ARTICLES

Transvascular delivery of small interfering RNA to the central nervous system

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A major impediment in the treatment of neurological diseases is the presence of the blood–brain barrier, which precludes the entry of therapeutic molecules from blood to brain. Here we show that a short peptide derived from rabies virus glycoprotein (RVG) enables the transvascular delivery of small interfering RNA (siRNA) to the brain. This 29-amino-acid peptide specifically binds to the acetylcholine receptor expressed by neuronal cells. To enable siRNA binding, a chimaeric peptide was synthesized by adding nonamer arginine residues at the carboxy terminus of RVG. This RVG-9R peptide was able to bind and transduce siRNA to neuronal cells *in vitro*, resulting in efficient gene silencing. After intravenous injection into mice, RVG-9R delivered siRNA to the neuronal cells, resulting in specific gene silencing within the brain. Furthermore, intravenous treatment with RVG-9R-bound antiviral siRNA afforded robust protection against fatal viral encephalitis in mice. Repeated administration of RVG-9R-bound siRNA did not induce inflammatory cytokines or anti-peptide antibodies. Thus, RVG-9R provides a safe and noninvasive approach for the delivery of siRNA and potentially other therapeutic molecules across the blood–brain barrier.

The endothelial cells of brain capillaries form extremely tight junctions, providing a superfine filter that prevents the transport of most molecules from the vasculature into the brain parenchyma[1–3]. To overcome this, the conventional approach in gene therapy experiments has been to inject siRNA and viral or DNA vectors directly into the brain by stereotactic surgery (reviewed in refs 2, 3). However, these methods result only in localized delivery around the injection site, with no widespread effects within the brain; they are also too invasive for human therapy. If one could overcome the blood–brain barrier (BBB), intravenous administration would provide the ideal noninvasive means for delivery throughout the brain because of the rich vascularity of the brain, with capillaries encasing virtually every brain cell[3].

Because neurotropic viruses do cross the BBB to infect brain cells, we asked whether the strategy used by viruses to enter the central nervous system could also be used to enable delivery of siRNA to the brain. We chose rabies virus to test this hypothesis because it shows a high degree of neurotropism *in vivo* and the cellular entry mechanisms have been well characterized.

RVG pseudotyping confers neuronal cell specificity

RVG interacts specifically with the nicotinic acetylcholine receptor (AchR) on neuronal cells to enable viral entry into neuronal cells[4,5]. We therefore initially tested whether pseudotyping lentivirus with RVG, instead of the conventionally used vesicular stomatitis virus glycoprotein (VSV-G), could confer specificity for neuronal cells. Green fluorescent protein (GFP)-encoding lentiviral vector Lentilox pLL3.7 (ref. 6) pseudotyped with either RVG or VSV-G was tested for its ability to infect neuronal or non-neuronal cells. Whereas VSV-G pseudotyped lentivirus infected both cell types, RVG pseudotyping resulted exclusively in the infection of Neuro 2a cells, not HeLa cells (Supplementary Fig. S1a). Because RVG has been shown to mediate retrograde axonal transport and increase the spread of a viral vector within the brain[7], we also tested whether RVG pseudotyping of pLL3.7 encoding a short hairpin RNA (shFvE^J)[8] targeting Japanese encephalitis virus (JEV) increases its antiviral efficacy. Different concentrations of shFvE^J lentivirus, pseudotyped with RVG or VSV-G, were tested for protection efficacy in an intracranial JEV challenge assay[8]. Whereas at a high dose (2×10^5 transducing units) both lentiviruses afforded protection equally, at a lower dose (2×10^3 transducing units), all mice treated with RVG-pseudotyped lentivirus survived but all those treated with VSV-G-pseudotyped lentivirus succumbed to JEV infection (Supplementary Fig. S1b). Taken together, these results suggest that RVG confers neuronal cell specificity and in addition, by facilitating retro-axonal and trans-synaptic spread[7], enhances the transduction of neighbouring neuronal cells.

RVG peptide binds specifically to neuronal cells

The snake-venom toxin α-bungarotoxin (BTX) specifically binds to the AchR[9], and a short (29-residue) peptide derived from RVG competitively inhibits the binding of BTX to the AchR in solution[10]. We reasoned that this peptide might bind specifically to neuronal cells expressing the AchR. To detect binding, we synthesized a biotinylated RVG peptide or a control peptide of similar length derived from the rabies viral matrix protein (RV-MAT). When tested for cell binding, RVG peptide was found to bind to the AchR-expressing Neuro 2a cells[11,12] but not to the receptor-negative HeLa cells, whereas RV-MAT peptide bound to neither cell type (Fig. 1a). RVG peptide also did not bind several other non-neuronal cells tested (Fig. 1b). To confirm AchR-mediated binding specificity, we tested whether BTX could inhibit RVG peptide binding to Neuro 2a cells. Indeed, BTX inhibited RVG binding in a dose-dependent manner (Fig. 1c). Moreover, BTX was also able to displace prebound RVG from Neuro 2a cells (not shown). Next we tested whether RVG peptide could also specifically bind primary neuronal cells. Freshly isolated mouse brain cells but not splenocytes bound the RVG peptide, and neither cell

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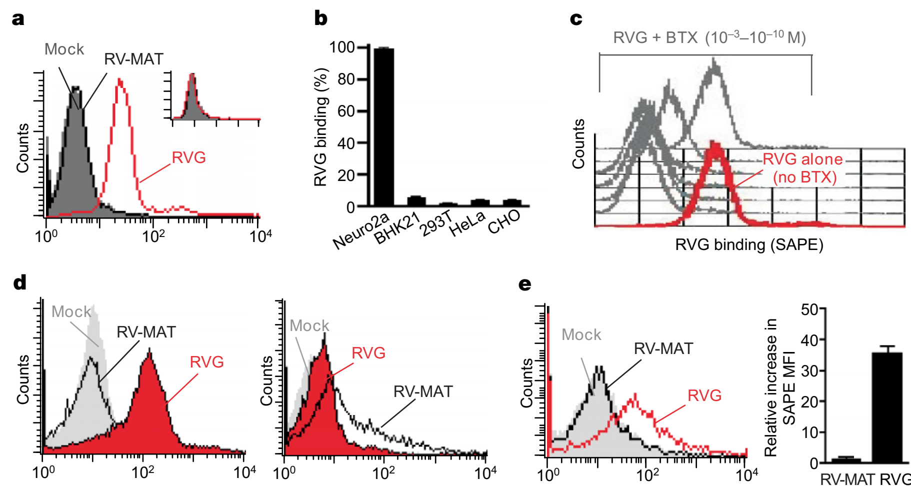

Figure 1 | A short RVG peptide binds to neuronal cells *in vitro* and *in vivo*. **a**, Neuro 2a and HeLa cells (inset) were incubated with biotinylated RVG or RV-MAT peptides, stained with SAPE and examined by flow cytometry. **b**, Peptide binding was also tested with the indicated cell lines in triplicate. Error bars indicate s.d. RV-MAT did not bind any of the cell lines (not shown). **c**, Neuro 2a cells were stained with biotinylated RVG in the absence (red histogram) or presence (grey histograms) of decreasing concentrations of BTX. **d**, Freshly isolated mouse brain (left) and spleen (right) cells were tested for peptide binding. **e**, Mice were injected intravenously with biotinylated RVG or RV-MAT peptide; 4 h later, isolated brain cells were stained with SAPE. Error bars indicate s.d. ($n = 6$).

type bound RV-MAT (Fig. 1d). Because AchR is widely expressed in the brain, including the endothelial cells of brain capillaries[13], we also examined the ability of RVG peptide injected intravenously to cross the BBB and enter brain cells. Mice were injected with biotinylated RVG or control RV-MAT peptides and, 4 h later, brain cell suspensions were examined by flow cytometry. As shown in Fig. 1e, brain cells from mice injected with RVG, but not those injected with RV-MAT peptide, were positive for peptide uptake, indicating that the RVG peptide might cross the BBB to enter brain cells.

RVG-9R peptide delivers siRNA to neuronal cells

Although RVG peptide can bind to neuronal cells, it does not bind nucleic acids and therefore cannot be used to transport siRNA. However, short, positively charged, cell-penetrating peptides bind negatively charged nucleic acids by charge interaction[14–16]. A nona(L-arginine) peptide was reported to be highly efficient in facilitating the cellular uptake of nucleic acids, and replacement of L-arginine with D-arginine (to form 9dR) enhanced the uptake even further[17]. Moreover, a cholesterol-conjugated oligo(D-arginine) has been used to deliver siRNA to a transplanted tumour in mice[18]. Thus, we tested whether RVG fused to 9dR could bind and deliver siRNAs to neuronal cells. For this we used RVG-spacer-9dR (designated RVG-9R) and control RV-MAT-spacer-9dR (RV-MAT-9R) chimaeric peptides. Both peptides were able to bind siRNA in a dose-dependent manner in a gel-shift assay (Fig. 2a). RVG-9R was also able to transduce fluorescein isothiocyanate (FITC)-labelled siRNA into neuronal cells in a dose-dependent manner and, in agreement with siRNA binding studies, a 1:10 molar ratio of siRNA to peptide was found optimal for maximal transduction (Fig. 2b). To determine the neuronal specificity of siRNA delivery, Neuro 2a and HeLa cells were transduced with FITC-siRNA complexed to RVG-9R or RV-MAT-9R, and Lipofectamine transfection was used as a positive control. Lipofectamine enabled siRNA uptake by both cells, and RV-MAT-9R was unable to transduce either cell type (Fig. 2c). In contrast, RVG-9R transduced Neuro 2a cells, but not HeLa cells, to a similar degree to Lipofectamine. Thus, RVG-9R allows neuronal cell-specific siRNA delivery.

Although RVG-9R could transduce siRNA to Neuro 2a cells in the above assay, siRNA is not functional unless it is delivered into the cytoplasm. Thus, we also assessed the gene-silencing ability of the siRNA delivered by RVG-9R. Neuro 2a cells stably expressing high levels of GFP were transduced with anti-GFP siRNA, bound to RVG-9R or RV-MAT-9R or transfected with siRNA by using Lipofectamine, and GFP expression was determined 2 days later. RV-MAT-9R-complexed siRNA was unable to decrease GFP levels, whereas RVG-9R/siRNA silenced GFP expression to a similar extent to Lipofectamine transfection (Fig. 2d), suggesting that the RVG-9R-delivered siRNA was indeed functional. The RVG-9R/siRNA

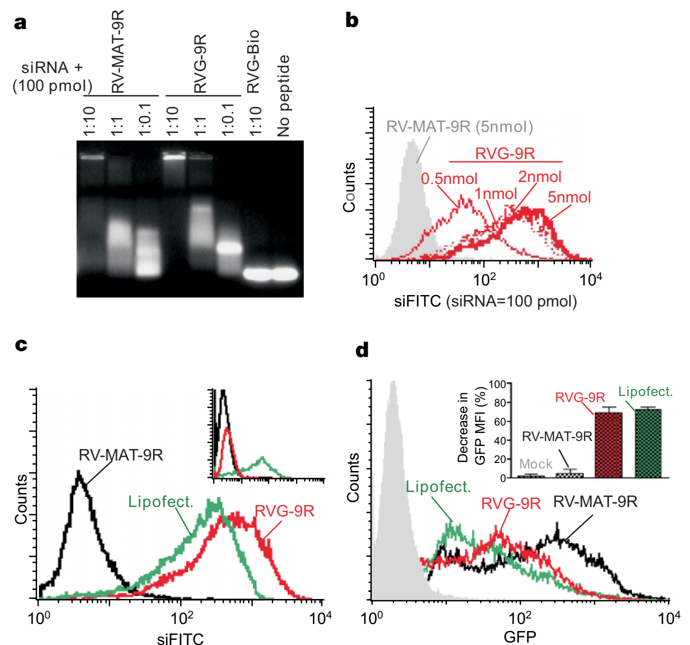

Figure 2 | RVG-9R peptide binds and delivers siRNA to neuronal cells *in vitro*, resulting in gene silencing. **a**, Mobility of free or peptide-complexed siRNA was analysed by agarose-gel electrophoresis. **b**, Neuro 2a cells were examined for uptake of FITC-siRNA complexed with RVG-9R at the indicated concentrations. **c**, Neuro 2a and HeLa (inset) cells were examined for uptake of FITC-siRNA complexed with RVG-9R or RV-MAT-9R peptides at a 1:10 molar ratio. Lipofectamine transfection (Lipofect.) was used as a positive control. **d**, Neuro 2a cells stably expressing GFP were transduced with GFP siRNA complexed with RVG-9R or RV-MAT-9R peptides, and GFP silencing was tested 2 days later. A representative histogram and cumulative data from three independent experiments (inset) are shown. The grey filled histogram represents Neuro 2a cells not expressing GFP. MFI, mean fluorescence intensity. Error bars indicate s.d.

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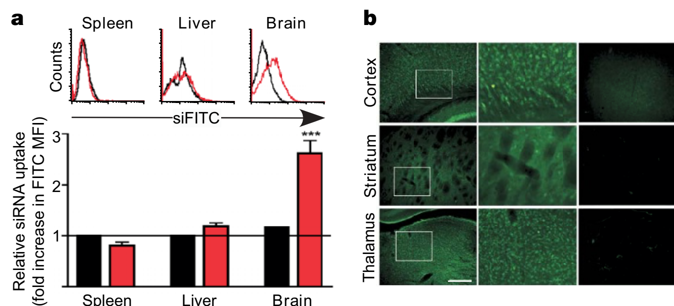

Figure 3 | RVG-9R enables transvascular delivery of siRNA to the central nervous system. a, Mice were injected intravenously with FITC-siRNA/peptide complexes, and uptake by brain, spleen and liver cells was examined by flow cytometry. Representative histograms (top) and cumulative data (bottom) are shown. Black, RV-MAT-9R; red, RVG-9R. Error bars indicate s.d. ($n = 4$). Three asterisks, $P = 0.001$. **b**, Coronal sections of brain from FITC-siRNA/RVG-9R-injected mice ($n = 6$) were stained with anti-FITC antibody and examined by fluorescence microscopy. Images of FITC-positive cells in the cortex, striatum and thalamus at lower magnifications (left panel) and higher magnifications of the boxed regions (middle panel) are shown. Right panel, images from control immunoglobulin-stained brain sections at the higher magnification. Scale bar, 200 μm.

complex was also found to be non-toxic in a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay (more than 90% viability at 48 h after treatment of Neuro 2a cells with RVG-9R at up to 25:1 peptide:siRNA ratio; data not shown).

RVG-9R enables transvascular siRNA delivery to the brain

For potential delivery *in vivo*, we first examined whether RVG-9R binding protects the siRNA against degradation from serum nucleases. Unlike naked siRNA, RVG-9R-bound siRNA was at least partly stable for up to 8 h (Supplementary Fig. S2). Next we tested whether RVG-9R could transport siRNA to brain cells *in vivo*. Mice were injected intravenously with FITC-siRNA complexed to RVG-9R or RV-MAT-9R; after 10 h, single-cell suspensions from the brain, spleen and liver were examined by flow cytometry. As shown in Fig. 3a, FITC fluorescence was detected in the brain only when the siRNA was complexed to RVG-9R. However, no FITC uptake was seen in the spleen or liver, suggesting that RVG-9R allows specific targeting of brain cells. The presence of FITC-positive cells in different regions throughout the mouse brain was also confirmed by microscopic examination of brain sections stained with anti-FITC antibody (Fig. 3b).

To test brain-specific gene silencing, GFP transgenic mice were injected intravenously with GFP siRNA bound to RVG-9R or RV-MAT-9R on three consecutive days; their brain, spleen and liver cells were examined for GFP expression 2 days after the last injection. GFP expression was much greater in the brain than in the spleen and liver in the transgenic mice. Despite this, a significant decrease in GFP expression was seen after treatment with RVG-9R-bound siRNA but not with RV-MAT-9R-bound siRNA (Fig. 4a). Moreover, GFP silencing was seen only in the brain and not in the liver or spleen, confirming the specificity of brain targeting. To confirm these results in a different system, we also targeted an endogenous gene. Wild-type Balb/c mice were injected intravenously with an siRNA targeting the mouse gene encoding Cu-Zn superoxide dismutase 1 (SOD1; ref. 19) complexed to RVG-9R or RV-MAT-9R, and mRNA and protein levels of SOD1 in the brain, spleen and liver were measured by quantitative polymerase chain reaction (PCR) and western blotting, respectively. Although no changes in SOD1 levels were detected in any organ in RV-MAT-9R/siRNA-treated animals, both messenger RNA and protein levels of SOD1 were significantly decreased in the brain, but not in other organs, in the RVG-9R/siRNA-treated mice (Fig. 4b).

To confirm that the observed knockdown was due to specific delivery of siRNA within the brain, we also tested for the presence of *SOD1* siRNA by northern blot analysis. siRNA was detected in the brain but not in the spleen or liver of treated mice (Fig. 4c). Both the gene silencing effect and siRNA detectability in the brain cells gradually decreased over a 9-day period (Fig. 4d and data not shown), in agreement with the duration of silencing reported after local administration of siRNA in the brain[20]. Repeated administration of RVG-9R/siRNA complex neither induced inflammatory cytokines nor elicited an anti-peptide antibody response (Supplementary Fig. S3), attesting to the viability of this delivery approach. Taken together, our results show that RVG-9R enables the intravenous delivery of siRNA to silence gene expression within the brain.

RVG-9R/siRNA treatment for viral encephalitis

We have reported that intracranial treatment with antiviral siRNAs can robustly protect mice from fatal flaviviral encephalitis[8]. However, a noninvasive intravenous treatment method would be optimal for

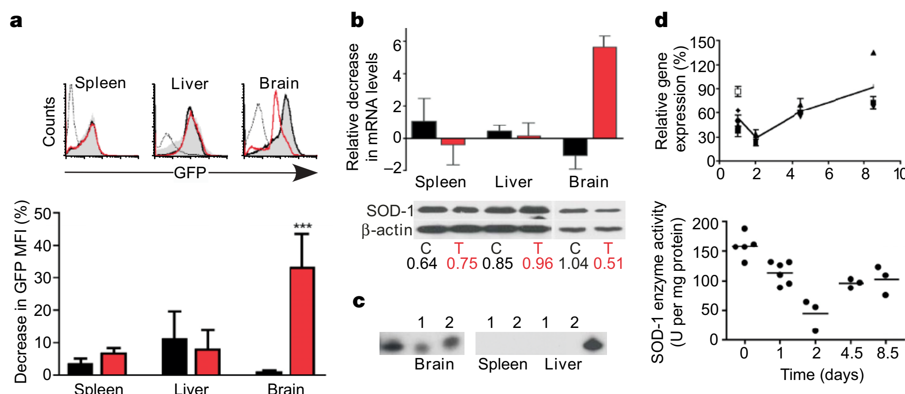

Figure 4 | Brain-specific gene silencing by intravenous injection of RVG-9R/siRNA complex. a, GFP transgenic mice were injected intravenously with GFP siRNA/peptide complexes, and their brain, spleen and liver cells were analysed for GFP expression. Representative histograms (top) and cumulative data (bottom) are shown. Error bars indicate s.d. ($n = 5$); asterisks, $P = 0.004$. Dotted lines in the upper panel, cells from wild-type mice; grey fill, mock; black lines and columns, RV-MAT-9R; red lines and columns, RVG-9R. **b**, Balb/c mice were injected intravenously with *SOD1* siRNA/peptide complexes, and their brain, spleen and livers were examined for *SOD1* mRNA (top) and SOD1 protein levels (bottom). Black columns, RV-MAT-9R (C); red columns, RVG-9R (T). Error bars indicate s.d. ($n = 3$). The numbers below the western blot represent the ratios of band intensities of SOD-1 normalized to that of β-actin. **c**, Small RNAs isolated from different organs of RVG-9R/*SOD1* siRNA-injected mice were probed with siRNA sense strand oligonucleotide. Antisense strand oligonucleotide was used as positive control (first and last lanes). **d**, Mice were injected intravenously with *SOD* siRNA bound to RVG-9R, and the duration of gene silencing was determined by quantification of *SOD1* mRNA levels (top) and SOD1 protein enzyme activity (bottom) on the indicated days after siRNA administration. Error bars in the upper panel indicate s.d. ($n = 4$); the horizontal lines in the lower panel represent mean values.

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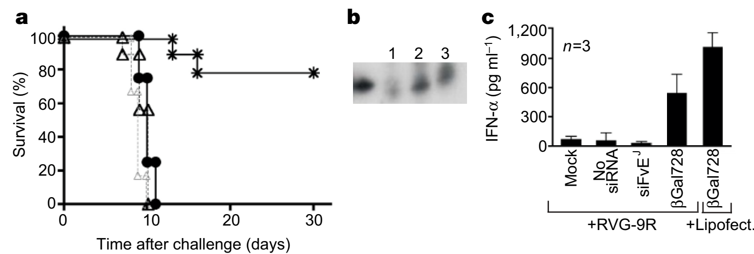

Figure 5 | Intravenous treatment with antiviral siRNA/RVG-9R complex protects mice against JEV encephalitis. a, JEV-infected mice were treated intravenously with siLuc or siFvE^J complexed to either RVG-9R or RV-MAT-9R daily for 4 days and monitored for survival. Grey triangles, mock (no siRNA); black triangles, RVG-9R plus siLuc; circles, RV-MAT-9R plus siFvE^J; stars, RVG-9R plus siFvE^J. $n = 9$. **b**, RNA isolated from the brains of RVG-9R/siFvE^J-treated mice were probed with siFvE^J sense strand to examine for the presence of siRNA antisense strand by northern blotting. Antisense strand of siFvE^J served as a positive control (first lane). **c**, Balb/c mice were injected intravenously with siFvE^J bound to RVG-9R peptide, and 7 h later their serum samples were tested for IFN-α levels by enzyme-linked immunosorbent assay. The immunostimulatory βgal 728 siRNA complexed with RVG-9R or Lipofectamine was used as a positive control. Error bars indicate s.d.

clinical use. We therefore tested whether intravenous treatment with siRNA bound to RVG-9R could protect mice from JEV-induced encephalitis. Unlike wild-type mice, immunodeficient mice are uniformly susceptible to peripheral infection with flaviviruses[21,22]. We therefore infected NOD/SCID mice with JEV (5 LD_{50}) intraperitoneally followed 4 h later by intravenous treatment with antiviral FvE^J siRNA (siFvE^J)[8] or an irrelevant luciferase siRNA (siLuc), complexed with RVG-9R or RV-MAT-9R. The siRNA treatment was repeated on three successive days and the mice were observed for survival for at least 30 days. Untreated mice and mice treated with siFvE^J complexed to RV-MAT-9R or with siLuc complexed to RVG-9R all died within 10 days, showing that neither the chimaeric peptides by themselves nor the irrelevant siRNA bound to RVG-9R affected the course of the disease. In contrast, treatment with siFvE^J complexed to RVG-9R resulted in about 80% survival (Fig. 5a). The presence of siFvE^J siRNA in the brains was also confirmed by northern blot analysis (Fig. 5b). To rule out the possibility that non-specific interferon (IFN) production mediated the protection observed, we measured serum IFN levels after administration of RVG-9R/siFvE^J. Although IFN levels were higher when mice were treated with a known immunostimulatory siRNA[23], IFN was not induced in RVG-9R/siFvE^J-treated animals (Fig. 5c), suggesting that the protection was mediated by RNA interference. Thus, intravenous treatment with RVG-9R/siRNA can be used for the treatment of viral encephalitis.

Discussion

Taken together, our results suggest that RVG-9R peptide may enable transvascular delivery of siRNA to the central nervous system. The relatively modest (about 50%) knockdown obtained is comparable to that reported after prolonged infusion of siRNA in the central nervous system[24,25]. However, many aspects of this delivery system could be refined to enhance the delivery efficacy. For instance, because RVG-9R-bound siRNA was only partly protected against degradation in the serum (Supplementary Fig. S2), the use of chemically stabilized siRNA[26] may enhance the efficacy of delivery. Moreover, encapsulation of even a stabilized siRNA within a liposomal nanoparticle greatly enhances serum half-life and bioavailability[27,28], and liposomal and polymeric nanopraticles coated with targeting ligands have been used for delivery in previous studies[29–31]. Thus, a combination of these methods to generate stabilized siRNA-encapsulated nanoparticles, coated with RVG peptide as a targeting ligand, may provide an ideal method to enhance delivery and decrease the requirement for siRNA and peptide for effective gene silencing. Moreover, RVG-coated nanoparticles may also provide a method for targeted brain delivery of other gene therapy vectors and small-molecule drugs. Direct conjugation of siRNA to the peptide[32] might be an alternative strategy to improve delivery.

Further studies to localize the presence of siRNA and gene silencing in different cell types within the brain are also needed to understand the mechanism by which RVG-9R enables delivery to the brain. However, because RVG peptide alone (without 9R) was also detectable in the brain after intravenous injection (Fig. 1e), it is likely that receptor-mediated transcytosis by means of the α7 subunit of the AchR (which is widely expressed in the brain, including by capillary endothelial cells[13]) is involved in the process. The fact that RVG-9R, but not RV-MAT-9R, facilitated crossing of the BBB also indicates that specific receptor binding might be important. Although cell-penetrating peptides might also enable covalently conjugated cargo to cross membranes[33,34], receptor clustering mediated by a 1:10 molar ratio of siRNA/RVG-9R binding may be required (particularly when the siRNA is non-covalently bound to the peptide) for efficient transport of siRNA to neuronal cells. This may explain the neuronal cell specificity of targeting by RVG-9R. Because RVG-9R-delivered siRNA was functional in gene silencing in multiple systems, siRNA seems to detach from the peptide inside the cell, although how exactly this happens is unclear. Similarly, siRNA complexed with protamine has been reported to be effective in gene silencing[35]. Thus, although further studies are needed to explain the exact mechanisms, our study highlights the potential of RVG-9R to mediate transvascular delivery of siRNAs to the central nervous system. RVG-mediated delivery might also allow the use of RNA interference for the systematic analysis of gene function in brain cells under experimental settings. In principle, RVG-assisted delivery might also be used for the brain-directed transport of other therapeutic molecules such as gene therapy vectors and small-molecule drugs.

METHODS SUMMARY

For peptide binding studies, cells were incubated for 20 min with biotinylated peptides, washed and then stained with streptavidin–phycoerythrin (SAPE). For all siRNA delivery studies, siRNA was incubated with peptides at a 1:10 molar ratio for 10–15 min at room temperature (20 °C) in serum-free DMEM medium (for *in vitro* studies) or 5% glucose (for *in vivo* studies) before use. For all *in vivo* delivery experiments, mice were injected into the tail vein with siRNA/peptide complexes in 100–200 μl of 5% glucose, and the mice received 50 μg of siRNA in each injection. All statistical analyses comparing groups of mice treated with test and control peptides were performed by one-way analysis of variance followed by Bonferroni's post hoc test. $P < 0.05$ was considered significant.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

Lentiviral experiments. shFvE[J] and shLuc lentiviral constructs and experiments to test protection against intracranial JEV challenge in mice have been reported previously[8]. Lentiviruses pseudotyped with VSV-G or RVG were generated by transfection of the lentiviral vector plasmids together with the helper plasmid pHR′8.9ΔVPR (core protein) and either the pVSV-G or pLTR-RVG envelope constructs into 293T cells. Culture supernatants were harvested after 48 h, and viral particles were concentrated by ultracentrifugation. Lentiviruses were spin-infected onto Neuro 2a or HeLa cells in the presence of Polybrene, and after 48 h the transduction efficiency was determined by analysing GFP expression by flow cytometry.

Peptides and siRNAs. Peptides RVG (YTIWMPENPRPGTPCDIFTNSRGK-RASNG), RV-MAT (MNLLRKIVKNRRDEDTQKSSPASAPLDDG), RVG-9R (YTIWMPENPRPGTPCDIFTNSRGKRASNGGGGRRRRRRRRR) and RV-MAT-9R (MNLLRKIVKNRRDEDTQKSSPASAPLDGGGGRRRRRRRRR) were synthesized and purified by high-performance liquid chromatography at the Tufts University Core Facility. RVG and RV-MAT peptides were also biotinylated at the carboxy terminus. In RVG-9R and RV-MAT 9R peptides, the C-terminal nine arginine residues were D-arginine.

siRNAs used in the studies included those targeting GFP (siGFP), firefly luciferase (siLuc), the envelope gene of JEV (siFvE[J]) described in ref. 8 and those targeting murine Cu-Zn superoxide dismutase (SOD-1)[19], and β-galactosidase (βGal728) bearing a motif eliciting interferon production[23]. For some experiments, siRNA with FITC label at the 3′ end of the sense strand was used. siRNAs were synthesized at Samchully Pharm. Co. Ltd or obtained from Dharmacon, Inc.

Peptide binding assay. For peptide binding studies, Neuro 2a, HeLa, CHO, 293T and BHK21 cell lines and single-cell suspensions made from freshly isolated mouse brain, spleen or liver were used. Cells were incubated in PBS with 2.5 μM biotinylated peptides for 20 min at 4 °C, washed three times with PBS and then treated with SAPE (BD Pharmingen) before analysis by flow cytometry. For competition experiments, cells were incubated with 2.5 μM biotinylated RVG peptide in the absence or presence of different concentrations of BTX (Sigma).

EMSA. For gel mobility-shift assays, 100 pmol of siRNA was incubated with peptides at 10:1, 1:1 and 1:10 molar ratios of siRNA to peptide for 15 min, subjected to electrophoresis on 2% agarose gels and stained with ethidium bromide. siRNA without peptide or incubated with biotinylated RVG (without 9R) served as controls.

Cytotoxicity assay. To test the cytotoxicity of RVG-9R/siRNA complexes, Neuro 2a cells (triplicates of 2×10^5 cells per well in 12-well plates) were incubated with different concentrations of peptide/siRNA complexes for 24–48 h before viability was determined with a standard MTT assay.

siRNA transduction and gene silencing *in vitro*. Uptake of siRNA into cells was monitored with FITC-labelled siLuc. siRNA (100 pmol) was incubated with different concentrations of RVG-9R or RV-MAT-9R in serum-free DMEM for 15 min at room temperature. The complexes were then added to Neuro 2a and HeLa cell cultures (plated at 5×10^4 cells per well in 12-well plates on the previous day). After incubation for 4 h at 37 °C the medium was replaced with 2 ml of fresh medium supplemented with 10% fetal bovine serum (Invitrogen) and the cells were cultured for a further 8–10 h before being examined by flow cytometry. Transfection with Lipofectamine 2000 was performed in accordance with the manufacturer's instructions.

To test gene silencing, Neuro 2a cells stably expressing GFP after transduction with the pLL3.7 lentiviral vector were incubated with 100 pmol of siGFP complexed with peptides at a 10:1 peptide/siRNA ratio and GFP expression analysed 48 h after transduction.

Animal experiments for testing siRNA delivery and gene silencing. Balb/c, C57BL/6-Tg(ACTB-EGFP)1Osb/J and NOD/SCID mice were purchased from Jackson Laboratories and used at 4–6 weeks of age. All mouse experiments had been approved by the CBR Institute (CBRI) institutional review board, and animal infection experiments were performed in a biosafety level 3 animal facility at the CBRI.

To test peptide uptake by brain cells, 200 μg of biotinylated peptides in 0.2 ml of PBS were injected into tail veins of Balb/c mice; 4 h later, single-cell suspensions of brains were permeabilized, treated with SAPE and analysed by flow cytometry. For all siRNA delivery experiments, peptide/siRNA complexes (at a peptide to siRNA molar ratio of 10:1) were prepared in 100–200 μl of 5% glucose and injected intravenously at 50 μg of siRNA per mouse per injection. To test FITC-siRNA delivery, Balb/c mice were injected twice with siRNA, 6 h apart, and organs were harvested after a further 10 h. To test GFP silencing, C57BL/6-Tg(ACTB-EGFP)1Osb/J mice were injected with peptide/siRNA complexes on three consecutive days and organs were harvested 2 days later. For SOD-1 silencing, Balb/c mice were given three injections of siRNA/peptides at 8-h intervals and organs were harvested at various time points. For testing protection against JEV encephalitis, NOD/SCID mice were challenged intraperitoneally with 5 LD_{50} of JEV (LD_{50}, the lethal dose for half of the mice, was predetermined by using serial dilutions of the virus) 4 h before treatment with intravenous peptide/siRNA was started. The siRNA treatment was repeated at 24-h intervals for a total of 4 days.

Staining of brain sections. Mice were injected twice with RVG-9R-bound siRNA-FITC; brains were harvested 10–12 h later. Brains were sectioned frozen on a sliding microtome to a thickness of 40 μm and incubated for 48 h at 4 °C with mouse anti-FITC antibodies (20 μg ml^{-1}; Jackson Immuno Research) or isotype controls (IgG1 from murine myeloma; 20 μg ml^{-1}; Sigma). Sections were washed and FITC immunoreactivity was detected with Alexa-488 goat anti-mouse secondary antibodies (dilution 1:500; Invitrogen). The antibody enhancement was performed because only one in six mice revealed FITC-positive cells by direct examination.

Quantitative RT–PCR. Total RNA was isolated from different organs of peptide/SOD1 siRNA-treated mice by using an RNeasy RNA isolation kit (Qiagen). The RNA was reverse transcribed with Superscript III and random hexamers (Invitrogen) in accordance with the manufacturer's protocol. Real-time PCR was performed on 1 μl of complementary DNA, or on a comparable amount of RNA without reverse transcriptase, with the QuantiTect SYBR Green PCR kit (Qiagen) in accordance with the manufacturer's instructions. Amplification conditions were as follows: 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s with a Bio-Rad iCycler. Primers for GAPDH and SOD-1 have been described previously[36]. Specificity was verified by melt-curve analysis and agarose-gel electrophoresis. SOD-1 mRNA levels from the test animals, normalized with glyceraldehyde-3-phosphate dehydrogenase levels, were divided by the equivalent values from untreated mice to calculate relative changes.

Western blot analysis. Cell suspensions from mouse tissue were homogenized in buffer containing 25 mM HEPES pH 7.5, 300 mM NaCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM EDTA and 0.5 mM dithiothreitol and protease-inhibitor cocktail (Complete-Mini; Roche Diagnostic). The samples (10 μg of protein each) were subjected to electrophoresis on 15% SDS–polyacrylamide mini gels (Bio-Rad) and transferred to a poly(vinylidene difluoride) membrane. The membrane was probed with anti-β-actin antibodies (Sigma) or anti-SOD1 antibodies (Stressgen Biotechnologies) and detected with an enhanced chemiluminescence western blot system (Pierce Biotechnologies). The blots were scanned and the ratio of band intensities of SOD-1 normalized to β-actin was calculated with Image J software.

Determination of SOD1 enzyme activity. The level of Cu/Zn SOD-1 enzyme activity in brain tissue was measured with the SOD-1 Assay Kit-WST (Cell Technologies, Inc.) in accordance with the manufacturer's instructions. Frozen brain tissues were homogenized in ice-cold sucrose buffer (50 mM sucrose, 200 mM mannitol, 1 mM EDTA in 10 mM Tris-HCl buffer pH 7.4) and used in ELISA after inactivation of Mn/Fe SOD-1 with chloroform/ethanol. The enzyme activity is denoted as units per milligram of total protein in the brain tissue.

Northern blot to detect siRNA. Small RNA (5 μg) extracted from cell suspensions with an miRNeasy mini kit (Qiagen) were subjected to electrophoresis on 15% TBE-urea PAGE gels (Invitrogen), transferred to a positively charged nylon membrane (BrightStar-plus; Ambion) and probed with sense siRNA probes as described previously[8]. Antisense strand of synthetic SOD-1 siRNA (200 fmol) (first and last lanes in Fig. 4c) was used as a positive control.

IFN response. Balb/c mice were injected intravenously with 50 μg of either siFvE[J] or siβGal728 complexed with RVG-9R peptide. siβGal728 complexed to Lipofectamine-2000 served as a positive control. Serum samples obtained 7 h after siRNA treatment were tested for IFN-α levels with a mouse type-I IFN detection ELISA kit (PBL Biomedical Laboratories), in accordance with the manufacturer's instructions.

Serum stability. Naked and RVG-9R-complexed siRNA (100 pmol) were incubated at 37 °C in 50% FBS (Invitrogen), 90% human serum or 90% mouse serum. Aliquots taken at different time points were treated with Proteinase K and frozen in 2 × urea TBE-loading buffer. All samples were subjected to electrophoresis on 15% TBE-urea polyacrylamide gels under non-denaturing conditions and detected by staining with SYBR gold.

Immunogenicity studies. Balb/c mice were injected intravenously with 50 μg of siLuc complexed to RVG-9R peptide or, for positive control, with 25 μg of trinitrophenol-conjugated keyhole-limpet haemocyanin-biotin (TNP-KLH-biotin) peptide (Biosearch Technologies). The injection was repeated on days 3, 10 and 22, and serum samples were collected on days 21 and 30. To detect the presence of mouse antibodies to RVG or biotin (in the TNP-KLH-biotin-injected mice), 1:10 and 1:50 dilutions of sera were incubated in 96-well microtitre plates coated with biotinylated RVG peptide (1 μg per well). The presence of binding mouse antibodies was detected with anti-mouse immunoglobulins conjugated to

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horseradish peroxidase. A mouse cytokine/chemokine array kit (Ray Biotech Inc.) was used to detect a panel of 56 secreted cytokines and chemokines in the serum obtained 1 day after the fourth injection of RVG-9R/siRNA. For positive control, mice were injected with 50 μg of lipopolysaccharide and serum was collected after 15 h. The manufacturer's recommended protocol was used to perform the assay.

Quantification and statistical analysis. Western blots and cytokine array profiles were quantified by determining band intensities with ImageJ public domain software from the National Institutes of Health (http://rsb.info.nih.gov/ij/).

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