Supporting Information

Title: Induction of oxidative stress and cell death in neural cells by silica nanoparticles.

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EXPERIMENTAL PROCEDURES

PI Staining. PI (Nacalai Tesque, Inc., Kyoto, Japan) is a nuclear and chromosomal dye with red fluorescence. Live cells are not permeable to PI, so it is commonly used to detect dead cells in a population.[1] We measured the intensity of PI fluorescence as an index of neuronal degeneration. Cultured cells were incubated with 10 μM PI in medium for 30 min and exposed to SiNPs for 2 h. Cell images were captured with identical incident light intensity and exposure time on an IX71 fluorescence microscope (Olympus) equipped with a 60× objective lens and an Orca-ER cooled CCD camera (Hamamatsu Photonics). The PI fluorescence intensity was measured using MetaMorph software.

MTT Assay. MTT reduction is often used to assess cellular metabolic activity.[2] For cell-viability analysis, we used the MTT Assay Kit (Nacalai Tesque), according to the manufacturer's recommendations. Briefly, after incubation with SiNPs, the cells were incubated with MTT for 3 h before reading the absorbance at 570 nm after complete solubilization of formazan crystals in lysis buffer (0.04 M HCl/isopropanol)

GSH-Glo and GSH/GSSG-Glo assay. To measure intracellular GSH and GSSG, we used the GHS-Glo and GSH/GSSG-Glo assay system (Promega), according to the manufacturer's recommendations. Briefly, dissociated hippocampal cells were plated in a PEI-coated, white-walled 96-well microplate at a density of 1×10^4 cells/well and incubated at 37°C for 6–8 days. Subsequently, a one-tenth volume of 10× NP suspension was administrated to culture medium. After removing medium, prepared reagents was added to each well. The luminescent signal was acquired using microplate luminometer (SpectraMax L).

Electrophoresis and Immunoblotting. Cultured hippocampal cells and HEK293 cells were solubilized in ice-cold RIPA buffer [1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 25 mM Tris-HCl, pH 7.5, 137 mM NaCl, and 3 mM KCl with a protease inhibitor cocktail (Complete, Roche Diagnostics, Mannheim, Germany)]. Samples were resolved by SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Immobilon-P, Millipore). After blocking with 5% skim milk in TBST (1× Tris-buffered saline with 0.1% Tween 20) membranes were incubated at 4 ˚C overnight or 25 ˚C for 2 h with primary antibodies: anti-Nrf2 (rabbit, 1:500 dilution; Santa Cruz, sc-722), anti-HSP40 (mouse, 1:1000 dilution; Neomarkers, MS-255-P),

anti-inducible HSP70 (mouse, 1:200 dilution; Santa Cruz, sc-66048), anti-pan-HSP70 (goat, 1:1000 dilution; Santa Cruz, sc-24), and anti-HSP90 (goat, 1:1000 dilution; Santa Cruz, sc-8262). After four changes of 1× TBST and three 5-min washed, membranes were incubated in horseradish peroxidase-conjugated secondary antibodies. After four washes, membranes were incubated with ECL solution (Thermo Fisher Scientific, MA, USA). For quantification, chemiluminescence light signals in Super Signal Dura substrate (Thermo Fisher Scientific) were captured by a cooled charge-coupled device camera system (LAS-3000plus; Fuji Film, Japan) that ensured wide ranges of linearity. Densitometric quantification of synaptic protein expression normalized to β-actin.

Protein carbonylation assay. Protein carbonyls were detected using the Protein carbonyls western blot detection kit (Shima Laboratory, Japan) according to manufacturer instructions using cell lysate from cultured hippocampal cells in 12-well plate. Briefly, Proteins were separated by 8 % SDS-PAGE and transferred to PVDF membranes using a semi-dry transfer. Membranes were incubated with 2,4-Dinitrophenylhydrazine (DNPH) solution and then with anti-DNP antibody (Shima Laboratory) 4 ˚C overnight. As a negative control, the membranes were incubated in 2%

HCl without DNPH before incubation with the primary antibody. Detection and quantification of carbonyl proteins were done as described above.

REFERENCES

1. Lecoeur, H., Nuclear apoptosis detection by flow cytometry: influence of endogenous endonucleases. *Exp Cell Res* **2002,** *277* (1), 1-14.

2. Mosmann, T., Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* **1983,** *65* (1-2), 55-63.

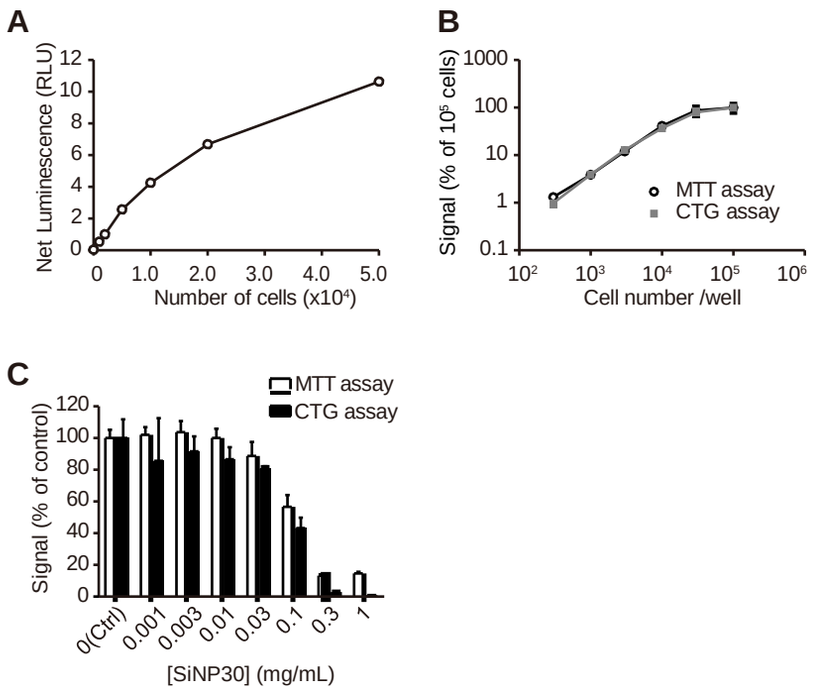

Figure S1. Comparison of cell-viability assay systems. (A) Dissociated hippocampal cells were seeded onto 0.1% PEI-coated 96-well microplates ($1 \times 10^3 – 5 \times 10^4$ cells/well). Cultures were maintained in B27-supplemented Neurobasal medium for 6 days. The CTG Luminescent Cell Viability Assay system was used to measure ATP in each well (n = 4). (B) HEK293 cells were seeded in 96-well microplates ($3 \times 10^2 – 1 \times 10^5$ cells/well). The CTG Luminescent Cell Viability Assay (open circles, n = 4 wells) or MTT assay (gray squares, n = 4 wells) was performed in 96-well plates. (C) Cultured hippocampal cells (1×10^4 cells/well) were treated with 0.001 to 1 mg/mL of SiNP30 or the vehicle control (PBS) for 2 h, and viability was assessed using the CTG Luminescent Cell Viability Assay system (open columns, n = 4 wells) or MTT assay system (solid columns, n = 4). Data are shown as the mean ± SD.

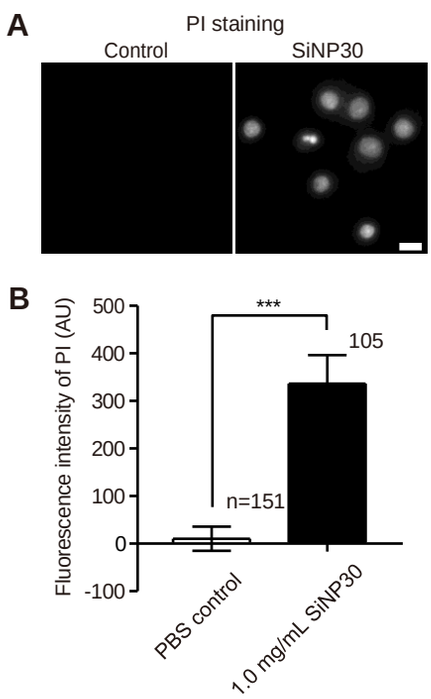

Figure S2. Propidium Iodide (PI) staining of hippocampal cells treated with SiNP30. Cultured hippocampal cells (6 DIV) were incubated with PI, a maker of dead or dying cells, and then treated with SiNP30 (1.0 mg/mL) or the vehicle control (PBS) for 2 h (A). Scale bar, 10 μm. Quantitative analysis of PI fluorescence in cell nuclei (B). Statistical significance was tested by performing an unpaired *t*-test. ***, $p < 0.001$. Data are shown as mean ± SD.

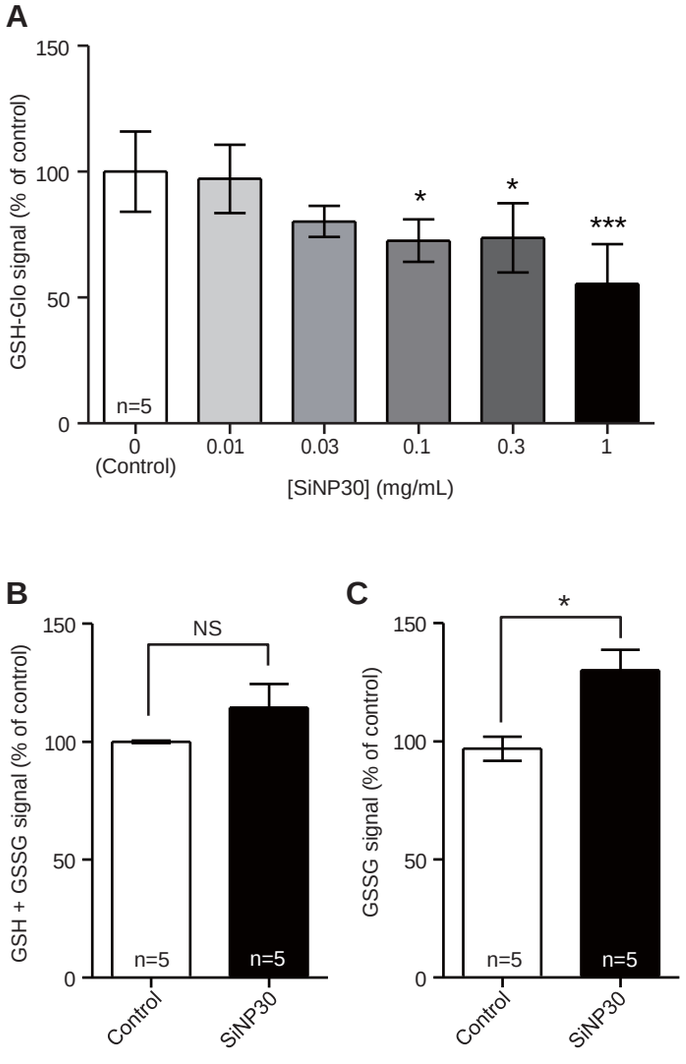

Figure S3. SiNP treatment reduced the intracellular levels of GSH. (A) GSH-Glo assay. Cultured hippocampal cells were treated for 30 min with 0.01 to 1 mg/mL of SiNP or vehicle control (PBS), and intracellular GSH was assessed using the GSH-Glo Luminescent Assay system. Statistical significance was tested by one-way ANOVA, and pairwise comparison was performed according to Dunnett's multiple-comparisons test. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ vs Control. (B, C) GSH/GSSG-Glo assay. Cultured hippocampal cells were treated with SiNP (n=4) or vehicle control (PBS) of 30 min, and intracellular total GSH (GSH + GSSG) and GSSG was assessed using the GSH/GSSG-Glo Luminescent Assay system. Statistical significance was tested by performing an unpaired *t*-test. *, $p < 0.05$, NS, $p > 0.05$. Data are shown as the mean ± SEM.

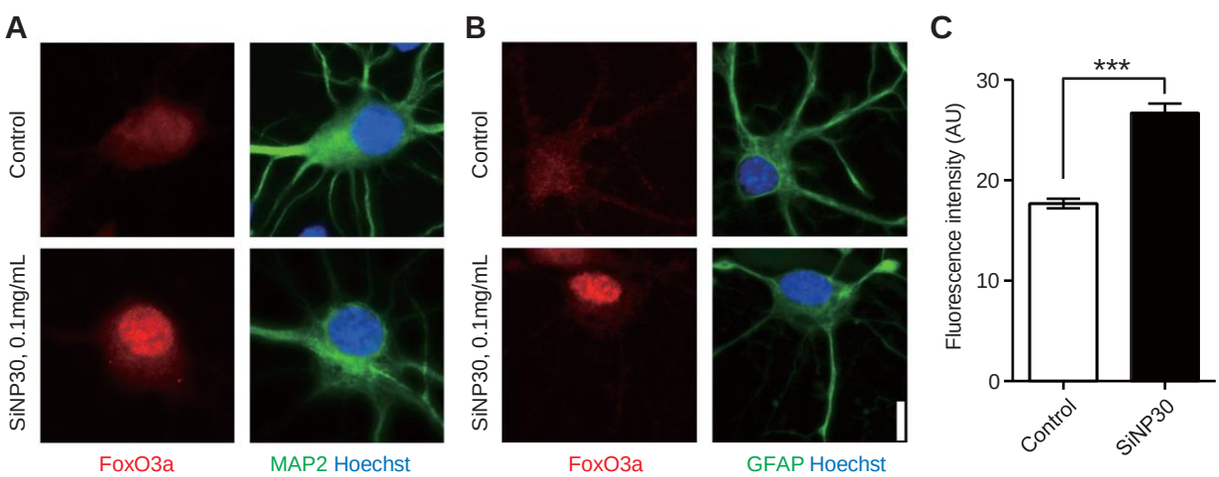

Figure. S4. FoxO3a nuclear translocation after SiNP treatment. Cultured hippocampal cells were treated with 0.1mg/mL SiNP30 for 30min. After fixing and washing, cells were and stained with Hoechst 33342 (blue), anti-FoxO3a (red), and anti-MAP2 (A) or anti-GFAP (B) (green) antibodies. Scale bar, 10 μm. (C) Fluorescence intensity of nuclear FoxO3a. Statistical significance was tested by Student's *t* test. ***, $p<0.001$. Data are mean ± SEM.

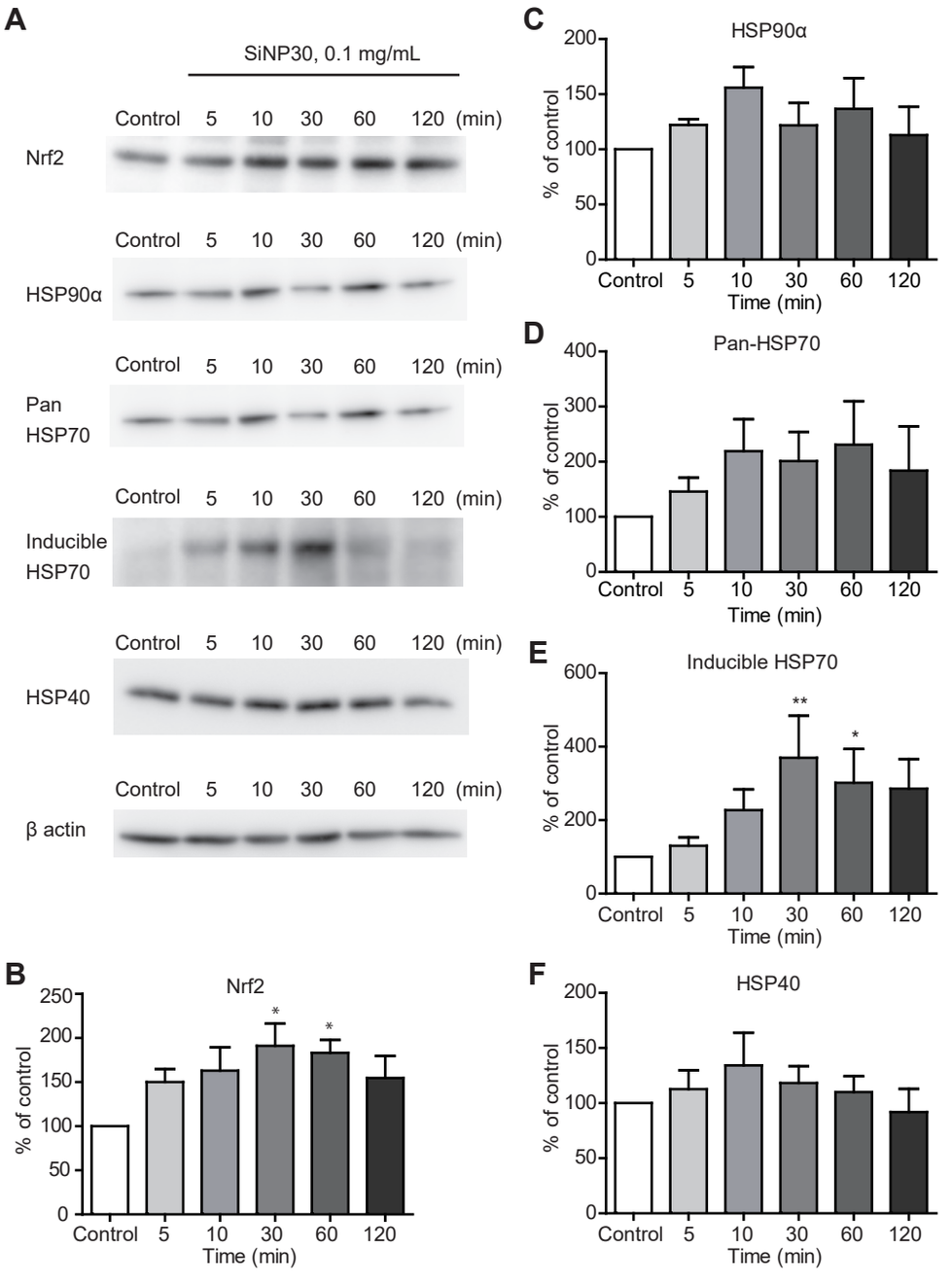

Figure. S5. SiNP treatment increased intracellular levels of Nrf2 inducible HSP. Cultured hippocampal cells were treated with 0.1mg/mL SiNP30 from 5 to 120 min. (A) Labeled proteins were detected by immunoblotting with antibodies against anti-Nrf2, HSP90α, HSP70 (inducible or pan), HSP40, and β-actin. (B-F) Quantitative analysis of expression levels of Nrf2, HSPs, normalized to β-actin served as a loading control. Statistical significance was tested by one-way ANOVA, and pairwise comparison was performed according to Dunnett's multiple-comparisons test. *, $p < 0.05$; **, $p < 0.01$ vs. control. Data are mean ± SEM based on four independent experiments (n = 4).

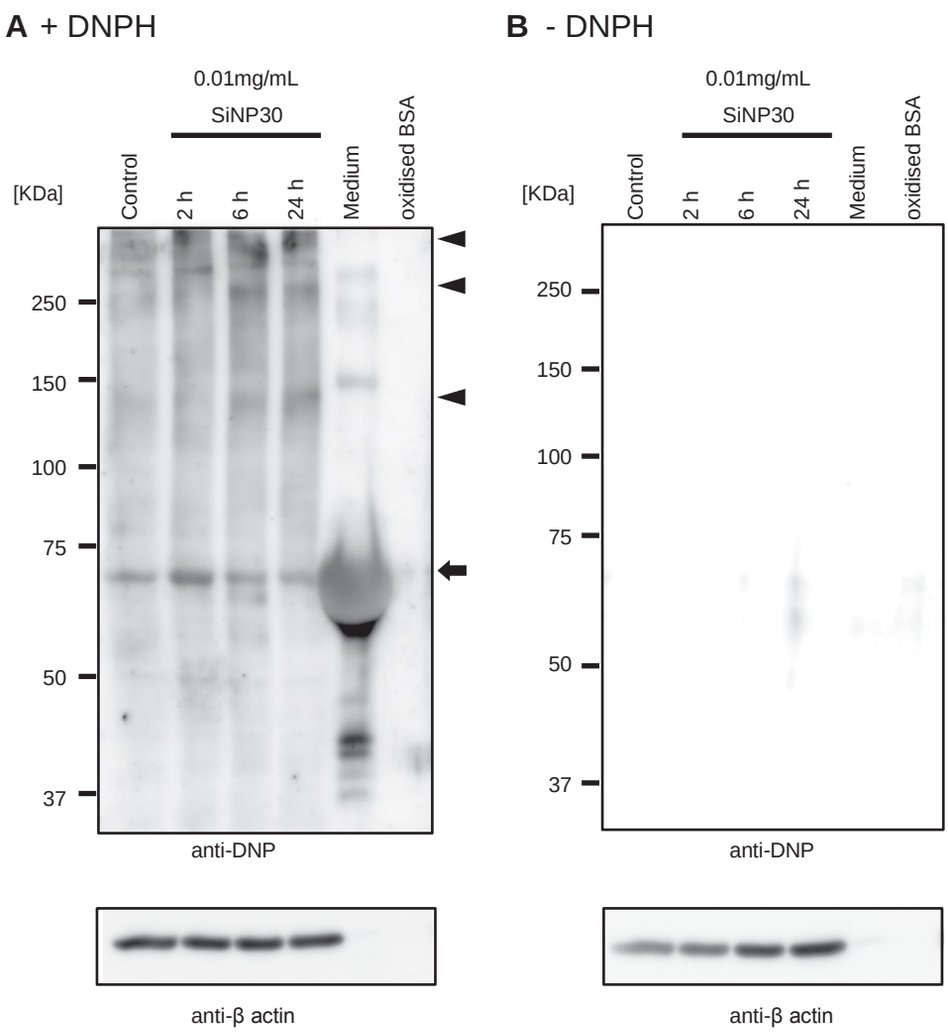

Figure. S6 Elevation of protein carbonyls in cultured hippocampal cells by SiNP treatment. Cultured hippocampal cells were treated with 0.01mg/mL SiNP30 for 2 to 24h. The cell lysate were resolved by SDS-PAGE and transferred onto PVDF membranes.The PVDF membranes were incubated 2M HCl with 2,4-Dinitrophenylhydrazine (DNPH) solution (A) or without DNPH (B), as a negative control. Carbonylated proteins and β-actin were detected using anti-DNP and β-actin. Protein carbonyl (arrowhead), oxidized BSA as a positive control (arrow). The strong signal in medium may be drived from albmin in medium.

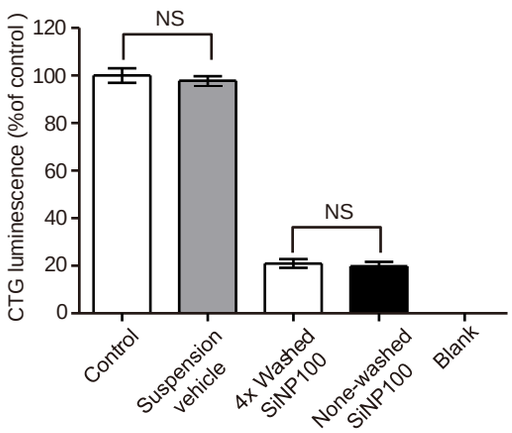

Figure S7. The suspension vehicle of SiNPs did not affect cell viability. Cultured hippocampal cells (n = 8 wells) were treated for 2 h with control vehicle (H_2O), suspension vehicle (supernatant of the SiNP100 suspension), 4×-washed SiNP100s (0.1 mg/mL), and non-washed SiNP100s (0.1 mg/mL), after which cell viability was assessed using the CTG Luminescent Cell Viability Assay system. Control and suspension vehicle were administered at the same concentration. Statistical significance was tested by one-way ANOVA, and differences between pairs were analyzed by using Tukey's post-hoc test. NS, $p > 0.1$. Data are shown as the mean ± SD.

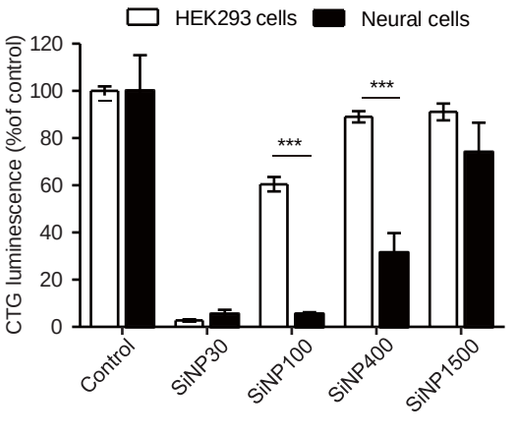

Figure S8. Particle size-dependent toxicity in HEK293 and hippocampal cells. HEK293 cells (n = 4 wells) or dissociated hippocampal cells (n = 4 wells) were treated with 0.1 mg/mL SiNP of various sizes for 2 h, and the cell viability was assessed with the CTG Luminescent Cell Viability Assay. CTG signals with HEK293 cells were decreased significantly by SiNP treatment (control vs. SiNP30, control vs. SiNP100, and control vs. SiNP400: $p < 0.001$; control vs. SiNP1500: $p < 0.01$). CTG signals of hippocampal cells were decreased significantly (control vs. SiNP30, control vs. SiNP100, control vs. SiNP400, and control vs. SiNP1500: $p < 0.001$). Statistical significances between control and SiNPs were analyzed by one-way ANOVA, and pairwise comparisons were performed using the Tukey's post-hoc test. CTG signals were significantly lower in hippocampal cells than in HEK 293 cells treated with SiNP100 ($p < 0.001$) or SiNP 400 ($p < 0.001$). The statistical significances of differences between hippocampal and HEK293 cells were analyzed by two-way ANOVA, and differences between pairs were analyzed by Bonferroni's post-hoc test. ***, $p < 0.001$. Data are shown as the mean ± SD.

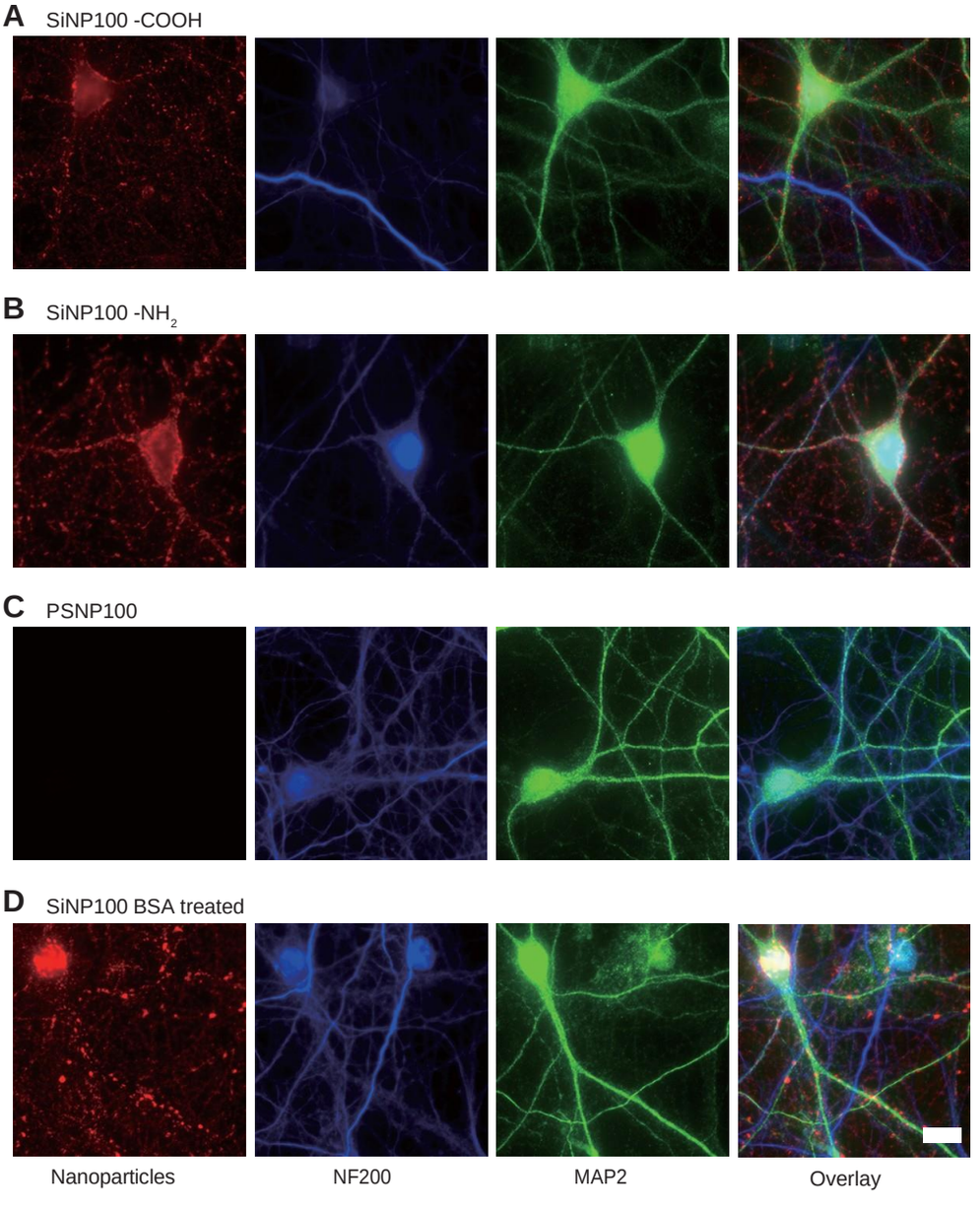

Figure S9. Fluorescence images of cultured neurons treated with NPs. Cultured hippocampal cells (7DIV) were treated for 15 min with 1 mg/mL fluorescently labeled (red) SiNP100-COOH (A), SiNP100-NH_2 (B), PSNP (C), and BSA-pretreated SiNP100 (D). After fixing and washing, cells were and stained with anti-NF200 (blue) and anti-MAP2 (green) antibodies. Scale bar, 20 μm