Environmental Science & Technology

Article

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Differential Effects and Potential Adverse Outcomes of Ionic Silver and Silver Nanoparticles in Vivo and in Vitro

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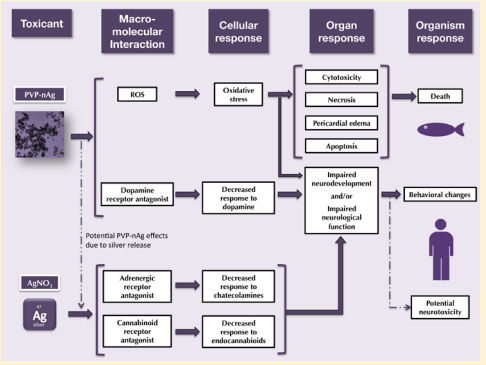
Supporting Information

ABSTRACT: Nanoparticles are of concern because of widespread use, but it is unclear if metal nanoparticles cause effects directly or indirectly. We explored whether polyvinylpyrrolidone-coated silver nanoparticles (PVP-AgNPs) cause effects through intact nanoparticles or dissolved silver. Females of the model species fathead minnow (*Pimephales promelas*) were exposed to either 4.8 μg/L of $AgNO_3$ or 61.4 μg/L of PVP-AgNPs for 96h. Microarray analyses were used to identify impacted receptors and toxicity pathways in liver and brain tissues that were confirmed using in vitro mammalian assays. $AgNO_3$ and PVP-AgNP exposed fish had common and distinct effects consistent with both intact nanoparticles and dissolved silver causing effects. PVP-AgNPs and $AgNO_3$ both affected pathways involved in Na^+, K^+, and H^+ homeostasis and oxidative stress but different neurotoxicity pathways. In vivo effects were supported by PVP-AgNP activation of five in vitro nuclear receptor assays and inhibition of ligand binding to the dopamine receptor. $AgNO_3$ inhibited ligand binding to adrenergic receptors α1 and α2 and cannabinoid receptor CB1, but had no effect in nuclear receptor assays. PVP-AgNPs have the potential to cause effects both through intact nanoparticles and metal ions, each interacting with different initiating events. Since the in vitro and in vivo assays examined here are commonly used in human and ecological hazard screening, this work suggests that environmental health assessments should consider effects of intact nanoparticles in addition to dissolved metals.



INTRODUCTION

With increasing use of engineered nanomaterials, incidental exposure during manufacture and processing or intentional exposure through personal care products or therapeutics are likely.[1] Despite recent studies suggesting that silver nanoparticles (AgNP) have the potential to cause toxicity in humans and wildlife,[2−9] there is still significant uncertainty regarding the specific biological impacts of such substances.

The potential adverse effects of nanoparticles (NP) in the environment at the population or ecosystem levels is still widely unexplored, but recent studies suggest that the impact could be more significant than previously suspected.[1,10] To date researchers have primarily focused on bioaccumulation and biomagnification of NP, indicating that there is potential concern for organisms higher in the food chain.[11−13] However, vertebrates and invertebrates in the lower trophic levels could also be dramatically affected by exposure to NPs.[1] For example, NP can affect the ability of the water flea *Daphnia magna* to detect the presence of predators and influence population growth and dynamics of freshwater phytoplankton.[10,14] In mammals, inhaled NP can reach the nervous system via the olfactory nerves or the blood-brain barrier.[15−19] Silver nanoparticles reaching the circulatory system may be distributed to different organs, including the brain,[18−21] implying a potential route of exposure.

The reactivity of nanomaterials depends on complex physicochemical properties (e.g., size, polydispersity, agglomeration state, dissolution kinetics, capping agent, ζ potential, specific surface area and coating material) with characterization

Received: September 22, 2013
Revised: March 12, 2014
Accepted: March 14, 2014
Published: March 14, 2014

of these properties being critical for predictive assessment of biological impacts. Toxicity due to AgNP is generally thought to be due to dissolution of the silver.[22−24] However some studies suggest that toxicity of AgNPs cannot be explained exclusively by the effects of ionic silver.[4,25,26] Therefore, more focused studies are needed to determine to what extent toxicity is due to direct AgNP interactions.

In this work, we exposed female fathead minnows (*Pimephales promelas*) to polyvinylpyrrolidone (PVP)-coated AgNPs or silver nitrate ($AgNO_3$) to examine whether observed effects were due to PVP-AgNPs, dissolved silver, or a combination of the two. We also aimed to identify molecular initiating events that could lead to adverse outcomes because of exposure to either PVP-AgNP or $AgNO_3$ both in fish and higher vertebrates, such as humans. We chose PVP because it is one of the most common coatings used to stabilize dispersed NP.

EXPERIMENTAL SECTION

Exposure. Three month old juvenile fathead minnows were obtained from Aquatic Biosystems (Fort Collins, CO, U.S.A.), held for 4 months (aged to 7 months) in aerated dechlorinated tap water, and fed three times daily with Zeigler AquaTox Feed (Gardners, PA, U.S.A.). Fish were exposed in five tanks per treatment (two fish per tank), to either 4.8 μg/L of $AgNO_3$ or 61.4 μg/L of PVP-AgNPs (Luna Innovations, Blackburn, VA, U.S.A.) for 96 h at 24 °C ± 1 with a 90% water change at 48 h. The water change conducted was done with fresh media containing either the PVP-AgNP or $AgNO_3$. Moderately hard reconstituted water (MHRW) formulated according to U.S. EPA guidelines[27] at a hardness of 80 mg/L as $CaCO_3$ was used as the diluent for $AgNO_3$ and PVP-AgNP dilutions. Test concentrations were chosen based on No Observed Adverse Effect Concentration (NOAEC) levels on survival (48 h) of 3.43 μg/L $AgNO_3$ and 69.9 μg/L PVP-AgNPs previously observed for larval fish.[22] Fish were fed 48 h prior to exposure and again before the 48 h water change where they were allowed to feed for five minutes to minimize partitioning of Ag to the food particles to ensure the exposure was from the water. At the exposure conclusion, fish were anesthetized in buffered tricaine methanesulfonate (MS-222; Finquel; Argent, Redmond, WA, USA). Gill tissues were removed and stored at −80 °C for chemical analysis. Brain and liver tissues were removed and stored in RNAlater (Ambion, Austin, TX, U.S.A.) at −80 °C until further use for gene expression analysis.

Analytical Chemistry. A detailed description of analytical chemistry procedures and particle characterization utilized in this study can be found in ref 22. PVP-AgNPs were prepared by Luna Innovations (Blacksburg, VA) using previously described methods.[22] Briefly, PVP-AgNPs were fabricated by addition of 75 mL of ethylene glycol to a round-bottom flask in a heating mantle. PVP (1.5 g, molecular weight) was allowed to dissolve with magnetic stirring and 0.050 g of $AgNO_3$ was added before heating and stirring in a sealed flask (1 °C/min to 120 °C, maintained for 1 h). The mixture was immersed in a room temperature water bath and dialyzed against deionized water to reduce ethylene glycol, unbound PVP, and Ag^+. The stock solution was bath sonicated for 30 min to disperse and homogenize the particles. The average hydrodynamic diameter size of PVP-AgNPs was measured by field flow fraction (FFF; PostNova F-1000 symmetrical flow FFF, Salt Lake City, UT, U.S.A.) interfaced with an inductively coupled plasma mass spectrometer (ICP-MS; Elan DRC-II, Perkin-Elmer, Waltham, MA, U.S.A.). Transmission electron microscopy (TEM; Zeiss 10CA, 60 kV, Oberkochen, Germany) and scanning electron microscopy (SEM) was used to verify the morphology and primary particle size of the PVP-AgNPs. Dynamic light scattering (DLS; 90Plus/BI-MAS, Brookhaven Instruments, Holtsville, NY, U.S.A.) was also performed to determine particle size distribution and surface charge.

Water samples were taken at 0, 24, 48, 72, and 96 h from the fish exposure tanks and tested for total and dissolved silver by ICP-MS as described in ref 22. Briefly, water samples were taken directly from testing chambers and 8 mL were placed into polycarbonate centrifuge tubes (Beckman Coulter, Inc., Catalog # 355603, Brea, CA, U.S.A.), spun in an ultracentrifuge (Beckman Optima XL-80K ultracentrifuge, Rotor 70.1 Ti, Brea, CA, U.S.A.) for 60 min at 100 000*g*, and analyzed by ICP-MS to determine the dissolved fraction. Overlying water (1.5 mL) was sampled, acidified 3% by volume with 100% nitric acid (Thermo Fisher Scientific, CAS No. 7697−37−2, Catalog# A200-212, Fairlawn, NJ, U.S.A.) and analyzed for total silver by ICP-MS. Total silver body burden analysis, performed by ICP-MS (Elan DRC-II, Perkin-Elmer, Waltham, MA, U.S.A.), was conducted on 45 whole fish. Additionally, gills from 15 fish per exposure were analyzed for total silver using ICP-MS according to Kennedy et al.[22]

RNA Extraction. Total RNA was isolated from samples using RNA extraction kits (RNeasy, Qiagen, Valencia, CA, U.S.A.). Microfluidic gel electrophoresis was used to assess RNA degradation (Agilent 2100 Bioanalyzer, Agilent, Wilmington, DE, U.S.A.) and quantity was determined using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, U.S.A.). Total RNA was stored at −80 °C until analyzed.

Microarray Analysis. RNA from each of five biological replicates per treatment was analyzed using custom fathead minnow 60 000 gene arrays (GPL15775). The arrays were created using fathead minnow sequences available in GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) and Wiseman et al.[28] and purchased from Agilent Technologies (Palo Alto, CA, U.S.A.). One microgram of total RNA was used for all hybridizations. Probe labeling, amplification and hybridization were performed using kits following the manufacturer's protocols (Quick Amp Labeling Kit and One-Color Microarray Hybridization Protocol, version 6.5; Agilent) and scanned with a high-resolution microarray scanner (Agilent). Data were resolved from microarray images using Agilent Feature Extraction software version 10.7 (Agilent). Raw microarray data from this study have been deposited at the Gene Expression Omnibus Web site (http://www.ncbi.nlm.nih.gov/geo/; GSE46520).

Real-time PCR Analysis. To verify microarray analysis and test selected genes of interest, real-time quantitative polymerase chain reaction (qPCR) was performed on the same samples used for microarray analysis. Briefly, 800 ng of total RNA was used to synthesize DNase-treated cDNA in a 20 μL reaction containing 250 ng of random primers and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.) following the manufacturer's protocol. The qPCR assays were performed on an ABI Sequence Detector 7900 (Applied Biosystems, Foster City, CA). Each 20 μL reaction was run in duplicate and contained 6 μL of synthesized cDNA template along with 2 μL of each forward and reverse primer (5 μM/μL) and 500 nM SYBR Green PCR Master Mix (Applied Biosystems). Cycling parameters were 95 °C for 15 min, 40

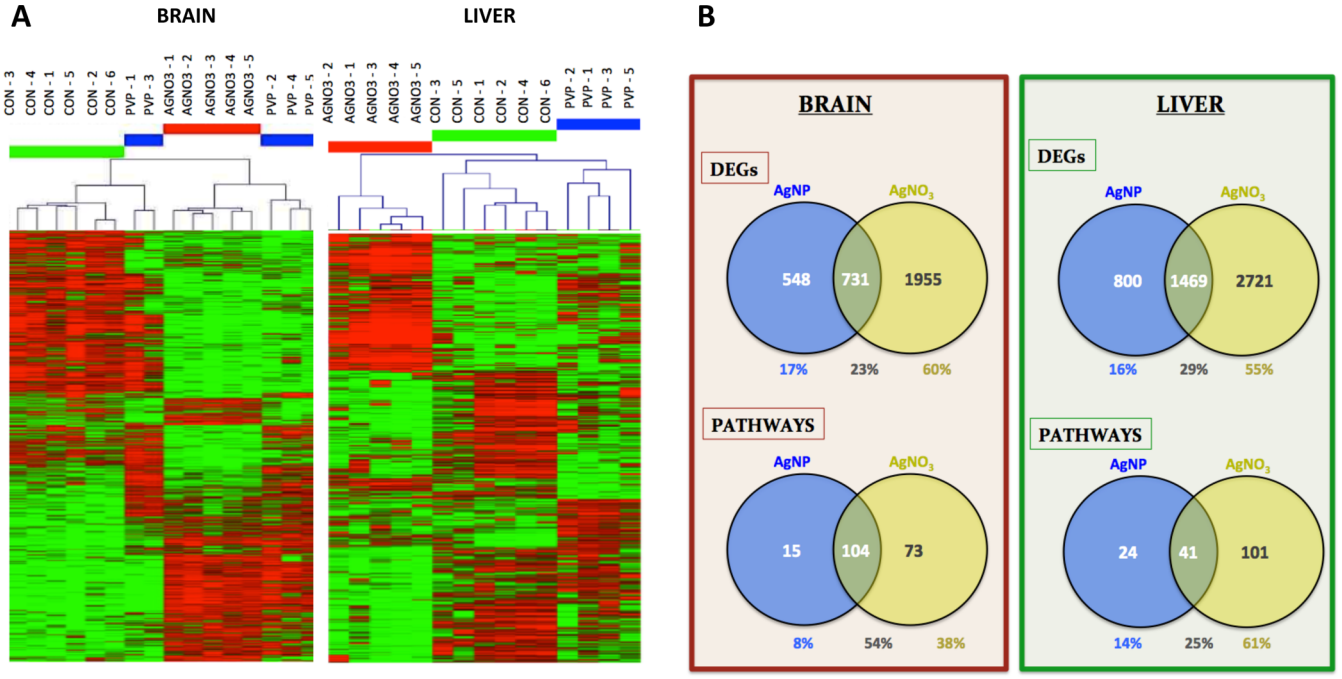


Figure 1. (a) hierarchical clustering of DEGs ($p < 0.05$) in the brain and liver of FHM females exposed to NPs or $AgNO_3$. Red color represents up-regulated genes relative to control, and green represents down-regulated genes relative to control. (b) Venn diagrams showing number of common and distinct differentially expressed genes (DEGs) and significant pathways in the brain and liver of FHM females exposed to NP (PVP) or $AgNO_3$; percentage of common and distinct genes and pathways is also shown.

cycles of 95 °C for 15 s, and 60 °C for 1 min. Primers were designed using Primer Express (Applied Biosystems) and synthesized by Operon Biotechnologies (Huntsville, AL, U.S.A.). The sequences for all the primers can be found in Supporting Information Table 1.

Bioinformatics. Raw microarray data was imported into the analysis software GeneSpring version GX11 (Agilent), and normalized using quantile normalization followed by median scaling across all samples. One-way Analysis of Variance (ANOVA) was performed followed by pairwise comparison ($p < 0.05$) to identify differentially expressed genes. Hierarchical clustering was performed with GeneSpring. Functional analysis and identification of upstream regulators of pathways was performed using the human ortholog genes of fathead minnow differentially expressed genes and the software Ingenuity Pathway Analysis (IPA, Redwood City, CA). IPA calculates the pathway enrichment significance using the right-tailed Fisher Exact Test. Venn diagrams were constructed using Venny (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

In Vitro Functional Assays. Human nuclear receptor and human transcription factor assays (Supporting Information, Tables S2 and S3) were performed by Attagene (Chapel Hill, NC, USA) as described in Romanov et al.[29] Briefly, cells from the human cell line HepG2 were transiently transfected with an optimized cis- or trans-factorial library. Twenty-four hours after transfection, cells were washed and supplied with fresh low serum culture medium and treated with either PVP-AgNPs or $AgNO_3$ for 24 h (2, 5, 6, 20, 70, or 211 μg/L for each of the compounds). A profile of the activities was determined as fold induction values versus vehicle-treated control (ttest, $p < 0.05$).

Receptor binding assays (rat adrenergic receptor, alpha 1A; rat adrenergic receptor alpha 1B; human cannabinoid receptor, CB1; rat GABA receptor, nonselective; rat dopamine receptor, DA nonselective; human nicotinic receptor, a-BnTx insensitive neuronal; rat opioid receptor, non selective; rat 5HT non selective serotonin receptor) and enzymatic assays (human protein kinase MAPK11 (p38beta); human protein kinase MAPK14 (p38alpha); and human CYP3A4) were performed by NovaScreen (Hanover, MA, USA). Briefly, reactions for the neurotransmitter assays were performed with a radioligand ([3H] 7-MeOxy-Prazosin (70−87 Ci/mmol)) and a reference compound. Reactions were carried out in duplicate in 50 mM TRIS-HCl (pH 7.7) at 25 °C for 60 min. The reaction was terminated by rapid vacuum filtration onto glass fiber filters. Radioactivity trapped onto the filters was determined and compared to control values in order to ascertain any interactions of test compound with the receptors binding site. Reactions for the kinase assays were performed in duplicate with the fluorescence-labeled peptides and a reference compound. Reactions were carried out in 100 mM HEPES (pH 7.5), 10 mM MgCl2, 1 mM DTT, 0.015% Brij-35. The reaction was started by addition of 94 μM ATP and incubated for 60 min at room temperature. The reaction was terminated by the addition of stop buffer containing 100 mM HEPES (pH 7.5), 30 mM EDTA, 0.015% Brij-35, 5% DMSO. Phosphorylated and unphosphorylated substrates were separated by charge using electrophoretic mobility shift. Product formed was compared to control wells to determine inhibition or enhancement of enzyme activity. Reactions for the CYP3A4 assay were performed in duplicate with the fluorescence-labeled peptides and a reference compound (ketoconazole). Reactions were carried out in 500 mM KPO4 (pH 7.4) containing 2% acetonitrile. Cofactors were preincubated for 10 min at 37 °C. Enzyme was then added and incubated for 10 min at 37 °C. Reactions were terminated by the addition of 2 M NaOH. The amount of fluorescent product formed was compared to control values to ascertain any interactions of test compounds with the CYP3A4 enzyme.

dx.doi.org/10.1021/es4042258 | Environ. Sci. Technol. 2014, 48, 4546−4555

Table 1. Impact of $AgNO_3$ and PVP-AgNP on Toxicity Pathways in Fathead Minnow[a]

Toxicity pathways	
$AgNO_3$	PVP-AgNP
Brain	
Common pathways: Renal Necrosis/Cell Death, PPARα/RXRα Activation, Liver Proliferation, Cardiac Hypertrophy, Mechanism of Gene Regulation by Peroxisome Proliferators via PPARα, p53 Signaling, RAR Activation, Decreases Permeability Transition of Mitochondria and Mitochondrial Membrane, Increases Cardiac Proliferation, NRF2-mediated Oxidative Stress Response, Decreases Depolarization of Mitochondria and Mitochondrial Membrane, Decreases Respiration of Mitochondria	
Different pathways: TR/RXR Activation, Cardiac Fibrosis, Cell Cycle: G2/M DNA Damage Checkpoint Regulation	**Different pathways:** Acute Renal Failure Panel (Rat), Cardiac Necrosis/Cell Death, Liver Necrosis/Cell Death, Protection from Hypoxia-induced Renal Ischemic Injury (Rat), Aryl Hydrocarbon Receptor Signaling, LPS/IL-1 Mediated Inhibition of RXR Function, Decreases Permeability Transition of Mitochondria and, Mitochondrial Membrane, Increases Transmembrane Potential of Mitochondria and Mitochondrial Membrane, Increases Renal Proliferation, Hepatic Fibrosis, Increases Liver Damage, Hormone Receptor Regulated Cholesterol Metabolism, VDR/RXR Activation, Increases Liver Hyperplasia/Hyperproliferation.
Liver	
Common pathways: TR/RXR Activation, Hypoxia-Inducible, p53 Signaling, Factor Signaling, TGF-β Signaling, Liver Proliferation, Cardiac Hypertrophy, RAR Activation, Increases Liver Hyperplasia/Hyperproliferation, Liver Necrosis/Cell Death, Mitochondrial Dysfunction	
Different pathways: Cell Cycle: G2/M DNA Damage Checkpoint Regulation, FXR/RXR Activation, PPARα/RXRα Activation, Renal Necrosis/Cell Death, NRF2-mediated Oxidative Stress Response, Cell Cycle: G1/S Checkpoint Regulation, Nongenotoxic Hepatocarcinogenicity Biomarker Panel, Mechanism of Gene Regulation by Peroxisome Proliferators via PPARα, VDR/RXR Activation, Hormone Receptor Regulated Cholesterol Metabolism, Pro-Apoptosis, Aryl Hydrocarbon Receptor Signaling, LXR/RXR Activation, Swelling of Mitochondria, Biogenesis of Mitochondria, Primary Glomerulonephritis Biomarker Panel (Human)	**Different pathways:** Decreases Depolarization of Mitochondria and Mitochondrial Membrane, Anti-Apoptosis, Increases Heart Failure -

[a]Pathways represent toxicity lists significantly enriched with a p value <0.05 in differentially expressed genes found in $AgNO_3$ or PVP-AgNP exposed fathead minnow tissue. Lists were identified using Ingenuity Pathway Analysis (IPA, Redwood City, CA).

RESULTS

Nanoparticle and Exposure Characterization. PVP-AgNPs (Supporting Information, Figure S1) were analyzed by several different methods to provide multiple lines of evidence of primary particle size (SEM, TEM) and hydrodynamic diameter (DLS, FFF). The TEM mean was 30 ± 3 nm, FFF was 36 nm, the DLS (intensity) in MQ (Milli-Q) water was 42 nm with a distribution range of 9−98 nm, and the DLS in dechlorinated tap (DT) water mean was 43 nm (23−126 nm distribution range). The hydrodynamic diameter was only slightly greater than measured primary particle size (SEM: 26 nm, TEM: 20 nm). $AgNO_3$ was present in the exposure water at 3.7 ± 0.4 μg/L and PVP-AgNPs were present in the exposure water at a total silver concentration of 46.1 ± 0.8 μg/L with 12.5 ± 3.4 μg/L present as dissolved silver. Silver was not detectable in the control exposure water. After 96 h, gill tissues of $AgNO_3$ exposed fish accumulated 213 ± 88 μg/kg silver and control fish accumulated 49 ± 15 μg/kg. PVP-AgNP accumulation was variable with average total silver content in gills of 119 ± 53 μg/kg tissue. These measurements represent an average of measurements at 24, 48, and 72h.

Gene Expression. Transcriptional analysis was performed on six fish for the control treatments and five fish each for the $AgNO_3$ and PVP-AgNP treatments and identified differentially expressed genes (Figure 1 and Supporting Information, Tables S5 and S6). In most cases we were able to use fish from 4 to 5 different replicate exposures, except for the liver PVP-AgNP, were we could only use fish from 3 replicate exposures (due to quality of the samples). After hybridization, one sample was determined to be poor quality and was removed from further analysis. Hierarchical analysis of all samples showed very distinct gene expression patterns effects for all treatments in livers of exposed fish (Figure 1a). Effects on brain gene expression were less distinct between treatments, possibly due to differential distribution of PVP-AgNP and $AgNO_3$ in the tissues. PVP-AgNP and $AgNO_3$ affected many of the same genes in both the brain and liver. However, 43% of genes affected by PVP-AgNP in brain and 35% of those affected by PVP-AgNP in liver were different from those affected by $AgNO_3$ treatment (Figure 1 b).

Analysis of pathways, which were generally more common between treatments because they were composed of several genes, indicated that $AgNO_3$ and PVP-AgNP exposure had several common effects in the liver and brain (Figure 1b). In brain, these were principally composed of signaling pathways and in liver were composed of a diverse range of functions including signaling, gene activation, protein ubiquitination, and metabolism (Supporting Information, table S6 and S7). Approximately 10% and 37% of biological pathway level effects caused by PVP-AgNP were different from those of $AgNO_3$ in the brain and liver, respectively (Figure 1b).

Both common and different pathways were observed when toxicity pathways were examined (Table 1 and Supporting

Information, Tables S6 and S7 for details). However PVP-AgNPs had more impact than $AgNO_3$ in affecting toxicity pathways in the brain and less in the liver. The high degree of similarity of PVP-$AgNO_3$ toxicity pathways in liver to those affected by $AgNO_3$ (10 of 13) suggests that many of the effects observed in the liver due to PVP-$AgNO_3$ exposure are likely due to dissolved silver. Conversely, the observation that 14 of 24 toxicity pathways affected by PVP-AgNP are different from those impacted by $AgNO_3$ exposure suggests that a significant portion of effects observed in the brain due to PVP-$AgNO_3$ exposure may be due to intact nanoparticles rather than dissolved silver.

The Nrf2-mediated oxidative stress pathway was significantly enriched in liver and brains of both $AgNO_3$ and PVP-AgNP exposed fish (Table 1, Supporting Information, Tables S6 and 7). Consistent with this, the gene for the antioxidant superoxide dismutase (SOD3), was confirmed to be up-regulated in livers of fish exposed to $AgNO_3$ and PVP-AgNP (Table 2). Drug

Table 2. Real Time Quantitative PCR Results[a]

	liver		brain	
	$AgNO_3$	PVP-AgNP	$AgNO_3$	PVP-AgNP
gene name and symbol	fold change	fold change	fold change	fold change
cytochrome p450 3A (CYP3A)	**2.4***	1.4	−1.48	1
flotillin 1 (FLOT1)	1.76	**2.57***	nt	nt
glutathione-S-transferase (GST)	1.7	2	−1	−1.24
glutathione-S-transferase (GSTA)	1.42	1.02	1.14	−1
hypoxia-inducible factor 1-alpha (HIF1)	**2.04***	**1.94***	1.23	1.4
P38 mitogen-activated protein kinase (P38MAPK)	**1.63***	1.18	**1.75***	1.49
superoxide dismutase (SOD3)	**337.8***	**13.18***	2.07	1.17
signal transducers and activators of transcription family 1 (STAT1)	**1.88***	**3.55***	nt	nt
signal transducers and activators of transcription family 3 (STAT3)	**2.78***	**2.25***	nt	nt
tumor protein p53 (TP53)	**2.98***	**2.93***	−1.38	1.32

[a]Values are fold change relative to controls. Asterisks denote values where significant up regulation was observed (p value <0.05). nt means not tested.

metabolizing enzymes appeared to be affected only in livers of $AgNO_3$ as evidenced by increased expression of Cytochrome p450 3A (Table 2). Expression of Flot1, encoding Flotillin 1, a protein previously proposed as involved in nanoparticle transport into cells,[30] was significantly increased in livers of fish exposed to PVP-AgNP but not $AgNO_3$. Up regulation of the cell cycle and tumor suppressor p53 pathway was confirmed by PCR (Table 2).

Several neurological disease pathways were impacted in the brains of exposed fish indicating that both $AgNO_3$ and PVP-AgNPs have the potential to impact neurological function (Table 3 and Supporting Information, Table S9 for details), therefore we examined what types of endogenous chemicals or proteins may be potential upstream regulators of pathways and genes differentially expressed in the brains of exposed fish (Supporting Information, Table S8). Since specific endogenous chemicals control neurological pathways in the brain by

Table 3. Neurological Disease Functions Impacted in Brains of $AgNO_3$ and PVP-AgNP Exposed Fathead Minnow[a]

Neurological disease functions in brains of exposed fish	
$AgNO_3$ exposed	**PVP-AgNP exposed**
Common functions: Movement disorder, dementia, tauopathy, neurodegenerative disorder, Alzheimer's disease, neurological signs, dyskinesia, Huntington's disease, disorder of basal ganglia, exencephaly of rhombencephalon	
Different functions: Chorea, neuromuscular disease, neural tube defect, ataxia, congenital anomaly of brain, dementia, progressive myoclonic epilepsy, neurodegeneration of cerebral cortex cells, X-linked mental retardation, gliosis, astrocytoma, dystrophy of neurites	**Different functions:** Roussy-Levy syndrome, ectopia of granule cells, brain cancer, tumorigenesis of brain cancer cell lines, familial advanced sleep phase syndrome, hereditary liability to pressure palsies, paroxysmal dyskinesia, benign nerve tumor, neurodegeneration of hippocampal neurons

[a]Neurological disease functions are listed that were significantly enriched with a p value <0.005 in differentially expressed genes found in $AgNO_3$ or PVP-AgNP exposed fathead minnow brain tissue. Functions were identified using Ingenuity Pathway Analysis (IPA, Redwood City, CA).

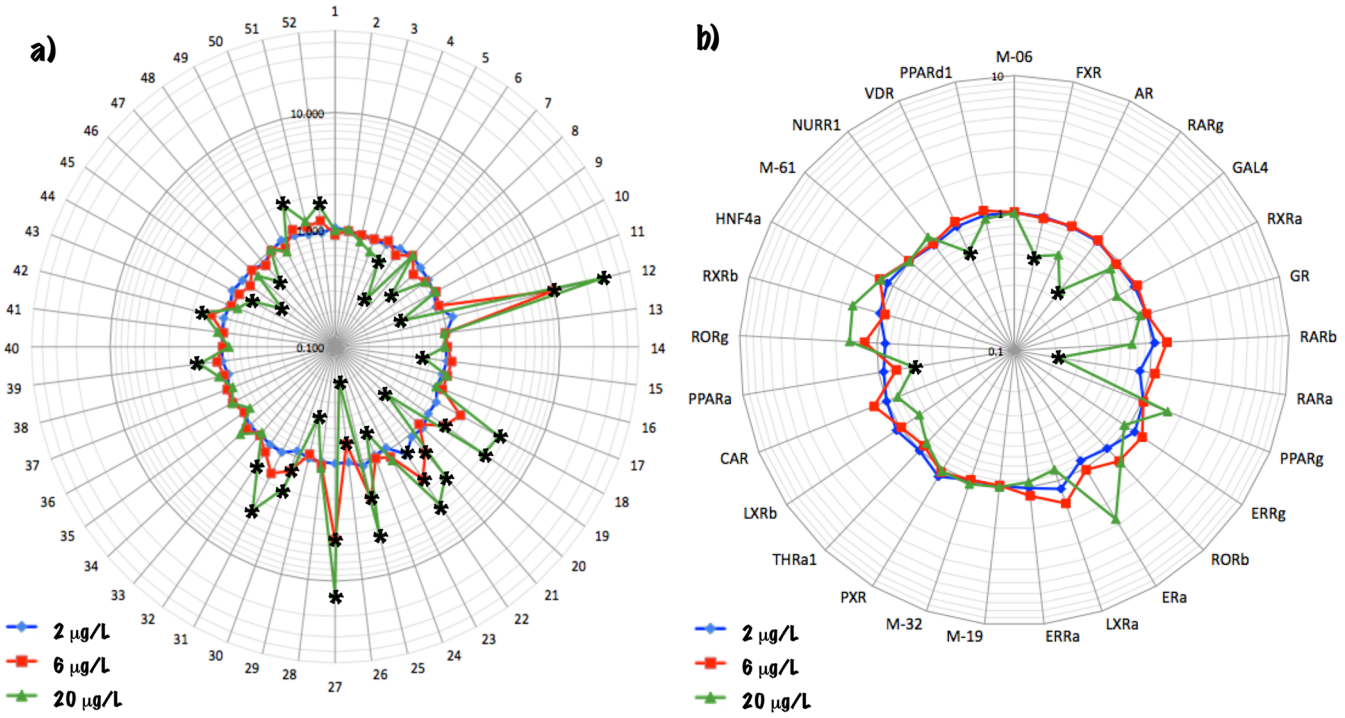
interacting with receptors, channels and key proteins, one could potentially identify which of these key proteins are affected by $AgNO_3$ or PVP-AgNPs by identifying what molecules regulate the pathways perturbed by $AgNO_3$ or PVP-AgNPs. Twenty-one upstream regulators were found to be common to both treatments, while 12 were specific to $AgNO_3$ and 64 were specific to PVP-AgNP treatments (Supporting Information, Table S8).

Molecules common to both exposures that were also receptors or ligands of receptors were: β-estradiol, dopamine which binds to the dopamine receptor D1 (DRD1), the G protein coupled estrogen receptor 1(GPER), the 5-hydroxytryptamine (serotonin) receptor 2A (HTR2A), L-triiodothyronine, which binds to the thyroid receptor, norepinephrine which binds to the adrenergic receptor, progesterone which binds to the progesterone receptor, the sweet and amino acid taste receptor (TAS1R3), and the urotensin 2 receptor (UTS2R).

Regulators unique to $AgNO_3$ exposure with activation z-scores greater than one were: aldosterone which binds to the aldosterone mineral corticoid receptor, anandamide, which binds to the cannabinoid receptor, and arachidonic acid which is part of the arachadonic acid signaling cascade. Regulators that were unique to PVP-AgNP exposure were the angiotensin I receptor (AGTR1), quinolinic acid which binds to the NMDA receptor, and sphingosine-1-phosphate, which binds to the lysophospholipid receptor.

Impacts of $AgNO_3$ and PVP-AgNP on Mammalian Receptor, Transcription Factor, and Enzymatic Functional in Vitro Assays. Functional effects of $AgNO_3$ and PVP-AgNP were examined using in vitro rat and human nuclear receptor and transcription factor assays, receptor binding assays, p38 kinase assays, and a CYP3A4 assay (Table 2). Effects of PVP-AgNP stock solution on human transcription factor assays was highly reproducible at 1 and 30 days after preparation indicating that the preparation was stable over time (Supporting Information, Figure S3).

Dissolved silver was tested at a higher concentration range (5−211 μg/L) than PVP-AgNP (2-20 μg/L) in these assays with minimal effects, while PVP-AgNP tested at levels of 70 μg/L and higher were toxic to the cells used in the assays. The PVP-AgNPs displayed a concentration response in range and number of human transcription factor assays affected with 1, 8, and 26 of 52 transcription factors impacted at 2, 6, and 20 μg/L



#	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Name	TGFb	M-06	HNF6	TCF/b-CAT	E-Box	PPRE	NFI	PXR	GRE	AP-1	ISRE	MRE	STAT3	TAL	NF-kB	FoxA2	CMV	Xbp1
#	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Name	CRE	AhR	EGR	NRF2/ARE	TA	ERE	Oct-MLP	DR4/LXR	HSE	SREBP	p53	BRE	Pax6	HIF1a	M-19	VDRE	RORE	M-32
#	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52		
Name	Ets	GLI	NRF1	GATA	E2F	C/EBP	Myb	PBREM	IR1	AP-2	DR5	M-61	FoxO	Sox	Sp1	Myc		

Figure 2. Effects of NPs on transcription factor (a) or nuclear receptor (b) assays. Transcription factor assays are represented with a number, the corresponding name can be found in the table at the bottom of the figure. More information on those assays can be found in Supporting Information Table S2 and S3. Activities are shown as fold induction values versus vehicle-treated control cells. Graph shows average fold-induction data plotted in logarithmic scale; * represents significantly different from control ($p < 0.05$).

respectively (Figure 2a). PVP-AgNPs interacted with several nuclear receptors in vitro whose pathways were also significantly enriched in fish exposed to either $AgNO_3$ or PVP-AgNP in vivo fish exposures (Figure 2b). $AgNO_3$ had no activity in transcription factor assays, but did decrease activity in 2 of 29 receptor assays (RARα and hepatocyte nuclear factor-4α HNF4α/NP2A1 (Supporting Information, Figure S2).

Furthermore, direct interactions of $AgNO_3$ and PVP-AgNP with several neurotransmitter receptors implicated in fathead minnow exposures or in previous zebrafish exposures were tested (Table 2).[25] Clozapine binding to dopamine receptors, a potential upstream regulator related to both stressors, was inhibited by PVP-AgNPs in vitro, but not by $AgNO_3$. $AgNO_3$ significantly inhibited ligand binding in rat adrenergic receptors α1 and α2 and the rat cannabinoid receptor CB1 consistent with upstream regulator predictions in fathead. $AgNO_3$ also inhibited activity in human p38 and CYP3A4 assays, while PVP-AgNP had no effect.

DISCUSSION

Possible Modes of Action for Silver and PVP−Silver Nanoparticle Toxicity. Recent research suggests that metallic NP toxicity is likely due to a combination of both the NP and released metal ions, as toxicity could not be explained solely by soluble metal ions.[4,30,31] In this study, transcriptional analyses found that a significant number of enriched biological and toxicological pathways in both the liver and brain of fathead exposed to PVP-AgNPs were different from those in $AgNO_3$ exposed fish (Figure 1), suggesting that not all effects were likely due to silver released from PVP-AgNPs. It should be noted that only one concentration was analyzed and concentrations of total silver in gills of PVP-AgNP fish were lower than gills of $AgNO_3$ exposed fish suggesting that in vivo concentrations of total silver, and hence dissolved silver, were lower in PVP-AgNP exposed fish than in $AgNO_3$ exposed fish. As a result, some of the differential expression could also be related to different amounts of dissolved silver, although the greater impact of PVP-AgNP exposure on toxicity pathways in the brain supports direct effects of PVP-AgNP rather than concentration dependent effects of dissolved silver.

We identified several toxicity pathways common to both exposures that are consistent with known effects of dissolved silver on fish indicating that dissolved silver causes a significant proportion of effects in PVP-AgNP exposed fish. Silver ions can cause toxicity to freshwater fish by compromising Na^+ and Cl^- transport across the gills, loss of Na^+ and Cl^- from blood, and ultimately death.[32] All tissues exhibited signs of silver toxicity, including disruption of Na^+ transport and down-regulation of

dx.doi.org/10.1021/es4042258 | Environ. Sci. Technol. 2014, 48, 4546−4555

Table 4. Effect of Polyvinylpyrrolidone-Coated Silver Nanoparticles and Silver Nitrate on Mammalian in Vitro Assays[a]

type	assay	$AgNO_3$			PVP-AgNP		
neurotransmitter	adrenergic α1	**20 μg/L**	**200 μg/L**	**2 mg/L**	**20 μg/L**	**200 μg/L**	**2 mg/L**
			101.34%			11.22%	
	adrenergic α2		***97.52%***			−5.28%	
	cannabinoid (CB1)	***53.84%***	***109.03%***		−1.02%	−1.79%	
	GABA, nonselective		0.83%				
	dopamine, nonselective	36.35%	−1.46%	24.56%	−16.59%	39.08%	***68.64%***
	nicotinic, neuronal	−2.49%			0.82%		
	opioid receptor, nonselective	11.65%			2.39%		
	serotonin receptor, 5HT1	5.58%			−7.90%		
kinases	MAPK11(p38b)		***60.72%***			8.41%	
	MAPK14(p38a)		***72.29%***			7.36%	
ADME-TOX	CYP3A4	***100%***			2.25%		

[a]Neurotransmitter assays were rat or human receptor natural ligand binding competition assays. Kinase assays were human mitogen-activated protein kinases (MAPK). The ADME-TOX assay was a metabolic cytochrome p450 3A4 (CYP3A4) enzyme assay. NP = polyvinylpyrrolidone-coated silver nanoparticles. AgNO3= silver nitrate. Values in bold are considered significant and correspond to percent of inhibition. Missing values were not tested.

the aldosterone signaling involved in maintaining Na^+, K^+, and H^+ homeostasis. Fish exposed to $AgNO_3$ also had decreased expression of the renin-angiotensin signaling pathway, a feedback circuit regulating blood pressure and fluid balance with aldosterone.[33] $AgNO_3$ exposures impacted a larger number of pathways related to Na^+, K^+, and H^+ homeostasis than PVP-AgNP exposures. This is consistent with the total silver detected in gills of PVP-AgNP exposures being composed of both intact nanoparticles and dissolved silver and implies a lower overall in vivo concentration of dissolved silver than in $AgNO_3$ exposures in the organisms.

A second major effect of dissolved silver is the production of reactive oxygen species (ROS) which has been linked to oxidative stress.[34−39] Increased ROS activates MAPK signaling pathways leading to a number of cellular responses including increased proliferation, differentiation, inflammatory responses, and apoptosis.[40] Furthermore, Massarsky et al [41] showed that both Ag^+ and AgNP increased ROS production causing depressed heart rate, delayed hatching, physical deformities, and mortality in zebrafish (*Danio rerio*) embryos. Here, both PVP-AgNP and $AgNO_3$ exposures resulted in oxidative stress as evidenced by enrichment of the NRF2-mediated oxidative stress pathway and upregulation of the antioxidant SOD3, in agreement with the above studies.

Another potential impact of silver is the interference with cellular stress response pathways that use p38 MAPK signaling. Lim et al. [39] demonstrated that PVP-AgNP caused reproductive toxicity in *C. elegans* through ROS induction of p38 MAPK signaling, in addition to increasing of hypoxia-inducible factor 1-alpha (HIF1α) which plays an essential role in cellular responses to hypoxia. In fathead minnow, $AgNO_3$ caused similar effects with increased expression of genes involved in cellular stress response pathways (MAPK signaling, p38MAPK, p38 responsive transcription factors STAT1 and 3, and HIF1α, Table 1 and 2). These observations are in contrast to inhibition of human p38 activity in vitro by $AgNO_3$ at 200 μg/L (Table 4). Species differences in p38 silver inhibition or partial inhibition by lower levels of silver could explain these apparent differences. PVP-AgNP exposures had no effect on p38 or MAPK signaling pathway gene expression nor did it affect p38 activity in vitro but did increase expression of STAT1, STAT3, and HIF1α suggesting that PVP-AgNP may activate these genes by a pathway other than the cellular stress response pathways impacted by dissolved silver.

In Vitro Assays Provide Functional Support for in Vivo Transcriptional Effects. A broad suite of fish oriented in vitro assays are not commercially available to enable test whether or not PVP-AgNP or $AgNO_3$ directly alter function of critical regulatory proteins, receptors or enzymes. Therefore we tested the equivalent mammalian in vitro assays for effects of PVP-AgNP and $AgNO_3$ and extrapolated those results to effects on fathead minnow. This approach can be used to extrapolate effects between species if sufficient similarity between the proteins used exists.[46] PVP-AgNPs, and not dissolved silver, interacted with several human transcription factors in a concentration dependent manner when tested in vitro, further supporting observations that PVP-AgNP, and not dissolved silver, may be directly responsible for some transcriptional effects observed in vivo (Figure 2). Observations that a large number of transcriptional changes were found in brains and livers of fathead minnow exposed to $AgNO_3$ (Figure 1), but no interaction of transcription factors with $AgNO_3$ using in vitro was seen (Supporting Information, Figure S2a), suggest that transcriptional effects due to $AgNO_3$ are likely secondary responses to other mechanisms such as oxidative stress, ROS, and Na^+ imbalance, rather than activation of specific transcription factors.

In vitro assays demonstrated that PVP-AgNP could directly interact with transcription factors and nuclear receptors that are involved in pathways found to be affected by PVP-AgNP in fathead minnows. For example, cAMP-responsive DNA-binding protein (CRE) was activated by PVP-AgNP in vitro (Figure 2a) and the cAMP signaling regulation pathway was activated by PVP-AgNP in fathead; NRF2 was activated in vitro and the NRF2 oxidative stress pathway was activated in vivo; HIF1α was activated in vitro and the hypoxia-inducible factor signaling pathway was activated in vivo; and bone morphogenetic protein (BRE) was activated in vitro and the TGF beta signaling pathway, which includes BRE, was activated in vivo. PVP-AgNP also directly interacted with a number of mammalian nuclear receptors in vitro (Figure 2b) consistent with observed effects in fathead where NP exposure affected pathways for RAR activation (both in brain and liver), PPARα/RXRα activation (brain), and VDR/RXR activation (brain). $AgNO_3$ inhibited activity of RARα in vitro receptor assays consistent with

$AgNO_3$ affecting the RAR activation pathway of exposed fathead. The congruence of effects between mammalian in vitro assays and pathways that were found to be affected in fathead minnow provides strong evidence that PVP-AgNP can directly interact with receptors and transcription factors to cause biological effects.

PVP-AgNP and AgNO3 Impact Expression of Neurological Pathways in Fathead Minnow and Interact with Mammalian Neuroreceptors in Vitro. We identified potential molecular interaction events responsible for the neurotoxic effects of PVP-AgNP and $AgNO_3$ through analysis of differentially expressed pathways in brain of fathead minnow and confirmed these interactions by use of mammalian receptor binding assays (Table 4). In fathead minnow, $AgNO_3$ significantly decreased expression of pathways activated by the neurotransmitters norepinephrine and anandamide. $AgNO_3$ likely competes with, or prevents, binding of these ligands to their respective receptors in the fathead minnow brain as $AgNO_3$ inhibited ligand binding to the rat adrenergic receptors $\alpha 1$ and $\alpha 2$ and cannabinoid receptor CBR1 in vitro (Table 4).

PVP-AgNPs have been found to affect dopamine signaling in rat PC12 cells and caused hyperactivity in exposed zebrafish.[25] Here we demonstrate that PVP-AgNP can inhibit ligand binding in rat dopamine receptor assays (Table 4) and that dopamine and dopamine receptor D1 are potential regulators of genes affected in brains of fathead exposed to PVP-AgNP suggesting that PVP-AgNP could cause hyperactivity by competing for, or inhibiting, dopamine binding to dopamine receptors.

None of the neuroreceptors affected by dissolved silver were inhibited by PVP-AgNP, or vice versa, supporting the hypothesis that observations of different pathway level effects in PVP-AgNP exposed versus $AgNO_3$ exposed fathead minnows is due to different receptors specificities. The release of silver ions from PVP-AgNPs is the most likely reason that upstream regulators such as adrenergic receptors were identified in PVP-AgNP fathead minnow exposures, as PVP-AgNP did not significantly interact with adrenergic receptors in vitro, while $AgNO_3$ did. It is worth noting that the type of exposure and the ability of the system to interact with NP, such as cellular uptake, interactions, biodistribution, etc., could also affect the biological activity or toxicity of PVP-AgNP and be responsible for differences observed in the literature, particularly when comparing in vitro versus in vivo exposures.[42]

Our data show that both dissolved silver and PVP-AgNP are potential neurotoxicants and cause different effects in vivo and in vitro. PVP-AgNP released significant amounts of silver ions in solution with the consequence of stressing fish with both nanoparticles and dissolved silver. $AgNO_3$ and PVP-AgNP impacted expression of genes in liver and brains of exposed fathead minnow through both common and distinct pathways (Tables 1 and 3). Both $AgNO_3$ and NPs elicited effects in the brain of exposed fish related to oxidative stress, neurotransmitters and neurological diseases. Exploration of potential upstream regulators of differentially expressed pathways in brains of exposed fish identified potential receptor targets of $AgNO_3$ and NP. The interaction with these targets was validated using mammalian in vitro assays demonstrating that PVP-AgNPs but not $AgNO_3$ activated some human transcription factors. NPs and $AgNO_3$ were also found to interact with different nuclear and neurotransmitter receptors further supporting the concept that nanoparticles, not just silver ions, may contribute to biological effects upon exposure. Furthermore, the fact that we observed PVP-AgNP effects in vitro at smaller concentrations than those of $AgNO_3$ indicates that there is a specific effect of the NP, as the dissolved Ag ion concentrations would be much less than those in the ion only exposure.

Adverse Outcome Pathways. Adverse outcome pathways (AOP) provide a framework that organizes mechanistic or predictive relationships between molecular initiating events, pathways and adverse phenotypic outcomes relevant to hazard assessment.[43] The AOP framework has also been proposed as a way to show how human-focused assays can be useful in identifying key initiators and predicting effects in non-mammalian species.[44,45]

Neurotoxicity and changes in behavior have already been linked to adverse outcomes.[45,46] Here, PVP-AgNPs were identified as dopamine receptor antagonists using in vitro assays. Dopamine receptor antagonism has been linked to a decline of food intake and locomotor activity in sea bass,[47] as well as decreased locomotor activity in zebrafish.[48,49] Both effects could eventually lead to death, particularly in a natural environment. Dopamine antagonists also decrease locomotor activity in mammals [50] and have been linked to behavioral pathologies such as Parkinson's disease, schizophrenia, and attention deficit hyperactivity disorder.[51−53] Adrenergic receptors are involved in the *fight-or-flight* response, a physiological reaction that occurs in response to attacks or threats to survival. They are also involved in muscle contraction, heart beat rate, and central neuronal activities.[54] Cannabinoid receptors may affect memory, cognition, and pain perception[55] and have been shown to affect swimming behavior in zebrafish.[56] Therefore impacts on the dopaminergic, cannabinoid, or adrenergic receptors could lead to adverse outcomes (behavioral changes, inability to scape from threat, etc) and could have significant impacts both in fish and mammals. These similarities support the use of fish as alternate species for both ecological and human health.

As mentioned earlier, ROS can lead to oxidative stress, which could result in cytotoxicity, pericardial edema, or apoptosis at the organ level.[32,39,57,58] These effects could potentially lead to death of the organism.

Our results reflect potential in vitro/in vivo toxicity similarities and differences that should be further studied and taken into account when using in vitro/in vivo extrapolation for risk asssessment. Our results also show that combinations of in vivo exposures, in vitro assays, and predictive computational tools are effective and could potentially be used to explore environmental and human hazard assessment.

■ ASSOCIATED CONTENT

Supporting Information

Transmission electron microscopy images of the PVP-AgNP used in this study, potential neurological diseases related to DEGs in the brain of FHM exposed to $AgNO_3$ or NPs, in vitro transcription factor or nuclear receptor assays and their activation by silver nitrate ($p < 0.05$), effect of long-term storage on NP activity in HepG2 cells, list of primer sequences used in real-time PCR, human nuclear receptors included in trans-factorial assays, Transcription factor assays end points, differentially expressed genes in the liver and brain of FHM females exposed to $AgNO_3$ ($p < 0.05$), differentially expressed genes in the liver and brain of FHM females exposed to NP ($p < 0.05$), functional analysis from liver and brain DEGs of FHM females exposed to $AgNO_3$, including significant biological and

toxicological pathways ($p < 0.05$), functional analysis from liver and brain DEGs of FHM females exposed to NP, including significant biological and toxicological pathways ($p < 0.05$), significant upstream regulators related to DEGs in the brain of FHM exposed to $AgNO_3$ and PVP-AgNP, including type of molecule, *z*-score, and target, and neurological disease related to DEGs in the brain of FHM exposed to $AgNO_3$ and PVP-AgNP ($p < 0.005$). This information is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was funded by the US Army Environmental Quality Research Program (including BAA 11-4838 to N.G.R.). Permission was granted by the Chief of Engineers to publish this information.

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