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Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes

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To realize the therapeutic potential of RNA drugs, efficient, tissue-specific and nonimmunogenic delivery technologies must be developed. Here we show that exosomes—endogenous nano-vesicles that transport RNAs and proteins[1,2]—can deliver short interfering (si)RNA to the brain in mice. To reduce immunogenicity, we used self-derived dendritic cells for exosome production. Targeting was achieved by engineering the dendritic cells to express Lamp2b, an exosomal membrane protein, fused to the neuron-specific RVG peptide[3]. Purified exosomes were loaded with exogenous siRNA by electroporation. Intravenously injected RVG-targeted exosomes delivered *GAPDH* siRNA specifically to neurons, microglia, oligodendrocytes in the brain, resulting in a specific gene knockdown. Pre-exposure to RVG exosomes did not attenuate knockdown, and non-specific uptake in other tissues was not observed. The therapeutic potential of exosome-mediated siRNA delivery was demonstrated by the strong mRNA (60%) and protein (62%) knockdown of *BACE1*, a therapeutic target in Alzheimer's disease, in wild-type mice.

Despite recent advances in delivering siRNA, targeting specific tissues or cell types while avoiding nonspecific delivery, especially to the liver, remains challenging. Another major barrier is immunogenicity of the siRNA or delivery vehicle, especially if repeated dosing is needed to treat chronic or degenerative diseases[4]. Naturally occurring RNA carriers, such as exosomes, might provide an untapped source of effective delivery strategies. Exosomes are natural transport nano-vesicles (40–100 nm) secreted by numerous cell types. The concept of using exosomes for gene delivery was previously suggested in two reports demonstrating transport of endogenous mRNAs and microRNAs expressed in the exosome-producing cells to other cells in culture[5,6]. If exosomes are to be exploited for therapeutic applications, it will be necessary to deliver exogenous cargoes, nucleic acid or otherwise, to specific tissues or cell types *in vivo*. This will require establishment of a source of 'self' exosomes, the ability to load them with therapeutics, such as chemically modified siRNAs, and the ability to introduce targeting moieties (**Fig. 1a**).

To obtain a pool of immunologically inert exosomes, we harvested bone marrow from inbred C57BL/6 mice with a homogenous major histocompatibility complex (MHC) haplotype. As immature dendritic cells produce large quantities of exosomes devoid of T-cell activators such as MHC-II and CD86 (ref. 7), we selected for dendritic cells with granulocyte/macrophage-colony stimulating factor (GM-CSF) for 7 d[8]. Exosomes were purified from the culture supernatant the following day using well-established ultracentrifugation protocols[9]. The exosomes produced were physically homogenous, with a size distribution peaking at 80 nm in diameter as determined by nanoparticle tracking analysis (NTA) and electron microscopy (**Supplementary Fig. 1a**,**b**). We obtained 6–12 μg of exosomes (measured based on protein concentration) per 10^6 cells.

To confer targeting capabilities, we fused targeting peptides for muscle and brain, two tissues affected by degenerative diseases amenable to gene therapy, to the extra-exosomal N terminus of murine Lamp2b, a protein found abundantly in exosomal membranes[10] (**Fig. 1b** and **Supplementary Fig. 2a**). Three different peptides—the central nervous system–specific rabies viral glycoprotein (RVG) peptide (YTIWMPENPRPGTPCDIFTNSRGKRASNG) that specifically binds to the acetylcholine receptor[3], a muscle-specific peptide (MSP) identified by *in vivo* phage display (ASSLNIA)[11] and a FLAG epitope—were cloned into Lamp2b. Plasmids encoding the Lamp2b constructs were transfected into the dendritic cells 4 d before exosome purification. FLAG-Lamp2b was strongly expressed in dendritic cells and was incorporated into the dendritic cell–derived exosomes based on western blots with anti-FLAG and anti-Lamp2b antibodies (**Fig. 1c**). As the topology of Lamp2b on exosomes was unknown, we used a pulldown assay with anti-FLAG beads to establish that the epitope is localized to the external exosomal surface (**Fig. 1d** and **Supplementary Fig. 2b**). Lamp-1, another exosomal protein, served as a marker for the presence of exosomes.

Expression of the RVG and MSP Lamp2b constructs was confirmed by quantitative PCR (qPCR) analysis of transfected dendritic cells (**Fig. 1e**). These modifications do not appear to affect the physical properties of the modified exosomes based on NTA (**Fig. 1f**) and electron microscopy (**Fig. 1g**) of RVG exosomes.

Next, we investigated the possibility of loading modified exosomes with exogenous cargoes using electroporation protocols adapted for nanoscale applications. As electroporation for membrane particles at the nanometer scale is not well-characterized, nonspecific Cy5-labeled siRNA was used for the empirical optimization of the electroporation protocol. The amount of encapsulated siRNA was assayed after ultracentrifugation and lysis of exosomes (**Supplementary Fig. 3a**,**b**). Electroporation at 400 V and 125 μF resulted in the greatest retention

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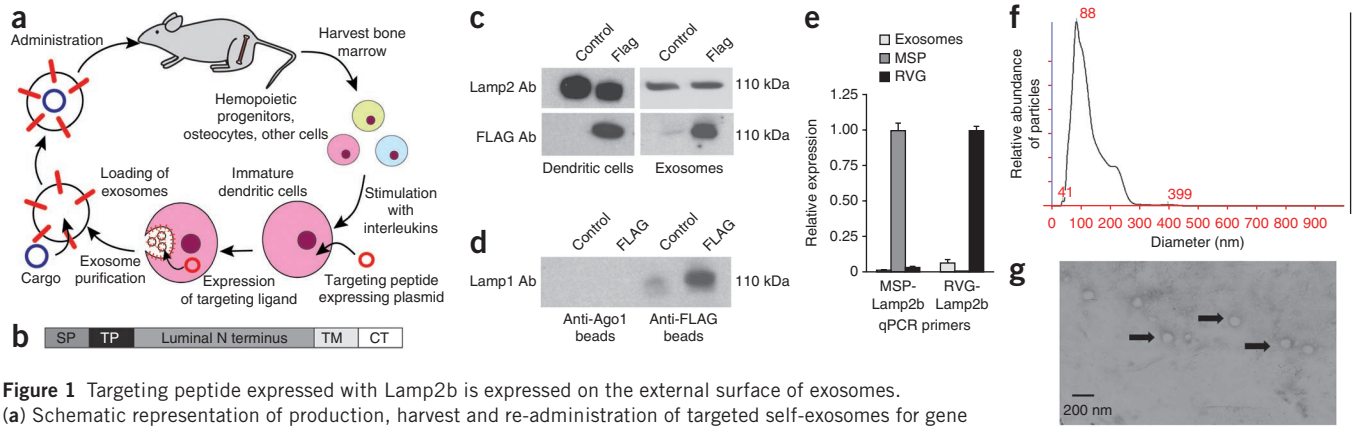

Figure 1 Targeting peptide expressed with Lamp2b is expressed on the external surface of exosomes. (**a**) Schematic representation of production, harvest and re-administration of targeted self-exosomes for gene delivery. (**b**) Schematic representation of the modified Lamp2b protein. SP, signal peptide; TP, targeting peptide; TM, transmembrane domain; CT, C terminus. (**c**) FLAG and Lamp2b western blots of dendritic cells and their corresponding exosomes. (**d**) Lamp-1 western blots of samples after exosome pulldown with either anti-Ago1 or anti-FLAG beads. Anti-FLAG beads retain FLAG-exosomes better than unmodified exosomes and anti-Ago1 does not retain either type of exosome. (**e**) qPCR of dendritic cells transfected with MSP-Lamp2b (MSP) or RVG-Lamp2b (RVG) with primers specific for MSP-Lamp2b or RVG-Lamp2b. (**f**) Size distribution of RVG exosomes as measured by NTA peaking at 88 nm diameter. (**g**) Electron micrograph of phosphotungstic acid stained RVG exosomes. Control is untransfected dendritic cells and exosomes, FLAG is dendritic cells transfected with FLAG-Lamp2b and derivative exosomes. Full-length blots are presented in **Supplementary Figure 9a**,**b**.

of siRNA and was used for all subsequent experiments. The electroporation methods used did not substantially alter the physical properties of RVG exosomes electroporated with *GAPDH* siRNA (**Supplementary Fig. 4a**,**b**).

To assess whether exosomes loaded with siRNAs would be able to specifically deliver their cargoes *in vitro*, we used murine muscle (C2C12) and neuronal cells (Neuro2A). These cell lines are predicted to be targeted by MSP exosomes and RVG exosomes, respectively. Both cell lines were treated for 48 h with Cy5-labeled *GAPDH* siRNA alone (siRNA), siRNA plus transfection reagent (Lipofectamine 2000; siRNA + LP) or unmodified and modified exosomes electroporated with the siRNA. qPCR analysis demonstrated that delivery with targeted exosomes and transfection reagent achieved comparable gene knockdown (**Fig. 2a**,**b**). High delivery efficiency was confirmed by fluorescence microscopy (**Fig. 2c**,**d**), suggesting that exosome-mediated delivery of siRNA can be as efficient as state-of-the-art transfection reagents. The knockdown was cell-type specific, as the strongest knockdown in Neuro2A and C2C12 cells was achieved with RVG exosomes (**Fig. 2a**) and MSP exosomes (**Fig. 2b**), respectively, suggesting that the modified Lamp2b constructs successfully endowed exosomes with cell targeting capabilities. The experiment was repeated with siRNA against cyclophilin B and similar results were obtained (**Supplementary Fig. 4c**,**d**), confirming the results obtained with the *GAPDH* siRNA.

We also targeted BACE1—which is important for Alzheimer's disease pathogenesis—to evaluate the therapeutic potential of this technology. BACE1 is a protease responsible for N-terminal cleavage of the amyloid precursor protein (APP) that produces the aggregate-forming β-amyloid peptide. Two validated *BACE1* siRNAs were applied *in vitro* at increasing doses in Neuro2A cells and resulted in dose-dependent knockdown (**Fig. 2e**). Exosomes had a delivery

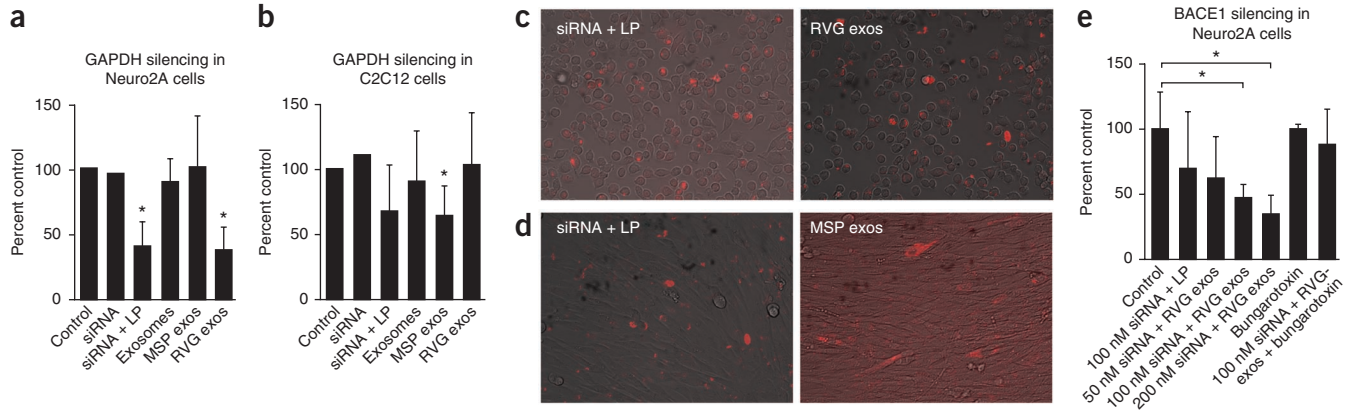

Figure 2 *In vitro* delivery of siRNA by targeted exosomes. (**a**,**b**) qPCR of GAPDH normalized against 18S in Neuro2A cells and C2C12 cells 2 d after application of medium (Control), naked siRNA (siRNA), siRNA delivered with Lipofectamine 2000 (siRNA + LP), unmodified exosomes (Exosomes), MSP exosomes or RVG exosomes. (**c**,**d**) Representative images of Neuro2A and C2C12 cells 2 d after application of Cy5-labeled GAPDH siRNA delivered with siRNA + LP, RVG exosomes (RVG exos) or MSP exosomes (MSP exos). (**e**) 25, 50 and 100 pmoles (50, 100 and 200 nM) of each *BACE1* siRNA was complexed with Lipofectamine 2000 or RVG exosomes (3 μg per 50 pmoles of siRNA) and applied to Neuro2A cells in the presence or absence of 500 nM of α-bungarotoxin. RNA was harvested 2 d later and assayed with qPCR normalized against 18S. * indicates $P < 0.05$ versus untreated control. The results represent the mean and s.d. of three independent experiments, each carried out with a new batch of exosomes to internalize batch-to-batch variation. For **a** and **b**, data are means plus s.d.

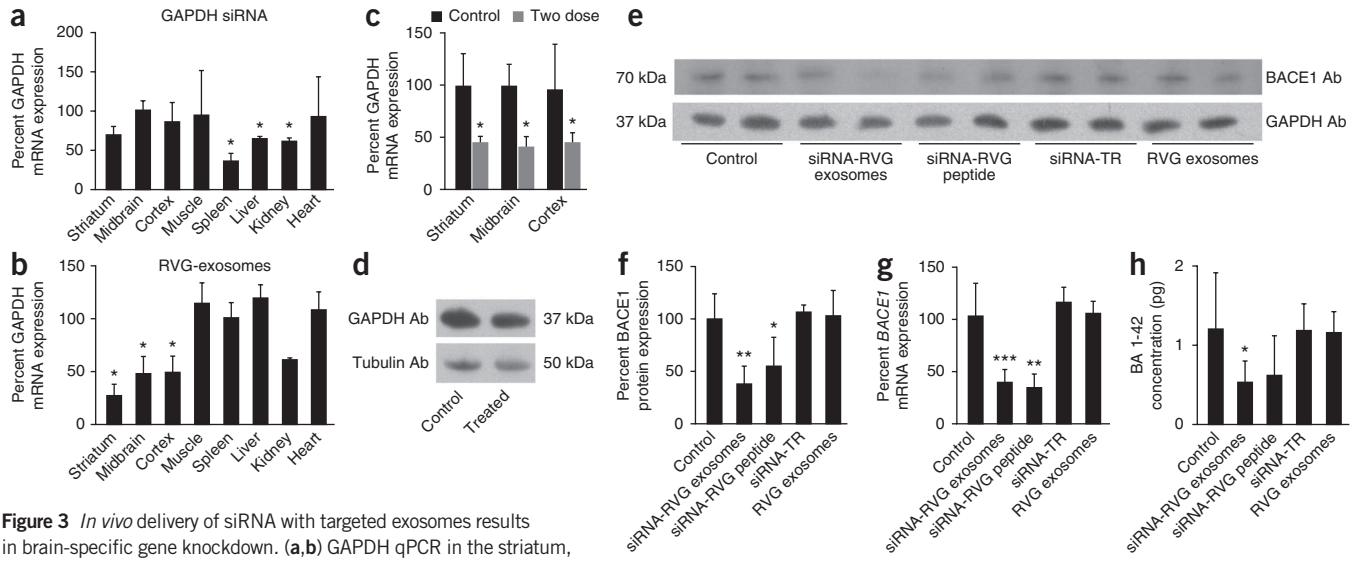

Figure 3 *In vivo* delivery of siRNA with targeted exosomes results in brain-specific gene knockdown. (**a**,**b**) GAPDH qPCR in the striatum, midbrain, cortex, quadriceps muscle, spleen, liver, kidney and heart of mice 3 d after intravenous injection of 150 µg naked GAPDH siRNA or siRNA encapsulated in RVG exosomes normalized to untreated controls (100%) ($n = 3$ for each group). (**c**,**d**) GAPDH qPCR and representative western blot of mice injected with RVG exosome–encapsulated GAPDH siRNA (150 µg) on two separate occasions 7 and 3 d before euthanization ($n = 3$). Mice were injected intravenously with RVG exosomes ($n = 5$), 150 µg each of two *BACE1* siRNAs encapsulated in 150 µg of RVG exosomes ($n = 8$), complexed with *in vivo* transfection reagent ($n = 5$) or RVG-9R peptide ($n = 5$) and compared with untreated control ($n = 8$). (**e**–**h**) Animals were euthanized 3 d after injection and cortical sections were assayed with *BACE1* qPCR (**g**), *BACE1* western blot (**e**,**f**) and β-amyloid 1-42 ELISA (**h**). All qPCR was normalized to 18S RNA levels. *, ** and *** indicates $P < 0.05$, $P < 0.01$ and $P < 0.001$ versus untreated control. All error bars reflect s.d. ($n = 5$). Full-length blots are presented in **Supplementary Figure 9c**,**d**.

efficiency comparable to cationic liposomes. Further, specifically blocking the acetylcholine receptor (the cellular target of the RVG-peptide) with α-bungarotoxin[3], substantially reduced the knockdown of BACE1, showing that the RVG exosomes required the receptor for cellular uptake and targeting specificity.

Delivery by exosomes is well tolerated *in vitro* as demonstrated by a MTT toxicity assay (**Supplementary Fig. 5a**,**b**). A carboxyfluorescein succinimidyl ester (CFSE) assay was used to indicate proliferation of syngeneic (C57BL/6 littermates) and allogeneic (BALB/C) $CD3^+$ T cells in a mixed splenocyte reaction after 3 d of stimulation with concanavalin A (ConA) as a positive control. Unmodified exosomes, RVG exosomes and RVG exosomes loaded with *GAPDH* siRNA failed to trigger syngeneic or allogeneic T-cell proliferation based on fluorescence activated cell sorting (**Supplementary Fig. 5c**). These experiments imply that exosomes themselves may be well tolerated *in vivo*, both from toxicological and immunological viewpoints.

Next, we investigated the potential for exosome-mediated systemic siRNA delivery *in vivo*. To characterize the tissue distribution, we initially chose the ubiquitously highly expressed housekeeping gene *GAPDH* as a target. We electroporated 150 µg of optimized *GAPDH* siRNA with 150 µg of either unmodified, RVG or MSP exosomes, then purified exosomes by ultracentrifugation and removed unencapsulated siRNA in the supernatant. Exosomes were resuspended in 80 µl of 5% glucose before intravenous injection in C57BL/6 mice. Injection of naked siRNA (150 µg) resulted in detectable *GAPDH* silencing in the spleen, liver and kidney (**Fig. 3a**), corresponding to reported siRNA sequestration after tail vein delivery[12]. By contrast, exosome-encapsulated siRNAs appeared to have no natural affinity to these organs and were resistant to nonspecific uptake. Systemic administration of unmodified exosomes did not induce GAPDH silencing in any organ analyzed (**Supplementary Fig. 6a**). Injection of MSP exosomes produced a slight, but nonsignificant GAPDH silencing in brain and kidney (**Supplementary Fig. 6b**), whereas injection of RVG exosomes resulted in significant ($P < 0.05$) knockdown of GAPDH mRNA in several brain regions (**Fig. 3b**), a tissue expressing the target of the RVG ligand–nicotinic acetylcholine receptors[3]. We also observed a nonsignificant decrease of GAPDH mRNA level in kidney 3 d after injection. Although *in vivo* tissue specificity was not seen with MSP exosomes, the relatively weak targeting capabilities of MSP *in vivo*[13] and the abundance of muscle tissue may have resulted in much lower average siRNA concentration within muscle cells and, hence, correspondingly lower gene knockdown. To validate the targeting with RVG and enhance the knockdown, we also injected three mice with two doses of RVG exosomes containing *GAPDH* siRNA at the same concentration 7 d and 3 d before assessing *GAPDH* mRNA and protein knockdown in the brain. Knockdown was observed with *GAPDH* mRNA (50.2 ± 9.1%, $P < 0.05$) and protein (19.6% ± 2.3%, not significant) in the cortex (**Fig. 3c**,**d**). Although increased knockdown was not observed, similar results from these two separate experiments suggested that specific knockdown was achieved.

To further investigate the brain targeting of RVG exosomes, transverse cortical sections from mice injected with Cy3-*GAPDH* siRNA encapsulated in RVG exosomes were stained with neural markers and imaged with confocal microscopy to determine distribution of the labeled siRNA. Co-localization of siRNA and specific cell markers suggests that RVG exosomes primarily delivered the siRNA to neurons, microglia, oligodendrocytes and their precursors (**Supplementary Fig. 7**).

We next examined whether re-administration of exosomes leads to decreased transgene delivery efficiency as previously described for viruses[14,15]. We studied whether pre-inoculating the animals with empty RVG exosomes would reduce the efficacy of delivery. The animals were injected with empty RVG exosomes at the same dose 3 and 2 weeks before injection of RVG exosomes containing *GAPDH* siRNA. Quantification of the *GAPDH* knockdown after 3 d indicated a minimal attenuation in silencing efficacy between unprimed and

primed mice (**Fig. 3b** versus **Supplementary Fig. 6c**), demonstrating that targeted exosomes can be re-administered multiple times without loss in delivery efficacy.

To confirm the therapeutic potential of RVG exosomes *in vivo,* we next investigated delivery of siRNAs that silence *BACE1* expression. Previous studies in transgenic APP overexpressing[16,17] and normal[18] mice suggest that *BACE1* is a strong candidate for anti-Alzheimer's disease therapeutics. *BACE1* knockout did not have an overt effect on the mouse phenotype unlike γ-secretase inhibition[19], suggesting BACE1 inhibition might be free of side effects. However, pharmacological inhibition of BACE1 has so far only been achieved with large molecules that cannot cross the blood-brain barrier[19], thus control of BACE1 expression levels with exosome-mediated siRNA delivery may be an alternative in Alzheimer's disease.

We administered 150 μg of each *BACE1* siRNA encapsulated in 150 μg of RVG exosomes to normal C57BL/6 mice and compared the knockdown efficiency to four controls: untreated mice, mice injected with RVG exosomes only, mice injected with *BACE1* siRNA complexed to an *in vivo* cationic liposome reagent and mice injected with *BACE1* siRNA complexed to RVG-9R, the RVG peptide conjugated to 9 D-arginines that electrostatically binds to the siRNA[3]. Cortical tissue samples were analyzed 3 d after administration and a significant protein knockdown (45%, $P < 0.05$, versus 62%, $P < 0.01$) in both siRNA-RVG-9R-treated and siRNA-RVG exosome-treated mice (**Fig. 3e**,**f**) was observed, resulting from a significant decrease in *BACE1* mRNA levels (66% ± 15%, $P < 0.001$ and 61% ± 13% respectively, $P < 0.01$) (**Fig. 3g**). Moreover, we demonstrated a significant decrease (55%, $P < 0.05$) in the total β-amyloid 1-42 levels, a main component of the amyloid plaques in Alzheimer's pathology, in the RVG-exosome-treated animals (**Fig. 3h**). The decrease observed was greater than the β-amyloid 1-40 decrease demonstrated in normal mice after intraventricular injection of BACE1 inhibitors[18]. We carried out 5′-rapid amplification of cDNA ends (RACE) on *BACE1* cleavage product, which provided evidence of RNAi-mediated knockdown by the siRNA (**Supplementary Fig. 8**).

Finally, we investigated whether siRNA-RVG exosomes induced immune responses *in vivo* by assessing IL-6, IP-10, TNF-α and IFN-α serum concentrations. Following siRNA-RVG exosome treatment, nonsignificant changes in all cytokines were registered similar to siRNA–transfection reagent treatment (**Supplementary Table 1**) in contrast to siRNA-RVG-9R, which potently stimulated IL-6 secretion, confirming the immunologically inert profile of the exosome treatment. Given that exosomes encapsulate only 20% of siRNA (**Supplementary Fig. 3**), delivery with RVG-exosome appears to be more efficient than RVG-9R delivery as comparable mRNA knockdown and greater protein knockdown was achieved with fivefold less siRNA without the corresponding level of immune stimulation. This experiment demonstrated the therapeutic potential of RVG-exosome technology, which is potentially suited for long-term silencing of genes related to neurodegenerative diseases.

Although exosome-mediated gene delivery *in vivo* demonstrated no overt side effects, the possible impact of dendritic cell–derived nucleic acids and proteins within the exosomes on the recipient will need to be further characterized. It is also unknown if modified nucleic acids, including other backbone chemistries, will be loaded and delivered as efficiently as unmodified siRNA.

One of the major challenges for clinical gene therapy applications is the development of gene therapy vehicles for diffuse delivery to the brain. The specificity imparted by targeted exosomes, the ability to load exogenous genetic cargoes, the ability to systemically administer the gene therapy and immune evasion by exosomes are valuable properties for future oligonucleotide therapy applications. RVG exosomes are especially capable of delivering siRNA specifically and safely after systemic administration and therefore represent a promising vehicle for gene therapies targeting chronic neurodegenerative disorders. Moreover, lack of nonspecific knockdown in the liver and other organs marks a major improvement over most current siRNA delivery strategies. Development of targeting moieties for other tissues and improvements in exosome production yield and efficiency will expand the utility of this gene delivery technology.

METHODS

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

Note: Supplementary information is available on the Nature Biotechnology website.

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AUTHOR CONTRIBUTIONS

L.A.-E., Y.S. and M.J.A.W. designed the experiments. L.A.-E. and Y.S. performed the experiments and analyzed the data except for intravenous injection, CFSE proliferation assay. H.Y. performed intravenous injections. C.B. assisted with dissection and harvesting of tissue. S.L. performed the CFSE proliferation assay. L.A.-E., Y.S. and M.J.A.W. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturebiotechnology/.

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ONLINE METHODS

All experiments relating to animal models were carried out in the animal unit, Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, UK according to procedures authorized by the UK Home Office.

Cell culture. C2C12 cells were cultured in DMEM Glutamax (Gibco-BRL) supplemented with 20% FBS and antibiotics. Neuro2A cells were cultured in EMEM (Gibco-BRL) supplemented with 10% FBS and antibiotics. All cells were incubated at 37 °C in 5% CO_2.

Dendritic cell isolation, transfection and exosome purification. Fetal calf serum (FCS) used for exosome production was spun at 25,000*g* for 90 min before preparation of medium. Primary dendritic cells harvested from murine bone marrow were cultured in DMEM Glutamax (Gibco-BRL), 10% FCS and antibiotics, supplemented with 10 ng/ml murine GM-CSF (Sigma-Aldrich). Dendritic cells were transfected 4 d after harvest with 5 μg of pLamp2b derivative plasmids and 5 μl of TransIT LT1 transfection reagent (Mirus Bio) as per manufacturer's instructions in six-well plates with 3×10^6 cells per well. Cell culture medium was changed on day 7 and cell culture supernatant harvested 24 h after, spun at 12,000*g* for 30 min to remove cell debris, then spun again at 120,000*g* for 1 h to pellet exosomes. Exosomes were then resuspended in 0.1 M ammonium acetate with a 27G syringe. The yield of exosomes was ~20–30 μg (based on Bradford measurement) per well.

Plasmids. Lamp2b was cloned with cDNA from C2C12 cells and XhoI and BspEI restriction sites were inserted after the signal peptide sequence together with glycine linkers using primers listed in **Supplementary Table 2**. The full construct was then cloned downstream of the CMV promoter with NheI and BamHI restriction sites into a pEGFP-C1 vector, removing the eGFP in the process. Primers designed to encode RVG[3], MSP[4] and FLAG tag were used to introduce the targeting ligands between XhoI and BspEI at the N terminus of Lamp2b (**Supplementary Fig. 9**).

siRNAs. siRNAs were purchased from Eurogentec or a kind gift from Novartis. Sequences of siRNAs used were (GAPDH sense CAGAAGACUGUGGAUGG CC(DTDT), antisense GGCCAUCCACAGUCUUCUG(DGDG); cyclophilin B sense UUUUUGGAACAGUCUUUCC(DTDT), antisense GGAAAGACU GUUCCAAAAA(DTDT); *BACE1*a[20] sense GCUUUGUGGAGAUGGUGGA (DTDT), antisense UCCACCAUCUCCACAAAGC(DT)(DT); *BACE1*b sense GUGGUAUUAUGAAGUGAUC(DTDT), antisense GAUCACUUCAUAAU ACCAC(DTDT)).

Exosome pulldown assay. We incubated 20 μl of Protein-A Sepharose beads (Sigma P9424) overnight at 4 °C in 500 μl PBS and 2 mg/ml BSA. The beads were subsequently washed three times with PBS. We loaded 1 μl rabbit anti-FLAG (Sigma F2555) or 1 μl of rabbit anti-Ago1 (Upstate 07-599) onto the beads in 100 μl of PBS with 2 mg/ml BSA for 4 h at 4 °C and the beads were subsequently washed three times as described above. Unmodified exosomes and FLAG-exosomes were spun at 200*g* for 10 min to remove contaminating debris and added to the beads to a protein concentration of 10 μg/ml in 200 μl of PBS. After incubation, the beads were subsequently washed three times as described above, then eluted in 0.1% SDS and 10 μl of supernatant was loaded onto a polyacrylamide gel. A western blot against Lamp-1, an exosomal protein, was used to detect the presence of exosomes pulled down by the antibodies.

Exosome loading. Exosomes at a total protein concentration of 12 μg (measured by Bradford Assay) and 400 nanomoles (for cell culture) or 12 μg (for *in vivo* injections) of siRNA were mixed in 400 μl of electroporation buffer (1.15 mM potassium phosphate pH 7.2, 25 mM potassium chloride, 21% Optiprep) and electroporated in a 4 mm cuvette. For *in vivo* experiments, electroporation was performed in 400 μl and pooled together for ultracentrifugation before resuspension in a volume of 5% glucose corresponding to 80 μl per injection.

Electron microscopy and NTA. Samples to be electroporated were incubated with 4% osmium tetraoxide for 30 min at 4 °C, then applied onto a copper grid and stained with 1% phosphotungstanic acid. NTA was carried out using the Nanosight LM10-HS system (NanoSight) on exosomes resuspended in PBS at a concentration of ~3 μg of protein/ml and were further diluted from 100- to 500-hundredfold for analysis. The system focuses a laser beam through a suspension of the particles of interest. These are visualized by light scattering, using a conventional optical microscope aligned normally to the beam axis which collects light scattered from every particle in the field of view. A 20–60 s video records all events for further analysis by NTA software. The Brownian motion of each particle is tracked between frames, ultimately allowing calculation of the size through application of the Stokes-Einstein equation.

Cytotoxicity assay. The cytotoxic potential of exosomes was assessed using the MTT assay, based on Mossman[21]. N2A and C2C12 cells were treated with exosomes in triplicate in a 96-well plate proportional to the scale listed above. After 48 h cell survival was analyzed by the MTT assay on a Wallac-Victor3 plate reader (PerkinElmer).

Mixed lymphocyte reaction. RBC-depleted spleen cells were incubated for 5 min in CFSE at 37 °C, washed three times with cold PBS with 0.2% FCS and cultured in triplicates at 5×10^4 cells/well in 96-well plates. We added 1.5 μg of unmodified/RVG exosomes by themselves or with 25 pmole of *BACE1* siRNA or concanavalin A (5 μg/ml) 1 h later. After 72 h of incubation, the proliferation response was measured by FACs analysis using anti-CD3-APC (Abcam). Analysis of proliferation response was performed using FlowJo.

Application of exosomes in cell culture and in animals. We added 100 μl of transfected or electroporated exosomes to cells in a 24-well plate in complete medium. The medium is changed after 5 and 24 h for transfected and electroporated exosomes, respectively. The cells were harvested 48 h after addition of the exosomes. C57BL/6 male 8- to 10-week-old mice were used for all experiments. Exosomes for *in vivo* experiments were spun down and resuspended in 80 μl of 5% glucose immediately before tail vein injection. We injected 150 μg of exosomes and the encapsulated siRNA (150 μg of GAPDH siRNA was electroporated) per animal except for experiments with *BACE1* siRNAs, where 150 μg each of two *BACE1* siRNA were added for electroporation. siRNA-transfection reagent complexes were prepared with cationic liposome-based *in vivo* transfection reagent as per manufacturer's protocol (Altogen Biosystems). For RVG-9R peptide delivery, peptide/siRNA complexes were prepared in 100 μl of 5% glucose (at a peptide/siRNA molar ratio of 10:1), as described previously[3].

ELISA. The release of β-amyloid 1-42 was assessed using the β-amyloid (42) Elisa Kit (Wako) as per manufacturer's protocol. We added 20 μg of brain lysate to each well and analyzed all samples in triplicate. Serum concentrations of IL-6, IP-10, TNFα and IFNα were measured using 50 μl of serum with Elisa Kit (Thermo Scientific (IFN), R&D (others)) as per manufacturer's protocol.

Statistics. Statistical analyses of the data were performed using SPSS program 16.0 by using the nonparametric Krusckal Wallis test followed by the Mann-Whitney U test. All experiments, unless otherwise stated, were performed in triplicate. All error bars used in this report are s.d.

Reverse transcription qPCR. RNA was harvested with Trizol (Invitrogen) or RNeasy kit (Qiagen) as per manufacturer's protocol. Reverse transcription was performed with Primer Design Reverse Transcriptase kit (Primer Design) as per manufacturer's instruction. qPCR experiments were performed on an ABI7000 thermal cycler in 20 μl reactions, with 10 nM of each primer and 2 μl of each cDNA preparation, using Precision qPCR Mastermix (Primer Design) as per manufacturer's instructions. qPCR primers used in this experiment are given in **Supplementary Table 2**.

Western blots and antibodies. Tissue were sectioned and lysed in 10 mM Tris pH 7.4 buffer containing 0.1% SDS, a protease inhibitor cocktail and DNase (Promega). Primary antibodies used for western blot analysis were rat anti-mouse Lamp2 antibody (Abcam), mouse anti-Lamp1 antibody (Abcam),

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anti-mouse GAPDH (Abcam), anti-mouse PPIB (Abcam), rabbit anti-FLAG (Sigma-Aldrich), rabbit anti-BACE1 (Abcam).

Immunohistochemistry. Animals were injected with Cy3-GAPDH siRNA encapsulated in RVG exosomes and euthanized 12 h later. Brain coronal sections (10 μM) were cut with a cryostat for the histopathological assessment. Slices were washed with PBS and fixed with 4% paraformaldehyde. Slices were treated with blocking solution (PBS containing 10% NGS and 0.05% Triton X-100). Slices were incubated overnight at 4 °C with the primary antibody; mouse anti-NeuN (1:100, Sigma), rabbit anti-glial fibrillary acidic protein (GFAP) (1:250, Sigma), rabbit anti-Iba1 (1:250, Dakocytomation), rabbit anti-OP1 (1:250, Abcam) and rabbit anti-oligodendrocyte specific protein (OSP) (1:205, Abcam). Slices were washed three times with PBS and were incubated with a secondary antibody of the appropriate species; Alexa 488 goat anti-mouse (1:200) and Alexa 488 goat anti-rabbit. The microscope analyses were performed using an epifluorescence microscope (Zeiss).

5′ Rapid amplification of cDNA ends. RNA was harvested from cortical sections of the brain of mice injected with *BACE1* siRNAs encapsulated in RVG exosomes, as mentioned previously. 5′ RACE was performed with Invitrogen GeneRacer kit as per manufacturer's instruction on 10 μg of total RNA. Briefly, an RNA linker (5′-CGACUGGAGCACGAGGACACUGACAUGGACUGAA GGAGUAGAAA-3′) was ligated to the unprotected phosphorylated 5′ end of RNA and the product was reverse transcribed using a specific primer against *BACE1* (5′-CGACAAGAGCATTGTGGACAGTGGGAC-3′) using New England Biolabs ThermoPol Taq polymerase. Using a specific primer for the 5′ linker and the specific *BACE1* primer, the cDNA was amplified and TA-cloned into a vector and sequenced.