Original Article

Assessment of the developmental neurotoxicity of silver nanoparticles and silver ions with mouse embryonic stem cells in vitro

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Keywords
Developmental neurotoxicity, Embryonic stem cells, Silver ions, Silver nanoparticles, Stem cell toxicology.

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Funding Information
K. C. Wong Education Foundation; National Natural Science Foundation of China (21461142001, 21577166); Chinese Academy of Sciences (2015J20, QYZDJSSW-DQC017, XDB14040301)

Received: 13 June 2018;
Revised: 02 July 2018;
Accepted: 17 July 2018

Journal of Interdisciplinary Nanomedicine, 2018; 3(3), doi: 10.1002/jin2.49

Abstract
The wide applications of silver nanoparticles (AgNPs) have raised many concerns worldwide regarding their safety. The few previous studies on the developmental toxicity of AgNPs have been mostly dependent on animal experiments, which are time-consuming and costly. The rapid development of stem cell biology provides a new in vitro alternative to live animal assays for developmental toxicity studies. Here, we assessed the developmental neurotoxicity of AgNPs and Ag ions using a mouse embryonic stem cell (mESC) toxicology model. Our results showed that AgNP and Ag ion treatments did not affect mESC viability or cause accumulation of reactive oxygen species, at concentrations below 1 μg/mL. Conversely, AgNPs and Ag ions perturbed mESC global and neural progenitor cell-specific differentiation processes. In fact, both AgNPs and Ag ions induced the anomalous expression of neural ectoderm marker genes, such as Sox1, Sox3, Map2, NeuroD, Nestin, and Pax6, at concentrations lower than 0.1 μg/mL. Interestingly, AgNP effects manifested at earlier time points as compared with Ag ions. In addition, treatment with Ag ions generated neural progenitor cell abnormal morphology. Overall, our data proved that both AgNPs and Ag ions are toxicants, and their toxic effects are somehow different.

Introduction
Because of their extraordinary physical and chemical properties, nanomaterials are employed in many applications. Silver nanoparticles (AgNPs) are particles, containing silver element, generally spherical and with a diameter of less than 100 nm. They stand out for their...
AgNPs impair neural progenitor cell specification

Excellent antibacterial properties and are widely used in medical catheters, surgical instruments, nose drops, room spray, and so on (Huang et al., 2015). With so many applications, AgNPs have alimented serious safety concerns. In fact, we are exposed to AgNPs via inhalation (Takenaka et al., 2001), oral administration (Gaillet & Rouanet, 2015; Park et al., 2010), and percutaneous absorption (Recordati et al., 2016). After entering the body, AgNPs reach major organs such as the brain, liver, testes, lungs (Kim et al., 2008), through the body fluids (Tang et al., 2008) and then show a systemic distribution (Mao et al., 2016). The accumulation of silver causes argyria (Bowden et al., 2011), the irreversible bluish-black discoloration of the surface of the skin.

At present, animal studies have shown that AgNPs can enter the body and be transferred to the offspring (Lee et al., 2012a). Moreover, AgNPs can penetrate the blood-testis barrier and affect sperm (Kim et al., 2008; van der Zande et al., 2012). Similarly, two other groups demonstrated that AgNPs accumulate in the fetus through the placenta (Melnik et al., 2013) and in the visceral yolk sac of pregnant mice (Austin et al., 2018c). In that study, they also reported that the AgNP concentration in the fetal brain was about 0.0035% of the dose ingested (the exposure dose was 0.81 mg of Ag/kg of body weight). Those findings indicate that the potential exposure risk for AgNPs in pregnant women cannot be ignored.

Several developmental neural toxic effects of AgNPs have been reported. For instance, Xu et al. showed that AgNPs may produce oxidative stress and apoptosis in the developing brain of newborn mice (Fatemi et al., 2013). Moreover, after exposure to AgNPs in mice, the offspring showed significant neural behaviour changes such as spatial cognition disorders (Wu et al., 2015). All this evidence implies that AgNPs may have severe toxic effects during neural differentiation and cause genotoxicity in the developmental process. All the previous developmental neurotoxicity studies were based on live animal assays and may have missed some important toxicity information in the earliest stages of embryonic development. The recently established stem cell toxicology models (Faiola et al., 2015; Genschow et al., 2002; Liu et al., 2017; Yao et al., 2016; Yin et al., 2018c; Yin et al., 2018b; Yin et al., 2018a) may help solve these issues effectively. In fact, stem cell differentiation can mimic in vitro the developmental process and allow the accumulation of toxicity data at many time points in a time-efficient and cost-efficient way.

Here, we employed a mouse embryonic stem cell (mESC) neural differentiation model to study the developmental neurotoxicity of AgNPs and Ag ions and determine whether the AgNP toxicity was due to the NPs or Ag ions. The abnormal expression of six different neural ectodermal genes, Sox1, Nestin, Sox3, Pax6, Map2, and NeuroD, and atypical neural progenitor cell (NPC) morphology, during both embryoid body (EB)-based and monolayer-based neural differentiations, were used to evaluate neural toxic effects. Our results substantiate the potential toxic doses of both AgNPs and Ag ions and provide scientific bases for the safe applications of AgNPs.

Results

Silver nanoparticle characterization

Silver nanoparticles in solution were characterized by transmission electron microscope (TEM) and dynamic light scattering. The properties of our AgNPs are summarized in Table 1. Morphology was observed through TEM (Fig. 1a): AgNPs were homogeneous and spherical in shape. Dynamic light scattering analyses (Fig. 1b) show that the average size of our AgNPs was about 34.08 nm, very close to the most used commercial AgNPs (He et al., 2016). In Figure 1a, we also noted a small peak at about 350 nm, suggesting a very slight aggregation of the particles in solution. Moreover, the AgNP poly dispersity index was 0.326 and their zeta potential $-38.3 \pm 9.5$ mV (Table 1). The low poly dispersity index value and negative zeta potential indicate that the AgNPs were stable in aqueous solution.

Silver nanoparticles did not cause cytotoxicity in mouse embryonic stem cells at concentrations equal or below 1 μg/mL

Mouse embryonic stem cells were treated with AgNPs and Ag ions (or their respective solvent controls) at concentrations ranging from 0.01 to 1 μg/mL. Cell viability was tested at 24, 48, and 96 h by Alamar Blue assay. As shown in Figure 2a, for the first 96 h

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Table 1. Characterization of silver nanoparticles.

| Size       | Poly dispersity index | Zeta potential     |
|------------|-----------------------|--------------------|
| 34.08 nm   | 0.326                 | $-38.3 \pm 9.5$ mV |

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of culture, mESC viability did not have significant changes in treated groups, compared with control groups. Thus, 1-μg/mL AgNPs seem far from being lethal for self-renewing mESCs.

Because reactive oxygen species (ROS) generation and oxidative stress are the main mechanisms of toxicity for AgNPs, as previously reported (Arora et al., 2008), and can cause apoptosis (Martindale & Holbrook, 2002; Valko et al., 2007), we used the DCFH-DA assay to assess the intracellular ROS levels induced by AgNPs or Ag ions; 1-mM H₂O₂-treated cells were used as positive control. Owing to the fact that accumulation of ROS appears in the early stages after chemical exposure (Oh & Lim, 2006), we measured ROS levels up to 12 h after exposure to AgNPs/Ag ions. No obvious ROS generation was detected at or under 1-μg/mL AgNP or Ag ion exposure for up to 12 h (Fig. 2b). Thus, ROS generation may not be the key mechanism of toxicity at low AgNP/Ag ion concentrations in self-renewing mESCs, probably because of the antioxidant machinery activation in those cells, contrary to other cellular systems (Yin et al., 2015a).

Calcium ion (Ca²⁺) levels are important in many cellular processes. For instance, an instantaneous intracellular calcium ion flux increase generally occurs during cell stress responses (Ankarcrona et al., 1995; Duchen, 2000; Mattson, 2007). We used a Fura-2/AM probe to test the level of intracellular calcium ions in AgNP or Ag ion-treated cells. As depicted in Figure 2c,d, only 1-μg/mL Ag ions stimulated intracellular calcium ion flux, with a peak at about 20 min, while AgNPs did not. Although Ag ions generated calcium flux, probably because of their higher charge as compared with AgNPs, and thus cellular stress, there was no activation of ROS and the cellular homeostasis was maintained, likely because of the activation of a variety of antioxidant enzymes, such as superoxide dismutase (SOD) (Yan et al., 2006).

Figure 1. Characterization of silver nanoparticles (AgNPs). (a) Transmission electron microscopy image of 2-μg/mL AgNPs. The scale bar is 100 nm. (b) Particle size distribution of AgNPs analysed by dynamic light scattering.
Silver nanoparticles and silver ions affected the specification of the three primary germ layers during mouse embryonic stem cell global differentiation

The in vitro global differentiation of mESCs via EB formation can mimic the early stages of embryonic development. In fact, EBs are cell aggregates in suspension, with three-dimensional structure and can generate the three primary germ layers (endoderm, mesoderm, and ectoderm), from which all the cells of the body are derived. We used this model to start investigating the toxicity of AgNPs and Ag ions during development.

We chose 0.01- and 0.1-μg/mL AgNP and Ag ion concentrations, to include normal population and occupational exposures (Lee et al., 2012b; Limpiteeprakan & Babel, 2016; Wang et al., 1998). Those concentrations were far from being lethal.

Starting from Day 0 of the differentiation, mESCs were treated with the indicated concentrations of AgNPs and Ag ions (or their solvent controls), for 28 days (Fig. 3a). At Days 4, 12, and 28, EB samples were collected and the expression levels of endoderm (Hnf4, Gata6), mesoderm (Flk1, Hand1), and neural ectoderm (Nestin, Sox3, Pax6) markers were analyzed. As shown

Figure 2. Silver nanoparticles (AgNPs) and Ag ions did not affect mouse embryonic stem cell (mESC) viability at or below 1 μg/mL. (a) Viability plot for cells incubated with 0- to 1-μg/mL AgNPs and Ag ions for 24, 48, and 96 h. (b) Reactive oxygen species (ROS) levels in mESCs treated with AgNPs or Ag ions for 2, 4, and 12 h. Intracellular calcium ion levels for AgNP-treated (c) or Ag ion-treated (d) mESCs. Data in panels (a–d) are averages of three independent experiments.

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in Figure S1a, the expression of these markers increased, as expected during normal development (Loebel et al., 2003; Zhang et al., 2018), along the differentiation process; 0.1 μg/mL AgNP treatment stimulated the expression of all the markers analysed at Day 12 (around twofold changes), but that was not evident for the lower concentration. No significant changes were observed at the other time points (Figs. 3b,d,f and S2a,c,e). Conversely, Ag ion treatments seemed to down-regulate the expression of the mesoderm markers at Day 12, and Gata6 and neural ectoderm markers at Day 28 (around twofold to threefold). No significant differences were observed elsewhere, except perhaps for the up-regulation of Gata6, Hand1, and Flk1 at Day 4, and the neural ectoderm markers at Day 12 (Figs. 3c,e,g and S2b,d,f). These results suggest that AgNPs and Ag ions slightly affected the early specification of the primary germ layers in different manners. Moreover, our findings are in agreement with previous reports describing AgNP neurotoxicity (Haase et al., 2012; Tang et al., 2008; Xu et al., 2013; Yin et al., 2013; Yin et al., 2015b; Zieminska et al., 2014) and imply that the effects of AgNPs on the neural ectoderm differentiation may be serious.

**Silver nanoparticles and silver ions affected mouse embryonic stem cell neural differentiation**

To further assess the potential developmental neurotoxicity of AgNPs and Ag ions, we used two different in vitro mESC neural-specific differentiation models. The first one involved the addition of retinoic acid
(RA) during EB differentiation, to promote neural commitment (Jones-Villeneuve et al., 1982). At the differentiation Day 8, after 4-day RA treatment, EBs were transferred into poly-L-lysine/laminin-coated plates or collected for RNA extraction. Cells were cultured for another day in N2 medium before final collection. Because, during the differentiation process, 1-μg/mL AgNPs and Ag ions caused cell death (data not shown), we only considered the other two concentrations to determine developmental toxicity. Figure 4 shows, by quantitative reverse transcription polymerase chain reaction (qRT-PCR), that six different neural ectoderm-specific markers, Sox1, Sox3, Map2, NeuroD, Nestin, and Pax6, were generally up-regulated by AgNPs as compared with controls, in a dose-dependent manner, at both Days 8 (aggregates in suspension) and 9 (neural progenitors in monolayer conditions). Conversely, Ag ions did not alter the expression of the markers analysed at Day 8 (except for a slight down-regulation of Sox1, Nestin, and Map2 in 0.01-μg/mL treated groups. However, similar to AgNP treatments, Ag ions increased the expression of Sox1, Sox3, and Map2 at Day 9 (Fig. 4b). Our results suggest that AgNPs might influence neural differentiation at earlier stages and more seriously, as compared with Ag ions.

In the other neural-specific differentiation procedure, in monolayer conditions, N2B27 medium was used as a neural induction medium. Analogously to the RA-directed process, at differentiation Day 8, the cells were replated into poly-L-lysine/laminin-coated dishes

Figure 4. Silver nanoparticles (AgNPs) or Ag ion treatment affected neural ectoderm specification during embryoid body-based neural progenitor cell (NPC) differentiation. Shown are the expression of neural ectoderm/NPC markers at differentiation Days 8 (a) and 9 (b) for AgNP and Ag ion exposures.
and cultured for four additional days (Ying et al., 2003). At Day 12, the NPC morphology was determined by immunofluorescence for MAP2, a microtubule-associated protein important for neuritogenesis (Shafit-Zagardo & Kalcheva, 1998). Clearly, more morphologically normal NPCs were observed in 0.01- and 0.1-μg/mL AgNP-treated groups, as compared with control (Fig. 5a). Conversely, 0.1-μg/mL Ag ion-treated samples generated very few MAP2-positive cells (Fig. 5b), whereas in the 0.01-μg/mL Ag ion-treated group, although NPCs could form, their synapses generally showed an abnormally curly shape (pointed with red arrows in Fig. 5b). Western blot analyses for MAP2 protein levels (Fig. S3a,b) confirmed the immunostaining results of Figure 5 and were consistent with the MAP2 mRNA levels (Fig. 4). Once again, 1-μg/mL AgNPs/Ag ions caused massive cell death under those conditions. These data prove that both AgNPs and Ag ions may generate developmental neural toxic effects, but the molecular mechanisms behind those effects may be different.

Silver nanoparticles and silver ions may exert neurotoxicity by regulating the Notch signalling pathway

Previous studies have attributed AgNP toxicity to their ability to generate oxidative stress and subsequently programmed cell death (Arora et al., 2008). However, our results, at least for AgNP/Ag ion concentrations up to 0.1 μg/mL, did not show any obvious change in ROS levels or cell death. To investigate the possible cause of AgNP developmental neural toxicity, we focused on the Notch pathway. This signalling cascade plays an important role in neural development (Kageyama & Ohtsuka, 1999; Kageyama et al., 2005; Ohtsuka et al., 1999; Sestan et al., 1999; Wang et al., 1998), and its alteration may lead to developmental disorders. qRT-PCR analyses of Day 8 samples of the EB-based RA-induced neural differentiation demonstrated that the expression of the Notch1 receptor, its ligand Dll (Kopan & Ilagan, 2009), and its target gene Hes1 (Kageyama & Ohtsuka, 1999) were upregulated after 0.1-μg/mL AgNP treatment and slightly down-regulated (except for Hes1) after 0.01-μg/mL AgNP treatment, while Ag ions only partially repressed those markers in 0.01-μg/mL treated samples (Fig. 6a). At Day 9, Hes1 was dose-dependently down-regulated after AgNP treatment, implying an inhibition of the Notch pathway and a consequent enhancement of neuronal specification. Conversely, the expression of all the three genes increased in the Ag ion group (Fig. 6b), corroborating their strong inhibition on NPC generation observed in Figure 5. In fact, it was demonstrated that the Notch signalling pathway had a remarkable influence on neuron morphology (Redmond et al., 2000).

Discussion

Silver nanoparticles are the most commercialized nanomaterials (Ahamed et al., 2010). However, the
mechanisms of their potential impact on neural development remain elusive. Also, there is controversy whether the toxicity of AgNPs is due to the nanoparticles themselves or the silver ions released from them (Chen et al., 2018).

In previous studies, the developmental toxicity of AgNPs was mostly assessed with live animal tests. Nowadays, in vitro toxicology, which is based on tissue culture techniques, has been slowly gaining ground as an alternative to animal assays. Although many different kinds of cells have been employed in toxicology, stem cells are the most promising tools, in particular for developmental toxicity investigations (Faiola et al., 2015; Genschow et al., 2002; Liu et al., 2017; Yao et al., 2016; Yin et al., 2018c; Yin et al., 2018b; Yin et al., 2018a).

We used mESCs to mimic the early developmental stages and study the toxicity of AgNPs and Ag ions, at not lethal doses, during those processes. In fact, we employed concentrations not higher than 0.1 μg/mL, which are far from the IC_{50} values for AgNPs and Ag ions in self-renewing and differentiating mESCs. Those doses, which reflect the actual exposure values in humans, affected the developmental process, in our in vitro conditions, implying serious toxicity for AgNPs and Ag ions. To our knowledge, there were only a few previous studies focusing on the potential health risks of low-dose AgNPs. For instance, a human embryonic stem cell-derived neural stem/progenitor cell model was used to study the difference in gene and miRNA expressions after exposure to AgNPs (Oh et al., 2016). In that study, the authors proposed several potential mechanisms for AgNPs’ toxicity. However, the high (10–200 μg/mL) doses and short exposure time (24 h) employed may not fully reflect the real health risk for AgNPs during development. Another neurotoxicity study utilized neural stem cells treated with 1-, 5-, 10-, and 20-μg/mL AgNPs for 24 h. There was no obvious ROS accumulation and cell death under 1-μg/mL AgNP exposure (Liu et al., 2015a). An additional neurotoxicity study employed low concentration of AgNPs in a mouse model (Dabrowska-Bouta et al., 2016). They showed that 0.2-mg/kg body weight silver ions induced behavioural issues while AgNPs did not. Nevertheless, both AgNPs and Ag ions prompted myelin sheath morphological alterations.

Our results indicate that AgNPs and Ag ions affected global mESC differentiation. They both altered the expression of markers of all the three primary germ layers (endoderm, mesoderm, and ectoderm), implying significant and potentially negative effects on neural development, at least at early developmental stages.
Moreover, these alterations were somehow different between AgNP and Ag ion treatments, suggesting that both the nanoparticles and Ag ions possess specific ways of exerting their toxicities. Those may be due to charge/particle effects and how the two different forms of silver are differentially up-taken by the cells. AgNP and Ag ion dysregulation on ectoderm appeared as early as Day 4 of the differentiation, as compared with the other two germ layers. This urged us to further investigate the effects of these substances on the development of ectoderm and its lineages, in particular, neural lineages.

We employed two different mESC neural differentiation protocols, via EB formation or monolayer conditions. At selected time points, we investigated the effects of AgNPs and Ag ions on the expression of the following markers: Sox1 and Sox3, neural ectoderm marker genes, which play key roles in the development of neural tissues (Sarkar & Hochedlinger, 2013); Nestin, which is specifically expressed in neural epithelial stem cells and neural stem cells (Neradil & Veselska, 2015); Pax6, highly homologous in humans and rats, which regulates the directionality differentiation of neurons and has an important role in the central nervous system division (Engelkamp et al., 1999); Map2, a neural dendritic marker gene; and NeuroD, the master regulator of brain development (Pataskar et al., 2016). We demonstrated that AgNPs and Ag ions, at concentrations as low as 0.01 μg/mL, affected the expression of all the previous markers, implying a strong developmental toxicity. Our findings are in agreement with previous reports. In fact, Xin et al. (2015) found that the neural development in zebrafish was affected by AgNPs. Studies on the neurotoxicity mechanisms of AgNPs have showed that the generation of ROS (Arora et al., 2008) and autophagy (Mao et al., 2016) may be the two major offenses induced by AgNPs. Also, it has been reported that AgNPs have the ability to induce cell death, such as apoptosis and necrosis (Volker et al., 2013). Our study, however, demonstrates that low concentrations of AgNPs as well as Ag ions, do not induce ROS generation and/or cell death. Our findings suggest that the neurotoxicity of AgNPs, manifested by the abnormal enhancement of neural lineage-specific markers, may be mediated by the blockade of the Notch pathway. In fact, it has been showed that the activation of Notch stimulates NPC self-renewal but inhibits the neuronal differentiation process (Louvi & Artavanis-Tsakonas, 2006).

Silver nanoparticles (20 μg/mL) were acquired from Sigma-Aldrich, USA. Sodium citrate was used as a stabilizer according to the manufacturer’s instructions, and stock solutions were stored at 4°C. The morphology of AgNPs impair neural progenitor cell specification

Interestingly, we found that AgNPs displayed a somehow different toxicity as compared with Ag ions. This was more evident when we analysed the effects of AgNPs and Ag ions on the morphology of NPCs (Fig. 5), inferring axon guidance may be a likely phenomenon affected more specifically by the Ag ions, but it remains to be verified. The differences mentioned previously are consistent with several previous studies (Charehsaz et al., 2016; Strickland et al., 2016; Sun et al., 2016). Nevertheless, others have reported similar toxic effects for AgNPs and Ag ions (Hadrup & Lam, 2014; Hadrup et al., 2012) likely due to leakage of Ag ions from the nanoparticles. Those incongruences may be because of the different systems and/or different concentrations of AgNPs/Ag ions used.

Conclusion

Stem cell-based toxicity evaluations allow for quick assessments of the developmental toxicity of environmental pollutants in a controllable and dynamic way. With our stem cell toxicology system, we demonstrated that AgNPs and Ag ions, at environmental and human relevant doses, affected the induction of neural ectoderm and the morphology of NPCs. This potential developmental neurotoxicity was in part mediated by the dysregulation of the Notch signalling cascade. In addition, we provided evidence that the toxicity of AgNPs may not just be due to the leakage of Ag ions.

Experimental Section

Cell culture

J1 mESCs were purchased from the Cell Bank/Stem Cell Core Facility, SIBCB, CAS, and cultured in 0.1% gelatin (Sigma-Aldrich, USA) coated plates, at 37°C and 5% CO₂. The culture medium consisted in high glucose KnockOut DMEM medium (Gibco, USA), supplemented with 15% fetal bovine serum (Corning, USA), 1% nucleosides (Merck Millipore, German), 2-mM GlutaMAX™ (Gibco, USA), 0.1-mM non-essential amino acids (Gibco, USA), 10⁻⁴ M β-mercaptoethanol (Solarbio, China), 10⁻³ U/mL leukaemia inhibitory factor (Merck Millipore, Germany), and 2% Pen/Strep antibiotics (Gibco, USA).

Silver nanoparticle characterization

Silver nanoparticles (20 μg/mL) were acquired from Sigma-Aldrich, USA. Sodium citrate was used as a stabilizer according to the manufacturer’s instructions, and stock solutions were stored at 4°C. The morphology of
AgNPs was determined by TEM (Hitachi H-7500, Japan). AgNP size distribution and zeta-potential were measured with a Malvern Zetasizer Nano ZS (Malvern, UK) at 25°C.

**Cytotoxicity assay**

Mouse embryonic stem cells were seeded in gelatin-coated 96-well plates and incubated with 0.001- to 1-μg/mL AgNPs (or sodium citrate solvent control) and AgNO₃ (Sigma, USA) (or H₂O solvent control). After 24-, 48-, and 96-h exposures, cell viability was determined by the Alamar Blue assay (Sigma-Aldrich, USA) (Strickland et al., 2016). Fluorescence was measured using a Varioskan LUX (Thermo, USA) at the excitation wavelength of 530 nm and emission wavelength of 590 nm.

**Intracellular reactive oxygen species level determination**

Intracellular ROS was measured by using the fluorescent probe DCFH-DA. Briefly, mESCs were treated for 2, 4, and 12 h with AgNPs/Ag ions (0.001-1 μg/mL), or related solvent controls first, and then loaded with the DCFH-DA probe (100 μM, Sigma-Aldrich, USA) for 30 min at 37°C and 5% CO₂. H₂O₂ (1 mM) was used as a positive control. Fluorescence was measured with a Varioskan LUX (Thermo, USA) at the excitation and emission wavelengths of 485 and 530 nm, respectively.

**Intracellular calcium ion level measurement**

Mouse embryonic stem cells were seeded in 24-well plates and loaded with Fura-2/AM (5 μM, Sigma-Aldrich, USA) for 30 min in DPBS at 37°C with 5% CO₂. Then cells were treated with AgNPs and Ag ions (or their respective solvent controls). Changes in intracellular Ca²⁺ concentration, measured by the 340 nm over 380-nm excitation fluorescence intensity ratio (F340/F380) detected with an emission wavelength of 510 nm, were obtained every 50 s, for 2 h in the dark.

**Embryoid body-based global differentiation assay**

Embryoid body differentiation assay was performed as previously described (Yin et al., 2015a). Trypsinized mESCs were seeded into 90-mm non-coated petri dishes in a 10-mL EB differentiation medium (mESCs medium without leukaemia inhibitory factor), at a cell density of 4 × 10⁶ cells per dish. Cells were incubated with 0.01- to 0.1-μg/mL AgNPs and Ag ions (or their solvent controls) from Day 0. Culture media were replaced every other day. EB samples were collected at Day 0 (undifferentiated mESCs), Day 4, Day 12, and Day 28, for RNA isolation and qRT-PCR analyses.

**Embryoid body-based neural differentiation assay**

Cell were seeded in 90-mm petri dishes in EB differentiation medium, as described previously. Media were replaced every other day. At Day 4 of EB formation, RA (Sigma, USA, at a working concentration of 5 μM) was added to the medium to stimulate neural ectoderm specification. At Day 8, EBs were dissociated into single cells with 0.05% trypsin (Gibco, USA) and seeded into plates coated with poly-L-lysine/laminin (Roche, Switzerland) in N2 medium (DMEM/F12 (Gibco, USA), 1% Glutamax, 1% N2 supplement (Gibco, USA), and 2% Pen/Strep antibiotics). Samples were collected at Days 0, 8, and 9 for total RNA extraction and qRT-PCR analyses.

**Monolayer neural differentiation assay**

Monolayer neural differentiation was performed as previously described (Liu et al., 2015b; Ying et al., 2003). The differentiation medium consisted in 50% DMEM/F12 and 50% Neurobasal medium (Gibco, USA) supplemented with N2 and B27 supplements (Gibco, USA), 1-mM GlutaMAX™, 0.1-mM β-mercaptoethanol, 0.1% bovine serum albumin fraction V (Sigma-Aldrich, USA), 10⁻⁴-U/mL penicillin, and 10⁻⁴-μg/mL streptomycin. Cells were seeded at a density of 1 × 10⁵ cells per well into gelatinized six-well plates, and culture media changed every other day. At Day 8 of differentiation, cells were trypsinized and replated as single cells in poly-L-lysine/laminin-coated plates and cultured in differentiation medium as previously, until Day 12.

**RNA extraction and quantitative reverse transcription polymerase chain reaction analyses**

Total RNA was extracted with Trizol (Life Technologies, USA), following the instructions of the manufacturer. RNAs were converted to cDNAs with the PrimeScript RT Master Mix Kit (Takara, Japan) according to the manufacturer’s recommended procedures. Quantitative PCR was performed with the SYBR Premix Ex Taq kit (Takara, Japan). Cycling conditions were performed 40 times, except for the initial denaturation and final cycle, as follows: initial denaturation, 95°C, 30 s; denaturation, 95°C, 5 s; annealing, 60°C, 30 s; and final cycle, 95°C, 5 s, 60°C 1 min, 95°C 30 s. Relative gene
expression, normalized to the Gapdh gene, was calculated with the comparative Ct method. Measures were obtained in triplicates. Results are shown as mean ± standard deviation. The primers used are listed in Table S1.

Immunofluorescence staining

Cells from Day 10 of the monolayer differentiation were fixed in 4% formaldehyde (Sigma, USA) for 15 min, washed with PBS (Gibco, USA), blocked with goat serum (Solarbio, China) and 0.3% Triton X-100 (Amresco, USA) for 1 h at room temperature, and then incubated with the primary anti-MAP2 (Cell Signaling Technology, USA) antibody, overnight at 4°C. The next day, after washing with DPBS (Gibco, USA) three times, samples were incubated with AlexaFluor488-tagged anti-rabbit IgG secondary antibody (Cell Signaling Technology, USA) antibody, overnight at 4°C. The next day, after washing with PBS (Gibco, USA), samples were incubated with AlexaFluor488-tagged anti-rabbit IgG secondary antibody (Cell Signaling Technology, USA) for 2 h at room temperature and in the dark. Images were obtained with an inverted microscope (Olympus IX73, Japan).

Western blot assay

Day 12 monolayer differentiation samples were lysed with RIPA buffer (Solarbio, China). Then, equal amounts of proteins were separated by sodiumdodecyl sulphate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (BIO-RAD, USA). Membranes were incubated with the primary antibodies anti-MAP2 (Cell Signaling Technology, USA) and anti-GAPDH (Santa Cruz Biotechnology, USA) overnight first and then with a horseradish peroxidase-linked goat anti-rabbit secondary antibody (Cell Signaling Technology, USA) for 1-2 h. After extensive washes, the membranes were treated with the Pierce ECL Western Blotting Substrate (Thermo, USA) and scanned for quantification with a ChemiDoc Touch imaging system (BIO-RAD, USA).

Acknowledgments

This research was funded by grants from the Chinese Academy of Sciences Strategic Priority Research Program (XDB14040301), the National Natural Science Foundation of China (21577166 and 21461142001), the Chinese Academy of Sciences Hundred Talent Program (29[2015]30), and the Key Research Program of Frontier Sciences, CAS (QYZDJSSW-DQC017). We would also like to thank the K. C. Wong Education Foundation. Nuoya Yin and Bowen Hu contributed equally to this work.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Gene expression profiling during global differentiation for endoderm (Hnf4 and Gata6), mesoderm (Flk1 and Hand1) and ectoderm (Nestin, Sox3 and Pax6) marker genes.

Figure S2 AgNP and Ag ion treatments affected the specification of the three primary germ layers during global differentiation. Shown are the expressions of endoderm marker genes for AgNP (a) and Ag ion (b) exposures, mesoderm marker genes for AgNP (c) and Ag ion (d) exposures, and ectoderm marker genes for AgNP (e) and Ag ion (f) exposures. Data are averages of three independent experiments.

Figure S3 MAP2 protein levels of monolayer neural differentiation day 12 samples treated with the indicated concentrations of AgNPs and Ag ions. Shown are the Western blot results (a) quantified by ImageJ (b). GAPDH serves as loading control.

Table S1 Primers used in qRT-PCR analyses