Supporting Information

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SI Methods

Characterization of GNRs and GNR-siRNA nanoplexes using transmission electron microscopy (TEM) and UV-visible absorbance and photoluminescence (PL) spectroscopy: TEM images were obtained using JEOL model JEM-100CX microscope, operating with acceleration voltage 80 kV. The specimens were prepared by drop-coating the sample dispersion onto a holey carbon-coated 200 mesh copper grid, which was placed on filter paper to absorb excess solvent.

The absorption spectra were collected using a Shimadzu model 3101PC UV-vis-NIR scanning spectrophotometer over the range of 300 to 800 nm. The samples were measured against water as reference. The PL spectra were collected using a Spectrofluorometer (Fluorolog-3, Horiba Jobin Yvon).

Agarose Gel Electrophoresis. Nanoplexes of GNR with either $siRNA^D$ or $siRNA^F$, and equivalent amounts of free siRNA, were added in individual wells (100 pmols of siRNA per well) in 1.5% agarose gel casted in Tris acetate-EDTA (TAE) buffer. The gel was run for 1 h at 100 volts, stained with ethidium bromide (in the case of $siRNA^D$ only) and documented using a UVP bioimaging system. An LM-20E UV benchtop transilluminator (UVP) was used in conjunction with an Olympus Digital Camedia C-4000 zoom color camera with a UV filter and a lens. Documentation was completed using the DOC-IT system software.

Cell Culture. Human dopaminergic neuronal precursor (DAN) cells were obtained from Clonexpress. Actively growing populations of cells were tested for tyrosine hydroxylase (TH) expression by immunocytochemistry. DAN cells are supplied with a proprietary growth factor supplement (DNCS) as a 100× stock solution, which is added to DMEM:F12 (50:50) containing 5% FBS and 10 ng/mL of bFGF and 5 ng/mL of GDNF to make DAN cell growth medium. These cells differentiate into neurons within a week, when plated on polylysine (PLL)-coated plates at a density of approximately 10^4 cells per cm^2 in DMEM/F12 (50:50) supplemented with DNC5, 5% FBS, 10 ng/mL of bFGF, 10 ng/mL of EGF, and 100 μM dibuturyl cAMP.

Imaging of Nanoparticles in Vitro. The cellular uptake of the *nanoparticle conjugated with DARPP-32 siRNA* ($siRNA^D$) was visualized using dark-field microscopy. The light-scattering images were recorded using an upright Nikon Eclipse 800 microscope with a high numerical dark-field condenser (NA 1.20–1.43, oil immersion) and a 100/1.4 NA oil Iris objective (Cfi Plan Fluor). In the dark-field configuration, the condenser delivers a narrow beam of white light from a tungsten lamp and the high NA oil immersion objective collects only the scattered light from the samples. The dark-field imaging was captured using a QImaging Micropublisher 3.3 RTV color camera. The Qcapture software from the camera manufacturer was used for image acquisition and has a feature for adjusting the white color balance for accurately capturing the color differences in samples.

Fluorescence Studies from Cell Lysates. DAN cells incubated with 100 pmols of free $siRNA^F$, and GNR-$siRNA^F$, and siPORT-$siRNA^F$ nanoplexes and 24 h later cells were processed for fluorescence measurements. The medium was removed and the cells were lysed using M-PER (mammalian protein extraction reagent Pierce) and analyzed using a spectrofluorometer.

Cell Viability Assay. Cell viability assay measures the reduction of a tetrazolium component (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, or MTT) into an insoluble formazan product by the mitochondria of viable cells. Cells in a 24-well plate (10,000 cells/mL/well) were incubated with the MTT reagent for ≈3 h, followed by addition of a detergent solution to lyse the cells and solubilize the colored crystals. The samples were read using an ELISA plate reader at 570 nm wavelength.

Transfection. Transgene expression is monitored at 24–96 h posttransfection and followed up to a period of 2 weeks post-transfection. SiRNA delivery takes a minimum of 12–24 h. An appropriate transfection reagent control and a scrambled sequence negative control are used in all experiments. Twenty-four hours before siRNA transfection, 2×10^5 dopaminergic neuronal cells are seeded onto 6-well plates in OPTI-MEM containing 4% FBS with no antibiotics to give 30 to 50% confluence at the time of transfections. The siRNA is reconstituted in DNase–RNase free water to a final concentration of 0.1 μM and mixed with 20 μL of solution of GNR synthesized as described above in a ratio empirically determined for each siRNA, incubated at room temperature for 15 min and used for in vitro transfection of dopaminergic neurons at a final concentration of 200 pmol of siRNA. Levels of mRNA were monitored at 12–120 h posttransfection and at up to 2 weeks. The commercially available siRNA delivery agent, siPORT (Ambion) was used as the positive control in our experiments.

RNA Extraction. Cytoplasmic RNA is extracted by an acid guanidinium-thiocyanate-phenol-chloroform method as described using TRIzol reagent (Invitrogen-Life Technologies) (1). The amount of RNA is quantitated using a Nano-Drop ND-1000 spectrophotometer (Nano-Drop) and isolated RNA is stored at −80 °C until used.

Real-time quantitative PCR (Q-PCR): Relative abundance of each mRNA species is quantitated using real-time quantitative PCR using specific primers using the Brilliant SYBR green Q-PCR master mix from Stratagene. RNA is reverse transcribed to cDNA using the reverse transcriptase kit from Promega. Relative expression of mRNA species is calculated using the comparative threshold cycle number (C_T) method (2, 3). All data are controlled for quantity of RNA input by performing measurements on an endogenous reference gene, β-actin.

Western Blot Analysis. Total protein is extracted using mammalian protein extraction reagent (Pierce) and 20 μg of protein is loaded per lane and separated by a 4–20% SDS-Tris/glycine PAGE. After blocking of nonspecific binding sites with 5% nonfat milk, membranes are probed with commercially available monoclonal antibodies. Protein levels of DARPP-32, and ERK1 are measured in cell lysates from primary neuronal cells treated with GNR-conjugated DARPP-32 siRNA using DARPP-32 antibody (Cell Signaling Technologies) and antibodies against ERK1 (Santa Cruz Biotechnology), respectively.

Immunocytochemistry Staining. Ten thousand DAN cells, treated with the various agents, were plated on 35-mm glass bottom Petri dishes with a 14-mm microwell (Mat Tek Corporation) and allowed to attach. Cells were then rinsed briefly in PBS (PBS) and then fixed in 100% ice-cold methanol for 15 min at room temperature. After fixation, cells were washed twice in ice-cold

PBS. Cells are then permeabilized by incubating them with PBS containing 0.5% saponin. Nonspecific binding was blocked using 1% BSA in PBS with 0.1% Tween 20 (PBST) for 30 min. Cells are then incubated in primary antibodies (DARPP-32 antibody, Cell Signaling Technologies; ERK1, Santa Cruz Biotechnology) diluted in 1% BSA in PBST for 1 h at room temperature, washed with PBST, and incubated with a Alexa Fluor 647-labeled secondary antibody. Nuclei are stained using DAPI (0.1 μg/mL) for 1 min and coverslip mounted with a drop of aqueous mounting media (4).

In vitro BBB model: The in vitro BBB model used primary cultures of human BMVEC and normal human astrocytes (NHA), both of which were obtained from Applied Cell Biology Research Institute (ACBRI) Kirkland, Washington. Characterization of BMVEC demonstrated that >95% cells were positive for cytoplasmic VWF/Factor VIII. BMVEC were cultured in CS-C complete serum-free medium (ABCRI) with attachment factors (ABCRI) and Passage Reagent Group (ABCRI). NHAs were cultured in CS-C complete serum-free medium (ABCRI), supplemented with 10 μg/mL human EGF, 10 mg/mL insulin, 25 μg/mL progesterone, 50 mg/mL transferrin, 50 mg/mL gentamicin, 50 μg/mL amphotericin, and 10% FBS, and characterized by >99% of these cells being positive for glial fribrillary acidic protein (GFAP). The BBB model, based on 2 compartments separated by a 3-μm polyethylene terephthalate (PET) insert (surface area = 4.67 cm^2), was generated as described previously (5). Formation of an intact BBB was measured by determining transendothelial electrical resistance (TEER), (Table S1) using an ohm meter Millicell ERS system (Millipore).

Transmigration Assay. All transmigration experiments are conducted on day 6 of BBB culture (5–7). Fluorescence (FAM)-labeled siRNA-GAPDH (or $siRNA^F$, Ambion), GNR-$siRNA^F$ nanoplexes, siPORT-conjugated $siRNA^F$, and free GNR were added to the upper chamber of the in vitro BBB, along with a fixed volume of media (2 mL), which was added to the upper and lower chambers. After addition of the $siRNA^F$, GNR-$siRNA^F$, siPORT-$siRNA^F$, and free GNR and incubation at 37 °C for 3 h, 1 mL of media was aspirated from both the upper and the lower chambers and analyzed spectrofluorometrically.

1. Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162(1):156–159.
2. Radonić A, et al. (2004) Guideline to reference gene selection for quantitative real-time PCR. *Biochem Biophys Res Commun* 313(4):856–862.
3. Bustin SA (2002) Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): Trends and problems. *J Mol Endocrinol* 1(29):23–39.
4. Coligan JE (2007) *Current Protocols in Immunology* (Wiley, New York).
5. Mahajan SD, et al. (2008) Methamphetamine alters blood brain barrier permeability via the modulation of tight junction expression: Implication for HIV-1 neuropathogenesis in the context of drug abuse. *Brain Res* 1203:133–148.
6. Mahajan SD, et al. (2008) Tight junction regulation by morphine and HIV-1 Tat modulates blood–brain barrier permeability. *J Clin Immunol* 28(5):528–541.
7. Persidsky Y, Gendelman H (1997) Development of laboratory and animal model systems for HIV-1 encephalitis and its associated dementia. *J Leukoc Biol* 62(1):100–106.

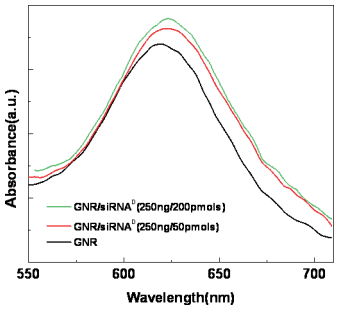

Fig. S1. The localized longitudinal surface plasmon resonance peak of GNRs red shift upon complexation with siRNA. This plasmonic shift of gold nanorods is a powerful tool for studying their interaction with various biomolecules such as nucleic acids and proteins.

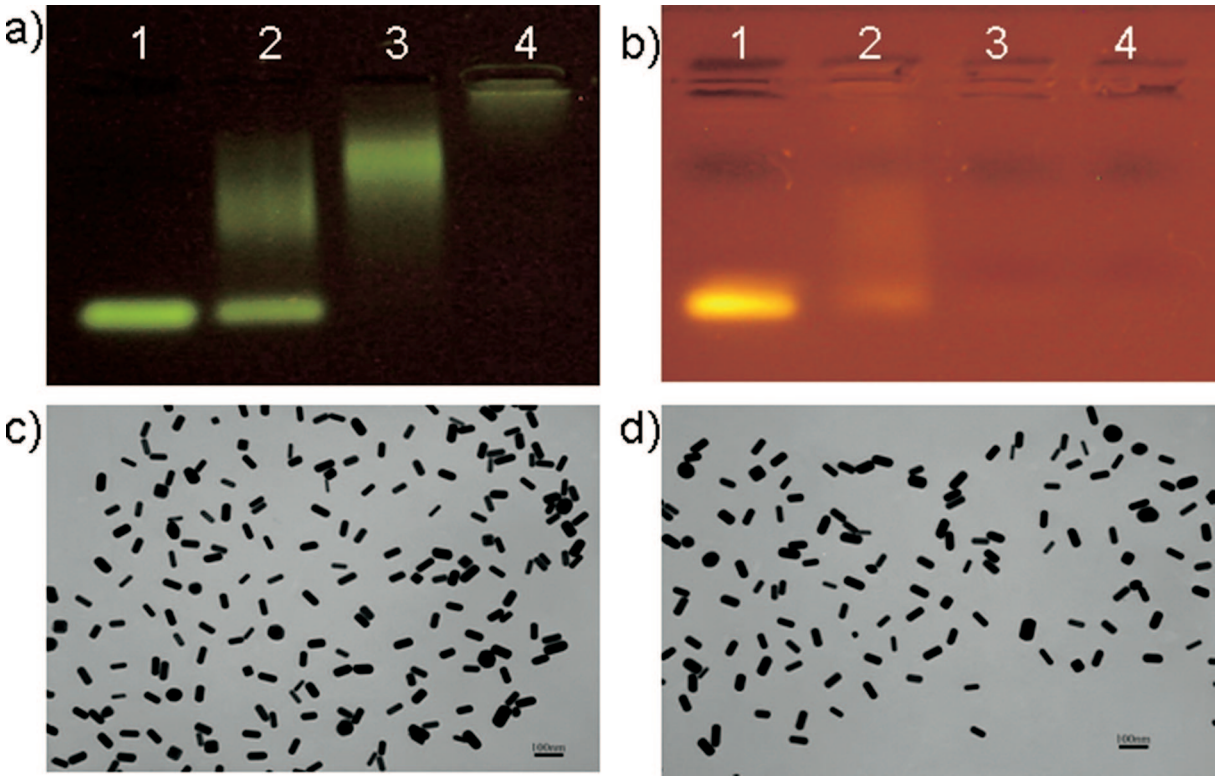

Fig. S2. Binding efficiency of siRNA^{F} (*a*) and siRNA^{D} (*b*) with GNR studied by agarose gel electrophoresis. Results show retarded mobility of the siRNA upon complexation with increasing amounts of GNRs. Lane 1, siRNA (50 pmols); lane 2, GNR-siRNA (250 ng GNR/50 pmols siRNA); lane 3, GNR-siRNA (500 ng GNR/50 pmols siRNA); lane 4, GNR-siRNA (750 ng GNR/50 pmols siRNA). TEM images of (*c*) free GNR and (*d*) GNR-siRNA nanoplexes, showing no sign of aggregation of GNRs upon complexation with siRNA.

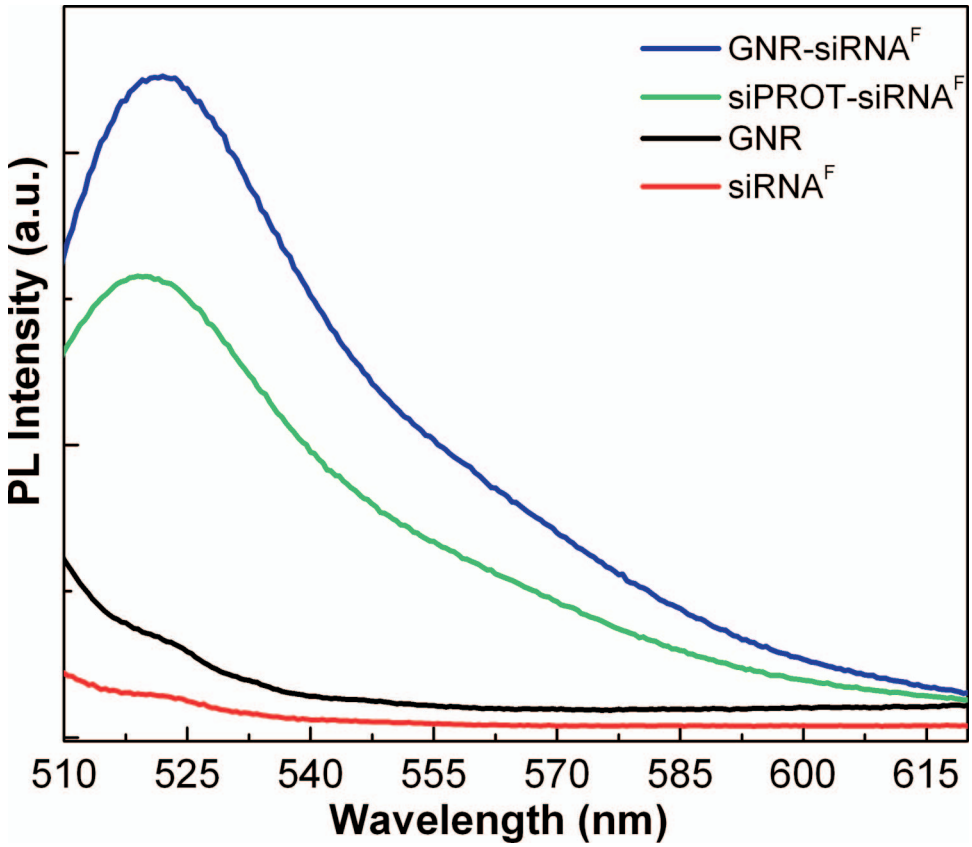

Fig. S3. Fluorescence spectra of siRNA^F taken from lysates of DAN cells after transfection using GNR-siRNA^F nanoplexes, SiPORT-siRNA^F complex, free siRNA^F, and GNRs only. Data show transfection with the GNR-siRNA^F nanoplexes result in the highest cellular uptake.

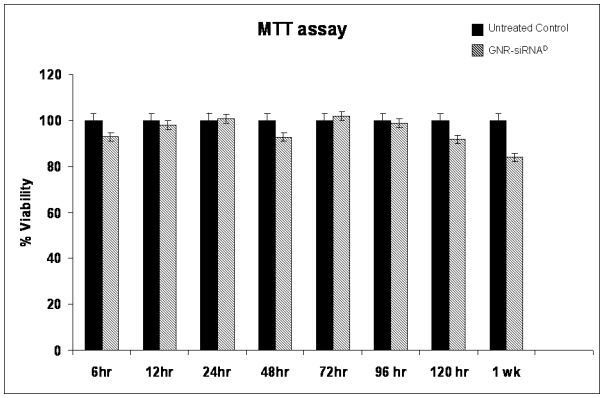

Fig. S4. Cell viability (MTT) assay of DAN cells following treatment with GNR-siRNA^{D} nanoplexes for various time points. Results show no indication of toxic effects on the cells following treatment with the nanoplexes, which were observed up to 1 week posttreatment. The results are the mean ± SD of 3 separate experiments.

Table S1. Transmigration measurement: Transendothelial electrical resistance (TEER) values of the BBB, from the BBB models, either untreated or treated with GNR-siRNA^{D} nanoplexes

	Pretreatment	Posttreatment
Untreated	215.67 ± 6.19	218 ± 3.51
GNR-siRNA^{D}	214 ± 2.52	211.33 ± 2.80

The similarity of these 2 values indicates that the functional integrity of the BBB remains unmodified upon treatment with the nanoplexes. Data are the mean ± SD of 3 measurements.