SUPPLEMENTAL FIGURES

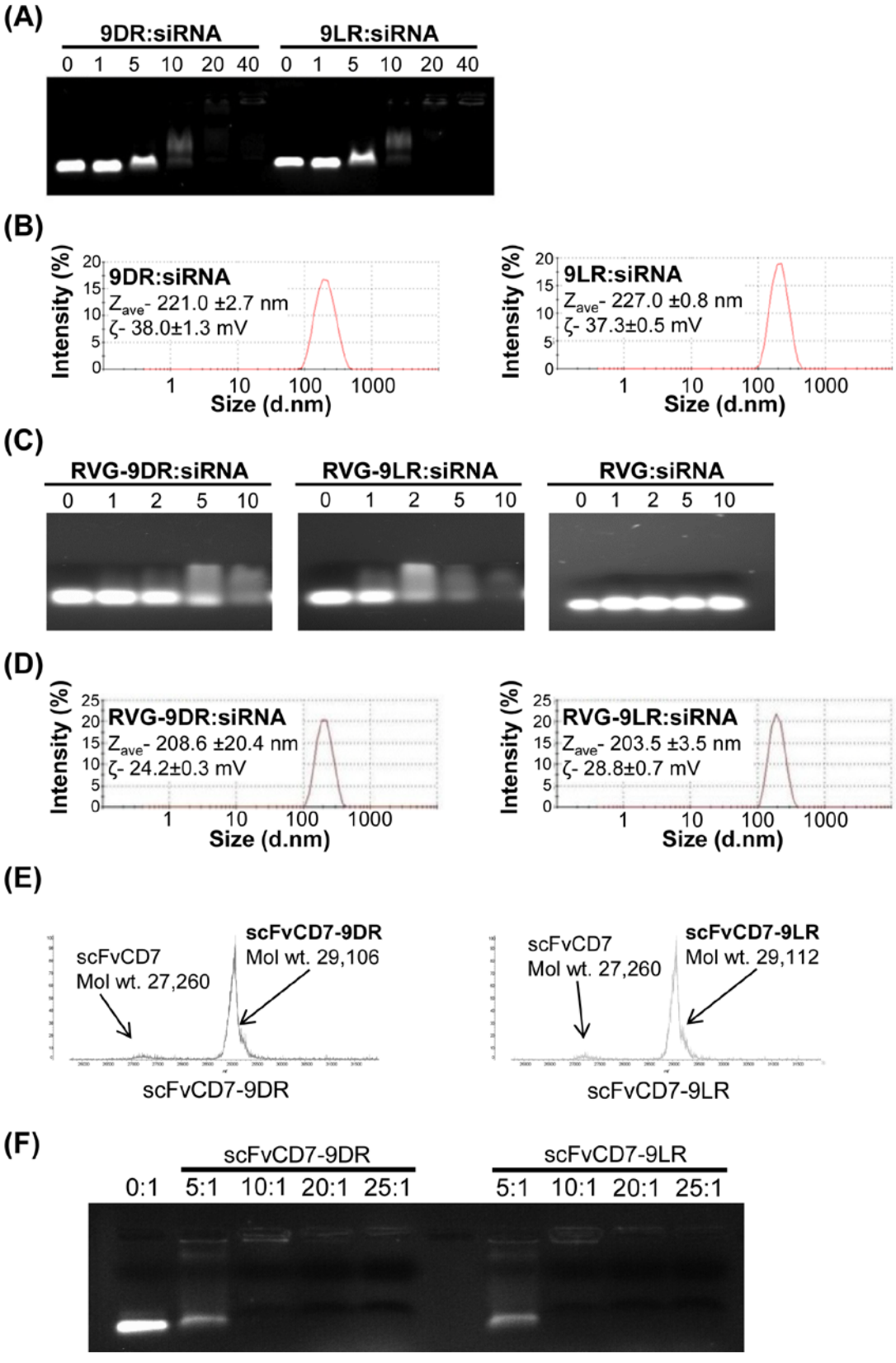

Figure S1, related to Figures 1 and 2. 9R and RVG-9R bind siRNA to form nanoparticles.

Electrophoretic gel mobility shift assay of 100pmol siRNA complexed to 9D/LR (A), RVG-9D/LR (C) and scFvCD7-9D/LR (F) at the indicated molar excess of peptide/antibody. Note: the RVG peptide by itself does not bind siRNA in (C). (B/D) Average particle diametric size distribution in nanometer (Z_{Ave}) and surface charge distribution (ζ) of peptide-siRNA complexes (100 pmol siRNA, 10:1 peptide:siRNA) measured by dynamic light scattering. (E) MALDI-TOF MS spectrum of scFvCD7-9D/LR conjugates.

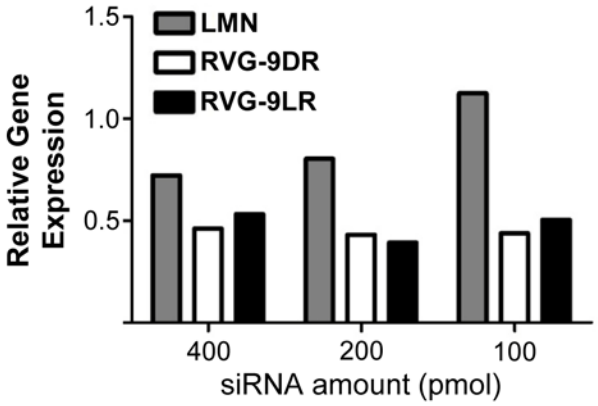

Figure S2, related to Figure 2. Functional siRNA delivery by RVG9R. QPCR analysis of SOD-1 mRNA levels in Neuro2a cells 4h after exposure to siRNA complexes (100 pmol siRNA, 10:1 peptide:siRNA).

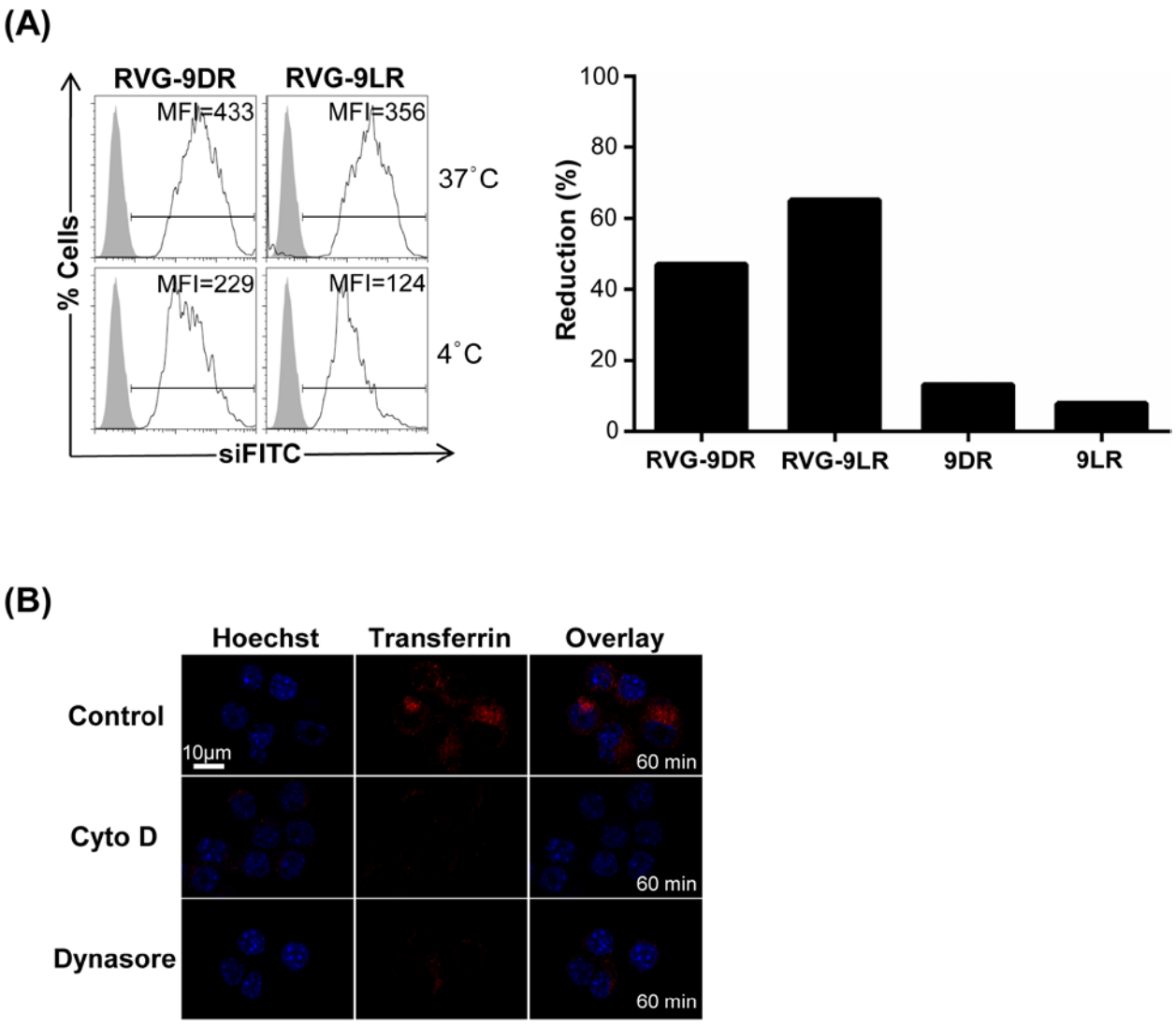

Figure S3, related to Figure 3. Analysis of cellular uptake mechanisms of RVG-9R:siRNA.

(A) Flow cytometric analysis of Neuro2a cells exposed to RVG9D/LR:siFITC (10:1 peptide:siRNA molar ratio) after incubation at the indicated temperatures. The filled histograms correspond to cells transfected with siFITC alone and the values represent MFIs of the open histograms. The graph on the right is a quantitation of reduction in siRNA uptake measured as percent fold reduction in MFIs at 4°C compared to that at 37°C. (B) Confocal images of Neuro2a cells demonstrating pretreatment with inhibitors cytochalasin-D or dynasore inhibits internalization of A_{568}-labeled transferrin.

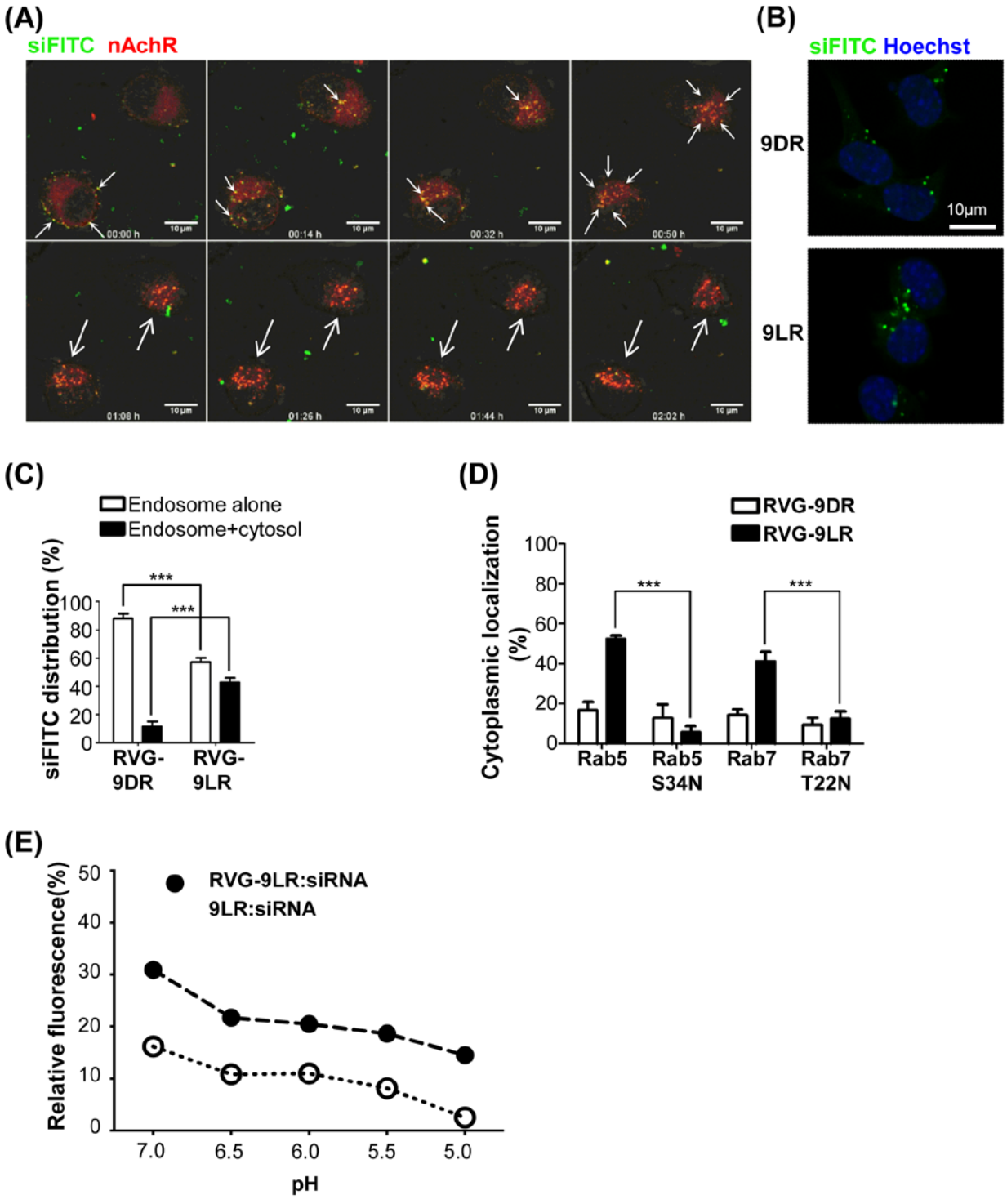
(A)
siFITC nAchR
00:00 h
00:14 h
00:32 h
00:50 h
01:08 h
01:26 h
01:44 h
02:02 h
10 μm
(B)
siFITC Hoechst
9DR
9LR
10μm
(C)
Endosome alone
Endosome+cytosol
siFITC distribution (%)
***
***
RVG-9DR
RVG-9LR
(D)
RVG-9DR
RVG-9LR
Cytoplasmic localization (%)
***
***
Rab5
Rab5 S34N
Rab7
Rab7 T22N
(E)
RVG-9LR:siRNA
9LR:siRNA
Relative fluorescence(%)
pH

Figure S4, related to Figure 5 and Video S6. (A) Time-lapse images of Neuro2A cells co-expressing α6-mCherry, α4 and β2 subunits of nAchR pre-incubated in medium containing siFITC at the indicated times after exposure to RVG-9LR. Fluorescent and DIC images in the same fields were merged. Arrows indicate site of peptide:siRNA binding and receptor aggregation. (B) Confocal microscopy of Neuro2a cells 24h after incubation with 9D/LR:siFITC complexes. Nuclei are stained with Hoechst 33342 (blue). (C and D) Analysis of intracellular fluor distribution in Neuro2A cells in Figures 5A and 5B at 24h. Data shown is cumulative of 3 independent experiments and represents cell numbers as percentages calculated with a sample size of ~50 cells. Error bars depict SEM. ***P<0.001. (E) Ethidium bromide (EtBr) exclusion assay conducted with peptide:siRNA complexes (400 pmol siRNA, 2.5:1 peptide:siRNA ratio) in phosphate buffers at the indicated pH values. The plot shows the amount of free siRNA (not complexed with peptides) detected in solution using EtBr. The fluorescence value for free siRNA without peptide at each pH value tested was set to 100%. Note: The 9LR peptide associates more strongly with siRNA than RVG-9LR suggesting poor availability to interact with cellular membranes. This was also reflected in the observation that ligand attachment lowered the average zeta potential of siRNA complexes suggesting a weaker association of siRNA in the ligand-9R format (Figure S1). Reducing the pH to 5.0 increased association of RVG-9LR with siRNA making it comparable to 9LR at pH 6.5 in this assay. Thus, under low pH conditions in late endosomes, RVG-9R-siRNA complex may need complex destabilization by action of endosomal proteases to promote membrane interaction and translocation of siRNA into the cytosol.

SUPPLEMENTARY VIDEOS

Supplementary Videos S1-S4, related to Figure 3. RVG-9R induces rapid cytosolic translocation of siRNA. Representative time-lapse videos of Neuro2a cells incubated with RVG9DR:siFITC (Movie S1), RVG9LR:siFITC (Movie S2), 9DR:siFITC (Movie S3) and 9LR:siFITC (Movie S4) complexes. LysoTracker (red) was used to label late endosomes and a 10:1 peptide:siRNA ratio was used.

Supplementary Video S5, related to Figure 4. RVG-9LR binding induces topical membrane inversion. Representative time-lapse videos of Neuro2A cells pre-incubated with A_{568}-labeled annexin V and siFITC followed by exposure to RVG-9LR. Upper left and right panels show fluorescent images of RVG-9LR:siFITC and A_{568}-labeled annexin V in the same fields respectively. The lower left and right panels are merged images of fluorescence and DIC images in the same fields.

Supplementary Video S6, related to Figures 5 and S4. RVG-9LR binding induces receptor aggregation. Representative time-lapse videos of Neuro2A cells co-expressing α6-mCherry, α4 and β2 subunits of nAchR pre-incubated in medium containing siFITC and then exposed to RVG-9LR. Upper left and right panels show fluorescent images of RVG-9LR:siFITC and mCherry-fused nAchR in the same fields respectively. The lower left and right panels are merged images of fluorescence and DIC images in the same fields.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Peptides and siRNA

Purified 9R peptides were purchased from Anaspec (Freemont, CA). HPLC-purified Rabies virus glycopeptide (Kumar et al., 2007) chimeric with 9DR and 9/LR (RVG-9D/LR) were synthesized at the Tufts University Peptide Synthesis Core Facility (Medford, MA). RVG was synthesized with L-amino acids while the 9 arginine residues were either the D or L isoforms siGFP, siLuc, siCD4 and siSOD-1 were purchased from Dharmacon, Thermofisher Scientific and have been described previously (Kumar, et al., 2008; Kumar, et al., 2007). siRNA uptake experiments used FITC- or Cy5-labeled siLuc (siFITC, siCy5).

Generation of 9R conjugates

9D/LR were labeled at the N-terminus using the Alexa Fluor® 488 5-SDP ester kit (Invitrogen). For 9R-GFP conjugates, 9D/LR peptides were C-terminally conjugated to recombinant purified GFP with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and sulfo-hydroxysuccinimide (NHS) in MES buffer, pH 5.0 using the standard protocol. Antibody-9R conjugates were generated as previously described (Kumar, et al., 2008).

Peptide uptake experiments

Mouse neuroblastoma Neuro2a cells at 5×10^4 cells per well in 24 well plates were incubated with A488 fluor-labeled or GFP-conjugated 9D/LR peptides at 37°C in DMEM/10%FBS. Cells were washed, fixed with 4% paraformaldehyde and analyzed using a BD FACS Calibur flow cytometer.

siRNA transfection experiments

siRNA uptake was assessed using FITC- or Cy5-labeled siLuc (siFITC, siCy5). To make siRNA complexes, 9D/LR or RVG-9D/LR was incubated with siRNA (100 pmol) in serum-free DMEM at molar ratios ranging between 5 and 25 for 20 min at room temperature. The complexes were then added to Neuro2a cells in DMEM/10%FBS plated as above in a total volume of 0.5ml yielding final peptide concentrations between 1 and 5μM. Experiments with scFvCD7-9D/LR similarly used human Jurkat T cells plated in a 200μl volume RPMI/10%FBS at 5×10^4 per well in 96-well plates. Carrier to siRNA ratio was maintained at 10:1 resulting in a final concentration of 5μM scFvCD7-9D/LR in culture media in transfection experiments. For experiments of >1h duration, the medium was refreshed after 1h exposure to peptide:siRNA complexes and cells cultured at 37°C for the indicated periods of time. Transfections with Lipofectamine 2000 (Invitrogen) were performed using manufacturer's instructions. Some experiments involved incubation at 4°C for 20 minutes with peptide:siRNA complexes prior to flow cytometric analysis. For inhibition of actin/dynamin dependent pathways, Neuro2a cells were pre-treated for 1h with 5μM Cytochalasin D (Sigma) or 80μM Dynasore (Sigma). For inhibition of endosomal proteolysis, Neuro2a cells were pre-treated for 1h with 40μM E-64d (Sigma) and peptide:siRNA complexes was internalized and chased in media containing E-64d for the duration of the experiment. For blocking nAchR, Neuro2a cells were pre-treated for 30 min on ice with 10^{-5}M A_{568}- labeled α-bungarotoxin prior to addition of peptide:siRNA complexes. Mammalian expression plasmids encoding (i) wild-type Rab5, Rab7, Rab5(S34N) and Rab7(T22N) fused in frame to the enhanced green fluorescent protein (EGFP) coding sequence and (ii) α4, β2 and α6 (the latter fused to mCherry) were transfected into Neuro2a cells using Lipofectamine 2000 48h prior to treatment with RVG-9R:siRNA complexes.

To test gene silencing, Neuro2a cells stably expressing GFP were incubated with 9R:siRNA or ligand-9R:siRNA complexes as described above and analyzed 72 h later by flow cytometry. For qPCR, mRNA was harvested from treated cells at the indicated time points using the Nucleospin RNA II purification kit (Machery-Nagel), reverse transcribed with Verso cDNA kit (Thermo Scientific) and quantified with the SYBR Fast qPCR kit (Kapa Biosystems) on the 7500 Fast Real-Time PCR system (Applied Biosciences). Murine SOD1 and human CD4 mRNA levels, quantified with the appropriate primer sets (Kumar, et al., 2008; Kumar, et al., 2007) were normalized to murine GAPDH and human beta-actin respectively. In all cases, relative gene expression was calculated in comparison to normalized target mRNA levels in cells treated with siRNA alone in the absence of transfecting peptide or Lipofectamine.

Size and surface charge measurements

Particle Z-average size and charge were measured with Malvern's Zetasizer Nano ZS (Malvern instruments, Worcestershire, UK). Peptide:siRNA complexes were prepared using 9D/LR or RVG-9D/LR and 100 pmol siRNA to attain a 10:1 molar ratio in filtered DI water for 20 min. The resulting mixtures were made up to 1 ml with 0.2 μm filtered deionized water. Five readings of Z-average size (nm), polydispersity (25°C, measurement angle 170°) and zeta potential (mV, 25°C, measurement angle 12.8°) were taken. For data analysis, the viscosity (0.8872 mPa.s) and refractive index (1.33) of water were used to determine Z-average size.

Confocal Microscopy

Neuro2a cells seeded and cultured in complete medium on poly-L-lysine (Sigma-Aldrich) treated coverslips were incubated with 9D/LR-GFP (1μM) or peptide:siRNA complexes (300pmol siRNA, final concentration- 3μM, 10:1 molar ratio). Confocal microscopy was performed in most cases, without washing or fixing, using Volocity spinning disc Nikon

TE2000 confocal microscope equipped with an environmental chamber (LIVE CELL; Pathology Devices) and a Nikon Perfect Focus with a 60X Nikon objective (ND 1.4) or a Leica TCS SP5 Spectral Confocal Microscope at 63X magnification. Hoechst 33342 was used to distinguish nuclei, LysoTracker Red to stain late endosomal vesicles. For experiments visualizing membrane-interactions of ligand-9R:siRNA complexes, siFITC (300 pmol, final concentration- 3μM) and/or $Alexa_{568}$-labeled annexin V was first added to Neuro2A cells followed by addition of RVG-9LR (10:1 peptide:siRNA ratio). Early endosomes were detected in cells fixed with 4% paraformaldehyde by staining with rabbit anti-EEA1 and mouse anti-rabbit FITC (green) antibodies. All images were processed using Volocity 6.2.1 software (Improvision/PerkinElmer). Quantification of image intensities was performed with Image J 1.49b software.

Visualization of Annexin V binding

For experiments visualizing membrane-interactions of ligand-9R:siRNA complexes, siFITC (300 pmol, final concentration- 3μM) and/or $Alexa_{568}$-labeled annexin V (Cat# A13202, Life Technologies) at 1/50 vol/vol was first added to Neuro2A cells followed by addition of RVG-9LR (a final of a 10:1 peptide:siRNA ratio) and cells imaged by live confocal imaging as above.

For cytometric analysis, cells in complete medium were treated with preformed peptide:siRNA complexes for the indicated periods of time and Annexin V added at 1/50 vol/vol for the last 30 minutes of incubation at 37°C.

Electrophoretic gel mobility shift assays (EMSA)

100pmol siRNA was incubated with RVG/scFvCD7-9R at the indicated molar ratios for 20 min. siRNA complexes were electrophoresed on 2% agarose gels with ethidium bromide.

Ribonuclease A and serum protection assays

Peptide:siRNA complexes (100 pmol siRNA, 10:1 ratio) were incubated at 37°C in 100ng RNAse A or 50% human AB serum (not heat inactivated) contained in DMEM. Timed aliquots were treated with proteinase K (50μg/ml, 10min at 25°C) and RNA extracted with phenol chloroform-isoamyl alcohol (25:24:1 v/v). All samples were subjected to electrophoresis on a 2% agarose gel with ethidium bromide. Band intensities estimated using the Image J software.

Ethidium Bromide Exclusion Assay

400 pmol siRNA complexed to RVG-9LR or 9LR peptides (at a 2.5 molar excess) was added to phosphate buffers of pH 5.0, 5.5, 6.0, 6.5 and 7.0 containing 1.1 μg/ml ethidium bromide and the free siRNA, not involved in complexation estimated by fluorescence measurements in a fluorometer (Molecular Devices) at wavelengths set for emission 531nm/ excitation 590 nm. Controls included solutions with no siRNA (0%) and siRNA alone (100%).

Statistical Analysis

Results are presented as mean ± SD or mean ± SEM as indicated. Statistical significance was calculated using data from three independent experiments using one-way ANOVA with Bonferroni's multiple-comparison test for pairwise comparisons within groups. Otherwise, comparisons were made using a two-tailed t-test.