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Developmental Exposure to Silver Nanoparticles at Environmentally Relevant Concentrations Alters Swimming Behavior in Zebrafish (*Danio rerio*)

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Abstract: Silver nanoparticles (Ag-NPs) are ubiquitous in household and medical products because of their antimicrobial activity. A consequence of the high volume of Ag-NP production and usage is increased amounts of Ag-NPs released into the environment. Their small size (1–100 nm) results in unique physiochemical properties that may increase toxicity relative to their bulk counterpart. Therefore, the goal of the present study was to assess the potential toxicity of environmentally relevant concentrations of Ag-NPs in zebrafish (*Danio rerio*). Wild-type tropical 5D zebrafish embryos were exposed to Ag-NPs from 4 to 120 h postfertilization at 0.03, 0.1, 0.3, 1, and 3 ppm (mg/L). Inductively coupled plasma–mass spectrometry confirmed concentration-dependent uptake of Ag into zebrafish as well as bioaccumulation over time. A morphological assessment revealed no significant hatching impairment, morphological abnormalities, or mortality at any concentration or time point examined. However, assessment of photomotor behavior at 3 d postfertilization (dpf) revealed significant hyperactivity in the 0.3, 1, and 3 ppm Ag-NP treatment groups. At 4 dpf, significant hyperactivity was observed only in the 3 ppm treatment group, whereas 5 dpf larvae exposed to Ag-NPs displayed no significant abnormalities in photomotor behavior. These findings suggest that nonteratogenic concentrations of Ag-NPs are capable of causing transient behavioral changes during development. *Environ Toxicol Chem* 2018;37:3018–3024. © 2018 SETAC

Keywords: Silver; Nanoparticles; Neurotoxicity; Zebrafish; Development; Behavior

INTRODUCTION

Nanoparticles (NPs), defined as any material with at least one dimension between 1 and 100 nm, have become ubiquitous in the environment. Their small size results in unique physiochemical properties that allow them to behave differently from their bulk counterpart, including an increased ability to interact with macromolecules and cross otherwise impenetrable biological barriers (Horie et al. 2012). These properties make NPs highly desirable for medical and industrial applications but may also increase their potential for toxicity (Zoroddu et al. 2014).

Silver nanoparticles (Ag-NPs) are highly efficacious antimicrobials, making them one of the most widely used nanomaterials (Nowack et al. 2011). They are used extensively in both household and medical items such as clothing, mattresses, children's toys, medical ointments, tube linings, and surface coatings. The high rate of Ag-NP usage and disposal has resulted in increased release into aquatic environments (Benn and Westerhoff 2008; Geranio et al. 2009). Recent reports demonstrating that Ag-NPs may be significantly more toxic than their bulk counterpart (Recordati et al. 2016; Abramenko et al. 2018) highlight the need to investigate their potential ecological impacts.

Although there are limited studies evaluating Ag-NP levels in aquatic systems, Ag-NPs have been reported in surface waters in the United Kingdom at concentrations >30 ppb (μg/L; O'Brien and Cummins 2010) and in wastewater treatment plant effluent in Germany at concentrations >1 ppb (Li et al. 2013). Because of the rapidly increasing use and production of Ag-NPs (Ge et al. 2014), environmental Ag-NP levels in both Europe and the United States are expected to exceed 4 ppm (mg/L), with the potential for annual exponential increases (Fabrega et al. 2011). Consistent with this prediction, a recent publication reported environmental concentrations of Ag-NPs in Malaysia as high as 10 to 20 ppm (Syafiuddin et al. 2018). Despite this, the US Environmental Protection Agency does not monitor the use of Ag-NPs and regulates them as bulk Ag (Nowack et al. 2011). Therefore, understanding the potential toxicity associated with

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these compounds, especially at environmentally relevant concentrations, is of increasing importance.

The zebrafish is a vertebrate model that has been used extensively to evaluate NP toxicity (Harper et al. 2015; Wehmas et al. 2015; Sheng et al. 2016; Ginzburg et al. 2018; Nix et al. 2018). It was recently reported that exposure of embryonic zebrafish to Ag-NPs at concentrations far greater than those observed in the environment caused morphological and physiological abnormalities (Cunningham et al. 2013; Gao et al. 2015). Zebrafish models have also been used to show that Ag-NPs accumulate in brain tissue (Asharani et al. 2008) and alter the expression of genes related to central nervous system development (Xin et al. 2015). Because of their ability to penetrate the chorion (Lee et al. 2007, 2013), Ag-NPs pose a significant threat to early-stage zebrafish embryos when many neurodevelopmental processes are at their peak (Miller et al. 2018).

In addition, developmental exposure to Ag-NPs has been associated with functional impairments in zebrafish, including altered expression of genes related to sensorimotor function (Osborne et al. 2016), as well as disruptions in normal swimming behavior (Powers et al. 2011; Asmonaite et al. 2016). However, because of the limited number of studies, variability in experimental designs, and differences in NP properties, it is important that the functional consequences of developmental exposures to Ag-NPs, particularly at environmentally relevant levels, be confirmed by independent laboratories. Therefore, the goal of the present study was to evaluate whether environmentally relevant Ag-NP concentrations produce functional behavioral abnormalities in developing zebrafish. To address this, embryonic zebrafish were exposed from 0 to 5 d postfertilization (dpf) to vehicle or increasing concentrations of Ag-NPs and monitored for hatching success, morphology, and mortality. At 3, 4, and 5 dpf, hatched larvae were assessed for photomotor function using a light–dark photomotor assay. Our results suggest that embryonic exposure to Ag-NPs at relatively low concentrations that cause no significant hatching impairment, morphological abnormalities, or mortality can alter normal swimming behavior.

MATERIALS AND METHODS

Animal husbandry and care

Fish husbandry, spawning, and all experiments with zebrafish were performed in accordance with University of California-Davis Institutional Animal Care and Use Committee protocol 19391. Wild-type tropical 5D adult zebrafish (*Danio rerio*; obtained from R. Tanguay, Oregon State University) were maintained in a standalone aquatic flow-through system (Aquaneering) in deionized water further purified by reverse osmosis at 28 ± 0.5 °C. The "system" water was supplemented with 20 g/L $NaHCO_3$ to maintain a pH of 7.2 ± 0.4 and 40 g/L sea salt solution (Instant Ocean) to maintain conductivity at 600 µS ± 100. Adult fish were kept on a 14:10-h light:dark cycle and fed twice daily with Gemma Micro fish food (Skretting). Embryos were obtained by natural group spawning, collected within 30 min of fertilization, and kept in an incubator at 28.5 ± 0.5 °C until exposed to vehicle or Ag-NPs beginning at 4 h postfertilization (hpf).

Nanomaterials and exposure

The Ag-NPs were manufactured by QSI-Nano (Quantum-Sphere Inc.) and obtained from the University of California Center for Environmental Implications of Nanotechnology. Detailed characterization of these particles has demonstrated a particle size ranging from 20 to 40 nm and an average diameter of 25 nm (Jin et al. 2010). This particle size was determined in a medium with very similar salt concentration, pH, and conductivity as the medium used in the present study to expose the fish. Stock solutions of Ag-NPs at 1 ppt, 50 ppm, and 1 ppm were dissolved in 10 ppm alginate (Millipore Sigma) in deionized water and sonicated for 45 min. Alginate was used to bind to the surface of the Ag-NPs and prevent aggregation of the particles over time. At 4 hpf, embryos were individually plated into 96-well plates filled with standardized Embryo Medium (Westerfield 2007). All embryos were exposed from 4 to 120 hpf to either vehicle (alginate at 3 ppm) or Ag-NPs at 0.03, 0.1, 0.3, 1, or 3 ppm (Figure 1A and B). Embryos were maintained on a 14:10-h light: dark cycle and incubated at 28.5 ± 0.5 °C in 100 µL of total treatment solution with one embryo per well.

Analysis of tissue concentrations of Ag

Zebrafish samples from vehicle-exposed and 0.03, 0.3, and 3 ppm Ag-NP exposure groups were analyzed at 3 and 5 dpf to quantify tissue concentrations of Ag. Samples were analyzed in triplicate with 22 to 26 larval zebrafish/sample obtained from 3 separate spawnings. Zebrafish samples, triplicate method blanks (200 µL 18.2 MΩ/cm water), and triplicate NIST1640a digestion quality control standards (200 µL NIST1640a Trace Elements in Natural Water; National Institute of Standards and Technology) were digested by adding 30 µL concentrated trace metal–grade HNO_3 (Fisher Scientific) and 100 µL concentrated trace metal–grade HCl (Fisher Scientific), then heating in a hot block that was ramped up to 95 °C over 30 min, followed by digestion at 95 °C for 1 h. 550 µL 30% ULTREX II H_2O_2 (J.T. Baker) was added incrementally to room temperature samples with heating ramped up to 95 °C between additions. The following increments were used: 50 µL at 40 °C for 15 min, 100 µL at 67 °C for 10 min, 100 µL at 85 °C for 45 min, 150 µL at 95 °C for 25 min, and 150 µL at 95 °C for 1 h. The samples were allowed to cool, then brought to a final volume of 1 mL with 18.2 MΩ-cm water prior to analysis by the Interdisciplinary Center for Plasma Mass Spectrometry at the University of California-Davis using an Agilent 8900 inductively coupled plasma mass spectrometry (ICP-MS; Agilent Technologies).

Mortality and malformation assessment

Hatching success was monitored from 0 to 5 dpf to evaluate whether Ag-NP exposure resulted in hatching delays or impaired hatching. Impaired hatching was defined as the failure of an

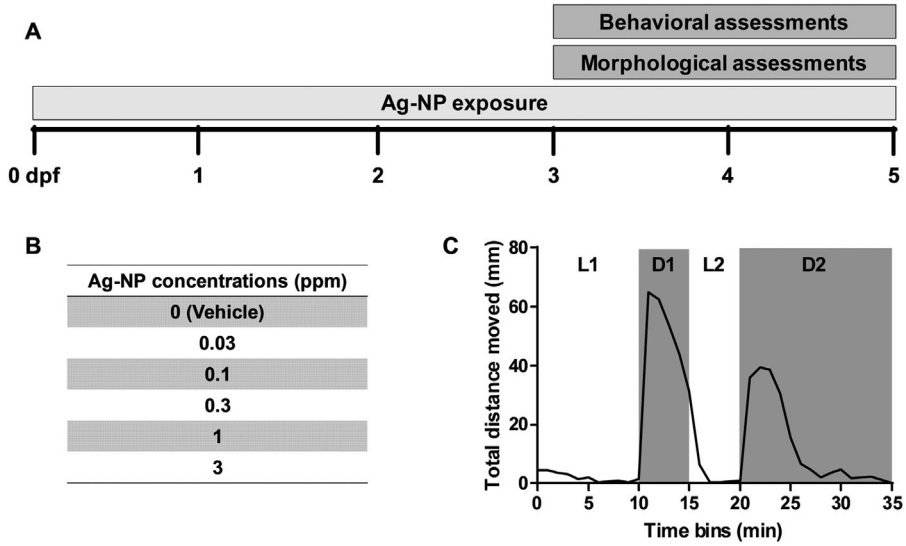


FIGURE 1: Experimental design. (**A**) Schematic illustrating the exposure paradigm used to evaluate the morphological and behavioral effects of silver nanoparticles in developing zebrafish. (**B**) Silver nanoparticle concentrations tested in larval zebrafish. (**C**) Representative trace of typical zebrafish behavior in the photomotor behavioral assay. Ag-NP = silver nanoparticle; D1/D2 = dark phases 1/2; dpf = days postfertilization; L1/L2 = light phases 1/2.

embryo to completely hatch from its chorion by 3 dpf. Embryos and hatched larvae were evaluated daily under a dissection microscope for mortality and malformations, primarily spinal curvature and yolk-sac edema ($n = 48$/group from 3 separate spawning events). There were no statistically significant differences between control fish from different spawning events (data not shown). All assessments were conducted by researchers blinded to experimental group.

Photomotor assessment

Individual larval zebrafish were assessed for locomotor response to changing light conditions at 3, 4, or 5 dpf. Larvae in 96-well plates were placed in a DanioVision behavior system (Noldus) and subjected to a 35-min light/dark locomotor test ($n = 48$/group from 3 separate spawning events). Temperature of the system and plates was maintained at $28.5 \pm 0.5\,^{\circ}C$ using a Noldus Temperature Control Unit. The behavioral test consisted of a 10-min light period (light 1; ~1900 lux) to allow for acclimation and to record baseline swimming behavior, followed sequentially by a 5-min dark period (dark 1; ~0 lux) to stimulate increased swimming behavior, a 5-min light period (light 2; ~1900 lux) to stimulate freezing behavior, and a 15-min dark period (dark 2; ~0 lux) to observe increased swimming behavior following acclimation to the dark condition (Figure 1C). Larval behavior was recorded by the DanioVision camera with an infrared filter to allow visibility in both light and dark conditions. Total distance moved was automatically tracked using EthoVisionXT software (Ver 10.1; Noldus), and all data were exported to GraphPad Prism (Ver 7.03) for analysis. Dead and/or malformed larvae were excluded from the behavior analysis. There were no statistically significant differences between control fish from different spawning events (data not shown).

Statistical analysis

Total distance moved for individual experimental groups was calculated for each test period (light 1, dark 1, light 2, and dark 2). Photomotor behavior and morphology/mortality data were analyzed using a one-way analysis of variance (ANOVA) to compare data across all treatments (vehicle control, 0.03, 0.1, 0.3, 1, and 3 ppm Ag-NP), and when applicable, a post hoc Bonferroni-Holm multiple comparisons test was used to identify statistically significant differences between groups. Significance was determined at $p < 0.05$. All statistical analyses were conducted using GraphPad Prism (Ver 7.03).

RESULTS

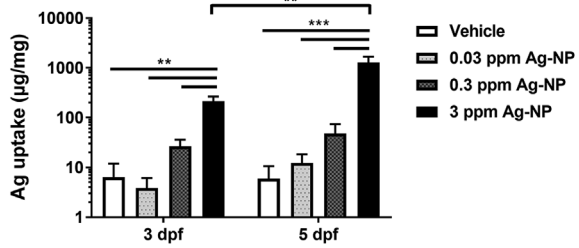
Ag uptake

We used ICP-MS to measure total Ag in the tissue of embryonic zebrafish exposed to Ag-NPs for up to 5 dpf (Figure 1). Concentrations of Ag were measured in vehicle control fish at the time of dosing (time 0) to obtain background levels and in 3- and 5-dpf zebrafish exposed to vehicle or Ag-NPs at 0.03, 0.3, or 3 ppm. Quantifiable Ag uptake was observed in the 0.3- and 3-ppm groups at 3 and 5 dpf (Figure 2A and B). At both time points, the 3-ppm group showed higher Ag levels than the 0.3-ppm group, demonstrating concentration-dependent uptake. Higher levels of Ag were detected at 5 than at 3 dpf within experimental groups, demonstrating accumulation of Ag in fish tissue over time.

Mortality and malformations

At 3 dpf, incidence rates for unhatched, malformed, or dead embryos remained ≤3, 0, and 5%, respectively, across all concentrations (Figure 3). At 4 dpf, new cases remained ≤3, 3,

A



B

	Ag uptake (µg Ag^+/mg tissue)		
	Time 0	3 d	5 d
Vehicle	1.4 ± 1.1#	6.4 ± 7.9#	6.0 ± 6.5#
0.03 ppm		3.9 ± 3.2#	12.2 ± 8.8#
0.3 ppm		26.7 ± 12.9	47.8 ± 37.1
3 ppm		213.7 ± 72.1	1278.1 ± 564.6

FIGURE 2: Uptake of silver nanoparticles (Ag-NPs) is concentration-dependent. (**A**) Total silver ion concentrations in 3- and 5-d postfertilization (dpf) zebrafish exposed to vehicle or Ag-NPs at 0.03, 0.3, or 3 ppm beginning at 0 dpf. Data are presented as mean ± standard error. (**B**) Total silver ion concentrations in vehicle controls at baseline (time 0) and in exposure groups at 3 and 5 dpf. Data are presented as mean ± standard deviation. $n = 3$ replicate samples/group with 22 to 26 fish/sample. $^{**}p < 0.01$, $^{***}p < 0.001$, #below limit of quantitation (~0.5 ppb in solution).

and 0% for unhatched, malformed, and dead embryos, respectively. At 5 dpf, no new cases of unhatched, malformed, or dead embryos were observed. These rates were not statistically different from vehicle at any time point or concentration of Ag-NP examined (Figure 3).

Behavioral assessment

The total distance zebrafish moved was calculated for each light and dark period (Figure 1C). Data from the light 1 period were not statistically analyzed because they represent acclimation of the zebrafish to the test chamber. Statistics were performed using data generated during the dark 1 period, representing the first activity period and acclimation to the dark condition, and the light 2 period, representing freezing behavior. The primary period of interest was the dark 2 period, when total movement was assessed following acclimation to both dark and light periods.

No significant differences between experimental groups were observed in the dark 1 (Figure 4A and B) or light 2 (Figure 5A and B) period. This suggests that there were no differences in baseline activity or freezing behavior between vehicle and Ag-NP-treated larvae. However, effects of Ag-NPs were observed in the dark 2 period (Figure 5C). At 3 dpf, significant hyperactivity was observed in larvae exposed to 0.3, 1, and 3 ppm Ag-NP relative to vehicle controls. At 4 dpf, significant hyperactivity was observed only in larvae exposed to 3 ppm Ag-NP. At 5 dpf, the total distance moved by Ag-NP-exposed larvae was no longer significantly different from vehicle controls.

DISCUSSION

In the present study, relatively low concentrations of Ag-NPs were used to assess the toxicity of environmentally relevant concentrations. The concentrations used in the present study are more than 6 times lower than those reported in the environment of Malaysia and below predicted levels in the United States and Europe. Of note, Ag-NPs were observed to disrupt photomotor behavior at concentrations that caused no mortality, morphological abnormalities, or hatching impairment, highlighting the potential for Ag-NPs to induce behavioral changes in the absence of overt toxicity. This observation is consistent with the finding that behavior is among the most sensitive endpoints in zebrafish toxicity screening (Gerhardt 2007).

The present study found significant concentration-dependent hyperexcitability in zebrafish following developmental exposures to Ag-NPs. This phenotype is consistent with recent literature describing hyperactivity in zebrafish following relatively low-concentration (~3 ppm) Ag^+ exposure (Powers et al. 2011). In addition, several other studies have reported hyperexcitability in zebrafish following low-concentration

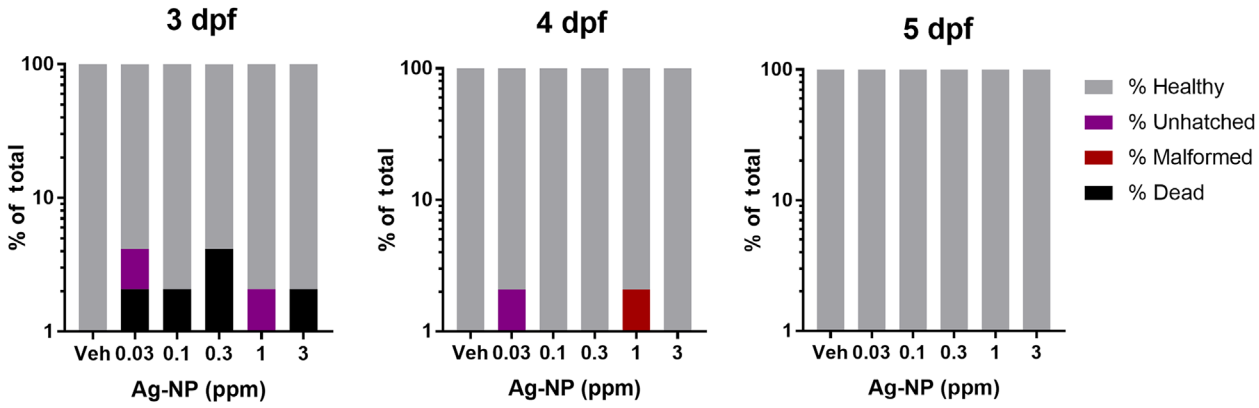


FIGURE 3: Silver nanoparticle (Ag-NP) exposure does not induce significant hatching impairments, malformations, or mortality. Incidence rates of unhatched, malformed, and dead zebrafish embryos following exposure to increasing concentrations of Ag-NPs or alginate vehicle. Embryos were scored at 3, 4, and 5 d postfertilization. No significant differences between groups were identified using one-way analysis of variance. $n = 48$/group from 3 separate spawning events. dpf = days postfertilization; veh = vehicle.

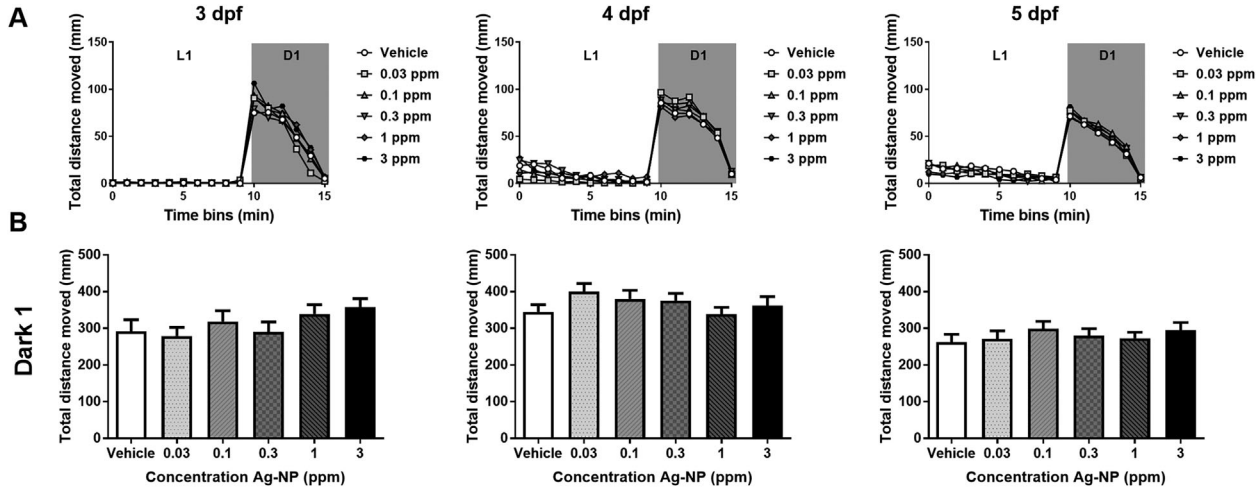


FIGURE 4: Silver nanoparticle (Ag-NP) exposure does not alter locomotion in the light 1 (L1) or dark 1 (D1) phase. (**A**) Behavioral traces showing mean movement of zebrafish larvae at each 1-min time bin during the L1 acclimation phase (0–10 min) and the D1 phase (10–15 min). (**B**) Quantitative summary of photomotor activity showing total distance moved during the D1 phase. No significant differences between groups were observed at any D1 period as determined by one-way analysis of variance. Data are presented as mean ± standard error. $n = 48$/group from 3 separate spawning events. dpf = days postfertilization.

exposure to diverse chemicals, including lead (Chen et al. 2012), bifenthrin (Frank et al. 2018), ethanol (Irons et al. 2010; Blaser and Peñalosa 2011), *d*-methamphetamine (Irons et al. 2010), and bisphenol A (Saili et al. 2012). Interestingly, a recent study evaluating behavioral deficits following Ag-NP exposure reported hypoactivity in exposed zebrafish (Asmonaite et al. 2016). Although the concentrations used were similar to those in the present study (2.15 ppb–2.15 ppm), a critical difference is

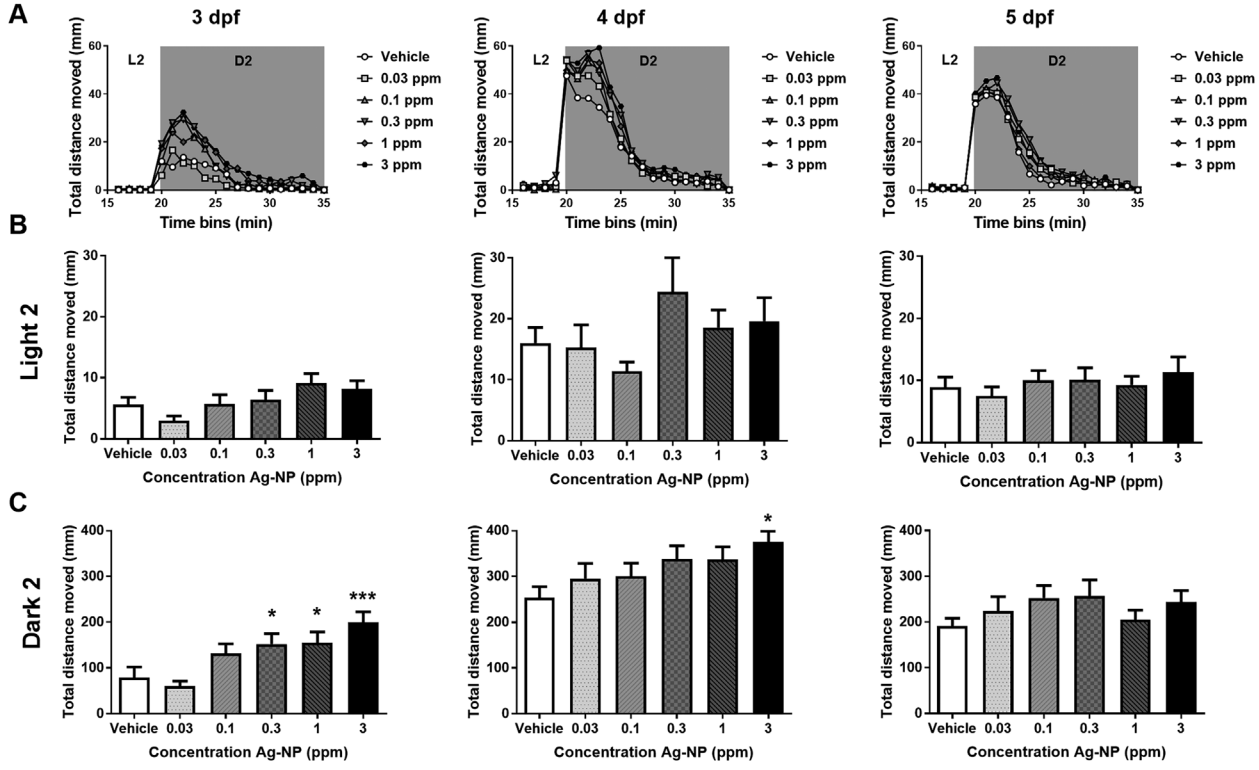


FIGURE 5: Silver nanoparticle (Ag-NP) exposure induces significant hyperactivity in the dark 2 (D2) testing phase. (**A**) Behavioral traces showing mean movement of zebrafish larvae at each 1-min time bin during the light 2 (L2) phase and the D2 phase (20–35 min). Quantitative summary of the total distance moved during the (**B**) L2 phase and (**C**) D2 phase for each experimental group. Significant hyperactivity was observed in the D2 phase in zebrafish exposed to Ag-NPs at 3 and 4 d postfertilization as determined by one-way analysis of variance and post hoc Bonnferroni-Holm multiple comparisons test. Data are presented as mean ± standard error. $n = 48$/group from 3 separate spawning events. $^{*}p < 0.05$, $^{***}p < 0.001$. dpf = days postfertilization.

that the authors reported hypoactivity only at concentrations that also caused teratogenicity. Overtly toxic concentrations of Ag-NPs have been reported to degrade the motor neurons of exposed zebrafish (Muth-Kohne et al. 2013), which potentially explains Ag-NP-induced hypoactivity. The authors did not measure zebrafish tissue concentrations of Ag, so it is possible that the teratogenicity and hypoactivity they observed reflect greater Ag uptake than was observed in the present model. Additional differences between the present study and Asmonaite et al. 2016 include the zebrafish strain used and the age at which the behavioral tests were performed. It is well documented that temporal, age-related, and genetic factors can influence zebrafish behavior in response to chemical exposure (MacPhail et al. 2009; Padilla et al. 2011).

The behavioral abnormalities in our model were most robust at 3 dpf and diminished over time, suggesting that the effects are transient. This does not rule out the possibility that other behavioral abnormalities manifest at later times in development. For example, in other zebrafish models of chemical-induced hyperactivity, exposed animals also showed impaired learning and memory (Chen et al. 2012; Saili et al. 2012; Knecht et al. 2017). Hyperactivity has also been observed in various zebrafish models of intellectual disability (Kim et al. 2014; Chen et al. 2018; Lange et al. 2018). Therefore, it may be that the hyperactivity observed in zebrafish exposed to Ag-NPs is in fact an impaired acclimation to the dark 2 period. This interpretation is consistent with the absence of behavioral differences during the dark 1 period as well as the diminishment of the phenotype over time, potentially suggesting a delayed learning process in Ag-NP-exposed larvae compared to vehicle controls. However, further experiments are needed to evaluate whether Ag-NP exposure impairs learning and memory processes.

Alternatively, Ag-NPs may be inducing hyperactivity via effects on the sensorimotor system. It has been reported that Ag-NPs alter transcription of genes associated with photoreception and central nervous system development at concentrations of 0.01 to 2 ppm (Xin et al. 2015; Cambier et al. 2018) and induce apoptosis in developing zebrafish at 50 to 60 ppm (Chakraborty et al. 2016; Verma et al. 2018). Exposure to Ag-NPs has also been shown to disrupt development of the lateral line system, a critical component of sensorimotor function in the zebrafish central nervous system. More specifically, they have been shown to alter gene transcription in the neuromasts (Osborne et al. 2016) and impair rheotaxis at 0.2 to 1 ppm, demonstrating a functional consequence of lateral line system disruption (McNeil et al. 2014). These adverse effects induced by Ag-NPs represent potential mechanisms contributing to the hyperexcitability observed in the present model.

CONCLUSIONS

Developmental exposure to Ag-NPs can cause hyperexcitability in developing zebrafish at concentrations that do not cause mortality, malformations, or hatching impairments. Of note, this behavioral phenotype was observed at concentrations below those found in some parts of the aquatic environment. Because of the rapidly increasing use of Ag-NPs, the present study highlights the importance of continued mechanistic research into the behavioral effects of Ag-NP exposure.

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Data Accessibility—Data pertaining to the present study are deposited in FigShare.com at DOI: 4275.

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