Published in final edited form as:

Neurotoxicol Teratol. 2011 November ; 33(6): 708–714. doi:10.1016/j.ntt.2011.02.002.

Silver Nanoparticles Alter Zebrafish Development and Larval Behavior: Distinct Roles for Particle Size, Coating and Composition

Christina M. Powers¹, Theodore A. Slotkin¹, Frederic J. Seidler¹, Appala R. Badireddy², and Stephanie Padilla³

¹Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC 27710 USA

²Department of Civil and Environmental Engineering, Pratt School of Engineering Center for the Environmental Implications of NanoTechnology (CEINT), Duke University, Durham, North Carolina 27708 USA

³Integrated Systems Toxicology Division, National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, NC 27709 USA

Abstract

Silver nanoparticles (AgNPs) act as antibacterials by releasing monovalent silver (Ag⁺) and are increasingly used in consumer products, thus elevating exposures in human and wildlife populations. In vitro models indicate that AgNPs are likely to be developmental neurotoxicants with actions distinct from those of Ag⁺. We exposed developing zebrafish (*Danio rerio*) to Ag⁺ or AgNPs on days 0–5 post-fertilization and evaluated hatching, morphology, survival and swim bladder inflation. Larval swimming behavior and responses to different lighting conditions were assessed 24 hr after the termination of exposure. Comparisons were made with AgNPs of different sizes and coatings: 10 nm citrate-coated AgNP (AgNP-C), and 10 or 50 nm polyvinylpyrrolidone-coated AgNPs (AgNP-PVP). Ag⁺ and AgNP-C delayed hatching to a similar extent but Ag⁺ was more effective in slowing swim bladder inflation, and elicited greater dysmorphology and mortality. In behavioral assessments, Ag⁺ exposed fish were hyperresponsive to light changes, whereas AgNP-C exposed fish showed normal responses. Neither of the AgNP-PVPs affected survival or morphology but both evoked significant changes in swimming responses to light in ways that were distinct from Ag⁺ and each other. The smaller AgNP-PVP caused overall hypoactivity whereas the larger caused hyperactivity. AgNPs are less potent than Ag⁺ with respect to dysmorphology and loss of viability, but nevertheless produce neurobehavioral effects that highly depend on particle coating and size, rather than just reflecting the release of Ag⁺. Different AgNP formulations are thus likely to produce distinct patterns of developmental neurotoxicity.

Keywords

Silver; Nanoparticles; Zebrafish

Correspondence: Dr. Theodore Slotkin, Box 3813 DUMC, Durham, NC 27710, Tel 919 681 8015, t.slotkin@duke.edu.

disclaimers

None of the other authors has any actual or potential competing financial interests.

INTRODUCTION

Silver nanoparticles (AgNPs) release monovalent silver ion (Ag^+) to produce an antimicrobial effect and thus, are increasingly incorporated in consumer products [17]. The rapid rise in commercial AgNP use raises concern for exposures to Ag^+ , particularly because the developing brain is a specific target for the adverse effects of heavy metals [3,4]. Silver crosses the placental barrier, accumulates in the human fetus [9], and alters neuroanatomy in developing rodents [16]. In an *in vitro* model of neuronal development, we recently found that Ag^+ has adverse effects on cell replication and viability, and interferes with the emergence of specific neurotransmitter phenotypes [13]. Using the zebrafish model, we demonstrated that Ag^+ has corresponding effects on neurodevelopment: at high concentrations, Ag^+ impairs embryonic survival, growth and development, but at lower concentrations that are devoid of effects on morphology or survival, Ag^+ nevertheless still depresses larval swimming behavior [14].

Formulation of toxic metals into nanoparticles results in adverse effects in both adult and developing zebrafish that reflect, in part, the toxicity of the metal itself [5,19]. In the case of AgNPs, high concentrations decrease survival, increase malformations in the embryo, and delay hatching [1,2,5]. Equally important, our studies with Ag^+ point to potential effects on brain development and behavior at lower exposures that do not produce outright toxicity; further, the *in vitro* studies suggest that AgNPs may differ from Ag^+ in their spectrum of effects on neurodevelopment [12,13]. AgNPs enter the developing zebrafish brain, cause apoptosis in the head region and alter expression of developmental genes [1,18]. There are, however, conflicting reports as to whether the effects of AgNPs are separable from those of Ag^+ [2,5], and until now no one has assessed behavioral outcomes that could point to specific consequences of AgNP developmental neurotoxicity. Here, we evaluated the neurobehavioral outcomes after embryonic exposure to Ag^+ compared to three types of AgNPs, so as to determine the potential differences due to particle size, coating and composition. The approach paralleled our cell culture work, which found dissimilarities between Ag^+ and AgNPs, as well as specific roles for AgNP particle coating and size [12]. We first compared the effects of Ag^+ to those of 10 nm citrate-coated AgNPs (AgNP-C) for their effects on zebrafish development, survival and morphology over a wide range of concentrations. We then evaluated whether a concentration that had little or no effect on these parameters affected larval swimming responses to different lighting conditions. Finally, we assessed the roles of coating and size in the outcomes by comparing 10 nm AgNP-C to 10 and 50 nm polyvinylpyrrolidone-coated silver nanoparticles (AgNP-PVP).

MATERIALS AND METHODS

Reagents

Silver nitrate (AgNO_3) was purchased from Sigma Chemical Co. (St. Louis, MO). AgNP-C was synthesized at Duke University using established methods [8]. AgNP-PVPs were purchased in powder form from Nanostructured & Amorphous Materials Inc. (Houston, TX). PVP was kindly provided by Dr. Mark Wiesner (Duke University, Pratt School of Engineering). Particle stock preparation and characterization procedures are published [12]. All stocks were prepared at a nominal concentration of 1 mM Ag. The “nominal concentration” is defined as the Ag concentration that would be seen if all the Ag within the particle were freely dissolved; since only a small fraction actually dissolves from the nanoparticle, the true Ag^+ concentration in solution is much lower than the nominal concentration calculated from the nanoparticle composition [11]. Because the AgNPs have different sizes, the stock suspensions contained 2×10^{13} particles per ml for the 10 nm AgNP-C and AgNP-PVP, but 3×10^{11} particles per ml for the 50 nm AgNP-PVP. For experiments with PVP-coated particles, we made up an additional stock solution consisting

of 10% PVP based on our previous measurements of the PVP concentration in the particles [12]. The nominal Ag concentration of particles in embryo medium or H₂O was measured by taking an aliquot near the top of suspensions to mimic exposures in our 96-well plates; the wells have a mesh at the bottom that restricted fish to the upper portion. Samples were measured using inductively coupled plasma-optical emission spectroscopy (Prism ICP High Dispersion, Teledyne Leeman Labs, Hudson, NH).

Animals

All experiments were approved by and carried out in accordance with guidelines of the Institutional Animal Care and Use Committee at the U.S. EPA National Health and Environmental Effects Research Laboratory. The methods for housing, maintenance, breeding, embryo selection and rearing were as previously described [10]. Briefly, all studies used wild type zebrafish raised individually in 96-well mesh-bottom (40 µm nylon) microtiter plates (Multiscreen™ catalog #MANMN4050, Millipore Corp., Bedford, MA). Each well contained 250 µl of 10% Hank's Balanced Salt Solution (13.7 mM NaCl, 0.54 mM KCl, 24 mM Na₂PO₄, 44 mM KH₂PO₄, 130 mM CaCl₂, 100 mM MgSO₄, 420 mM NaHCO₃) with or without addition of test substances. Exposures covered final concentrations from 0.01 to 100 µM nominal Ag.

Embryonic toxicity

Embryonic exposures began at approximately 6 hours post-fertilization. We renewed the test solutions and observed fish every 24 hr, up to 4 days post-fertilization (dpf). On 5 dpf, larvae were placed in 10% Hank's solution alone and observations were made over the ensuing 48 hr period to characterize mortality, dysmorphology, hatching, and swim bladder inflation. On 6 or 7 dpf (either at the end of behavioral testing or on the next day), we evaluated dysmorphology in six categories: 1) spine (e.g. stunted skeletal growth, kink in tail), 2) fins (e.g. stunted fins), 3) cranial/facial (e.g. ocular edema, small otoliths), 4) thorax (e.g. distension, tube heart), 5) abdominal (e.g. distension, emaciation), and 6) position in the water column (e.g. fluttering/trembling, lying on side or back). If an animal had a malformation in any of the six categories, a score of 1–4 was assigned to that deformity, with higher scores signifying greater severity. A number of different malformations could occur in each of the six categories and thus scores for individual malformations were summed to generate a net category score. The scores for all of the categories were then summed, resulting in a total score ranging from 0–34, with higher scores denoting more severely malformed fish. Those with a score of 0–3 were deemed “normal”, whereas those with a score of 4–34 were deemed “abnormal”. This categorization was determined based on the observation that the vast majority of the control animals score in the range of 0–3. Animals that had not hatched by the time of assessment were not scored and these animals were counted as “dead.” Of the 1920 fish included in this study, three live fish had not hatched by 6 dpf, two of which had been treated with AgNP-C at a 30 µM nominal Ag concentration, and one with AgNP-C at a 10 µM nominal Ag concentration; these fish generally never hatched and expired by the end of the observation period.

Larval behavior testing

Testing occurred on 6 dpf (i.e. 24 hr after the termination of Ag⁺ or AgNP exposure) using published procedures [10]. By 09:30 h on the day of testing, animals were transferred to a quiet, dark environment kept at 26°C (the same temperature as the incubator they were reared in) and evaluations were made between 12:30 and 16:30 h, the diurnal period with the most stable activity [10]. For testing, larvae were placed under a baffle that excluded extraneous light so as to permit experimental manipulation of light conditions. In accord with the known responses to changes in light levels [10] we recorded activity in five sequential epochs: 1) 20 min of darkness (infrared illumination), 2) 18 min of a visual acuity

test, (2 min of visible light at 0.0015 lux, below the level of zebrafish visual detection, followed by 4 min of darkness, then 2 min of visible light at 0.0020 lux, on the edge of detection, followed by 4 min of darkness, then, visible light at 0.0025 lux, above their detection level for 2 min, followed by 4 min of darkness), 3) 20 min of visible light at 5 lux, 4) 20 min of alternating low (0.005 lux) and high (5 lux) levels of visible light, and 5) 20 min of darkness. Fish movement was recorded and analyzed using the Noldus tracking system and EthoVision 3.1 software (Noldus Information Technology, Lessburg VA).

Data analysis

Occasionally, a batch of zebrafish (“batch” defined as embryos fertilized on the same day) displayed poor overall health and viability even in the absence of toxicant exposure. Accordingly we applied a criterion that, for a batch to be included in the study, the control group had to contain at least 85% of fish categorized as “normal” by the morphological criteria described above. We excluded three batches out of a total of 16. Treatment effects on general development were compared using Fisher’s Exact Test. Comparisons of toxicants to the control group were considered to be one-tailed because the exposures were expected only to impair or delay the developmental indices [1,2]; however for comparisons between toxicants, we used a two-tailed criterion, since the groups could differ in either direction.

For behavioral testing, we included only larvae that were deemed “normal” in the teratology scoring; that is, if a treated larva had an overall score >3 , we did not include its behavioral performance in the analysis. Swimming distances were compiled in two-minute periods for each treatment in the plate. We used nonparametric statistical comparisons for two reasons. First, examination of the data indicated a non-Gaussian distribution of swimming distance. Second, there were batch-to-batch differences, so that, although treatment effects were consistent across batches, the absolute values were variable. Because of these underlying plate-to-plate differences, all the larvae for a given treatment on a given plate were considered to be technical replicates of each other (i.e. the plate was considered $n=1$). For each plate, the values for all the larvae of a specified treatment group were averaged to make a single number corresponding to the average distance swum for each 2 min period; the number of larvae for each treatment on each plate was typically 16 but varied in individual experiments from 6–35, depending on the number of different treatments evaluated on the plate. Across all the plates, we then compared treated and control means over the entire testing period using the Wilcoxon Signed Rank Test to determine if there was a net increase or decrease caused by toxicant exposure; where this global test was statistically significant, we then evaluated whether the effects occurred within individual epochs of the test (e.g. dark, visible light, or alternating levels of visible light). The results are shown for a representative plate for each treatment but the statistical analyses incorporated all the plates, which are shown in the Supplement. However, to aid in the visualization of the results, where the Wilcoxon test indicated a significant difference, we also present chi-square analysis comparing the incidence of increases and decreases in activity from the paired control values; if exposure had no effect, then the expected frequency is 50% showing increases and 50% showing decreases. Significance for all tests was assumed at $p < 0.05$.

RESULTS

Particle characterization

We were concerned that AgNPs might fall out of suspension during the 24 hr exposure period between changes of solution. To test this, we prepared solutions with a nominal Ag concentration of 30 μM and then measured the total Ag concentration in the upper portion of suspensions after 24 hr. For AgNP-C, 55% of the Ag remained in suspension, whereas the proportion was much lower for AgNP-PVPs (~9%). We obtained similar values comparing

AgNPs suspended in Hank's solution or water. The difference between AgNP-C and AgNP-PVP suggested that AgNP-PVPs were aggregating faster than AgNP-C, which we confirmed using dynamic light scattering to characterize the size of the particles that remained in suspension. Compared to their measured sizes in water, all the AgNPs averaged larger sizes in Hank's solution, indicating aggregation of the particles in this medium, likely because of its higher salt content: AgNP-C, 12 nm in H₂O, 28 nm in Hank's solution; smaller AgNP-PVP, 45 nm and 160 nm, respectively; larger AgNP-PVP 63 and 324 nm, respectively. However, whereas the size of AgNP-C in Hank's solution remained relatively unchanged after 24 hr, both of the AgNP-PVPs showed a further increase in size (data not shown), indicating that these particles aggregated to a greater extent. Thus, when we make comparisons based on nominal Ag concentration (the total concentration if all the Ag were free in solution), the actual values are lower for AgNP-C compared to Ag⁺ and much lower for AgNP-PVPs, because the larger aggregated size reduces the surface-to-volume ratio. As seen below, however, the results for the AgNPs cannot be explained simply by a reduced amount of total Ag compared to freely-dissolved ions.

Embryonic toxicity of Ag⁺ and AgNP-C

Most of the control fish hatched by 3 dpf and all hatched by 4 dpf (Table 1). Both Ag⁺ and AgNP-C delayed hatching, but by 4 dpf virtually all fish had hatched regardless of treatment. Furthermore, there was a "ceiling" effect in that increases from 3 μ M to 30 μ M did not produce a corresponding increase in the incidence of delayed hatching. The development of swim bladder inflation was less sensitive to AgNP-C, with significant delays apparent only at levels corresponding to a nominal Ag concentration ≥ 10 μ M. However, the situation was different for Ag⁺, which delayed swim bladder inflation at a concentration where AgNP-C was ineffective (3 μ M). Furthermore, the effect of Ag⁺ on this parameter showed a progressive increase with higher concentrations, whereas that for AgNP-C did not. There were also clear dichotomies between Ag⁺ and AgNP-C in indices of dysmorphology and mortality (Figure 1). Silver ion produced a high rate of dysmorphology at 30 and 100 μ M but a much lower incidence of mortality. In contrast, AgNP-C was virtually equipotent against both these parameters and was significantly less potent toward dysmorphology, most notably at 30 μ M.

Larval behavior with Ag⁺ and AgNP-C

In general, when the light was switched off, control fish swam more than in the lighted conditions (Figure 2A), in agreement with earlier work [10]. We began the test in the dark (epoch 1). In the second epoch, we evaluated the threshold at which fish would detect light and thus show a rebound increase in swimming when the light was switched off; this first occurred with the intermediate light intensity. In the third epoch, the light was left on at the highest intensity, which initially suppressed swimming, followed by a gradual return of activity. In the fourth epoch, the light was switched repeatedly between the highest and intermediate intensities to produce a repetitive increase and decrease in swimming that would depend on their ability to discriminate between the two intensities. In the fifth epoch, the animals were again exposed only to dark to ensure that they would still show normal activation of swimming after the preceding test epochs; this also served to verify that any confounds in epoch 1 related to the initial transfer to the test apparatus had not generated artifactual changes in swimming behavior.

We first evaluated the effects of Ag⁺ at a concentration (30 μ M) that had a significant effect on morphology but not survival; we further restricted our determinations to include only those fish that had normal morphology, since behavioral abnormality in the absence of dysmorphology is a hallmark of neuroteratogens (Figure 2A). In the dark (epoch 1), the Ag⁺-exposed fish were markedly hyperactive and remained so during epoch 2. Nevertheless,

like the controls, the fish still showed a threshold of detection at the intermediate light level in epoch 2, indicating that their visual acuity was normal despite their overall hyperactivity. When the light remained on (epoch 3), fish in the Ag⁺ group showed suppressed swimming, just like the controls, except that now there was no evidence of hyperactivity, and in fact, across plates, the Ag⁺ group was significantly less active than the controls in the constant light (Supplement Figure 1). During epoch 4, the rapid switching of lighting conditions evoked the same increases and decreases in swimming activity that were seen in the controls, but the Ag⁺ group was consistently hyperactive at all points; finally, upon return to continuous dark (epoch 5), these fish again demonstrated marked hyperactivity. To summarize the effects across all plates, the chi-square analysis showed that Ag⁺ consistently increased activity levels in epochs 1, 2, 4 and 5 while suppressing activity in epoch 3 (Figure 2B).

In contrast to the effects of Ag⁺, AgNP-C at a nominal concentration of 30 µM Ag (a concentration that did not evoke significant dysmorphology or mortality) had no discernible effect on swimming behavior (Figure 2B). We also evaluated lower concentrations of AgNP-C (down to 1 µM nominal Ag) and found no effect (Supplemental Figure 1).

Embryonic toxicity and larval behavior with AgNP-C versus AgNP-PVP

We next compared the effect of 10 nm AgNP-C to 10 and 50 nm PVP-coated particles. Neither AgNP-PVP 10 nor 50 nm affected hatching or swim bladder inflation at a nominal concentration of 30 µM Ag (data not shown). Similarly, neither size PVP-coated particle altered morphology or survival (97% of controls designated as normal, 97% for AgNP-C, 95% for control with added freely-dissolved PVP, 98% for AgNP-PVP 10 nm, 97% for AgNP-PVP 50 nm). While PVP alone elicited no detectable effect on behavior, 10 nm AgNP-PVP nevertheless significantly depressed swimming behavior in the dark, the opposite effect of that seen with Ag⁺ (Figure 3A). This did not reflect a loss of visual acuity, since the 10 nm AgNP-PVP group displayed the same threshold in epoch 2 and also responded normally to having the light switched on continuously (epoch 3). Similar to controls, they also showed increases and decreases in activity in response to alternating intensities of light (epoch 4), although overall they were significantly hypoactive (Supplementary Figure 2), again opposite to the effect of Ag⁺. Upon return to dark (epoch 5), the fish exposed to 10 nm AgNP-PVP again displayed abnormally low activity. On the whole then, the smaller AgNP-PVP produced net hypoactivity in epochs 1, 4 and 5 (Figure 3B), opposite to the effects of Ag⁺.

The dissimilarities between Ag⁺ and AgNPs were reinforced when we examined the effects of the larger PVP-coated particles (Figure 3C). These fish showed hyperactivity in the dark (epoch 1), the opposite of what was seen with the smaller AgNP-PVP but, like all the treatment groups, had normal visual acuity with the appropriate threshold for detecting light-dark differences (epoch 2). Exposure to 50 nm AgNP-PVP had a unique effect in continuous light (epoch 3), evoking significant hyperactivity, an effect that was not seen with Ag⁺, AgNP-C or 10 nm AgNP-PVP. The 50 nm AgNP-PVP group showed normal changes in swimming activity with alternating intensities of light (epoch 4), though like Ag⁺ they were significantly more active. Upon return to the dark (epoch 5), fish exposed to the 50 nm AgNP-PVP again showed hyperactivity akin to that seen in the first epoch. Thus, the larger particles produced hyperactivity in epochs 1, 3, 4 and 5 (Figure 3D), an effect distinct from both the 10 nm AgNP-PVP and from Ag⁺.

DISCUSSION

This work in the zebrafish model provides some of the first evidence that AgNPs are neurobehavioral disruptors. Equally important, their actions do not simply reflect the release

of Ag^+ . Indeed, if that were the case, then all four test agents would elicit effects in the same direction but with a predictable magnitude, namely $\text{Ag}^+ > \text{AgNP-C} > 10 \text{ nm AgNP-PVP} > 50 \text{ nm AgNP-PVP}$. The reason for this prediction is that the AgNPs tested here are all chemically stable, so that the actual, freely-dissolved Ag^+ concentration for each of them is far less than the nominal concentrations added to the bath [11]. Accordingly, they would all have much smaller effects when compared to the same nominal concentration of Ag^+ . Superimposed on that basic pattern, AgNP-C would have greater effects than the other nanoparticles because the AgNP-PVPs aggregated to a greater extent, decreasing the surface-to-volume ratio, and thus reducing the proportion of Ag^+ in contact with the solution. For the same reason, the smaller AgNP-PVP would have a greater effect than the larger AgNP-PVP. Our results show a pattern totally unrelated to this predicted order.

Although AgNP-C was much less potent than Ag^+ toward disruption of swim bladder inflation, it impaired hatching with a comparable profile. If dissolution of the ion from the particles accounted for the effects of AgNP-C we would expect the same relationship between the particles and ion for both of these measures, and yet they are clearly distinct. The same dichotomy exists for the patterns of dysmorphology vs. mortality: Ag^+ elicited significant dysmorphology starting at $30 \mu\text{M}$ with lesser increases in mortality, whereas AgNP-C evoked significant mortality below this concentration and less dysmorphology at $30 \mu\text{M}$.

Our data further demonstrate that AgNPs alter neurobehavioral endpoints in the zebrafish in ways wholly different from Ag^+ . Across a variety of lighting conditions and challenges, Ag^+ exposure evoked hyperresponsiveness, with increased locomotion compared to controls in the dark, and decreased locomotion in continuous light; the latter finding is in agreement with our earlier report [14]. The lack of a comparable effect from AgNP-C versus Ag^+ exposure points to two possibilities: 1) AgNP-C did not release sufficient Ag^+ to elicit a change in behavior, or 2) the characteristics of the particles are critical determinants of their behavioral effects. The first option can be ruled out, since AgNP-C altered morphology and evoked embryonic death at lower concentrations than those tested for behavior, parameters shared by Ag^+ . Instead, our results support the second possibility, a conclusion verified by the findings with the two AgNP-PVPs. The larger AgNP-PVP evoked hyperactivity in virtually all lighting conditions, indicative of a generalized locomotor activation. This result is distinct not only from AgNP-C but also from Ag^+ . Thus, the particle releasing the lowest concentration of Ag^+ (larger AgNP-PVP) had effects greater than those of the nanoparticle releasing the most Ag^+ (AgNP-C), with both nanoparticles producing behavioral changes that were entirely different from those of Ag^+ alone. Finally, the smaller AgNP-PVP had yet another pattern of behavioral effects, eliciting hypoactivity in the dark and during alternating light levels, but no apparent effect under continuous light. These findings clearly show that AgNPs alter neurodevelopment in ways beyond the release of Ag^+ and that depend upon particle size and coating.

The key question is why these particle parameters are so important in determining the magnitude and type of effect on larval behavior. The differences could rest either in pharmacokinetics or pharmacodynamics. Nanoparticles with an approximate diameter of 50 nm are taken up into cells more readily than other sizes due to preferential endocytosis [6], which could explain greater effects of the AgNP-PVPs in general, as these aggregated to larger particle sizes than AgNP-C. However, this distinction is not uniform in all models; studies in worms suggest greater internalization of smaller nanoparticles, with preference for AgNP-C and the smaller AgNP-PVP compared to the larger AgNP-PVP [11]. It will thus be critical to determine the actual uptake and distribution of different nanoparticles in our model system in order to distinguish the actual internal cell exposure to these agents,

including the potential role of the chorion and the fluid within it, both of which can affect ion and particle uptake during early development [15].

In our earlier work *in vitro*, we similarly found that the biological actions were distinct for the three AgNPs and that each differed from the effect of Ag⁺ [12]. Notably, Ag⁺ was uniformly more disruptive than any of the AgNPs, likely reflecting the much higher availability of the heavy metal in the soluble form. Nevertheless, the current results in zebrafish embryos showed much less effect of AgNP-C than would have been expected from the *in vitro* model. Cell cultures have fewer diffusion barriers than do intact animals and therefore the relative insensitivity to AgNP-C seen here suggests that there are significant pharmacokinetic limitations imposed by diffusion barriers; the fact that we saw greater effects with either of the two AgNP-PVPs suggests that these are excluded to a lesser extent, again pointing to important considerations dependent on particle coating. Further, it is clear that size and coating influence pharmacodynamics in addition to pharmacokinetics. If access to the cell were the sole limitation, then there would be no difference in the direction of change elicited by the two AgNP-PVPs, and yet they had opposite effects on behavioral outcomes. Our results, therefore, show how the combined effects of AgNP coating and size can produce outcomes that are clearly different and that reflect their impact on both pharmacokinetics and pharmacodynamics. Thus, although it has been shown that other AgNPs can enter the zebrafish [1,7], it is not clear if that is equally true for particles of all coatings and sizes, nor is there quantitative information that would permit comparison of actual potencies based on internal cell concentrations.

To our knowledge, this study is the first demonstration that AgNPs act as neurobehavioral disruptors and that their effects are distinct from those of Ag⁺. Our results thus confirm and extend *in vitro* work showing that AgNPs disrupt neural cell replication and differentiation [12], demonstrating adverse outcomes at the levels of morphology, survival and behavior. It is especially important that behavioral abnormalities seen here in zebrafish occurred in the absence of dysmorphology, the hallmark of a true, neurobehavioral teratogen. These findings thus indicate a potential environmental impact of AgNPs in aquatic species, and point to the need to evaluate whether similar adverse effects occur in terrestrial species, extending to humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

AgNP-C	citrate-coated silver nanoparticles
AgNP-PVP	PVP-coated silver nanoparticles
AgNPs	silver nanoparticles
ANOVA	analysis of variance
dpf	days post-fertilization
hpf	hours post-fertilization

Acknowledgments

Research was supported by NIH ES011961 and GM007105, and by the U.S. Environmental Protection Agency. The information has been subjected to review by the National Health and Environmental Effects Research Laboratory and approved for publication. Approval does not signify that the contents reflect the views of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for

use. The authors thank Stella Marinakos, Beth Padnos, and Deborah L. Hunter for their expertise and technical assistance. TAS has provided expert witness testimony in the past three years for: The Calwell Practice (Charleston WV), Weltchek Mallahan & Weltchek (Lutherville MD), Finnegan Henderson Farabow Garrett & Dunner (Washington DC), Carter Law (Peoria IL), Pardieck Law (Seymour, IN), Gutglass Erickson Bonville & Larson (Madison WI), The Killino Firm (Philadelphia PA) Alexander Hawes (San Jose, CA) and the Shanahan Law Group (Raleigh NC).

REFERENCES

1. Asharani P, Wu Y, Gong Z, Valiyaveetil S. Toxicity of silver nanoparticles in zebrafish models. *Nanotechnology*. 2008; 19:255102–255110. [PubMed: 21828644]
2. Bar-Ilan O, Albrecht RM, Fako VE, Furgeson DY. Toxicity assessments of multisized gold and silver nanoparticles in zebrafish embryos. *Small*. 2009; 5:1897–1920. [PubMed: 19437466]
3. Bondy SC, Campbell A. Developmental neurotoxicology. *J Neurosci Res*. 2005; 81:605–612. [PubMed: 16035107]
4. Costa LG, Aschner M, Vitalone A, Syversen T, Soldin OP. Developmental neuropathology of environmental agents. *Annu Rev Pharmacol Toxicol*. 2004; 44:87–110. [PubMed: 14744240]
5. Griffitt RJ, Luo J, Gao J, Bonzongo JC, Barber DS. Effects of particle composition and species on toxicity of metallic nanomaterials in aquatic organisms. *Environ Toxicol Chem*. 2008; 27:1972–1978. [PubMed: 18690762]
6. Johnston HJ, Hutchison G, Christensen FM, Peters S, Hankin S, Stone V. A review of the in vivo and in vitro toxicity of silver and gold particulates: particle attributes and biological mechanisms responsible for the observed toxicity. *Crit Rev Toxicol*. 2010; 40:328–346. [PubMed: 20128631]
7. Lee KJ, Nallathamby PD, Browning LM, Osgood CJ, Xu XH. In vivo imaging of transport and biocompatibility of single silver nanoparticles in early development of zebrafish embryos. *ACS Nano*. 2007; 1:133–143. [PubMed: 19122772]
8. Lee PC, Meisel D. Adsorption and surface-enhanced raman of dyes on silver and gold sols. *J Phys Chem*. 1982; 86:3391–3395.
9. Lyon TD, Patriarca M, Howatson G, Fleming PJ, Blair PS, Fell GS. Age dependence of potentially toxic elements (Sb, Cd, Pb, Ag) in human liver tissue from paediatric subjects. *J Environ Monit*. 2002; 4:1034–1039. [PubMed: 12509062]
10. MacPhail RC, Brooks J, Hunter DL, Padnos B, Irons TD, Padilla S. Locomotion in larval zebrafish: Influence of time of day, lighting and ethanol. *Neurotoxicology*. 2009; 30:52–58. [PubMed: 18952124]
11. Meyer JN, Lord CA, Yang XY, Turner EA, Badireddy AR, Marinakos SM, Chilkoti A, Wiesner MR, Auffan M. Intracellular uptake and associated toxicity of silver nanoparticles in *Caenorhabditis elegans*. *Aquat Toxicol*. 2010; 100:140–150. [PubMed: 20708279]
12. Powers CM, Badireddy AR, Ryde IT, Seidler FJ, Slotkin TA. Silver nanoparticles compromise neurodevelopment in PC12 cells: critical contributions of silver ion, particle size, coating and composition. *Environ Health Perspect*. 2010
13. Powers CM, Wrench N, Ryde IT, Smith AM, Seidler FJ, Slotkin TA. Silver impairs neurodevelopment: studies in PC12 cells. *Environ Health Perspect*. 2010; 118:73–79. [PubMed: 20056586]
14. Powers CM, Yen J, Linney EA, Seidler FJ, Slotkin TA. Silver exposure in developing zebrafish (*Danio rerio*): persistent effect on larval behavior and survival. *Neurotoxicol Teratol*. 2010; 32:391–397. [PubMed: 20116428]
15. Rombough PJ. The influence of the zona radiata on the toxicities of zinc, lead, mercury, copper and silver ions to embryos of steelhead trout *Salmo gairdneri*. *Comp Biochem Physiol C*. 1985; 82:115–117. [PubMed: 2865049]
16. Rungby J, Slomianka L, Danscher G, Andersen AH, West MJ. A quantitative evaluation of the neurotoxic effect of silver on the volumes of the components of the developing rat hippocampus. *Toxicology*. 1987; 43:261–268. [PubMed: 3824394]
17. Wijnhoven SWP, Peijnenburg WJGM, Herberts CA, Hagens WI, Oomen AG, Heugens EHW, Roszek B, Bisschops J, Gosens I, Van De Meent D, et al. Nano-silver -- a review of available data

and knowledge gaps in human and environmental risk assessment. *Nanotoxicology*. 2009; 3:109–138.

18. Yeo M-K, Yoon J-W. Comparison of the effects of nano-silver antibacterial coatings and silver ions on zebrafish embryogenesis. *Mol Cell Toxicol*. 2009; 5:23–31.
19. Zhu X, Zhu L, Duan Z, Qi R, Li Y, Lang Y. Comparative toxicity of several metal oxide nanoparticle aqueous suspensions to Zebrafish (*Danio rerio*) early developmental stage. *J Environ Sci Health A*. 2008; 43:278–284.

Figure 1. Comparison of Ag⁺ and AgNP-C for survival and morphology. Asterisks denote a significant difference from the control group and daggers denote differences between Ag⁺ and AgNP-C (Fisher's Exact Test).

Figure 2.

Comparison of Ag^+ and AgNP-C for behavioral performance. Panel A shows a representative plate comparing swimming behavior (all 8 plates are shown in Supplemental Figure 1). Fish were exposed either to $30 \mu\text{M}$ Ag^+ or AgNP-C at a nominal Ag concentration of $30 \mu\text{M}$. The different lighting conditions for each epoch are shown schematically by the bar at the bottom: black denotes darkness, white denotes a high level of visible light, lightly shaded denotes increasing intensity of visible light, and striped denotes alternating 2-min intervals of intermediate level and high level visible light. Overall, the Ag^+ group is significantly more active than the control ($p < 0.0001$; Wilcoxon Test) whereas the AgNP-C group is not significantly different; notably, though the Ag^+ group is *hypoactive* specifically in epoch 3 (continuous light). For Ag^+ , the differences are also significant for each epoch individually ($p < 0.0001$ for all individual epochs), tested across all 8 plates (see Supplemental Figure 1). Panel B shows the results of chi-square analysis of the incidence of increases and decreases compared to control, carried out only for the treatment that was significant in the Wilcoxon Test (Ag^+). Note that there are only 9 time points in epoch 2, compared to 10 in the other epochs.

Figure 3.

Swimming behavior after exposure to 10 nm AgNP-PVP (A,B) or 50 nm AgNP-PVP (C,D), at a nominal Ag concentration of 30 μ M. Panels A and C show representative plates for each nanoparticle exposure compared to controls and to PVP alone (all 8 plates are shown in Supplemental Figure 2). The schematic representation of lighting in each epoch is as described in Figure 2 but note the change in the ordinate scale from Figure 2. Across all epochs, the 10 nm AgNP-PVP group is significantly less active than either the control or the PVP groups ($p < 0.0001$; Wilcoxon Test) whereas the 50 nm AgNP-PVP group is significantly more active ($p < 0.0001$). For the 10 nm AgNP-PVP group, differences were significant in individual epochs 1, 4 and 5 ($p < 0.01$, $p < 0.001$, $p < 0.0001$, respectively).

The larger particles were significant in epochs 1, 3, and 4 ($p < 0.01$, $p < 0.02$, $p < 0.01$, respectively) and on the cusp of significance ($p < 0.06$) in epoch 5. For both groups differences in individual epochs were compiled across all 8 plates (see Supplemental Figure 2). Panels B and D show the results of chi-square analysis of the incidence of increases and decreases compared to control for 10 and 50 nm AgNP-PVP respectively. The marginal significance for AgNP-PVP 10 nm in epoch 1 reflects the fact that the chi-square test is less sensitive than the Wilcoxon Test because it does not take the magnitude of the effect into account.

Table 1

Hatching and Swim Bladder Inflation with Ag⁺ or AgNP-C Exposure 0–5 dpf

Measure	Control (246)	Ag ⁺ (16)	AgNP-C (84)	Ag ⁺ (20)	AgNP-C (85)	Ag ⁺ (162)	AgNP-C (197)
	0	3 μ M		10 μ M		30 μ M	
%Hatched 3 dpf	69	50	57*	50	46*	57*	59*
%Hatched 4 dpf	100	100	99	100	99	96*	98
%Inflated 5 dpf	70	44*	68	35*	59*	8*	52
%Inflated 6 dpf	93	81	82*	75*	86	26*	82*

Data are calculated as a percentage of the number of live fish on 6 dpf.

Asterisks denote treatments that differ significantly from the control group and daggers denote where AgNP-C differs from Ag⁺ (Fisher's Exact Test). For Ag⁺ at 3 or 10 μ M, the percent hatched on 3 dpf is not distinguishable from either control or AgNP-C at the same nominal Ag concentration due to the relatively low n. For the same reason, the values are not shown for concentrations below 3 μ M or above 30 μ M, which had too few subjects to permit statistical interpretation.