013, Yu et al. , 2014) and accumulate in tissues, an accurate measure of true exposure is challenging. This is further compounded by a lack of studies to determine the levels of AgNPs found in the environment and by widely varying differences in exposure levels due inhalation or oral exposure through interaction with consumer or commercial products (Quadros and Marr, 2010, Wen et al. , 2002, Whiteley et al. , 2013). Our study focused on exposure levels that were shown to be nontoxic in cultured cortical neurons (Haase, Rott, 2012, Xu, Pielt, 2013) and several other cell types (Hadrup et al. , 2012). They are also comparable to levels of silver found in the brain of rats receiving chronic oral doses of AgNPs at levels below those eliciting pathological symptoms. This allows us to study the effects of sublethal concentrations of AgNPs on cellular mechanisms during neural differentiation, a key mechanism in neurogenesis and development.

2. Materials and Methods

2.1 SVZ-NSC isolation and maintenance of progenitor cells

Stem cells were isolated from female young adult (3-6 months old) Sprague-Dawley rats (Hilltop Lab Animals, Scottsdale, PA) according to established protocols (Muraoka et al. , 2008, Reynolds and Weiss, 1992, Wachs, Couillard-Despres, 2003). Rats were deeply anesthetized with inhaled isoflurane (Kent Scientific Corp., Torrington, CT) and decapitated. The brain was removed and sterilized in cold phosphate-buffered saline 1 (PBS1, in mM: NaH_2PO_4 , 2.69 (Sigma); Na_2HPO_4 , 11.9 (AMRESCO); NaCl , 137 (Fisher); KCl , 27 (AMRESCO); pH 7.4) containing 1% penicillin-streptomycin (P/S; Life Technologies, Grand Island, NY) before the subventricular zone was isolated. The tissue was chopped in PBS plus 4.5g/L glucose (Sigma, St. Lois, MO) with a scalpel blade, then enzymatically digested in fresh PDD (0.01% papain

(Fisher); 0.01% DNase I (Sigma); 0.1% Dispase II (Sigma); 12.4mM MgSO₄ (Sigma)) for 40 minutes with trituration every 10 min. After digestion, cells were collected by centrifugation, washed three times with DMEM/F12 (Life) and suspended in Neurosphere medium (DMEM/F12 + Glutamate, non-essential amino acids (NEAA), P/S, B27, 20ng/ml EGF, 5ng/ml bFGF, (all from Life) and 1.25U/ml Heparin sodium (Sigma)). Fungizone antimycotic (Life) was included in the medium for the first week of culture. Fresh medium was added every 2-3 days and neurospheres formed within two weeks of culture in non-TC flasks at 37°C, 5% CO₂. Cells in neurospheres were dissociated with accutase (Life) before reaching a diameter greater than 500µm (every 7-14 days) and gave rise to new neurospheres.

2.2 In vitro differentiation of SVZ-NSCs and Exposure to AgNP

Undifferentiated neurospheres (passage 4-18) were collected by centrifugation and plated on poly-L-lysine (0.01%) and laminin (10µg/mL) coated coverslips or wells in 24-well plates in differentiation medium (Neurobasal (Life), 2% B27 (Life), 1% NEAA, 1% Glutamax (Life), and 1% P/S) lacking EGF and bFGF. After being allowed to attach and differentiate for 2-3 days, medium was replaced with fresh medium containing 0, 0.05, 0.1, 0.5, 1.0, or 2.0 µg/mL AgNP stabilized in 2mM citrate (Aldrich). 2mM sodium citrate was used as a vehicle control. Cells were incubated for 48 hours unless otherwise indicated.

2.3 B35 Cell Culture

Undifferentiated B35 neuroblastoma (ATCC, Manassass, VA, United States) cells were maintained in TC-coated flasks in growth medium (DMEM, 10% fetal bovine serum, 1% P/S) and passaged at 90-95% confluence using trypsin. To induce differentiation, cells were plated in 24-well plates coated with poly-L-lysine and laminin in serum-free medium. Cells were allowed to adhere and differentiate for two days before exposure to 1.0µg/mL AgNP in serum-free medium.

2.4 Time lapse microscopy

Cells were grown in differentiation conditions for 2-3 days on poly-L-lysine and laminin-coated wells. Medium was replaced with either AgNP-containing medium (1.0µg/mL) or control medium. Immediately following media replacement, cells were moved to stage-top incubator at

37°C and 5% CO₂ and images were collected using transmitted light. Images of B35 cells were collected every hour for 24 hours, and images of SVZ-NSCs were collected every 15 minutes for 6 hours, with at least two positions collected per well in every experiment.

2.5 Immunocytochemistry

Following two days of AgNP exposure, cells were washed with PBS1, fixed on coverslips with 4% paraformaldehyde for 15min, washed with phosphate buffered saline 2 (PBS2; 100mM sodium phosphate, 150mM NaCl, pH 7.4) and then permeabilized with 0.3% Triton-X/PBS2 for 10 minutes. Preparations were blocked with 5% bovine serum albumin (BSA, Fisher) and 10% normal goat serum (NGS, Life) for one hour before being incubated overnight with primary antibodies specific to cell fate marker proteins. No-primary control preparations were included in each experiment. Primary antibodies were mouse- α -MAP2 (Millipore, Darmstadt, Germany), rabbit- α -MBP (Millipore), mouse- α - β -tubulin III (Phosphosolutions, Aurora, CO), mouse - α -GFAP (ABCAM, San Francisco, CA), and rabbit- α -DCX (ABCAM). Secondary antibodies were goat- α -mouse or goat- α -rabbit Alexa 488 (Life), and were co-incubated with phalloidin-Alexa 568 (Life) overnight in 5% BSA and 1% NGS solution. Cells were counterstained with DAPI and washed with D.I. H₂O before being mounted on slides using Prolong Gold (Life). Slides were allowed to cure at room temperature overnight and stored at 4°C. Images were collected using epifluorescent microscopy.

2.6 AgNP Enhancement

For AgNP enhancement, cells were cultured and exposed to AgNPs for two days before fixation and permeabilization as described for immunocytochemistry. Following permeabilization, cells were treated with phalloidin-Alexa 568 in PBS2 for 20 minutes, washed with PBS2, and images were recorded using epifluorescent microscopy. Image XY locations were saved, and then cells were treated with the Aurion R-Gent SE-LM Silver Enhancement kit (Electron Microscopy Sciences, Hatfield, PA), as per the manufacturer's instructions. Images were collected again from cells in the saved XY positions with transmitted light immediately after enhancement.

2.7 RT-PCR

Undifferentiated neurospheres were collected by centrifugation. Total RNA was isolated from cells using an Ambion PureLink kit with on-column DNA treatment (Life) followed by reverse transcription of 2µg of RNA to cDNA using an ABI high-capacity RT kit (Life). No RT control reactions were run in parallel for each sample. PCR reactions were run using Taq polymerase (Bioexpress, Kaysville, UT), and primers for DCX (F: TTGGACATTTTGACGAACGA, R: CCCTTCTTCCAGTTCATCCA; NM_053379; 353bp (Liu et al. , 2009)), GFAP (F: TAAGCTAGCCCTGGACATCG, R: TTTCTCGGATCTGGAGGTTG; NM_010277; 110bp (Liu, Chopp, 2009)), Sox2 (F: ACCAGCTCGCAGACCTACAT, R: TAGGAGTGGGAGGAAGAGGTA; NM_001109181; 154bp (Liu, Chopp, 2009)), Nestin (F: AGAGAAGCGCTGGAACAGAG, R: AGGTGTVTGCAACCGAGAGT; 234bp (Sun et al. , 2011)) or GAPDH (F: GATGCTGGTGCTGAGTATGTCG, R: GTGGTGCAGGATGCATTGCTCTGA; 197bp)(All 5'→3'). PCR products were separated by 10% poly-acrylamide gel electrophoresis, stained with ethidium bromide, and imaged under UV light.

2.8 Imaging

A Leica DM6000B microscope was used to obtain epifluorescent and transmitted light images with a 20x objectives. Images were acquired using LAS AF software (Leica, Buffalo Grove, IL). Adjustments to brightness and contrast were performed on entire images using ImageJ (NIH, Bethesda, MD). For the enhancement assay, open source software (GIMP) was used to manually align the images and the transmitted light images were color-reversed to allow overlaying in ImageJ and simultaneous visualization of silver enhancement and actin staining. All figures were created using Adobe Illustrator.

2.9 Data Analysis

Cells were counted in ImageJ (NIH, Bethesda, MD) and neurites were analyzed with the NeuronJ extension (Meijering et al. , 2004). Data in bar graphs are presented as mean ± standard deviation of at least three independent experiments as detailed. Graphs were generated and statistical significance was determined using Prism (Graphpad, San Diego, CA). Following one-way ANOVA tests, Tukey's multiple comparisons was used to determine significance between

groups. Following two-way ANOVA tests, Sidak's multiple comparisons was used to determine significance between groups.

3. Results

3.1 Characterization of SVZ-derived NSC

Cells isolated from adult rat SVZ formed non-adherent neurospheres when maintained in medium including EGF and bFGF (Fig 1A). These neurospheres regularly express Sox2, a proliferative marker, nestin and DCX, neural stem cell markers, and GFAP, an astrocyte and immature precursor cell marker (Fig 1B). When provided an extracellular matrix (laminin) and subjected to growth factor withdrawal, cells attach, migrate away from neurospheres and differentiate towards a mature neural lineage with complex morphologies. Differentiating cells expressed the mature neuron marker, MAP2 (Fig 1C), β -tubulin III (Fig 1D) a marker of immature neurons, and MBP (Fig 1E) a protein expressed by oligodendrocytes.

3.2 f-Actin inclusions are more frequently found in AgNP-treated cells

Following two days' exposure to AgNPs, exposed cells formed punctate f-actin inclusions (arrows). Control cells contained the inclusions very rarely (Fig 2A&D), whereas many cells contained inclusions following AgNP exposure (Fig 2B,C,E). The percentage of inclusion-containing cells in AgNP conditions was near double that of control (Fig 2F) and the proportion of cells containing actin inclusions increased in a dose-dependent fashion (Fig 2G).

3.3 f-Actin inclusions do not co-localize with AgNPs

A silver enhancement assay showed no staining of control cells (Fig 3A) while AgNP were visible as dark spots in association with treated cells (Fig 3B&C, arrows) and adherent to the coverslip in treated preparations. In double labeling experiments, individual AgNP-treated cells contained f-actin inclusions (Fig 3D, arrows) and enhanced AgNPs (Fig 3E, arrowheads). When the images were merged (Fig 3F), it was evident that most enhanced AgNPs did not regularly co-localize with f-actin inclusions (single arrowheads) although a few nonspecific co-localizations were observed (double arrowheads).

3.4 AgNP exposure interferes with neurite extension

To determine if exposure to AgNPs affected cytoskeleton-mediated processes, we performed time lapse microscopy experiments. B35 neuroblastoma cells, a model for neurite extension and cell migration, exhibited a significant reduction in maximum neurite length when exposed to AgNPs (Fig 4A). This effect was replicated in time lapse experiments with differentiating SVZ-NSCs, where AgNP exposure resulted in a reduction in maximum neurite length (Fig 4B).

A more detailed analysis of individual time points from SVZ-NSC time-lapse experiments demonstrated that control cells (Fig 5A) and AgNP-treated cells (Fig 5B) exhibited distinct morphological differences during six hours of exposure to AgNPs. The length of neurites on control cells (Fig 5C) remained variable and dynamic, with cells actively extending and retracting neurites throughout the six hour period. Conversely, AgNP-exposed cells exhibited a significant loss of average length during the AgNP treatment (Fig 5D). Moreover, control cells (Fig 5E) consistently extended complex, well-arborized neurites with multiple branches, but during AgNP exposure, cells demonstrated a significant loss of branching over time (Fig 5F).

4. Discussion

Although the cellular effects of AgNP exposure at cytotoxic levels have been well studied (reviewed in (Bartlomiejczyk et al. , 2013)), little is known about how cells might respond to lower, sublethal, levels of exposure that may be more representative of those resulting from contact with consumer products containing AgNP-based antimicrobial coatings. AgNPs accumulate in tissues, including brain, after chronic oral low-level exposure without associated signs of illness or discomfort. In this study, we investigated the effect of such exposure on adult NSCs, a population of cells that is key to normal brain function. Adult SVZ-NSCs provide an accessible culture model to investigate the effects of neurotoxins on the cellular mechanisms underlying neurogenesis in addition to providing more general insights on mechanisms operating in neurons and during development.

In cortical neurons, a two-day exposure to 1 μ g/mL AgNPs after four days in culture did not result in a significant loss of viability (Xu, Pielt, 2013). Indeed, even 5 μ g/mL was not toxic to

mixed neural cultures (Haase, Rott, 2012) or PC12 cells (Hadrup, Loeschner, 2012). In rats, ingestion of 100 or 500mg/kg AgNPs for 28 days caused no observable ill effects, but silver was detected in the brains of treated rats at 300-400ng/g brain tissue (Lee, Kim, 2013), corresponding to 0.3-0.4µg/mL. Even after one month of recovery with no additional treatments, the levels of silver in brain remained at 300-400ng/g, and in some cases increased to 400-500ng/g (Lee, Kim, 2013). Therefore, we performed our experiments using 0.05-2.0µg/mL, representing *in vitro* doses that mimic sublethal levels observed in rats *in vivo*, and elicit robust effects while remaining below reportedly toxic concentrations in cultured cells. These concentrations are also consistent with studies investigating incidental exposure; AgNP-containing burn dressings were found to release approximately 0.6µg/day (Bidgoli et al. , 2013). Similarly, human exposure from food packaging materials was estimated at 0.06-13µg/kg/day (Cushen et al. , 2013, 2014).

Our data demonstrate that exposure to sublethal levels of AgNPs resulted in formation of f-actin inclusions. These inclusions were similar to those seen when eukaryotic cells are exposed to the actin-stabilizing toxin jasplakinolide or the actin-depolymerizing toxin cytochalasin D (Lazaro-Diequez et al. , 2008, Muller et al. , 2013), indicating that the inclusions may point to a disruption of actin dynamics. AgNPs are already known to disrupt the actin cytoskeleton at toxic concentrations ranging from 5.0 to 400µg/mL (Asharani, Sethu, 2010, Schrand, Braydich-Stolle, 2008, Xu, Pielt, 2013), but our findings suggest that they can also do so in cultured neural cells at much lower concentrations.

AgNP exposure can result in equal distribution of AgNPs within cells (Asharani et al. , 2009a, Marano et al. , 2011) or in agglomeration and sequestration within membrane-bound compartments (Gliga et al. , 2014, Schrand, Braydich-Stolle, 2008). We found that in our cells, with low concentrations of exposure, AgNPs did not regularly co-localize with f-actin inclusions. This suggests that the inclusions do not represent sequestered agglomerates of AgNPs within the cells, and indicates that they may instead represent disruptions in the actin cytoskeleton.

Cytoskeleton dynamics are vital for numerous processes involved in neurogenesis, such as neurite extension and neurite arborization, where the cytoskeleton serves as a motor for extension and an anchor for adhesion (Bray and Chapman, 1985, Hotulainen and Lappalainen, 2006). Therefore, we wanted to investigate if these cytoskeleton-dependent processes were affected by AgNP exposure. In time course experiments, we found that maximum neurite length

was reduced in B35 neuroblastoma cells exposed to AgNPs, an effect that was replicated in SVZ-NSCs. Additionally, in SVZ-NSCs, neurite extension and neurite arborization were both significantly reduced over time following AgNP exposure. These findings support our hypothesis that AgNPs disrupt cytoskeleton dynamics at sublethal levels, and indicate a potential inhibition of neurogenesis following AgNP exposure.

Neurogenesis has been linked with cognitive functions such as learning and memory, and is thought to play a key role in these processes, especially the consolidation of short-term to long-term memory (Snyder, Kee, 2001, van Praag, Christie, 1999). Therefore, if AgNPs do cause a disruption of neurogenesis, deficits in these areas following AgNP exposure would be expected. Indeed, rats exposed to sublethal levels of AgNPs for 14 days exhibited a loss of spatial memory and learning (Liu et al. , 2012). This study did not investigate neurogenesis directly, although AgNP-dependent generation of reactive oxygen species (ROS) (AshaRani, Low Kah Mun, 2009b, Haase, Rott, 2012, Schrand, Braydich-Stolle, 2008) was proposed as a possible mechanism. In mice, however, neither hippocampal neurogenesis nor cognitive function was impaired following a 7-day exposure to AgNPs (Liu et al. , 2013). At toxic concentrations, AgNPs interact with cellular signaling pathways to induce an inflammatory response or apoptosis (reviewed by (Marano, Hussain, 2011)). This is also true of sublethal concentrations of AgNPs, which increase ERK and Akt signaling in SH-SY5Y cells, resulting in a corresponding increase in neuronal differentiation (Dayem et al. , 2014). Interestingly, activation of ERK signaling encourages NSC proliferation and neuronal differentiation (Huang et al. , 2014, Liu et al. , 2014), while Akt signaling activation encourages NSC proliferation and survival during differentiation (Conti et al. , 2001, Jin et al. , 2005). Conversely, our work suggests a potential loss of neurogenesis through decreased functional mobilization of the cytoskeleton. Clearly, more work examining the effects of sublethal concentrations of AgNPs on neurogenesis, and neurogenesis-dependent processes is necessary *in vitro* and *in vivo*. To that end, SVZ-NSCs are an excellent tool. The SVZ system itself is well characterized and easily accessible for both *in vitro* and *in vivo* assays, and the behavior of cultured SVZ-NSCs mirrors adult and developmental neurogenesis *in situ* (Ge, Sailor, 2008, Lois and Alvarez-Buylla, 1993, Parmar et al. , 2003). Therefore, disruptions to NSC differentiation *in vitro* following AgNP exposure could be indicative of a disruption of neurogenesis *in vivo*, leading to deficits in

neurodevelopment in early life, or impairing learning, memory, repair, and other healthy brain functions in the adult brain (Snyder, Kee, 2001, van Praag, Christie, 1999).

5. Acknowledgements

This work was supported by funding to N.S. from NSF Cooperative Agreement Award number EPS-1003907. Nancy Proper and Amy Parsons-White provided maintenance of neurosphere cultures and expert technical assistance. The authors thank Dusty Vance for assistance with performing experiments, and Ian Waddell, Kristen Chaffins, Samantha Clay, and Tamara Westfall for their help through the Teacher Research Experience for the advancement of Knowledge (TREK) Program at Marshall University.

Figure Captions

Figure 1: Adult rat SVZ-derived progenitor cells proliferate and form free-floating neurospheres that can be maintained in culture (A). RT-PCR of three separate cell lines shows that neurospheres express stem cell markers (B). SVZ-derived NSC differentiated *in vitro* express markers (green) of early neuronal (β -tubulin III; C), mature neuronal (Map2; D) and oligodendrocyte (MBP; E) fates. Cell morphology is delineated by staining f-actin with phalloidin (red), nuclei are blue (DAPI).

Figure 2: AgNP exposure induces formation of f-actin inclusions. In control conditions, some differentiated NSCs contained f-actin inclusions (A; arrow), though most did not (A&D; arrowheads). Cells exposed to 0.1 or 1 μ g/mL AgNPs also formed f-actin inclusions (B,C,E; arrows). When compared to control conditions, a higher percentage of cells exposed to 1 μ g/mL AgNP formed these inclusions (F; $p < 0.0001$ compared to control or citrate vehicle, ANOVA). Actin inclusions formed in a dose-dependent manner (G). Cytoskeletal f-actin is stained with phalloidin (red), and nuclei with DAPI (blue). D and E show outlined regions in A and C, respectively. Numbers in bars are number of independent coverslips analyzed. * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$; all values compared to 0 μ g/mL; Tukey's multiple comparisons.

Figure 3: AgNPs do not co-localize with f-actin inclusions. Transmitted light images of control cells (A) and cells treated with 1 μ g/mL AgNP (B) following treatment with a silver enhancement assay to allow visualization of AgNPs (black). Cells (arrows, A-C) become difficult to spot amidst enhanced AgNPs, so a higher-contrast image is also shown (C). When the same AgNP-exposed cells are stained with phalloidin to visualize f-actin (D; red) and the original transmitted light image is color-reversed (E), overlaying the two (F) reveals that the majority of f-actin inclusions do not co-localize with enhanced AgNPs (arrows, D&F). Conversely, most enhanced AgNPs within the cell do not co-localize with actin inclusions (arrowheads, E&F). While some random co-localization is observed (double arrowheads), enhanced AgNPs do not regularly co-localize with actin inclusions.

Figure 4: AgNP exposure correlates to a decrease in maximum neurite length in differentiating neural cells. B35 neuroblastoma cells (A) and SVZ-NSCs (B) develop shorter maximum neurite lengths when exposed to 1 μ g/mL AgNP in time lapse experiments. Numbers in bars are number of neurites analyzed; all neurites on a given cell were analyzed. Total number of cells analyzed: 22 control, 10 AgNP in two independent experiments for B35 and 16 control, 18 AgNP in three independent experiments for SVZ-NSCs. * $p < 0.0001$; two-tailed t-test.

Figure 5: Loss of neurite length and complexity is time-dependent in SVZ-NSCs. Representative images of control (A) and AgNP-exposed (B) cells were obtained using time-lapse microscopy over six hours. The length of control neurites remained highly variable and dynamic over time (C), whereas neurites on AgNP-exposed cells (D) significantly decreased over six hours ($p < 0.05$, 2-way ANOVA). Similarly, control neurites were consistently and variably branched (E), while AgNP-exposed cells (F) suffered from a significant loss of branching compared to control. $p < 0.05$, 2-way ANOVA; ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to same time point in control conditions, Sidak's multiple comparisons. Numbers in bars are number of neurites analyzed; all neurites on a given cell were analyzed. Y-axis breaks (C&D) do not exclude any data points.

References

- Ahamed M, Karns M, Goodson M, Rowe J, Hussain SM, Schlager JJ, et al. DNA damage response to different surface chemistry of silver nanoparticles in mammalian cells. *Toxicology and applied pharmacology*. 2008;233:404-10.
- Altman J, Das GD. Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *The Journal of comparative neurology*. 1965;124:319-35.
- Arora S, Jain J, Rajwade JM, Paknikar KM. Cellular responses induced by silver nanoparticles: In vitro studies. *Toxicology letters*. 2008;179:93-100.
- AshaRani PV, Hande MP, Valiyaveetil S. Anti-proliferative activity of silver nanoparticles. *BMC cell biology*. 2009a;10:65.
- AshaRani PV, Low Kah Mun G, Hande MP, Valiyaveetil S. Cytotoxicity and genotoxicity of silver nanoparticles in human cells. *ACS nano*. 2009b;3:279-90.
- Asharani PV, Sethu S, Vadukumpully S, Zhong S, Lim CT, Hande MP, et al. Investigations on the Structural Damage in Human Erythrocytes Exposed to Silver, Gold, and Platinum Nanoparticles. *Advanced Functional Materials*. 2010;20:1233-42.
- Bartłomiejczyk T, Lankoff A, Kruszewski M, Szumiel I. Silver nanoparticles -- allies or adversaries? *Annals of agricultural and environmental medicine : AAEM*. 2013;20:48-54.
- Bidgoli SA, Mahdavi M, Rezayat SM, Korani M, Amani A, Ziarati P. Toxicity assessment of nanosilver wound dressing in Wistar rat. *Acta medica Iranica*. 2013;51:203-8.
- Bray D, Chapman K. Analysis of microspike movements on the neuronal growth cone. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 1985;5:3204-13.
- Conti L, Sipione S, Magrassi L, Bonfanti L, Rigamonti D, Pettirossi V, et al. Shc signaling in differentiating neural progenitor cells. *Nature neuroscience*. 2001;4:579-86.
- Curtis MA, Low VF, Faull RL. Neurogenesis and progenitor cells in the adult human brain: a comparison between hippocampal and subventricular progenitor proliferation. *Developmental neurobiology*. 2012;72:990-1005.
- Cushen M, Kerry J, Morris M, Cruz-Romero M, Cummins E. Migration and exposure assessment of silver from a PVC nanocomposite. *Food chemistry*. 2013;139:389-97.
- Cushen M, Kerry J, Morris M, Cruz-Romero M, Cummins E. Evaluation and simulation of silver and copper nanoparticle migration from polyethylene nanocomposites to food and an associated exposure assessment. *Journal of agricultural and food chemistry*. 2014;62:1403-11.
- Dayem AA, Kim B, Gurunathan S, Choi HY, Yang G, Saha SK, et al. Biologically synthesized silver nanoparticles induce neuronal differentiation of SH-SY5Y cells via modulation of reactive oxygen species, phosphatases, and kinase signaling pathways. *Biotechnology journal*. 2014;9:934-43.

de Lima R, Seabra AB, Duran N. Silver nanoparticles: a brief review of cytotoxicity and genotoxicity of chemically and biogenically synthesized nanoparticles. *Journal of applied toxicology : JAT*. 2012;32:867-79.

Dobias J, Bernier-Latmani R. Silver release from silver nanoparticles in natural waters. *Environmental science & technology*. 2013;47:4140-6.

Doetsch F, Garcia-Verdugo JM, Alvarez-Buylla A. Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 1997;17:5046-61.

Doetsch F, Petreanu L, Caille I, Garcia-Verdugo JM, Alvarez-Buylla A. EGF converts transit-amplifying neurogenic precursors in the adult brain into multipotent stem cells. *Neuron*. 2002;36:1021-34.

Eriksson PS, Perfilieva E, Bjork-Eriksson T, Alborn AM, Nordborg C, Peterson DA, et al. Neurogenesis in the adult human hippocampus. *Nature medicine*. 1998;4:1313-7.

Gage FH. Mammalian neural stem cells. *Science*. 2000;287:1433-8.

Garcia-Reyero N, Kennedy AJ, Escalon BL, Habib T, Laird JG, Rawat A, et al. Differential effects and potential adverse outcomes of ionic silver and silver nanoparticles in vivo and in vitro. *Environmental science & technology*. 2014;48:4546-55.

Ge S, Sailor KA, Ming GL, Song H. Synaptic integration and plasticity of new neurons in the adult hippocampus. *The Journal of physiology*. 2008;586:3759-65.

Gerard C, Langlois X, Gingrich J, Doucet E, Verge D, Kia HK, et al. Production and characterization of polyclonal antibodies recognizing the intracytoplasmic third loop of the 5-hydroxytryptamine 1A receptor. *Neuroscience*. 1994;62:721-39.

Gliga AR, Skoglund S, Wallinder IO, Fadeel B, Karlsson HL. Size-dependent cytotoxicity of silver nanoparticles in human lung cells: the role of cellular uptake, agglomeration and Ag release. *Particle and fibre toxicology*. 2014;11:11.

Haase A, Rott S, Manton A, Graf P, Plendl J, Thunemann AF, et al. Effects of silver nanoparticles on primary mixed neural cell cultures: uptake, oxidative stress and acute calcium responses. *Toxicological sciences : an official journal of the Society of Toxicology*. 2012;126:457-68.

Hadrup N, Loeschner K, Mortensen A, Sharma AK, Qvortrup K, Larsen EH, et al. The similar neurotoxic effects of nanoparticulate and ionic silver in vivo and in vitro. *Neurotoxicology*. 2012;33:416-23.

Hotulainen P, Lappalainen P. Stress fibers are generated by two distinct actin assembly mechanisms in motile cells. *The Journal of cell biology*. 2006;173:383-94.

Huang JH, Cai WJ, Zhang XM, Shen ZY. Icaritin promotes self-renewal of neural stem cells: an involvement of extracellular regulated kinase signaling pathway. *Chinese journal of integrative medicine*. 2014;20:107-15.

Jin L, Hu X, Feng L. NT3 inhibits FGF2-induced neural progenitor cell proliferation via the PI3K/GSK3 pathway. *Journal of neurochemistry*. 2005;93:1251-61.

Kim S, Choi JE, Choi J, Chung KH, Park K, Yi J, et al. Oxidative stress-dependent toxicity of silver nanoparticles in human hepatoma cells. *Toxicology in vitro : an international journal published in association with BIBRA*. 2009;23:1076-84.

Kruszewski M, Brzoska K, Brunborg G, Asare N, Dobrzyńska M, Dušinská M, et al. Toxicity of Silver Nanomaterials in Higher Eukaryotes. In: James CF, editor. *Advances in Molecular Toxicology*; Elsevier; 2011. p. 179-218.

Lazaro-Diequez F, Aguado C, Mato E, Sanchez-Ruiz Y, Esteban I, Alberch J, et al. Dynamics of an F-actin aggresome generated by the actin-stabilizing toxin jasplakinolide. *Journal of cell science*. 2008;121:1415-25.

Lee JH, Kim YS, Song KS, Ryu HR, Sung JH, Park JD, et al. Biopersistence of silver nanoparticles in tissues from Sprague-Dawley rats. *Particle and fibre toxicology*. 2013;10:36.

Li Y, Bhalli JA, Ding W, Yan J, Pearce MG, Sadiq R, et al. Cytotoxicity and genotoxicity assessment of silver nanoparticles in mouse. *Nanotoxicology*. 2014;8 Suppl 1:36-45.

Liu F, Xuan A, Chen Y, Zhang J, Xu L, Yan Q, et al. Combined effect of nerve growth factor and brain-derived neurotrophic factor on neuronal differentiation of neural stem cells and the potential molecular mechanisms. *Molecular medicine reports*. 2014;10:1739-45.

Liu P, Huang Z, Gu N. Exposure to silver nanoparticles does not affect cognitive outcome or hippocampal neurogenesis in adult mice. *Ecotoxicology and environmental safety*. 2013;87:124-30.

Liu XS, Chopp M, Zhang XG, Zhang RL, Buller B, Hozeska-Solgot A, et al. Gene profiles and electrophysiology of doublecortin-expressing cells in the subventricular zone after ischemic stroke. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*. 2009;29:297-307.

Liu Y, Guan W, Ren G, Yang Z. The possible mechanism of silver nanoparticle impact on hippocampal synaptic plasticity and spatial cognition in rats. *Toxicology letters*. 2012;209:227-31.

Lois C, Alvarez-Buylla A. Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. *Proceedings of the National Academy of Sciences of the United States of America*. 1993;90:2074-7.

Luskin MB. Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone. *Neuron*. 1993;11:173-89.

Marano F, Hussain S, Rodrigues-Lima F, Baeza-Squiban A, Boland S. Nanoparticles: molecular targets and cell signalling. *Archives of toxicology*. 2011;85:733-41.

Meijering E, Jacob M, Sarria JC, Steiner P, Hirling H, Unser M. Design and validation of a tool for neurite tracing and analysis in fluorescence microscopy images. *Cytometry Part A : the journal of the International Society for Analytical Cytology*. 2004;58:167-76.

Muller P, Langenbach A, Kaminski A, Rychly J. Modulating the actin cytoskeleton affects mechanically induced signal transduction and differentiation in mesenchymal stem cells. *PloS one*. 2013;8:e71283.

Muraoka K, Shingo T, Yasuhara T, Kameda M, Yuen WJ, Uozumi T, et al. Comparison of the therapeutic potential of adult and embryonic neural precursor cells in a rat model of Parkinson disease. *Journal of neurosurgery*. 2008;108:149-59.

Pal S, Tak YK, Song JM. Does the antibacterial activity of silver nanoparticles depend on the shape of the nanoparticle? A study of the Gram-negative bacterium *Escherichia coli*. *Applied and environmental microbiology*. 2007;73:1712-20.

Parmar M, Sjoberg A, Bjorklund A, Kokaia Z. Phenotypic and molecular identity of cells in the adult subventricular zone. *in vivo* and after expansion *in vitro*. *Molecular and cellular neurosciences*. 2003;24:741-52.

Quadros ME, Marr LC. Environmental and human health risks of aerosolized silver nanoparticles. *Journal of the Air & Waste Management Association*. 2010;60:770-81.

Reynolds BA, Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science*. 1992;255:1707-10.

Scheffler B, Walton NM, Lin DD, Goetz AK, Enikolopov G, Roper SN, et al. Phenotypic and functional characterization of adult brain neurogenesis. *Proceedings of the National Academy of Sciences of the United States of America*. 2005;102:9353-8.

Schrand AM, Braydich-Stolle LK, Schlager JJ, Dai L, Hussain SM. Can silver nanoparticles be useful as potential biological labels? *Nanotechnology*. 2008;19:235104.

Sharma HS, Hussain S, Schlager J, Ali SF, Sharma A. Influence of nanoparticles on blood-brain barrier permeability and brain edema formation in rats. *Acta neurochirurgica Supplement*. 2010;106:359-64.

Snyder JS, Kee N, Wojtowicz JM. Effects of adult neurogenesis on synaptic plasticity in the rat dentate gyrus. *Journal of neurophysiology*. 2001;85:2423-31.

Sondi I, Salopek-Sondi B. Silver nanoparticles as antimicrobial agent: a case study on *E. coli* as a model for Gram-negative bacteria. *Journal of colloid and interface science*. 2004;275:177-82.

Sun Y, Hu J, Zhou L, Pollard SM, Smith A. Interplay between FGF2 and BMP controls the self-renewal, dormancy and differentiation of rat neural stem cells. *Journal of cell science*. 2011;124:1867-77.

Tang J, Xiong L, Wang J, Liu L, Li J, Wan Z, et al. Influence of silver nanoparticles on neurons and blood-brain barrier via subcutaneous injection in rats. *Applied Surface Science*. 2008;255:502-4.

Tang J, Xiong L, Wang S, Wang J, Liu L, Li J, et al. Distribution, translocation and accumulation of silver nanoparticles in rats. *Journal of nanoscience and nanotechnology*. 2009;9:4924-32.

van Praag H, Christie BR, Sejnowski TJ, Gage FH. Running enhances neurogenesis, learning, and long-term potentiation in mice. *Proceedings of the National Academy of Sciences of the United States of America*. 1999;96:13427-31.

Wachs FP, Couillard-Despres S, Engelhardt M, Wilhelm D, Ploetz S, Vroemen M, et al. High efficacy of clonal growth and expansion of adult neural stem cells. *Laboratory investigation; a journal of technical methods and pathology*. 2003;83:949-62.

Wen LS, Santschi PH, Gill GA, Tang D. Silver concentrations in Colorado, USA, watersheds using improved methodology. *Environmental toxicology and chemistry / SETAC*. 2002;21:2040-51.

Whiteley CM, Dalla Valle M, Jones KC, Sweetman AJ. Challenges in assessing release, exposure and fate of silver nanoparticles within the UK environment. *Environmental science Processes & impacts*. 2013;15:2050-8.

Xu F, Pielt C, Farkas S, Qazzaz M, Syed NI. Silver nanoparticles (AgNPs) cause degeneration of cytoskeleton and disrupt synaptic machinery of cultured cortical neurons. *Molecular brain*. 2013;6:29.

Yu SJ, Yin YG, Chao JB, Shen MH, Liu JF. Highly dynamic PVP-coated silver nanoparticles in aquatic environments: chemical and morphology change induced by oxidation of Ag(0) and reduction of Ag(+). *Environmental science & technology*. 2014;48:403-11.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5