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Effects of silica nanoparticles on endolysosome function in primary cultured neurons

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Abstract

Silica nanoparticles (SiNPs) have been used as vehicles for drug delivery, molecular detection, and cellular manipulations in nanoneuromedicine. SiNPs may cause adverse effects in brain including neurotoxicity, neuroinflammation, neurodegeneration and enhancing levels of amyloid beta protein (A β); all pathological hallmarks of Alzheimer's disease. Therefore, the extent to which SiNPs influence A β generation and the underlying mechanisms by which this occurs deserves investigation. Our studies were focused on the effects of SiNPs on endolysosomes which uptake, traffic, and mediate the actions of SiNPs. These organelles are also where amyloidogenesis largely originates. We found that SiNPs, in primary cultured hippocampal neurons, accumulated in endolysosomes and caused a rapid and persistent deacidification of endolysosomes. SiNPs significantly reduced endolysosome calcium stores as indicated by a significant reduction in the ability of the lysosomotropic agent GPN to release calcium from endolysosomes. SiNPs increased A β _{1–40} secretion whereas two agents that acidified endolysosomes, ML-SA1 and CGS21680, blocked SiNPs-induced deacidification and increased generation of A β _{1–40}. Our findings suggest that SiNP-induced deacidification of and calcium release from endolysosomes might be mechanistically-linked to increased amyloidogenesis. The use of SiNPs might not be the best nanomaterial for therapeutic strategies against AD and other neurological disorders linked to endolysosome dysfunction.

Keywords

Alzheimer's disease; amyloid beta protein; endolysosomes; nanoparticles; ML-SA1; silica nanoparticles; SiNP

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Conflict of interest
None declared

Introduction

The discovery of silica nanoparticles (SiNPs) has led, in part, to the growth of nanotechnology in various fields including electronics, aerospace engineering, health care, and biomedicine (Singh *et al.* 2009, Bitar *et al.* 2012). In biomedical applications, appealing physicochemical features of SiNPs (Bae *et al.* 2012, Li *et al.* 2012, Slowing *et al.* 2008, Trewyn *et al.* 2007) helped promote the promiscuous use of these inorganic nanoparticles as carriers for a diverse series of molecules and pharmaceuticals (Biju 2014). Indeed, SiNPs have been used successfully to enhance the actions of pharmaceuticals (Kwon *et al.* 2013, Bitar *et al.* 2012), enzymes (Coll *et al.* 2011), biosensors and bio-imaging agents (Korzeniowska *et al.* 2013), and theranostics (Vivero-Escoto *et al.* 2012). Because SiNPs are able to freely pass the blood-brain barrier and enter into the CNS (Klejbor *et al.* 2007), their clinical application has extended to the diagnosis of and possible therapeutic intervention against such neurological diseases as spinal cord and traumatic brain injury, and such neurodegenerative disorders as Alzheimer's and Parkinson's disease (Cho *et al.* 2010, Sharma *et al.* 2009).

Relatively little is known about the biocompatibility, clearance, biodegradation and safety profiles of nanotechnology in the CNS. Nanoparticles including SiNPs have possible adverse effects including the promotion of neuroinflammation and neurotoxicity (Migliore *et al.* 2015). Relevant to Parkinson's disease, SiNPs damaged dopaminergic neurons in rodent striatum (Wu *et al.* 2011) and induced alpha-synuclein formation and aggregation in cultured PC12 cells (Xie & Wu 2016). With relevance to Alzheimer's disease (AD), SiNPs increased the levels of amyloid beta protein (A β) in (Yang *et al.* 2014). Therefore, greater attention towards understanding underlying mechanisms by which SiNPs might lead to neurological complications appears warranted. Endolysosomes are acidic organelles that play important physiological roles in brain including energy homeostasis, plasma membrane repair, immune regulation and cellular signaling (Xu & Ren 2015). Pathophysiologically, endolysosomes have been implicated in the pathogenesis of lysosomal storage diseases, metabolic disorders and various neurodegenerative disorders including AD (Nixon 2007, Nixon *et al.* 2008, Nixon & Cataldo 1995, Nixon & Cataldo 2006). Evidence suggests that endolysosome de-acidification can increase amyloidogenesis within and the release of calcium from endolysosomes and may contribute to the pathogenesis of AD and other neurodegenerative disorders (Hui *et al.* 2012a, Chen *et al.* 2013, Chen *et al.* 2010). Clearly, nanoparticles can accumulate in endolysosomes and in doing so can affect significantly many important functions attributed to these organelles (Cupaioli *et al.* 2014). Very little is known about negative effects of SiNPs in brain, but it has been reported recently that SiNPs can alter autophagy and impair autophagic-lysosome functions in non-neuronal cells (Schutz *et al.* 2016). Accordingly, we determined here the extent to which SiNPs could affect endolysosomes in neurons including their ability to accumulate in endolysosomes, and their effects on neuronal viability, endolysosome pH, calcium stores and amyloidogenesis.

Materials and Methods

Synthesis of pure SiNPs and Rubpy-doped SiNPs

Pure and Rubpy-doped SiNPs were synthesized using a modified reverse-microemulsion method (Bagwe *et al.* 2004). An aliquot of 7.5 ml of cyclohexane, 1.8 ml of 1-hexanol, and 1.77 ml of Triton X-100 were mixed and stirred for 20 min to form a homogenous solution. Afterwards, an aliquot of 340 μ l H₂O or Rubpy solution was added for making pure SiNPs or Rubpy-doped SiNPs, respectively. The mixture was stirred for 20 min and then 100 μ l of tetraethylorthosilicate (TEOS, 98%) was added. 60 μ l of 29% NH₄OH was added to initiate the polymerization of TEOS and the solution was stirred for 24 hours to form SiNPs. The microemulsion was stopped by adding 10 ml of acetone and SiNPs were collected by centrifugation at 8,000 rpm for 20 min and washed three-times with ethanol and three-times with water. SiNPs underwent ultra-sonication (10 min) for re-dispersion prior to their application to primary cultured neurons.

Primary neuronal cultures

As previously described, primary cultures of hippocampal neurons were prepared from Sprague-Dawley rats (Charles River Laboratories) (Buscemi *et al.* 2007, Hui *et al.* 2012b). Pregnant dams (embryonic day 18) were sacrificed by asphyxiation with CO₂. The fetuses were removed, decapitated, and meninges-free hippocampi were isolated, trypsinized, and plated onto 35-mm poly-D-lysine-coated glass bottom tissue culture dishes (MatTek Corp, Ashland, US). Neurons grown in Neurobasal™ medium plus L-glutamine, antibiotic/antimycotic and B₂₇ supplement (ThermoFisher, Grand Island, US) were maintained at 37°C and 5% CO₂ for 10–14 days at which time they were taken for experimentation. Typically, the purity of the neuronal cultures was greater than 95% as determined by immunostaining with neuron markers mouse anti-NeuN and goat anti-MAP2 antibodies (Millipore, Bilerica, US), as well as with the astrocyte marker mouse anti-GFAP antibody (Sigma, St. Louis, US).

MTT assay

We determined cell viability with the colorimetric MTT metabolic activity assay. Primary neurons (1×10^4 cells/well) were cultured in 96-well plates at 37°C, and exposed to varying concentrations of SiNPs for 24 h. Cells treated with DMSO served as vehicle controls. After treatment, 10 μ l of MTT solution (5 mg/ml in PBS) was added to each well. After incubation for 4 h, the resultant formazan crystals were dissolved in DMSO (100 μ l) and the absorbance intensity was measured using a microplate reader (Molecular Devices, San Jose, US) set at 490 nm with a reference wavelength of 620 nm. Data were expressed as a percentage of viable cells relative to the total number of cells in each well.

Endolysosome accumulation of SiNPs

Neurons were incubated with RuBpy-doped SiNPs for 24 hours at 37°C and then further incubated for 15 min with LysoTracker. After washing with PBS, neurons were examined by confocal microscopy (Zeiss LSM800, Germany).

Endolysosome pH measurements

As previously described (Liu *et al.* 2008, Hui et al. 2012a), endolysosome pH was measured using a ratio-metric endolysosome pH indicator dye (LysoSensor Yellow/Blue DND-160, ThermoFisher Scientific, Eugene, US); a dual excitation dye that permits pH measurements in acidic organelles independently of dye concentration. Neurons were loaded with 2 μ M LysoSensor for 5 minutes at 37°C. Light emitted at 520 nm in response to excitation at 340 nm and 380 nm was measured for 20 msec every 30 seconds using a filter-based imaging system (Zeiss, Germany). The ratios of light excited (340/380 nm) versus light emitted (520 nm) were converted to pH using a calibration curve established using 10 μ M of the H⁺/Na⁺ ionophore monensin, and 20 μ M of the H⁺/K⁺ ionophore nigericin; both were dissolved in 20 mM 2-(N-morpholino) ethane sulfonic acid (MES), 110 mM KCl, and 20 mM NaCl adjusted to pH 3.0 to 7.0 with HCl/NaOH.

Endolysosome calcium store measurements

Levels of free intracellular Ca²⁺ were determined using the Ca²⁺-specific fluorescent probe Fura-2/AM (Invitrogen, Eugene, US). Neurons were incubated with 2 μ M Fura-2/AM for 30 min at 37°C, washed with calcium-free buffer (145 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 10 mM glucose, 0.2 mM EGTA and 10 mM HEPES, pH=7.4) to remove extracellular Fura-2/AM, and incubated at 37°C for another 10 min to allow for intraneuronal de-esterification of Fura-2/AM to Fura-2. Neurons were excited at 340 and 380 nm, and light emitted at 510 nm was measured using our filter-based calcium imaging system (Zeiss, Germany). Images were acquired every 2 seconds and at this acquisition rate we were able to measure baseline as well as peak increases in levels of free intracellular calcium. The ratio of 340/380 was used as a measurement of intracellular calcium levels. Endolysosome calcium levels were measured indirectly using the lysosomotropic agent glycyl-L-phenylalanine 2-naphthylamide (GPN) by measuring free intraneuronal calcium levels in the absence or presence of GPN (Penny et al. 2014; Lee et al. 2015). GPN causes brief permeabilization of the endolysosome membrane (Jadot et al. 1984), de-acidifies endolysosomes, and releases calcium from endolysosomes. SiNP-induced reduction in the ability of GPN to release calcium from endolysosomes was interpreted as indicating the ability of SiNPs to stimulate the release of calcium from endolysosomes and thereby decrease readily releasable calcium from the endolysosome pool.

Quantification of A β levels by ELISA

A β levels were quantified using human/rat A β _{1–40} and A β _{1–42} ELISA kits as per the manufacturer's protocol (Wako, Osaka, Japan). Media from cultured neurons were collected and diluted (1:2 for A β _{1–42}, 1:5 for A β _{1–40}) with standard diluent buffer. A β levels were measured in duplicate using a calorimetric sandwich ELISA method (Wako, Osaka, Japan). A β levels were normalized to total protein content in each sample as determined by a DC protein assay (Bio-Rad, US). For total protein determinations, neurons in each dish were lysed with RIPA buffer (Pierce, Rockford, US) containing 10 mM NaF, 1 mM Na₃VO₄ and Protease Inhibitor Cocktail (Sigma-Aldrich, USA). After centrifugation (14,000 X g for 10 min at 4°C), supernatants were collected for DC protein assays.

Reagents

Tetraethylorthosilicate (98%), cyclohexane, 1-hexanol, acetone, Triton X-100, MTT reagent, CGS21680, and GPN were purchased from Sigma-Aldrich (US). Ammonium hydroxide (28.0–30.0%), ethanol (99%), LysoTracker DND99, LysoSensor DND160, calcium fluorescent dye Fura-2/AM and DMSO were obtained from Fisher Scientific (USA). Sodium citrate, 1-pentanol, and poly (vinylpyrrolidone) (average molecular weight = 40,000) were purchased from Alfa Aesar. Tris(bipyridine)-ruthenium(II) dichloride (Rubpy) was purchased from ICN Biomedicals. Human/Rat ELISA kits were obtained from Wako (Japan). ML-SA1 was obtained from R&D (US).

Statistics

All data were expressed as means and SEM. Statistical significance was determined by one-way ANOVA plus a Tukey post hoc test, or by Student's-t test. $p < 0.05$ was considered to be statistically significant.

Results

Characterization of nanoparticles

The morphology of synthesized nanoparticles was characterized using a scanning electron microscope (Figures 1A and 1B). SiNPs were spherical in shape with diameters of 68 ± 4 nm (Figure 1A). Rubpy-doped SiNPs were spherical with diameters of 71 ± 5 nm (Figure 1B). To further confirm the surface properties of the SiNPs, zeta potentials were measured (Figures 1C and 1D). SiNPs displayed high negative zeta potentials at -38.33 ± 0.55 mV. After doping with Rubpy dye, the zeta potentials of Rubpy-SiNPs were less negatively charged (-7.46 ± 0.25 mV) compared to control SiNPs. In addition, an elemental analysis of SiNPs was conducted using energy-dispersive X-ray spectroscopy (EDS) (Figures 1E and 1F). The results of EDS clearly showed the presence of Si and O, both of which were from the SiNPs. In addition, peaks of C and Cu were detected.

Effects of SiNPs on neuronal viability

We first determined the extent to which SiNPs affected the viability of primary cultured neurons. Neurons incubated for 24 hours with concentrations of SiNPs ranging from 0.01 to 1 $\mu\text{g/ml}$ were tested for viability using the MTT assay. SiNPs were found to decrease neuronal viability significantly ($p < 0.05$) by about 40% at the highest concentrations tested (1 $\mu\text{g/ml}$) (Figure 2). Accordingly, for all subsequent studies of endolysosome function we used a non-toxic concentration (0.1 $\mu\text{g/ml}$) of SiNPs.

Endolysosome accumulation of SiNPs in primary cultured neurons

Because SiNPs were found by others to be endocytosed (Lesniak *et al.* 2012) and to accumulate in endolysosomes (Shi *et al.* 2010), we next determined the extent to which SiNPs were endocytosed into and accumulated by neuronal endolysosomes. Primary cultured neurons incubated for 24 hours with SiNP-tagged RuBpy and for 15 min with LysoTracker showed (Figure 3A) colocalization of SiNP-tagged RuBpy with LysoTracker. These results indicated that SiNPs were endocytosed by and accumulated in neuronal

endolysosomes. However, no attempt was made to quantify the amount of SiNP accumulation in the endolysosomes.

Effects of SiNPs on endolysosome pH in primary cultured neurons

Endolysosome pH is an established indicator of endolysosome function because the acidic environment critically controls a variety of cellular functions and homeostatic mechanisms (Appelqvist *et al.* 2013). After confirming that SiNPs trafficked to the endolysosome system, we determined the extent to which SiNPs affected endolysosome pH acutely (within 5 min) and persistently (24 h post treatment). Using an approach similar to that used by us to show that endolysosome de-acidification contributed to pathological features shared by several neurodegenerative disorders (Chen *et al.* 2013, Hui *et al.* 2012a), we found here that non-toxic concentrations of SiNPs (0.1 $\mu\text{g/ml}$) significantly ($p < 0.001$) de-acidified endolysosomes within 5 min of application (Figure 3B). 24 hours after application, SiNPs at 0.1 $\mu\text{g/ml}$ significantly ($p < 0.001$) de-acidified endolysosomes (Figure 3C). Thus, SiNPs at non-toxic concentrations could significantly de-acidify endolysosome acutely and persistently.

Effects of SiNPs on endolysosome calcium stores in primary cultured neurons

Others and we have shown that neuronal endolysosomes have readily releasable stores of calcium equivalent to those found in endoplasmic reticulum; levels sufficient to affect calcium homeostasis and neuronal function (Brailoiu *et al.* 2005, Dickinson *et al.* 2010, Pandey *et al.* 2009, Hui *et al.* 2015). Here, we determined the extent to which SiNPs affected endolysosome calcium stores using a lysosomotropic agent GPN that releases calcium from endolysosomes. Under nominal calcium-free conditions, GPN significantly ($p < 0.001$) increased levels of intracellular calcium shortly after its application to neurons (Figure 4A) indicating that GPN induced calcium release from endolysosomes. Next, we treated neurons for 24 hours with non-toxic concentrations of SiNPs (0.01 and 0.1 $\mu\text{g/ml}$) and measured effects on GPN-induced calcium release from endolysosomes. Compared with controls, SiNPs at 0.1 $\mu\text{g/ml}$ significantly ($p < 0.001$) decreased GPN-induced calcium release from endolysosomes (Figure 4B). This suggested that SiNPs at non-toxic concentrations released calcium from endolysosomes thus decreasing the pool of GPN-releasable calcium.

Effects of SiNPs on extra-neuronal levels of A β in primary cultured neurons

Endolysosomes are major sites where amyloidogenic processing of A β PP occurs following A β PP internalization, and we have shown that de-acidifying endolysosomes increases the formation of A β (Hui *et al.* 2012a). Because SiNPs were shown (see above) to de-acidify endolysosomes, we next determined the extent to which SiNPs, at non-toxic concentrations, affected extra-neuronal A β levels in cultured neurons. 24 hours after the application of SiNPs, we collected the media and determined levels of A β_{1-40} and A β_{1-42} . SiNPs at 0.1 $\mu\text{g/ml}$ modestly but statistically significantly ($p < 0.05$) increased levels of extra-neuronal A β_{1-40} compared to controls (Figure 5A). In contrast, SiNPs failed to significantly affect levels of extra-neuronal A β_{1-42} at concentrations of either 0.01 or 0.1 $\mu\text{g/ml}$ (Figure 5B).

Effects of endolysosome acidifying agents on SiNP-induced endolysosome de-acidification and increases in A β ₁₋₄₀ release in primary cultured neurons

Using ML-SA1, a TRPML channel agonist, and CGS21680, an agonist of adenosine A_{2a} receptors, that have been shown to acidify endolysosomes (Bae *et al.* 2014, Liu *et al.* 2008), we determined next the extent to which these endolysosome acidifying agents could reverse SiNP-induced endolysosome de-acidification and increases in A β ₁₋₄₀ levels. In confirmation of results noted above, SiNPs at 0.1 μ g/ml increased significantly endolysosome pH ($p < 0.001$) (Figure 6A) and levels of A β ₁₋₄₀ ($p < 0.05$) (Figure 6B). After 24 h treatment, ML-SA1 (20 μ M) and CGS21680 (100 μ M) did not significantly affect endolysosome pH but both significantly ($p < 0.001$) blocked SiNP-induced endolysosome de-acidification (Figure 6A) and SiNP-induced increases in secreted levels of A β ₁₋₄₀ ($p < 0.01$) (Figure 6B).

Discussion

The rapid development of and enthusiasm for the use of nanotechnology in health and disease has been accompanied by concerns about the potential toxicity of nanomaterials (Stone & Donaldson 2006). Among the variety of nanomaterials used, SiNPs have attracted attention in biomedical fields because of their favorable biocompatibility and their scalable synthetic capability (Lieberman *et al.* 2014). The importance of safety is tantamount to any use of SiNPs in nanomedicine (Elsaesser & Howard 2012, Mohamed *et al.* 2011, Fadeel & Garcia-Bennett 2010) and the study of their safety profiles is complicated by the variable procedures used to produce SiNPs and their diverse physicochemical properties (Fadeel & Garcia-Bennett 2010, Tang & Cheng 2013). Rather than regarding dosage levels as the only major toxic concern (Moss & Wong 2006, Oberdorster *et al.* 2007, Wittmaack 2007), many other important factors have been identified to influence safety such as cell types (Nan *et al.* 2008, Rabolli *et al.* 2010, Yu *et al.* 2011), physicochemical properties of SiNPs (Yu *et al.* 2012a, Yu *et al.* 2011, Yu *et al.* 2012b), and the apparent high tolerance of some experimental animals for even large doses of SiNPs (Liu *et al.* 2011, Lu *et al.* 2010). Here, we focused our studies on endolysosomes because these acidic organelles likely are affected first upon nanoparticle entry into cells and might serve as early indicators of the biological effects of SiNPs. We found that at non-toxic concentrations, SiNPs de-acidified endolysosomes, decreased readily releasable stores of calcium in endolysosomes, and increased amyloidogenesis in primary cultured neurons; findings that provide mechanistic insight into SiNP-induced neurotoxicity and enhanced amyloidogenesis.

Depending on the particle size and surface treatment, SiNPs enter cells via different pathways including phagocytosis, micropinocytosis, and receptor-mediated endocytosis (Zhang *et al.* 2015). SiNPs without conjugated ligands may also be endocytosed non-specifically. Although we did not explore the involvement of surface proteins that could mediate the endocytosis of SiNPs, we did observe that SiNPs were accumulated in endolysosomes of primary cultured neurons. Keeping this in mind, we also acknowledge the possibility that SiNPs may be present in other organelles besides the endolysosomes. SiNPs are often engineered so that the release of guest molecules is affected by acidic environments under physiological conditions in stomach and endolysosomes, and under such pathological conditions as tumors and pro-inflammatory conditions (Song *et al.* 2017,

Yang *et al.* 2012). However, more attention has been focused on how pH regulates the function of SiNPs, and little is known whether SiNPs themselves can impact pH. Thus, we explored the potential direct effects of SiNPs on functions of endolysosomes. As acidic intracellular organelles, endolysosomes are especially important for regulating neuronal functions because neurons are mainly long-lived post-mitotic cells that require the endolysosome system in turning over cellular components and obsolete organelles (Nixon & Cataldo 1995, Nixon & Cataldo 2006). Because maintaining an optimum acidic environment in endolysosomes is critical to maintaining endolysosome function, we determined the extent to which SiNPs affected endolysosome pH. We demonstrated that SiNPs, at non-neurotoxic concentrations, de-acidified endolysosomes acutely and persistently. Our findings suggest that SiNPs directly affect endolysosome pH, which could lead to neuronal cell death at higher concentrations. Currently, we do not know how SiNPs de-acidify endolysosomes, but their physiochemical properties could play a role.

Endolysosomes are important intracellular calcium stores containing high concentrations (400–600 μM) of readily releasable calcium. Given the dynamic properties of endolysosomes, calcium released from endolysosomes can regulate a variety of fundamental calcium-dependent processes including vesicular trafficking, endolysosome fusion, lysosome-related organelle biogenesis, membrane repair, and exocytosis-endocytosis coupling (Ruas *et al.* 2010, Lloyd-Evans *et al.* 2010, Huynh *et al.* 2004, Lima *et al.* 2012, Li *et al.* 2008, Hosoi *et al.* 2009). Indeed, for neurons, endolysosome calcium has been implicated in neurotransmitter release, neuronal excitability, synaptic plasticity, neurite extension, and neuronal viability (Brailoiu *et al.* 2005, Dickinson *et al.* 2010, Pandey *et al.* 2009, Hui *et al.* 2015). Moreover, decreased endolysosome calcium has been implicated in neurodegeneration (Feng *et al.* 2014) and neurological diseases including AD (McBrayer & Nixon 2013). Although the mechanisms responsible for calcium uptake into and release from endolysosomes are not fully understood, others and we have shown that decreased endolysosome calcium can be a direct consequence of endolysosome de-acidification (McBrayer & Nixon 2013, Hui *et al.* 2015). Here, we found that non-toxic concentrations of SiNPs could decrease the releasable pool of calcium in endolysosomes and increase cytosolic calcium levels, and this might lead to calcium dyshomeostasis and impact neuron function (Gilardino *et al.* 2015). It is possible that other calcium stores could be contributing to the SiNP-induced increase in cytosolic calcium levels, e.g. calcium released from endolysosomes might trigger the release of calcium from endoplasmic reticulum stores or induce calcium influx across the plasma membrane (Hui *et al.*, 2015). Moreover, further investigations are necessary to understand more fully how SiNPs affect highly coordinated calcium events including endolysosome calcium release, calcium uptake and release from other organelles, calcium levels in cytoplasm, and the influx of extracellular calcium.

Endolysosomes play an important and early role in the pathogenesis of sporadic AD that precede the development of amyloid plaques (Tate & Mathews 2006, Boland *et al.* 2008). Amyloid plaques are comprised of aggregated A β , which have two major isoforms 1–40 and 1–42; A β _{1–42} is more fibrillogenic than A β _{1–40} although the levels of A β _{1–40} are several fold higher than A β _{1–42} (Gu & Gou 2013). Endolysosomes are major sites where A β is generated following A β PP internalization, and A β is degraded in acidic lysosomes (Edgar *et al.* 2015, Miners *et al.* 2011, Vingtdeux *et al.* 2012). Even modest de-acidification can

compromise degradation capabilities of endolysosomes, resulting in increased intraneuronal accumulation and secreted levels of A β . Indeed, SiNPs have been shown to promote amyloidogenesis (Yang et al. 2014). Here, we explored the extent to which endolysosome de-acidification played a role in SiNP-induced amyloidogenesis. We demonstrated that SiNPs de-acidified endolysosomes and increased extra-neuronal levels of A β ₁₋₄₀ but interestingly did not affect extra-neuronal levels of A β ₁₋₄₂. It is possible that SiNP's might be increasing the intracellular production but also inhibiting the secretion of A β ₁₋₄₂. Further studies would need to be conducted to determine the effects of SiNPs on producing, processing, and releasing A β . To further explore the causal relationship between SiNP induced de-acidification and amyloidogenesis, we determined the extent to which acidifying endolysosomes prevented SiNP-induced amyloidogenesis, and ML-SA1 (a TRPML agonist) and CGS21680 (an adenosine A_{2A} receptor agonist) that have been shown to acidify endolysosomes were used (Bae et al. 2014; Liu et al. 2008). Acute treatment with ML-SA1 and CGS21680 acidifies endolysosomes, but longer-term 24 h treatment did not change endolysosome pH. One explanation might be because over the 24 h timespan the neuronal endolysosomes are able to homeostatically regulate pH levels back to normal. Similarly, 24 h treatment with ML-SA1 and CGS21680 did not significantly reduce extra-neuronal A β ₁₋₄₀ levels, but did block SiNP-induced endolysosome de-acidification and amyloidogenesis in neurons. Thus, endolysosome de-acidification appears to play an important role in SiNP-induced amyloidogenesis. Altogether, our findings have led us to suggest a proposed mechanism by which SiNP might induce neurotoxicity; SiNPs are trafficked into the endolysosome system and once within endolysosomes disrupt the function of these organelles thereby inducing calcium dyshomeostasis and increased levels of extra-neuronal A β .

Clearly, there is a great promise that SiNPs may benefit biomedical studies and therapeutic strategies. However, the utilization of SiNPs should proceed advisedly because of findings such as ours that these nanoparticles de-acidify endolysosomes, increase calcium release from endolysosomes and increase levels of beta amyloid protein.

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Abbreviation:

SiNPs	Silica nanoparticles
Aβ	amyloid beta protein
AD	Alzheimer's disease
GPN	glycyl-L-phenylalanine 2-naphthylamide
ML-SA1	a TRPML channel agonist
CGS21680	an adenosine A _{2A} receptor agonist

TEOS	tetraethylorthosilicate
ELISA	enzyme-linked immunosorbent assay
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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E **F** **Figure 1.**

Characterization of silica nanoparticles (SiNPs). Using a Hitachi SU8010 field scanning-electron microscope, sizes of synthesized SiNPs and Rubpy-doped SiNPs were detected. (A) The diameters of SiNPs were 68 ± 4 nm. (B) The diameters of Rubpy-doped SiNPs were 71 ± 5 nm. Zeta potentials of SiNPs (C) were -38.33 ± 0.55 mV and for Rubpy-doped SiNPs (D) were -7.46 ± 0.25 mV. EDS patterns for SiNPs (E) and Rubpy-doped SiNPs (F) were illustrated.

Figure 2.

Effects of SiNPs on neuronal viability. Primary neurons were grown in culture for 10–14 days and were then treated with SiNPs (Zhao *et al.* 2008) at various concentrations as indicated. Cell viability was determined by MTT assay 24 hours after SiNPs were applied. (* $p < 0.05$, vs control, $n = 6$).

Figure 3.

Endocytosis of SiNPs in endolysosomes and the effects of SiNPs on endolysosome pH in primary cultured neurons. (A) Cells were treated with RuBpy tagged SiNP (NP-RuBpy) for 24 h followed by incubation with LysoTracker; an endolysosome marker dye. Images shown were neurons labeled with LysoTracker and NP-RuBpy separately as well as a merger of the images. Images shown demonstrated that SiNPs were accumulated in endolysosomes (A). Scale bar = 10 μ m. (B-C) Endolysosome pH was measured ratio-metrically using LysoSensor dye within the first 5 minutes of (B) and 24 hours after (C) application of SiNPs (Zhao et al. 2008) at 0.1 μ g/ml. Application of SiNPs to neurons produced immediate endolysosome de-acidification (B) (n=13) and the endolysosome de-acidification was sustained over a 24 h treatment period (C) (n>10). (***)p<0.001, vs control).

[Ca²⁺]_{cyt}

Figure 4.

Effects of SiNPs on GPN-inducible release of calcium from endolysosomes in primary cultured neurons. (A) GPN increased significantly (***) levels of free intracellular calcium as a result of increased release of calcium from endolysosomes. (B) Compared to controls, SiNPs (0.01 μ g/ml and 0.1 μ g/ml) significantly (*) and concentration-dependently reduced GPN-inducible stores of calcium in endolysosomes.

Figure 5.

Effects of SiNPs on extra-neuronal levels of $A\beta$ in primary cultured neurons. Primary cultured neurons were treated for 24 hours with SiNPs at 0.01 and 0.1 $\mu\text{g/ml}$. At the end of the treatment period, media were collected and levels of $A\beta_{1-40}$ (A) and $A\beta_{1-42}$ (B) were determined by ELISA assay. (A) SiNPs at 0.1 $\mu\text{g/ml}$ modestly but statistically significantly ($*p < 0.05$, *vs* control, $n=6$) increased levels of extra-neuronal $A\beta_{1-40}$ compared to controls. (B) Neither of the SiNPs at the concentrations tested significantly affected levels of extra-neuronal $A\beta_{1-42}$ ($n=4$).

Figure 6.

Effects of endolysosome acidifying agents on SiNP-induced endolysosome de-acidification and increases in Aβ₁₋₄₀ release in primary cultured neurons. (A) SiNPs (0.1 μg/ml) induced deacidification of endolysosome pH was significantly (***p<0.001, vs control, n=20) decreased by ML-SA1 (20 μM) and CGS21680 (100 μM). (B) SiNP (0.1 μg/ml) induced increases in extra-neuronal Aβ₁₋₄₀ levels were reduced significantly (*p<0.05, **p<0.01 vs control, n=5) by ML-SA1 and CGS21680.

Figure 7.

Following endocytosis of SiNPs into neurons, endolysosome pH is increased (deacidification), calcium in endolysosomes is released into the cytosol, and $A\beta$ levels are increased extra-cellularly. It appears endolysosome pH is an important starting point for these actions because endolysosome acidification with the TRPML1 agonist ML-SA1 or the adenosine A_{2A} receptor agonist CGS21680 block the effects of SiNPs on calcium and $A\beta$.