PrP\textsuperscript{C} knockdown by liposome-siRNA-peptide complexes (LSPCs) prolongs survival and normal behavior of prion-infected mice immunotolerant to treatment

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Abstract

Prion diseases are members of neurodegenerative protein misfolding diseases (NPMDs) that include Alzheimer’s, Parkinson’s and Huntington diseases, amyotrophic lateral sclerosis, tauopathies, traumatic brain injuries, and chronic traumatic encephalopathies. No known therapeutics extend survival or improve quality of life of humans afflicted with prion disease. We and others developed a new approach to NPMD therapy based on reducing the amount of the normal, host-encoded protein available as substrate for misfolding into pathologic forms, using RNA interference, a catabolic pathway that decreases levels of mRNA encoding a particular protein. We developed a therapeutic delivery system consisting of small interfering RNA (siRNA) complexed to liposomes and addressed to the central nervous system using a targeting peptide derived from rabies virus glycoprotein. These liposome-siRNA-peptide complexes (LSPCs) cross the blood-brain barrier and deliver PrP\textsuperscript{C} siRNA to neuronal cells to decrease expression of the normal cellular prion protein, PrP\textsuperscript{C}, which acts as a substrate for prion replication. Here we show that LSPCs can extend survival and improve behavior of prion-infected mice that remain immunotolerant to treatment. LSPC treatment may be a viable therapy for prion and other NPMDs that can improve the quality of life of patients at terminal disease stages.

Introduction

Prion diseases are neurodegenerative, protein misfolding diseases that have an impact on a broad range of species from sheep (scrapie), cervids (chronic wasting disease (CWD)) and cattle (bovine spongiform encephalopathy (BSE)) to humans (Creutzfeldt-Jakob disease (CJD), among others). Plaque deposits, neuronal vacuolation, glial activation, neuronal cell death and
long incubation times characterize prion diseases [1–3]. Unlike other infectious agents, prions encode instructions for additional infectious particles within the structure of the misfolded form of a normal host protein, the cellular prion protein (PrP\textsuperscript{C}), rather than nucleic acid sequence [4]. Consumption of infected meat or exposure to infected bodily fluids or tissues likely transmits infectious prion diseases, including scrapie, BSE, variant CJD in humans, and CWD [5,6]. However, spontaneous prion generation also results from several PrP\textsuperscript{C} polymorphisms that increase the likelihood of PrP\textsuperscript{C} misfolding. Most prion diseases can result from, or be influenced by prnp polymorphisms, including scrapie, BSE, CWD, genetic CJD, and fatal familial insomnia in humans [7,8].

PrP\textsuperscript{C}, in its immature form, is a 250-amino acid protein that all mammals investigated express [9,10] throughout the body, with the highest levels of mRNA and protein detected in neurons of the central nervous system (CNS) [11,12]. PrP\textsuperscript{C} matures in the endoplasmic reticulum and the Golgi apparatus, where it is processed into a 208-amino acid protein with a glycosylphosphatidylinositol (GPI) anchor and two N-linked glycans [10,13]. Approximately half of the N-terminus of PrP\textsuperscript{C} adopts no formal structure, while the C-terminus folds into three α-helices and two short β-sheets. Its GPI anchor sequesters PrP\textsuperscript{C} within cholesterol and sphingolipid-rich rafts in the plasma membrane [14]. When PrP\textsuperscript{C} misfolds, it becomes known as PrP\textsuperscript{Sc} or PrP\textsuperscript{Res}, which correlates with prion infectivity [4,15,16].

Research over the last 30 years revealed many proposed PrP\textsuperscript{C} functions. PrP\textsuperscript{C} can be neuroprotective through antioxidant [17–19] and anti-apoptotic functions [20,21]. The ability of PrP\textsuperscript{C} to regulate Ca\textsuperscript{2+} homeostasis [22–24], leading to activation of the MAPK/ERK, PKA, and STAT1 cell signaling pathways to modulate responses to oxidative and apoptotic damage [25,26] supports this neuroprotective function theory. PrP\textsuperscript{C} also binds to Cu\textsuperscript{2+} via tandem octapeptide repeats in the unstructured N-terminus, which is thought to mediate oxidative stress damage [27–29]. Other proposed PrP\textsuperscript{C} functions include hematopoietic stem cell renewal [30], axonal myelination sensing [31], immune activation [32,33] and regulation of circadian rhythms [34]. However, PrP-null mice develop, breed and behave normally [35], suggesting a redundant or inducible function for PrP\textsuperscript{C}.

No known therapeutics improve quality of life or extend survival of humans afflicted with prion disease. Most early therapeutic compounds targeted conversion of PrP\textsuperscript{C} into PrP\textsuperscript{Res}, including polyanionic compounds HPA-23 [36,37], dextran sulfate [36,38,39], pentosan polysulphate [39–41], and congo red [42–44]; and polycationic compounds, including branched polyamines [45,46], lipopolyamines [47] and dendrimers [48]. Antiviral [49–51], antibacterial [51–53], antimalarial [54–56] and an anti-cancer drug [57] have also shown therapeutic promise \textit{in vitro}. Some of these compounds prolong survival in laboratory animals experimentally infected with prions, but showed little or no efficacy in human trials [58]. In recent years, anti-PrP antibodies administered as either active or passive immunization have also shown therapeutic promise [59–62], depending on the PrP epitope these antibodies recognize [63].

However, significant challenges remain before large-scale clinical trials are considered for these drugs. Most do not cross the blood-brain barrier (BBB) or target neuronal cells. Many polyionic compounds are toxic and cannot be given in large doses or over an extended time period. Some drugs only have anti-prion activity for specific prion strains. Most importantly, these compounds only have shown efficacy before or directly after prion inoculation, and few have shown any promise when given at late or clinical stages, when prion disease are typically diagnosed and invariably fatal.

We and others investigated a new approach to prion disease therapy based on the observation that 21% of mice heterozygous for the prion protein gene (prnp) and expressing approximately half the amount of PrP\textsuperscript{C} survived terminal prion disease, and the remaining mice lived 2.5 times longer than prnp homozygous mice [64–66]. We reasoned that therapeutics that
reduce the PrP\textsuperscript{C} substrate required for prion replication by 50% should significantly prolong survival of prion-infected mice.

RNA interference (RNAi) is a catabolic pathway that utilizes RNA molecules to decrease levels of mRNA encoding a particular protein [67–70]. These RNA molecules, short hairpin RNA (shRNA) or small interfering RNA (siRNA), activate the RNA-induced silencing complex that cleaves mRNA and enables endo- and exonucleases to degrade the targeted mRNA resulting in a decrease in translated protein levels. We and others have shown that both shRNA and siRNA treatment targeted towards PrP\textsuperscript{C} can reduce the level of PrP\textsuperscript{Res} in cultured and primary cells by decreasing the amount of PrP\textsuperscript{C} available for conversion [71–73]. A single stereotactic injection of PrP shRNA into the hippocampus of prion-infected mice resulted in prolonged survival and reversal of prion neuropathology [72]. However, stereotactic injections are highly invasive and can compromise the BBB. Lentiviral delivery of shRNA also irreversibly silences prnp expression, affects a relatively localized brain region and still presents significant safety concerns. We have previously reported using liposome-siRNA-peptide complexes (LSPCs) addressed to nicotinic acetylcholine receptor (nAchR)- expressing cells using a short, modified peptide from rabies virus glycoprotein (RVG-9r) to deliver PrP siRNA to neuronal cells \textit{in vitro} and \textit{in vivo} and cure neuroblastoma cells chronically infected with prions [73]. We now focus on using LSPCs \textit{in vivo} to treat prion-infected mice.

Here, we demonstrate that PrP siRNA LSPCs cross the BBB and decrease the amount of neuronal PrP\textsuperscript{C} 40–50\% \textit{in vivo} after a single intravascular injection in two different mouse models. We then treated prion-infected mice every two or four weeks starting midway through prion disease course with PrP siRNA LSPCs or control LSPCs and monitored mice for clinical and behavioral signs of prion disease. We found that repeated LSPC treatment significantly prolonged survival of 6/19 mice (responders) infected with prions, and significantly improved the behavior of all prion-infected mice at late stages of disease. Treated mice that did not survive significantly longer (non-responders) developed high anti-RVG-9r IgG titers, indicative of a significant immune response to the repetitive treatment protocol, while 5/6 responder mice did not seroconvert. Intranasal LSPC delivery doubled the response rate and decreased seroconversion rate by 50\%. Limiting LSPC treatment to three transvascular injections prevented seroconversion, prolonged survival of PrP\textsuperscript{C} overexpressing mice up to 22\%, and improved behavior of prion-infected mice compared to control-treated infected mice in this accelerated prion disease model. These results indicate that LSPC delivery of PrP siRNA significantly decreases PrP\textsuperscript{C} expression and subsequent prion replication in the brain that prolongs normal behavior and life span of prion-infected mice. \textit{In toto}, this report promotes LSPCs, delivering siRNA targeting expression of normal host proteins that act as substrates for misfolding, as a viable candidate to treat prion diseases and other NPMDs.

**Results**

**LSPCs deliver PrP\textsuperscript{C} siRNA to the brain**

To assess the number of targetable cells within the brain that express PrP\textsuperscript{C} and nAchRs, we incubated primary neuronal cells with RVG-9r and the anti-PrP\textsuperscript{C} antibody BAR-224 and performed flow cytometry. The majority (96.4\%) of neuronal cells stained double-positive for BAR-224 and RVG-9r, indicating that most brain cells express both PrP\textsuperscript{C} and nAchR (Fig 1A) and are targets for PrP LSPCs addressed with RVG-9r. We previously reported RVG-9r binding to cultured kidney cells [74]. To assess possible off-target effects of LSPCs \textit{in vivo}, we also incubated primary kidney cells with BAR-224 and RVG-9r. Flow cytometry revealed fewer kidney cells (80\% versus 98\%) expressing 10-fold less PrP\textsuperscript{C} and two-fold less nAchRs, indicating a diminished potential for LSPCs targeting the kidney compared to the brain.
Fig 1. LSPCs target the majority of neuronal cells and deliver PrP\textsuperscript{C} siRNA to the brain when injected intravenously. A) Flow cytometry of neuronal cells labeled with BAR-224, an anti-PrP\textsuperscript{C} antibody, and RVG-9r reveals that 98% of cells in the brain display PrP\textsuperscript{C} and nAchRs on their surfaces. Ninety-six percent of those cells express both PrP\textsuperscript{C} and nAchRs. A smaller proportion of kidney cells (78%) express both PrP\textsuperscript{C} and nAchRs. B) \textit{In vivo} live imaging revealed that RVG-9r increased LSPC delivery to the brain two minutes to ten days after intravascular injection compared to siRNA and peptide complexes without liposomes (SPCs). We controlled for background fluorescence using PBS-injected mice. C) Flow cytometry analysis 24 hours after fluorescent LSPC injection revealed siRNA delivery to 47% of brain cells and 15% of kidney cells (black histograms) compared to PBS-injected controls (gray histograms). Data are representative of at least three independent experiments.
We next combined *in vivo* whole animal imaging with flow cytometry to assess pharmacokinetics of LSPC delivery to the brain after intravascular injection. To observe the biodistribution of LSPCs, naïve FVB mice were injected intravenously with LSPCs containing Alexa 488-labeled PrP\textsuperscript{C} siRNA and RVG-9r labeled with Dylight 650. *In vivo* live imaging showed that mice injected intravenously with siRNA-peptide only complexes (SPCs) had a wide biodistribution and rapidly decreasing fluorescence within the body with little PrP\textsuperscript{C} siRNA signal in the brain, whereas mice injected intravenously with liposomes containing siRNA and the RVG-9r peptide (LSPCs) showed increased signal of PrP siRNA within the brain two minutes to ten days after injection (Fig 1B). Eighty-five percent of LSPCs were detected in the brain by two hours post injection, and 63% persisted in the brain ten days later. Flow cytometry of cellular targets in these mice revealed 47% of brain cells and 15% of kidney cells contained PrP siRNA (Fig 1C).

**Intravenous LSPC administration decreases neuronal PrP\textsuperscript{C} protein and mRNA levels**

To assess pharmacodynamics of PrP knockdown via LSPCs, wild type mice were injected intravenously with LSPCs and monitored for mRNA expression and PrP\textsuperscript{C} protein levels at serial time points after treatment using digital droplet PCR (ddPCR) and flow cytometry, respectively. FVB, CD-1 and C57Bl/6 mice expressed decreased levels of neuronal PrP\textsuperscript{C} mRNA (Fig 2A) and protein (Fig 2B) at multiple time points in both brains and kidneys of LSPC-treated mice. Additional control experiments revealed that mice injected with LSPCs carrying irrelevant siRNA or addressed with a control peptide (RVM) that does not target nAchRs, expressed normal amounts of PrP\textsuperscript{C} mRNA and protein in both brains and kidneys at 4 days post treatment (S1 Fig). Mice injected with LSPCs carrying scrambled PrP siRNA expressed more PrP mRNA, but not protein, in brains; and more PrP mRNA and slightly less PrP protein in kidneys at 4 days post-treatment.

**Repetitive LSPC treatment prolongs survival in a subset of prion-infected mice**

Based on these data, we treated prion-infected mice every two weeks with LSPCs and monitored them for behavioral, cognitive and clinical signs of prion pathogenesis. Because extra-neural prion exposure more closely mimics a natural prion infection, we inoculated WT mice intraperitoneally with RML-5 prions, then began treating mice approximately midway through prion disease course (see S1 Table), when onset of early behavioral and cognitive changes occur. We delivered LSPCs intravenously (IV) to most of the mice in this study. However, a subset of mice received LSPCs intranasally (IN, n = 5) to assess efficacy of a less invasive route previously shown to be an effective method to deliver drugs to the brain [75,76]. An additional cohort received LSPCs via both routes (n = 5). We used LSPCs to deliver two PrP\textsuperscript{C} siRNAs, 1578 or 1672, that both targeted the 3' untranslated region of the prnp locus [74]. Mice began exhibiting clinical signs of terminal prion disease after the seventh LSPC injection and were subsequently euthanized beginning 203 days post infection (DPI), while a minority of mice lived to receive nine (n = 6) LSPC treatments (> 217 DPI). We observed no difference in survival proportion or time between infected, treated (19/19 died with a mean survival time of 221, 95% confidence interval (CI) ± 5 DPI) and untreated mice (9/9, 220 ± 4 DPI, Fig 3A). However, 6 of 19 treated mice responded significantly better to treatment, surviving terminal prion disease significantly longer (235 ± 9 DPI) than non-responders (215 ± 3 DPI) and untreated mice. Of the 6 responder mice, 3 were treated IV (33% response rate) and lived to a mean survival time of 232 ± 5 DPI. The other 3 responders were treated IN (60% response
rate) and survived a mean 238 ± 7 DPI (p < 0.05, S2 Fig). Mice treated by both routes simultaneously did not survive significantly longer than untreated or non-responder mice (212 ± 8 DPI).

Proteinase K digestion and western blot analysis of brains from prion-infected mice showed PrPRes deposition at terminal disease, with brains from 5 of 6 responder animals appearing to harbor relatively less PrPRes than most non-responders or infected untreated controls, although densitometry revealed these differences to be not quite statistically significant (p = 0.057 and 0.072, respectively, Fig 3B and 3C). Consistently, responder mice exhibited significantly reduced vacuolation and astrogliosis in the cerebellum than non-responders and infected untreated control mice, although PrPRes accumulation was equivalent among the
The groups (Fig 4A). We observed more dramatic differences in the hippocampus, where we observed significantly less PrP\textsuperscript{Res}, GFAP and vacuoles in both responders and non-responders compared to infected, untreated mice (Fig 4B). We observed variable and inconsistent neuropathology in other brain regions with or without treatment.

Fig 3. Repeated LSPC treatment prolongs survival of and decreases PrP\textsuperscript{Res} accumulation in a subset of prion-infected mice. Wild type mice were injected intraperitoneally with RML-5 prions and treated intravenously, intranasally, or both with 1578 PrP\textsuperscript{C} siRNA LSPCs or 1672 PrP\textsuperscript{C} siRNA LSPCs. We observed no significant differences in survival times among different routes or siRNA used, so we present compiled data here. See S3 Fig for survival curves for each delivery route. A) We observed no significant difference between control infected untreated mice (dashed black line, n = 9) and prion-infected, LSPCs-treated mice (solid black line, n = 19). However, a subset of treated mice positively responded to LSPC treatment (both solid red lines, n = 6, one shown extending from solid black line of all infected treated mice, the other as its own group), surviving significantly longer than non-responders and untreated mice (*p<0.05, survival analysis). Three of five uninfected treated mice (dotted black line) also died unexpectedly. All infected mice harbored PrP\textsuperscript{Res} (B and C). All samples were treated with 50 \(\mu\text{g/mL}\) PK. Red asterisks denote samples from responder mice, 5 of 6 of which appeared to contain relatively less PrP\textsuperscript{Res}. Double asterisks denote responder #4. We detected no PrP\textsuperscript{Res} in uninfected (un)treated mice (D) All samples except lane 1 were treated with 50 \(\mu\text{g/mL}\) PK. Black lines to the left of each blot indicates the 25 kD molecular weight marker. NBH, normal brain homogenate. RML, Rocky Mountain Lab prion strain. Samples in all lanes were digested with 50 \(\mu\text{g/mL}\) Proteinase K, except NBH.

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Fig 4. LSPC treatment decreases neuropathology in prion-infected mice. Immunohistochemistry (IHC) of brain sections through the (A) cerebellum and (B) hippocampus in infected and uninfected mice reveal that (A) while we observed no difference in cerebellar PrP\(_\text{Res}\) accumulation among infected untreated, non-responder or responder mice, both GFAP stain intensity, a measure of astrogliosis, and vacuolation, a hallmark of prion-mediated neurodegeneration, were significantly decreased in LSPC responder mice. (B) In the hippocampus, we observed decreased PrP\(_\text{Res}\), GFAP and vacuolation in responder and non-responder mice compared to infected untreated mice. We detected no PrP\(_\text{Res}\) or vacuolation but significant GFAP expression in both cerebellum and hippocampus of uninfected treated mice compared to uninfected untreated mice. Boxed areas indicate magnified areas in the panel directly below. Scale bars, 100 \(\mu\)m. Quantitation of PrP\(_\text{Res}\) and GFAP is expressed as relative pixel intensity ± 95% CI per mm\(^2\). Vacuolation scores indicate number of vacuoles ± 95% CI per mm\(^2\). IHC images are representative of at least three mice per group. We collected data from at least three non-consecutive slides from at least two animals from each group. *p < 0.05, **p < 0.01, compared to scores from infected untreated mice, except where indicated otherwise.

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Repetitive LSPC treatment prolongs normal behavior in prion-infected mice

We subjected all mice to behavioral testing to determine if LSPC treatment abrogates early behavioral deficits observed in prion-infected mice. We started burrowing and nesting tests four weeks before the first LSPC treatment and continued every two weeks thereafter until terminal disease or end of the study. While we observed differences in survival and neuropathology among treated mice (responders versus non-responders), we observed no significant differences in behavior between responders and non-responders. We also observed no differences in LSPC-treated or untreated, male or female mice. We therefore compared all treated mice to the infected untreated control group. PrP siRNA LSPC treatment prolonged normal burrowing behavior in infected mice up to 169 DPI in infected mice compared to untreated infected mice. LSPC treatment improved nesting in prion-infected mice up to terminal disease compared to untreated infected mice, although not to normal levels observed for uninfected treated mice (Fig 5B). These data indicate that PrP siRNA LSPC treatment can prolong normal behavior, and significantly improve behavior of prion-infected mice up to terminal disease.

Anti-RVG-9r IgG detected in the serum of mice treated with LSPCs

Unexpectedly, 3 of 4 uninfected, LSPC-treated control mice died suddenly one hour after the ninth LSPC treatment, indicating possible toxicity of repeated LSPC administration, and were immediately and humanely euthanized. Neuropathology, including some vacuolation and
abundant astrogliosis, in the absence of PrP<sub>Res</sub> deposition in the brains of these mice (Fig 4A and 4B), corroborate this hypothesis. We did not observe acute, unexpected deaths in any other infected, treated or untreated mice. Given that a majority of uninfected LSPC-treated mice died at relatively young ages (287–329 days old), we reasoned that pathogenesis resulting from immune responses against LSPCs may have masked therapeutic benefit of LSPCs against prion disease. Necropsy of these control mice revealed enlarged, darkened spleens and kidneys and severe blood coagulation, indicative of extensive immune complex formation likely contributing to death of these animals. To determine the extent of immune activation against LSPCs, we collected serum samples from mice at time of euthanasia and measured anti-RVG-9r IgG levels by indirect ELISA. We detected significant anti-RVG-9r IgG titers in all uninfected treated (n = 4) and infected, LSPC non-responsive mice (n = 13, Fig 6A). Five of 6 LSPC-responsive mice and all untreated, RVG-9r naïve mice produced no significant anti-RVG-9r IgG titers. (Fig 6A). Only one IV-treated responder mouse (#4), the same mouse with increased PrP<sub>Res</sub> (Fig 3C, two red asterisks), produced significant anti-RVG-9r IgG titers. We initiated a second independent experiment, limiting treatment of mice to 4 LSPC injections 24 to 33 days apart to avoid immune response to treatment. We observed no significant difference in survival times between infected, untreated mice (223 ± 3 DPI, n = 13) and infected treated mice (219 ± 2, n = 18).

**More than three LSPC treatments increase anti-RVG-9r IgG levels and abrogates PrP<sup>C</sup> knockdown**

We next sought to determine the number of LSPC treatments that would result in the maximal decrease of PrP<sup>C</sup> protein and mRNA and minimal anti-RVG-9r IgG titers. We infected WT mice intraperitoneally with RML-5 prions then treated with LSPCs every two weeks for a total of ten weeks. After every treatment, we euthanized groups of treated and untreated mice (n = 5 per group) and analyzed their sera for anti-RVG-9r IgG titers, and brains and kidneys for PrP mRNA and protein expression. We detected no significant RVG-specific IgG titers in any of the LSPC-treated mice until after the third LSPC treatment, after which IgG titers rose steadily (Fig 6B). We observed maximal decrease of PrP mRNA and protein up to three repetitive LSPC treatments every two weeks, which resulted in a 3-fold decrease of neuronal PrP<sup>C</sup> levels (Fig 6C and 6D). After three treatments, PrP mRNA and protein levels steadily increased in the brain (Fig 6C and 6D), concomitant with detection of increasing RVG-9r-specific IgG titers (Fig 6B). Messenger RNA and protein levels in the kidney were more variable but followed a similar trend: initial decrease in PrP mRNA (after 2 treatments) and protein (after 3 treatments), followed by a steady rise to normal levels after subsequent treatments. These results strongly suggest that an immunological response to LSPCs in vivo abrogates their therapeutic effect.

**Limited PrP siRNA LSPC treatment extends survival time and normal behavior in an accelerated prion disease model**

Given that immune responses to the RVG-9r targeting peptide after three exposures limits LSPC efficacy, we performed an additional therapeutic study using an accelerated prion disease mouse model. TgA20 mice express 4-7-fold more PrP<sup>C</sup>, and succumb to prion disease 4–5 times faster than wild-type mice [77]. TgA20 mice are also the gold standard for titrating the RML-5 prion strain [65,78,79], so we can accurately assess prion replication with or without LSPC treatment. We leveraged accelerated disease kinetics and precise prion titration to determine whether LSPCs, in the absence of an immune response against them, could extend survival and normal behavior by reducing prion replication and subsequent neuropathology that we observed in a typical prion disease course. We infected TgA20 mice intracerebrally with 10<sup>6</sup> LD<sub>50</sub> units of RML-5 prions, then beginning at 20 DPI treated them 1–3 times with
PrP siRNA LSPC treatments 20 days apart. We detected no anti-RVG-9r IgG titers in any treated mice using this regimen (S3 Fig). While all infected mice still succumbed to terminal prion disease, we observed a significant 15–22% increase in mean survival time in infected mice treated once (70 ± 95% CI ± 1 DPI, n = 10), twice (72 ± 2 DPI, n = 15) or thrice (74 ± 1 DPI, n = 11) with LSPCs compared to untreated infected mice (61 ± 1 DPI, n = 15, p < 0.01, Fig 7A). Delays to terminal disease in infected treated mice equate to approximately one log less prion infectivity, equivalent to approximately 90% reduction in prion replication (Fig 7B). We detected PrPRes in brain homogenates from all infected mice but not uninfected treated controls (Fig 7C). Immunohistochemistry (IHC) revealed similar PrPRes accumulation in the cerebellum of all prion-infected mice, but significantly reduced PrPRes in the hippocampus of infected treated mice compared to untreated mice (Fig 7D). We also observed far less GFAP signal and vacuolation in the cerebellum and hippocampus of infected and uninfected treated mice compared to infected untreated mice. Normal nesting behavior was prolonged to midway through disease course in infected treated mice compared to infected untreated mice (Fig 7D). After this point, we observed impaired nesting behavior in prion-infected mice compare to
Fig 7. Limited LSPC treatment extends survival times and normal nesting behavior in an accelerated prion disease mouse model. (A) Survival of prion infected TgA20 mice was significantly extended with just one PrP siRNA LSPC treatment given 40 DPI (dashed line), and extended further with two (dashed dotted line, given 40 and 60 DPI) and three (dotted line, given 20, 40 and 60 DPI) treatments over infected untreated mice (solid black line). All infected treated mice appeared normal and survived over 100 days after three treatments (solid gray line). (B) LSPC treatment reduced prion replication up to 90%. All mice were infected with $10^6$ LD$_{50}$ units of RML-5 prions. The graph shows equivalent prion titers based on time to terminal disease. (C) Representative immunoblot showing infected, LSPC treated mice appeared to harbor less PrP$_{\text{Res}}$ in their brains compared to brain samples from infected untreated mice, although densitometry revealed no significant differences ($p = 0.074$), except in uninfected treated mice, which harbored no PrP$_{\text{Res}}$. All samples except lane 1 were treated with 50 μg/mL PK. (D) IHC revealed no PrP$_{\text{Res}}$ and little to no GFAP reactivity in uninfected mice treated three times with LSPCs (first column). While we observed no difference in cerebellar (Cb) PrP$_{\text{Res}}$ accumulation in infected mice untreated (second column) or treated (third column) with LSPCs, we did observe significantly less PrP$_{\text{Res}}$ in the hippocampus (Hp) of infected, treated mice compared to infected, untreated mice. We also observed significantly less GFAP and vacuoles overall in brains of infected and uninfected, treated mice compared to infected untreated mice. Scale bar, 100 μm. Quantitation of infected treated samples (third column) show values for Cb/Hp. All other values are combined Cb and Hp scores. We collected data...
uninfected controls, although LSPC-treated mice did exhibit improved nesting behavior compared to untreated mice.

Discussion

The blood-brain barrier (BBB) is a physical barrier composed of endothelial cells, pericytes, and astrocytes that protects neuronal cell types from infection, serum proteins and toxins. Crossing the BBB is the rate-limiting step for delivery of therapeutics to the brain and remains the biggest challenge in producing effective therapeutics for neurodegenerative disorders. The BBB prevents access to the central nervous system (CNS) in a number of ways, including tight junctions between endothelial cells, efflux pumps and cell-surface proteases [80–82].

While the BBB is formidable, therapeutic drugs can still cross this membrane if designed appropriately. Many strategies have been employed to transport drugs across the BBB, but the ‘Trojan Horse’ method is perhaps the best-known approach. A targeting ligand bound to a drug or delivery system binds to a cell-surface receptor on endothelial cells, actively transporting the drug across the BBB. Care must be taken to avoid the endocytotic pathway of the endothelial cells in favor of the transcytotic pathway for transport [80,81]. Multiple ligands and their cognate receptors have been used with varying degrees of success to transport drugs and delivery systems across the BBB, including transferrin [83], insulin [84], low-density lipoprotein [85], low-density lipoprotein receptor ligands [86], leptin [87], and brain-derived neurotrophic factor [81].

Here, we used the neuro-targeting peptide RVG-9r to guide our therapeutic PrP\textsuperscript{C} siRNA across the BBB. RVG-9r is a small peptide from the rabies virus glycoprotein that binds to the α\textsuperscript{7} subunit of nAChRs. Kumar et al. showed that RVG-9r dramatically increased siRNA delivery to the CNS when complexed together. siRNA bound to RVG-9r decreased exogenous GFP by 40%, and anti-viral siRNA against Japanese encephalitis virus increased survival times in mice infected with the virus [88]. We protect both PrP siRNA and RVG-9r in our formulation by complexing them with liposomes to increase serum half-life and decrease nuclease attack by serum proteins. We have previously characterized the ability of our LSPCs to deliver PrP\textsuperscript{C} siRNA to neuronal cells \textit{in vitro} [73]. LSPCs delivered PrP siRNA directly to mouse neuroblastoma cells without the need for lipofection reagents and decreased PrP levels by 50–75% in these cells. The liposomes protected the siRNA from serum degradation, while the RVG-9r peptide delivered PrP\textsuperscript{C} siRNA specifically to cells that expressed nAChRs and decreased PrP\textsuperscript{C} expression, both \textit{in vitro} and \textit{in vivo} [73]. In this report, we confirmed these preliminary \textit{in vivo} results and optimized LSPC dosing regimen to treat prion-infected mice.

We evaluated LSPC pharmacodynamics in two inbred wild-type mouse strains, C57Bl/6 and FVB, and outbred CD-1 mice. Most of the PrP siRNA LSPCs reached the brain by two hours post-injection and remained there for at least 10 days. LSPCs decreased neuronal and renal PrP\textsuperscript{C} mRNA and protein in both mouse lines by up to 50%. We observed no overt biological effects of decreasing PrP\textsuperscript{C} in kidneys, which normally express 10-fold less PrP\textsuperscript{C} and 2-fold less nAChRs than brain cells. However, prudence dictates monitoring these off-target effects in future therapeutic trials.

We first performed a two-week treatment regimen of prion-infected mice given that neuronal PrP\textsuperscript{C} is decreased from four to fifteen days after a single LSPC injection. We also opted to
start our LSPC therapeutic midway through prion disease course to determine whether the
treatment could reverse the early neuronal changes seen in prion-infected mice. When we
observed that repeated LSPC injections every two weeks were causing significant side effects,
we performed another LSPC treatment study with intervals ranging from 22–33 days. Unfortu-
nately, repeated LSPC treatments at extended intervals did not extend the survival times of
prion-infected mice. However, we discovered a subset (6/19) of infected mice treated every
two weeks responded positively to LSPC therapy, living approximately 7% longer than infected
untreated control and 9% longer than infected, non-responder treated mice. Five of six
responder mice appeared to accumulate less PrP[^Res] and GFAP, and contained fewer vacuoles
in their brains compared to infected untreated and non-responder mice. We observed a more
pronounced decrease in neuropathology in the hippocampus, involved in learning, memory
and behavior; compared to the cerebellum, which functions in motor control and coordina-
tion. These neuropathologic observations correlate with behavioral assessments revealing pro-
longed, improved burrowing and nesting in all infected mice treated with LSPCs, including
non-responders, over infected untreated controls. Improvement in nesting, but not burrowing
behavior, was sustained until terminal disease. More severe destruction of the cerebellum may
have impaired the ability of treated mice to coordinate movement required for burrowing,
while nesting requires less motor coordination and strength. Improvements in these behav-
ioral tests, which model human activities of daily living and social engagement [89–92], may
be an important predictor of therapeutic success for LSPCs and other drugs combating neuro-
degenerative diseases. That LSPC treatment improved behavior may be important for at least
sustaining quality of life for afflicted individuals, if not extending survival time.

Deaths of the uninfected control group were unexpected. Three of four mice in this group
died or were euthanized one hour after LSPC treatment due to severe morbidity. Observations
noted during necropsy and the sudden morbidity after treatment suggested that these mice
died of an acute Type III immune complex response. Total IgG levels against RVG-9r were
increased in most treated mice, indicating that RVG-9r is stimulating the immune system.
Increased astrogliosis in these mice supports this contention. However, 5 of 6 responder mice
expressed no significant anti-RVG-9r titers and reduced astrogliosis. Immune responses
against the RVG-9r peptide were not observed previously [88], but did not entail repeated
administrations utilized here. Indeed, we did not detect anti-RVG-9r titers until after the third
exposure, consistent with potentiating an immune response. Moreover, PrP siRNA may act as
a TLR agonist, especially when complexed with liposomes, creating a powerful adjuvant for
antibody production [93,94].

Scientists developing LSPCs and similar therapeutics with immunogenic potential must
consider strategies to avoid or circumvent these immune responses. Here we tested IN LSPC
delivery as a more direct route to the central nervous system that avoids LSPC exposure to the
spleen and most other peripheral lymph nodes en route. We observed double the response rate
(60 versus 33%) and half the seroconversion rate (40 versus 78%) among IN versus IV treated
mice, respectively. Simultaneous IV and IN treatment potentiated seroconversion to 100%,
suggesting spatiotemporal cross-priming and further emphasizing the need to monitor and
control unwanted immune responses to LSPCs. We are currently exploring transient immuno-
suppression during IN delivery to circumvent the immune response that seemed to dampen
therapeutic benefit of LSPCs. We are also exploring liposome modifications, include adding
PEGylated groups to make them less available to bind to serum proteins and generate immune
complexes [95,96].

To investigate whether this immune-mediated suppression of LSPC can be circumvented,
we conducted a third LSPC treatment study using an accelerated disease progression model
wherein we can administer fewer LSPCs treatments. We intracerebrally inoculated TgA20
mice, who die from prion infection in 60 days, with prions and treated them once, twice, or thrice. As expected, three LSPC treatments did not induce anti-RVG-9r titers but did result in significantly prolonged survival (up to 22%) and improved behavior that correlated with decreased neuropathology in treated mice compared to untreated mice. These results are consistent with previous studies using lentiviral delivery directly to the brain via stereotactic injection [72] (up to 24%), while using a far less invasive and potentially safer delivery system, if modifications to avoid unwanted immune responses can be achieved. LSPC treatment also impaired prion replication in the brains of infected TgA20 mice by up to 90%. A peculiarity of prion disease is that accumulation of PrP\text{Res}, as assessed by PK digestion and western blotting, does not always correlate to infectious prion titers, as assessed by mouse bioassay [97–102]. This anomaly could explain why LSPC treated mice replicated less prions and lived longer despite detecting no significant difference in PrP\text{Res}. Additionally, PrP\text{Res} distribution in infected mice is not uniform: we detected most PrP\text{Res} in the cerebellum, and far less in the hippocampus.

In summary, these data support LSPCs as an efficient vehicle to deliver therapeutic drugs across the BBB to the CNS. Additionally, the siRNA knockdown of PrP\text{C}, the substrate for prion replication, can reduce prion replication and neuropathology, and extend survival times in RVG-9r immunotolerant mice. Importantly, LSPC therapy may also significantly prolong normal behavior, and ameliorate cognitive decline associated with prion diseases. LSPC therapy could be extended to treat other protein misfolding diseases, like Alzheimer’s, Parkinson’s and Huntington Diseases, and amyotrophic lateral sclerosis. Therapeutics that can improve quality of life and daily living activities of afflicted patients would represent tremendous progress in treating these increasingly prevalent diseases. However, immune responses to these therapeutics must be carefully considered and avoided to prevent exacerbating disease-mediated neuropathology with immune-mediated neuroinflammation.

**Materials and methods**

**Mice**

C57Bl/6, FVB and CD-1 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). TgA20 mice overexpressing PrP\text{C} were created as previously described [77]. Mice were euthanized using CO\text{2}. All mice were bred and maintained at Lab Animal Resources, accredited by the Association for Assessment and Accreditation of Lab Animal Care International, in accordance with protocols approved by the Institutional Animal Care and Use Committee at Colorado State University.

**Generation of liposomes**

1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) LSPCs consist of a 1:1 DOTAP:cholesterol (Avanti Lipids 890890 and 700000) ratio in a 1:1 chloroform:methanol solution. The solvents were evaporated using N\text{2} gas and the resultant dry lipid film was placed under vacuum for a total of 8 hours to remove any excess solvent. A stock solution of liposomes was made at an 8 mM (40 μmole total) concentration by resuspending the lipid film in 5 mL of 10% sucrose heated at 55˚C. All components (lipid film and sucrose) were kept at this temperature during rehydration. One milliliter of heated sucrose was added to the lipid cake every 10 minutes and the lipid film swirled every 3 minutes to promote lipid mixing. Resulting liposomes were stored at 4˚C.

**Generating LSPCs and treating mice**

PrP\text{C} 1578 siRNA sequence: GAAAGTGGCTCCATTTCCAAA (Qiagen)
PrP\textsuperscript{C} 1672 siRNA sequence: ACATAAACTGCGATAGCTTC (Qiagen).

RVG-9r peptide: YTAIMPENPRPGTPCDIFTNSRGKRASNGGGGrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrr
5’-AGACAGCAGCTGTGGTCGAT-3’. Supermix (BioRad) was added to the cDNA/primer solution to generate a final reaction volume of 20 μL. Droplet generator oil (BioRad) was added to the reaction mix and droplets were generated using a QX-100 droplet generator. Droplets were transferred to a 96-well plate and sealed with pierceable sealing foil sheets (BioRad). PCR amplification was performed using a C1000 Touch Thermal Cycler (BioRad) with the following cycling parameters: enzyme activation at 95˚C for 5 minutes, denaturation at 95˚C for 30 seconds, annealing/elongation at 57˚C for 1 minute for 40 cycles, signal stabilization at 4˚C for 5 minutes and 95˚C for 5 minutes, and hold at 4˚C. Following amplification, the droplets were transferred to a QX100 droplet reader and analyzed using Quantasoft (BioRad) software.

**Prion infections, clinical scoring and mouse dissections**

RML-5 prions were prepared as previously described [79]. 10% RML-5 brain homogenates were diluted 1:10 in 1X PBS supplemented with 100 units/mL of Penicillin and 100 μg/mL of Streptomycin (Gibco) immediately before inoculation. Prion titers were determined using the relationship:

\[ y = 11.45 - 0.088x, \]

where y is lethal dose 50 (LD\(_{50}\)) units and x is the incubation time in days to terminal disease [78]. We injected 100 μL of inoculum containing 3.3 x 10\(^6\) LD\(_{50}\) units, in the left or right bottom quadrant of the intraperitoneal cavity, or 30 μL (10\(^6\) LD\(_{50}\) units) injected intracerebrally 3 mm deep through the coronal suture 3–5 mm lateral of the sagittal suture, with a 29-gauge insulin syringe (BD). Mice were monitored daily and sacrificed at the onset of terminal disease or specified time points. We scored mice for clinical prion disease as previously described [103]. Briefly, we employed a scoring system to assess the severity of disease, including: tail rigidity (0–2), akinesia (0–4), ataxia (0–4), tremors (0–4), and weight loss (0–4). Mice scored above 10 or 4 in any single category were considered terminally ill and immediately euthanized via CO\(_2\) inhalation, replacing 20% of air per minute to effect. Brain, spleen, and sera were collected from each mouse at time of euthanasia. Half of the brain and spleen were frozen at -20˚C, and the other halves were fixed in 4% paraformaldehyde.

**Behavioral testing**

Behavioral tests were started approximately midway through disease course. For burrowing, mice were given approximately 100 grams of food stuffed into a 6-inch plastic PVC pipe. Mice were allowed to burrow out the food for 30 minutes. Rate of burrowing was calculated by the number of grams of food removed divided by total time burrowed. For nesting, mice were given a small cotton nestlet and allowed to build a nest overnight. Mice were scored on a scale from 0–4, with 0 being no nest and 4 being a normally built nest. Average nesting scores were calculated for each treatment group.

**Immunohistochemistry**

Brains from prion-infected mice from the 1st LSPCs terminal study were sent to Colorado State University’s Veterinary Diagnostic Laboratory (VDL) for paraffin embedding, sectioning and GFAP staining. Unprocessed sections were stained for PrP\(^{Res}\) using the following protocol. Slides were incubated at 53˚C for 30 minutes before being immersed in xylene twice for 10 minutes. The slides were then rehydrated through an ethanol gradient consisting of 100%, 95% and 70% concentrations for 5 minutes each and then immersed in 88% formic acid for 10 minutes. After washing the slides in running water for 10 minutes, the slides were processed through antigen retrieval while in citrate buffer, pH of 7.4. The slides were allowed to cool before being washed.
twice in a 0.1% PBS-Triton buffer for 5 minutes on a rocker. Slides were immersed in a 3% hydrogen peroxide preparation in methanol for 30 minutes to extinguish exogenous peroxidase activity of the tissues before undergoing another wash cycle. Tissues were then encircled with a hydrophobic barrier and allowed to incubate with Superblock (Pierce) for 30 minutes. The excess block was tapped off each slide and the slides were incubated overnight in 4°C with D18 anti-PrP monoclonal antibody at a 1:1000 dilution. Slides were washed and incubated with a biotinylated anti-human Ig (1:1000) for 1 hour at room temperature. They were then washed and incubated with streptavidin solution for 30 minutes at room temperature. After 3 wash cycles, the slides were incubated with diaminobenzidine reagent for 5 minutes to develop the staining. Slides were then washed and counterstained in hematoxylin for 5 minutes, and then immersed in water for 10 minutes to deactivate the hematoxylin. Slides were dehydrated through the alcohol gradient and xylene before being mounted with a coverslip. Slides were visualized using a BX-60 microscope and pictures recorded using a cooled charge-coupled diode camera (Olympus). We quantified PrP<sub>Res</sub> and GFAP signal intensities using the CMYK color model and Graphic Converter 10 (Lemke Software) as previously described [79,104,105]. We quantified vacuolation by manual counting in at least three non-consecutive sections per mouse and at least two mice per group.

**ELISA for RVG-9r-specific IgG**

Serum samples from terminally ill mice were collected by heart stick after euthanasia. Samples were stored at -20°C until assay was performed. 1 μg of RVG-9r was coated into 96-well ELISA plates (Nunc) using carbonate/bicarbonate buffer (Sigma). The plates incubated overnight at 4°C, then washed 2X with ELISA wash buffer (1X PBS + 0.05% Tween). All wells were blocked with SuperBlock (ThermoFisher) at room temperature for 2 hours. Plates were washed 2X with ELISA wash buffer. The following serum dilutions from LSPCs-treated mice were dispensed onto the plate: 1:50, 1:100, 1:250, 1:500, 1:1000, and 1:2000. The serum was incubated overnight at 4°C. All wells were washed 4X with ELISA wash buffer. A 1:5000 dilution of an anti-mouse IgG horseradish peroxidase secondary antibody (Cell signaling) in SuperBlock was added to each well and incubated at room temperature for 2 hours. All wells were washed again with ELISA wash buffer. TMB substrate (ThermoFisher) was added to each well and allowed to incubate until a deep blue color change developed. To stop the reaction, a stop solution (0.5 M H<sub>2</sub>S<sub>O</sub><sub>4</sub> in 1X PBS) was added to each well. Photometric analysis was performed at 450 nm using a Multiskan Spectrum plate reader (ThermoFisher).

**PK digestion and western blots**

Proteinase K (Roche) was added to western blot samples at a 1:10 dilution for a final concentration of 50 μg/mL. The samples were incubated at 37°C for 30 minutes with a 10-minute deactivation step at 95°C. Proteins were electrophoretically separated using 12% sodium dodecyl sulfate polyacrylamide gels (Invitrogen). Proteins were then transferred to a polyvinylidene difluoride membrane (Millipore). Membranes were blocked using 5% non-fat dry milk for 1 hour, washed 2X for 10 minutes each using 1X PBS with 0.2% Tween, then incubated with horseradish peroxidase-conjugated BAR-224 (SPI Bio) anti-PrP<sup>C</sup> antibody diluted 1:20,000 overnight at 4°C. Membranes were washed again 6X for 10 minutes each and incubated with enhanced chemiluminescent substrate (Millipore) for 5 minutes. Membranes were photographed using an ImageQuant LAS 4000 (GE).

**Statistical analysis**

We performed statistical analyses using GraphPad Prism and report specific tests, parameters, results and significance values for each experiment for which the test was used.
Supporting information

S1 Fig. Additional control LSPCs demonstrate PrP siRNA LSPC specificity. Naïve FVB mice were injected intravenously with PBS, PrP siRNA LSPCs, scrambled PrP siRNA LSPCs, irrelevant siRNA LSPCs or RVM-9r LSPCs. Protein and mRNA expression were analyzed four days after treatment by flow cytometry and ddPCR, respectively. Only PrP siRNA LSPCs significantly reduced PrP mRNA (A) and protein (B) in the brain. Scrambled PrP siRNA LSPCs significantly increased PrP mRNA in both brains and kidneys, but reduced PrP protein expression in kidneys. All other controls did not significantly affect PrP mRNA and protein expression. Error bars indicate 95% CI of the mean. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. One-way ANOVA with Dunnett’s multiple comparisons.

S2 Fig. We compared (A) IN versus (B) IV delivery of LSPCs on survival of prion infected mice. (A) IN responders survived significantly longer (dotted red line, n = 3, median survival, 238 DPI) than IN non-responders (dotted black line, n = 2, 217 and 227 DPI, p < 0.05). (B). IV responders similarly lived significantly longer (dotted red line, n = 3, median survival 232 DPI) than IV non-responders (dotted black line, n = 9, median survival 216 DPI).

S3 Fig. IgG levels against RVG-9r in infected TgA20 mice treated 1-3X with LSPCs. We detected no significant RVG-9r titers in any group, the data from which we compared to data from uninfected, treated wild type mice reported in Fig 6A.

S1 Table. Treatment groups. Breakdown of control and treated groups in the 1st (Panels A and B) and 2nd (Panels C and D) LSPC treatment studies, along with days post infection each LSPC treatment was given.

S2 Table. Treatment regimen. DPI of LSPC treatment and euthanasia of early time point mice treated with LSPCs to assess minimal LSPCs treatments required for an immune response.

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LSPCs as therapy for neurodegenerative protein misfolding diseases

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References

1. Aguzzi A, Nuvolone M, Zhu C. The immunobiology of prion diseases. Nature Reviews Immunology. 2013. https://doi.org/10.1038/nri3553 PMID: 24189576
2. Kovacs GG, Budka H. Prion diseases: From protein to cell pathology. American Journal of Pathology. 2008. https://doi.org/10.2353/apjpath.2008.070442 PMID: 18245809
3. Jeffrey M, McGovern G, Sisó S, González L. Cellular and sub-cellular pathology of animal prion diseases: Relationship between morphological changes, accumulation of abnormal prion protein and clinical disease. Acta Neuropathologica. 2011. https://doi.org/10.1007/s00401-010-0700-3 PMID: 20532540
4. Prusiner SB. Novel proteinaceous infectious particles cause scrapie. Science (80-). 1982; 216: 136–144. Available: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=6901762&retmode=r&cmd=prlinks
5. Aguzzi A, Sigurdsson C, Heikenwalder M. Molecular Mechanisms of Prion Pathogenesis. Annu Rev Pathol Mech Dis. 2007; 0: 070808165255001. https://doi.org/10.1146/annurev.pathol.3.121806.154326
6. Belay ED. Transmissible Spongiform Encephalopathies. International Encyclopedia of Public Health. 2016. https://doi.org/10.1016/B978-0-12-803678-5.00469–0
7. Imran M, Mahmood S. An overview of animal prion diseases. Virol J. 2011; https://doi.org/10.1186/1743-422X-8-493 PMID: 22044871
8. Mead S. Prion disease genetics. European Journal of Human Genetics. 2006. https://doi.org/10.1038/sj.ejhg.5201544 PMID: 16391566
9. Basler K, Oesch B, Scott M, Westaway D, Walchli M, Groth DF, et al. Scrapie and cellular PrP isoforms are encoded by the same chromosomal gene. Cell. 1986; 46: 417–428. Available: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=2873895&retmode=r&cmd=prlinks https://doi.org/10.1016/0092-8674(86)90662-6 PMID: 2873895
10. Oesch B, Westaway D, Walchli M, McKinley MP, Kent SBH, Aebersold R, et al. A cellular gene encodes scrapie PrP 27–30 protein. Cell. 1985; 40: 735–746. https://doi.org/10.1016/0092-8674(85)90333-2 PMID: 2859120
11. Manson J, West JD, Thomson V, McBride P, Kaufman MH, Hope J. The prion protein gene: a role in mouse embryogenesis? Development. 1992; 115: 117–122. PMID: 1353438
12. Miele G, Alejo Blanco AR, Baybutt H, Horvat S, Manson J, Clinton M. Embryonic activation and developmental expression of the murine prion protein gene. Gene Expr. 2003; 11: 1–12. Available: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12691521 PMID: 12691521
13. Locht C, Chesebrot B, Racet R, Keith JM, Fields BN. Molecular cloning and complete sequence of prion protein cDNA from mouse brain infected with the scrapie agent. 1986; 83: 6372–6376.
14. Naslavsky N, Stein R, Yanai A, Friedlander G, Taraboulous A. Characterization of detergent-insoluble complexes containing the cellular prion protein and its scrapie isoform. J Biol Chem. 1997; 272: 6324–6331. https://doi.org/10.1074/jbc.272.6.6324 PMID: 9045652
15. McKinley MP, Bolton DC, Prusiner SB. A protease-resistant protein is a structural component of the scrapie prion. Cell. 1983; 35: 57–62. https://doi.org/10.1016/0092-8674(83)90207-6 PMID: 6432339
16. Prusiner SB, Groth DF, Bolton DC, Kent SB, Hood LE. Purification and structural studies of a major scrapie prion protein. Cell. 1984; 38: 127–134. Available: http://www.sciencedirect.com/science/article/pii/0092867484905336 https://doi.org/10.1016/0092-8674(84)90533-6 PMID: 6432339
17. Brown DR, Wong BS, Hafiz F, Clive C, Haswell SJ, Jones IM. Normal prion protein has an activity like that of superoxide dismutase [published erratum appears in Biochem J 2000 Feb 1;345 Pt 3:767]. Biochem J. 1999; 344 Pt 1: 1–5.
18. Brown DR, Clive C, Haswell SJ. Antioxidant activity related to copper binding of native prion protein. J Neurochem. 2001; 76: 69–76. Available: http://www.ncbi.nlm.nih.gov/htbin-post/Entrez/query?db=m&form=6&dopt=PubMed&u id=11145979 https://doi.org/10.1046/j.1471-4159.2001.00009.x PMID: 11145979
19. Vassallo N, Herms JW. Cellular prion protein function in copper homeostasis and redox signalling at the synapse. Journal of Neurochemistry. 2003. https://doi.org/10.1046/j.1471-4159.2003.01882.x PMID: 12859667

20. Kuwahara C, Takeuchi AM, Nishimura T, Haraguchi K, Kubosaki A, Matsumoto Y, et al. Prions prevent neuronal cell-line death. Nature. 1999; 400: 225–226. https://doi.org/10.1038/22241 PMID: 10421360

21. Bounhar Y, Zhang Y, Goodyer CG, LeBlanc A. Prion protein protects human neurons against Bax-mediated apoptosis. J Biol Chem. 2001; 276: 39145–39149. Available: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11522774 https://doi.org/10.1074/jbc.C100443200 PMID: 11522774

22. Herms JW, Tings T, Dunker S, Kretzschmar HA. Prion protein affects Ca2+-activated K+ currents in cerebellar purkinje cells. Neurobiol Dis. 2001; 8: 324–330. Available: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11300727 https://doi.org/10.1006/nbdi.2000.0369 PMID: 11300727

23. Brini M. The Prion Protein and Its Paralogue Doppel Affect Calcium Signaling in Chinese Hamster Ovary Cells. Mol Biol Cell. 2005; https://doi.org/10.1091/mbc.E04-10-0915 PMID: 15785688

24. Fuhrmann M, Bittner T, Mitteregger G, Haider N, Moosmang S, Kretzschmar H, et al. Loss of the cellular prion protein affects the Ca2+ homeostasis in hippocampal CA1 neurons. J Neurochem. 2006; https://doi.org/10.1111/j.1471-4159.2006.04011.x PMID: 16945105

25. Chiariini LB, Freitas AR, Zanata SM, Brentani RR, Martins VR, Linden R. Cellular prion protein transduces neuroprotective signals. EMBO J. 2002; 21: 3317–3326. Available: http://emboj.oupjournals.org/cgi/content/full/21/13/3317 https://doi.org/10.1093/emboj/cfd32 PMID: 12093733

26. Chen S, Mange A, Don G, Lehmann S, Schachner M. Prion protein as trans-interacting partner for copper in vivo. Nature. 1997; 390: 684–687. https://doi.org/10.1038/37783 PMID: 9414160

27. Shiraiishi N, Ohta Y, Nishikimi M. The octapeptide repeat region of prion protein binds Cu(II) in the redox-inactive state. Biochem Biophys Res Commun. 2000; 267: 398–402. https://doi.org/10.1006/ bbrc.1999.1944 PMID: 10623631

28. Horshaw MP, McDermott JR, Candy JM, Lakey JH. Copper binding to the N-terminal tandem repeat region of mammalian and avian prion protein: structural studies using synthetic peptides. Biochem Biophys Res Commun. 1995; 214: 993–999. https://doi.org/10.1016/bbrc.1995.2384 PMID: 7575574

29. Zhang CC, Steele AD, Lindquist S, Lodish HF. Prion protein is expressed on long-term repopulating hematopoietic stem cells and is important for their self-renewal. Proc Natl Acad Sci U S A. 2006; 103: 2184–2189. https://doi.org/10.1073/pnas.0510577103 PMID: 16467153

30. Bremer J, Baumann F, Tiberi C, Wessig C, Fischer H, Schwarz P, et al. Axonal prion protein is required for peripheral myelin maintenance. Nat Neurosci. Nature Publishing Group; 2010; 13: 310–318. https://doi.org/10.1038/nn.2483 PMID: 20096419

31. Lotscher M, Recher M, Hunziker L, Klein MA. Immunologically induced, complement-dependent up-regulation of the prion protein in the mouse spleen: follicular dendritic cells versus capsule and trabeculae. J Immunol. 2003; 170: 6040–6047. Available: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12794132 https://doi.org/10.4049/jimmunol.170.12.6040 PMID: 12794132

32. Polymenidou M, Heppner FL, Pellicioli EC, Urich E, Miele G, Braun N, et al. Humoral immune response to native eukaryotic prion protein correlates with anti-prion protection. Proc Natl Acad Sci U S A. 2004; 101 Suppl: 14670–6. https://doi.org/10.1073/pnas.0404772101 PMID: 15292505

33. Tobler I, Gaus SE, Deboer T, Achermann P, Fischer M, Rulicke T, et al. Altered circadian activity rhythms and sleep in mice devoid of prion protein. Nature. 1996; 380: 639–642. Available: http://www.nature.com/nature/journal/v380/n6575/abs/380639a0.html https://doi.org/10.1038/380639a0 PMID: 8602267

34. Büeler H, Fischer M, Lang Y, Bluthmann H, Lipp HP, DeArmond SJ, et al. Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein. Nature. 1992; 358: 577–582. https://doi.org/10.1038/356777a0 PMID: 1373229

35. Kimberlin RH, Walker CA. Suppression of scrapie infection in mice by heteropolyanion 23, dextran sulfate, and some other polyanions. Antimicrob Agents Chemother. 1986; 30: 409–413. https://doi.org/10.1128/aac.30.3.409 PMID: 2430521

36. Kimberlin RH, Walker CA. The antiviral compound HPA-23 can prevent scrapie when administered at the time of infection. Arch Virol. 1983; 76: 9–18. PMID: 6986005
38. Ehlers B, Diringer H. Dextran sulphate 500 delays and prevents mouse scrapie by impairment of agent replication in spleen. J Gen Virol. 1984; 65: 1325–1330. https://doi.org/10.1099/0022-1317-65-8-1325 PMID: 6205119

39. Wong C, Xiong LW, Horiuchi M, Raymond L, Wehrly K, Chesebro B, et al. Sulfated glycans and elevated temperature stimulate PrP(Sc)-dependent cell-free formation of prion-resistant prion protein. EMBO J. 2001; 20: 377–386. Available: http://www.ncbi.nlm.nih.gov/htbin-post/Entrez/query?db=m&form=6&dopt=r&uid=11157745 https://doi.org/10.1093/emboj/20.3.377 PMID: 11157745

40. Shyng SL, Lehmann S, Moulder KL, Harris DA. Sulfated glycans stimulate endocytosis of the cellular isoform of the prion protein, PrPC, in cultured cells. J Biol Chem. 1995; 270: 30221–30229. https://doi.org/10.1074/jbc.270.50.30221 PMID: 8530433

41. Farquhar C, Dickinson A, Bruce M. Prophylactic potential of pentosan polysulphate in transmissible spongiform encephalopathies [letter]. Lancet. 1999; 353: 117.

42. Caughey B, Brown K, Raymond GJ, Katzenstein GE, Thresher W. Binding of the protease-sensitive form of PrP (prion protein) to sulfated glycosaminoglycan and congo red [corrected] [published erratum appears in J Virol 1994 Jun;68(6):4107]. J Virol. 1994; 68: 2135–2141. Available: http://jvi.asm.org/content/68/4/2135.short PMID: 7511169

43. Ingrosso L, Ladogana A, Pochiari M. Congo red prolongs the incubation period in scrapie-infected hamsters. J Virol. 1995; 69: 506–508. PMID: 7983747

44. Caspi S, HALIMI M, Yanai A, Sasson SB, Taraboulos A, GABIZON R. The anti-prion activity of Congo red. Putative mechanism. J Biol Chem. 1998; 273: 3484–3489. Available: http://www.ncbi.nlm.nih.gov/htbin-post/Entrez/query?db=m&form=6&dopt=r&uid=10937879 https://doi.org/10.1074/jbc.273.6.3484 PMID: 9452472

45. Supattapone S, Nguyen HO, Cohen FE, Prusiner SB, Scott MR. Elimination of prions by branched polyamines and implication for therapeutics. Proc Natl Acad Sci U S A. 1999; 96: 14529–14534. https://doi.org/10.1073/pnas.96.25.14529 PMID: 10588739

46. Supattapone S, Wille H, Uyechi L, Safar J. Branched polyamines cure prion-infected neuroblastoma cells. J. . . 2001; Available: http://jvi.asm.org/content/abstract/75/7/3453

47. Winklhofe KF, Tatzelt J. Cationic lipopolyamines induce degradation of PrPSc in scrapie-infected mouse neuroblastoma cells. Biol Chem. 2000; 381: 463–9. Available: http://www.ncbi.nlm.nih.gov/htbin-post/Entrez/query?db=m&form=6&dopt=r&uid=10937879 https://doi.org/10.1515/BC.2000.061 PMID: 10937879

48. Solassol J, Crozet C, Perrier V, Leclaire J, Bé ranger F, Caminade AM, et al. Cationic phosphorus-containing dendrimers reduce prion replication both in cell culture and in mice infected with scrapie. J Gen Virol. 2004; https://doi.org/10.1099/vir.0.19726–0

49. Haig DA, Clarke MC. The effect of beta-propiolactone on the scrapie agent. J Gen Virol. 1968; 3: 281–283. https://doi.org/10.1099/0022-1317-3-2-281 PMID: 4972403

50. Cochran KW. Failure of adenine arabinoside to modify scrapie in mice. J Gen Virol. 1971; 13: 353–354. https://doi.org/10.1099/0022-1317-13-2-353 PMID: 5168245

51. Tateishi J. Antibiotics and antivirals do not modify experimentally-induced Creutzfeldt-Jakob disease in mice. J Neurol Neurosurg Psychiatry. 1981; 44: 723–724. https://doi.org/10.1136/jnnp.44.4.723 PMID: 6170735

52. Pocchiari M, Casaccia P, Ladogana A. Amphotericin B: a novel class of antiscrapi drugs [published erratum appears in J Infect Dis 1990 Mar;161(3):591]. J Infect Dis. 1989; 160: 795–802. https://doi.org/10.1093/infdis/160.5.795 PMID: 2509571

53. Pocchiari M, Schmittinger S, Masullo C. Amphotericin B delays the incubation period of scrapie in intracerebrally inoculated hamsters. J Gen Virol. 1987; 68: 219–223. https://doi.org/10.1099/0022-1317-68-1-219 PMID: 2433887

54. Bian J, Kang HE, Telling GC. Quinacrine promotes replication and conformational mutation of chronic wasting disease prions. Proc Natl Acad Sci. 2014; 111: 6028–6033. https://doi.org/10.1073/pnas.132377111 PMID: 24711410

55. Wroe SJ, Pal S, Siddique D, Hyare H, Macfarlane R, Joiner S, et al. Clinical presentation and pre-mortem diagnosis of variant Creutzfeldt-Jakob disease associated with blood transfusion: a case report. Lancet. 2006; 368: 2061–2067. Available: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17161728 https://doi.org/10.1016/S0140-6736(06)69835-5 PMID: 17161728

56. Collinge J, Gorham M, Hudson F, Kennedy A, Keogh G, Pal S, et al. Safety and efficacy of quinacrine in human prion disease (PRION-1 study): a patient-preference trial. Lancet Neurol. 2009; https://doi.org/10.1016/S1474-4422(09)70049-3
57. Tagliavini F, McArthur RA, Canciani B, Giacone G, Porro M, Bugiani M, et al. Effectiveness of anthra
cycline against experimental prion disease in Syrian hamsters. Science (80-). 1997; 278: 1404–1405.
Available: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=9323198&retmode=ref&cmd=prlinks

58. Stewart LA, Rydzewska LHM, Keogh GF, Knight RSG. Systematic review of therapeutic interventions
in human prion disease. Neurology. 2008; https://doi.org/10.1212/01.wnl.0000308955.25760.c2
PMID: 18391159

59. Enari M, Flechsig E, Weissmann C. Scrapie prion protein accumulation by scrapie-infected neuroblas-
toma cells abrogated by exposure to a prion protein antibody. Proc Natl Acad Sci U S A. 2001; 98:
9295–9299. Available: http://www.pnas.org/cgi/content/full/98/16/9295 https://doi.org/10.1073/pnas.151242598
PMID: 11470893

60. Peretz D, Williamson RA, Kaneko K, Vergara J, Leclerc E, Schmitt-Ulms G, et al. Antibodies inhibit
prion propagation and clear cell cultures of prion infectivity. Nature. 2001; 412: 739–743. Available:
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11507642
https://doi.org/10.1038/35089090 PMID: 11507642

61. White AR, Enever P, Tayebi M, Mushens R, Linehan J, Brandner S, et al. Monoclonal antibodies
inhibit prion replication and delay the development of prion disease. Nature. 2003; 422: 80–83. Avail-
able: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11507642

62. Klohn PC, Farmer M, Linehan JM, O’Malley C, Fernandez de Marco M, Taylor W, et al. PrP
Antibodies Do Not Trigger Mouse Hippocampal Neuron Apoptosis. Science (80-). 2012; 335: 52.
https://doi.org/10.1126/science.1215579 PMID: 22223800

63. Sonati T, Reimann RR, Falsig J, Baral PK, O’Connor T, Hornemann S, et al. The toxicity of anti-
prion antibodies is mediated by the flexible tail of the prion protein. Nature. 2013; 501: 102–106.
https://doi.org/10.1038/nature12402 PMID: 23903654

64. Büeler H, Raeber a, Sailer a, Fischer M, Aguuzzi a, Weissmann C. High prion and PrPSc levels but
delayed onset of disease in scrapie-inocu lated mice heterozygous for a disrupted PrP gene. Mol Med.
1994; 1: 19–30. PMID: 8790598

65. Fischer M, Rülicke T, Raeber a, Sailer a, Moser M, Oesch B, et al. Prion protein (PrP) with amino-prox-
imal deletions restoring susceptibility of PrP knockout mice to scrapie. EMBO J. 1996; 15: 1255–64.
https://doi.org/10.1002/j.1460-2075.1996.tb00467.x PMID: 8635458

66. Mallucci GR, White MD, Farmer M, Dickinson A, Khatun H, Powell AD, et al. Targeting Cellular Prion
Protein Reverses Early Cognitive Deficits and Neurophysiological Dysfunction in Prion-Infected Mice.
Neuron. 2007; 53: 325–335. https://doi.org/10.1016/j.neuron.2007.01.005 PMID: 17270731

67. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interfer-
ence by double-stranded RNA in Caenorhabditis elegans. Nature. 1998/03/05. 1998; 391: 806–811.
https://doi.org/10.1038/35888 PMID: 9486653

68. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleot ide RNAs
mediate RNA interference in cultured mammalian cells. Nature. 2001; 411: 494–498. https://doi.org/
10.1038/35078107 PMID: 11373684

69. Paddison PJ, Caudy AA, Bernstein E, Hannon GJ, Conklin DS. Short hairpin RNAs (shRNAs) induce
sequence-specific silencing in mammalian cells. Genes & Dev. 2002/04/18. 2002; 16: 948–958.
https://doi.org/10.1101/gad.981002 PMID: 11959843

70. McCaffrey AP, Meuse L, Pham TT, Conklin DS, Hannon GJ, Kay MA. RNA interference in adult mice.
Nature. 2002; 418: 38–39. https://doi.org/10.1038/418038a [pii] PMID: 12097900

71. Daude N. Specific inhibition of pathological prion protein accumulation by small interfering RNAs. J
Cell Sci. 2003; 116: 2775–2779. https://doi.org/10.1242/jcs.00494 PMID: 12759373

72. White MD, Farmer M, Mirabile I, Brandner S, Collinge J, Mallucci GR. Single treatment with RNAi
against prion protein rescues early neuronal dysfunction and prolongs survival in mice with prion dis-
ease. Proc Natl Acad Sci. 2008; 105: 10238–10243. https://doi.org/10.1073/pnas.0802759105 PMID:
18632556

73. Pulford B, Reim N, Bell A, Veatch J, Forster G, Bender H, et al. Liposome-siRNA Peptide Complexes
Cross the Blood-Brain Barrier and Significantly Decrease PrPC on Neuronal Cells and PrPRES in
Infected Cell Cultures. Pulford B, Reim N, Bell A, Veatch J, Forster G, Bender H, et al., editors. PLoS
One. 2010; 5: e11085. https://doi.org/10.1371/journal.pone.0011085 PMID: 20559428

74. Pulford B, Reim N, Bell A, Veatch J, Forster G, Bender H, et al. Liposome-siRNA-peptide complexes
cross the blood-brain barrier and significantly decrease PrPC on neuronal cells and PrPRES in
infected cell cultures. PLoS One. 2010; 5: 1–13. https://doi.org/10.1371/journal.pone.0011085 PMID:
20559428
75. Bitko V, Musiyenko A, Shulyayeva O, Barik S. Inhibition of respiratory viruses by nasally administered siRNA. Nat Med. 2005; https://doi.org/10.1038/nm1164 PMID: 15619632

76. Zhang W, Yang H, Kong X, Mohapatra S, Juan-Vergara HS, Hellermann G, et al. Inhibition of respiratory syncytial virus infection with intranasal siRNA nanoparticles targeting the viral NS1 gene. Nat Med. Nature Publishing Group; 2005; 11: 56–62. https://doi.org/10.1038/nm1174 PMID: 15619625

77. Fischer M, Rulicke T, Raebber A, Sailer A, Moser M, Oesch B, et al. Prion protein (PrP) with amino-proximal deletions restoring susceptibility of PrP knockout mice to scrapie. EMBO J. 1996; 15: 1255–1264. Available: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=8635458&retmode=prlinks PMID: 8635458

78. Prusiner SB, Cochran SP, Groth DF, Downey DE, Bowman KA, Martinez HM. Measurement of the scrapie agent using an incubation time interval assay. Ann Neurol. 1982; 11: 353–358. https://doi.org/10.1002/ana.410110406 PMID: 6808890

80. Abbott NJ, Patabendige AAK, Dolman DEM, Yusof SR, Begley DJ. Structure and function of the blood-brain barrier. Neurobiology of Disease. 2010. https://doi.org/10.1016/j.nbd.2009.07.030 PMID: 19664713

82. Lemke A, Kiderlen AF, Petri B, Kayser O. Delivery of amphotericin B nanosuspensions to the brain and determination of activity against Balamuthia mandrillaris amebas. Nanomedicine Nanotechnology, Biol Med. 2010; https://doi.org/10.1016/j.nano.2009.12.004 PMID: 20060497

84. Gaskill BN, Karas AZ, Garner JP, Pritchett-Corning KR. Nest Building as an Indicator of Health and Welfare in Laboratory Mice. J Vis Exp. 2013; https://doi.org/10.3791/51012 PMID: 24429701

86. Torres-Lista V, Giménez-Lloret L. Impairment of nesting behavior in 3xTg-AD mice. Behav Brain Res. 2013; https://doi.org/10.1016/j.bbr.2013.03.021 PMID: 23523959

90. Dow SW, Fradkin LG, Liggitt DH, Charleston P, Heath TD, Potter T. Lipid-DNA complexes induce potent activation of innate immune responses and antitumor activity when administered intravenously. J Immunol. 1999; 163: 1552–1561. PMID: 10389235

94. Goodyear A, Keliihan L, Helle BO, Troyer R, Propst K. Protection from pneumonic infection with burkholderia species by inhalational immunotherapy. Infect Immun. 2009; 77: 1579–1588. https://doi.org/10.1128/IAI.01384-08 PMID: 19179415
95. Papahadjopoulos D, Allen TM, Gabizon A, Mayhew E, Matthay K, Huang SK, et al. Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. Proc Natl Acad Sci. 1991; 88: 11460–11464. https://doi.org/10.1073/pnas.88.24.11460 PMID: 1763060

96. Bender HR, Kane S, Zabel MD. Delivery of therapeutic siRNA to the CNS using cationic and anionic liposomes. J Vis Exp. 2016. https://doi.org/10.3791/54106 PMID: 27501362

97. Leske H, Hornemann S, Herrmann US, Zhu C, Dametto P, Li B, et al. Protease resistance of infectious prions is suppressed by removal of a single atom in the cellular prion protein. PLoS One. 2017; 12: 1–20. https://doi.org/10.1371/journal.pone.0170503 PMID: 28207746

98. Sajnani G, Silva CJ, Ramos A, Pastrana MA, Onisko BC, Erickson ML, et al. PK-sensitive PrPSc infectious and shares basic structural features with PK-resistant PrPSc. PLoS Pathog. 2012; 8. https://doi.org/10.1371/journal.ppat.1002547 PMID: 22396643

99. Cronier S, Gros N, Tattum MH, Jackson GS, Clarke AR, Collinge J, et al. Detection and characterization of proteinase K-sensitive disease-related prion protein with thermolysin. Biochem J. 2008; https://doi.org/10.1042/BJ20081235 PMID: 18684106

100. Wille H, Zhang GF, Baldwin MA, Cohen FE, Prusiner SB. Separation of scrapie prion infectivity from PrP amyloid polymers. J Mol Biol. 1996; https://doi.org/10.1006/jmbi.1996.0343 PMID: 8683568

101. Pastrana MA, Sajnani G, Onisko B, Castilla J, Morales R, Soto C, et al. Isolation and characterization of a proteinase K-sensitive PrPSc fraction. Biochemistry. 2006; https://doi.org/10.1021/bi0615442 PMID: 17176093

102. Hill AF, Antoniou M, Collinge J. Protease-resistant prion protein produced in vitro lacks detectable infectivity. J Gen Virol. 1999; https://doi.org/10.1099/0022-1317-80-1-11 PMID: 9934677

103. Wyckoff AC, Lockwood KL, Meyerett-Reid C, Michel BA, Bender H, VerCauteren KC, et al. Estimating Prion Adsorption Capacity of Soil by BioAssay of Subtracted Infectivity from Complex Solutions (BASICS). PLoS One. 2013; 8. https://doi.org/10.1371/journal.pone.0058630 PMID: 23484043

104. Pham NA, Morrison A, Schwock J, Aviel-Ronen S, Lakovlev V, Tsao MS, et al. Quantitative image analysis of immunohistochemical stains using a CMYK color model. Diagn Pathol. 2007; 2: 1–10. https://doi.org/10.1186/1746-1596-2-1

105. Zabel MD, Heikenwalder M, Prinz M, Arrighi I, Schwarz P, Kranich J, et al. Stromal Complement Receptor CD21/35 Facilitates Lymphoid Prion Colonization and Pathogenesis. 2010