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Therapy for prion diseases

Insights from the use of RNA interference

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Insights into the molecular basis and the temporal evolution of neurotoxicity in prion disease are increasing, and recent work in mice leads to new avenues for targeting treatment of these disorders. Using lentivirally mediated RNA interference (RNAi) against native prion protein (PrP), White et al. report the first therapeutic intervention that results in neuronal rescue, prevents symptoms and increases survival in mice with established prion disease.¹ Both the target and the timing of treatment here are crucial to the effectiveness of this strategy: the formation of the neurotoxic prion agent is prevented at a point when diseased neurons can still be saved from death. But the data also give new insights into the timing of treatment in the context of the pattern of spread of prion infection throughout the brain, with implications for developing the most effective treatments.

This perspective considers developments in the field that led to the rationale for targeting endogenous prion protein (PrP) in prion therapeutics and to the discovery of a window of reversibility of early neuronal damage in prion disease. It introduces RNA interference (RNAi) and its therapeutic use in this context and discusses insights into prion pathogenesis and future treatment strategies and goals. A key concept is targeting the critical brain regions for the spread of prion replication. This may have relevance in other neurodegenerative diseases due to protein misfolding, which recent literature suggests may also propagate throughout the brain in disease-specific patterns.

Prion Diseases: Background and Pathogenesis

Prion diseases, or transmissible spongiform encephalopathies, are fatal, neurodegenerative diseases that include Creutzfeldt-Jacob disease (CJD) and kuru in humans, scrapie in sheep and bovine spongiform encephalopathy (BSE) in cattle. They are transmissible within and between mammalian species and are unique in having sporadic, inherited and acquired origins.

Central to prion pathogenesis is the conversion of a host-encoded prion protein, PrP^C, into a partially-protease resistant isoform, PrP^{Sc}, which accumulates in the brain and is associated with infectivity. The ability of transmission to be effected both naturally and experimentally by disease-associated prion protein, in the absence of associated nucleic acid, led to the 'protein only hypothesis' of prion propagation and infectivity.² The conversion process is self-propagating, with PrP^{Sc} acting as a conformational template recruiting PrP^C for further conversion. The conversion reaction itself is critical to neurotoxicity in prion diseases: neither loss of PrP^C function,³⁻⁵ nor deposition of PrP^{Sc} is sufficient to cause pathology. However, the precise identity of the neurotoxic prion species and the exact mechanism of neurotoxicity are unknown. Key pathological features of prion disorders, in addition to the aggregation of PrP^{Sc} (often in the form of amyloid plaques), are spongiform degeneration of neurons, astrogliosis and neuronal loss.

Prion diseases in humans are rare, with an overall incidence of ~1/million

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worldwide, but their unique biology has resulted in their being amongst the best understood of the neurodegenerative disorders at a molecular level. Currently, there is no effective treatment for prion diseases and the recent evidence for prion spread through contaminated blood products,⁶⁻⁸ the identification of a novel type of vCJD with a protracted incubation time,⁹ and the emergence of atypical strains of BSE in cattle¹⁰ re-emphasize the importance of continued research into therapies for this ongoing threat to public health.

Therapeutic Strategies in Prion Disease: Rationale for Targeting PrP^C

Any successful therapy in prion disease should prevent the formation, or block the actions, of the neurotoxic species. While pathological changes are characterized by, and infectivity is associated with, PrP^{Sc} there is no evidence for *in vivo* toxicity caused by PrP^{Sc}.^{11,12} The existence of subclinical disease states where high levels of PrP^{Sc} are present without clinical symptoms¹³⁻¹⁵ also argue against its direct toxicity. Further, there are several inherited prion diseases in which PrP^{Sc} is not detected in significant amounts,¹⁶⁻¹⁸ and the degree of PrP^{Sc} accumulation in specific brain regions does not necessarily correlate with clinical features. Expression of host PrP^C is, however, essential for both prion propagation and pathogenesis: PrP-null mice are resistant to prion disease and unable to replicate infectivity,^{3,4} disease incubation time is determined by PrP^C expression levels,^{4,19} and prion neurotoxicity is confined to PrP-expressing neural tissue.²⁰ Thus the expression of host PrP^C is necessary for prion-induced neurotoxicity, suggesting the generation of a toxic intermediate form during prion conversion, or possibly the toxicity of early oligomeric forms of PrP^{Sc}. Removing PrP^C, the substrate for prion conversion, is therefore an appealing therapeutic target.

PrP^C is highly conserved across species. Its physiological function is not well understood and ablation of PrP^C is surprisingly well tolerated *in vivo*, at least in laboratory mice. Thus PrP-null mice are essentially normal phenotypically and behaviorally with only very subtle changes, including

altered intrinsic properties of specific cell types, detectable electrophysiologically,²¹ and reported alterations of circadian rhythms.²² Depletion of PrP^C in adult mice by transgene mediated deletion of PrP produces no further phenotypic consequences and effectively excludes loss of PrP^C function as a central mechanism in prion-mediated neurodegeneration.⁵

Proof of principle of the validity of strategies targeting PrP^C came from the use of the adult onset model of PrP^C depletion. Transgenic animals in which PrP was depleted in neurons at nine weeks of age provided a system for testing the effects of PrP depletion in prion infection. PrP-expressing mice were infected with prions at one week of age, eight weeks prior to transgene mediated PrP depletion. Prion infection was allowed to develop over this time, with establishment of early spongiform change, astrogliosis and PrP^{Sc} deposition by the time neuronal PrP knockout occurred. This transgene mediated removal of neuronal PrP^C during established prion infection allowed the animals to survive long term without symptoms and led to a reversal of early spongiform change. The animals were effectively clinically cured.¹² Control animals with equivalent early pathology at eight weeks post infection (wpi) progressed to full prion neurodegeneration and death, just four weeks after the onset of PrP knockout in 'treated' animals.

Timing of Treatment: Rescuing Early Neuronal Dysfunction, a Window of Reversibility

Spongiform change and synapse loss precede neuronal loss in mouse scrapie.²³ Changes in species-typical behaviors also occur long before end stage motor symptoms²⁴⁻²⁷ and correlate with early loss of presynaptic terminals in the dorsal hippocampus.²⁸ In other neurodegenerative disorders, such as Alzheimer and Huntington diseases, the loss of synapses precedes neuronal loss. Compromised synaptic function is proposed to underlie early symptoms and cognitive decline in these disorders.

In prion infected mice with PrP depletion early spongiform change was associated with cognitive and behavioral deficits

and impaired synaptic transmission in the hippocampus, which recovered just one week after neuronal PrP depletion had occurred.²⁹ The link between spongiosis and synaptic loss is not clear, but may reflect a stage of functional impairment of synapses before they are physically lost. The recovery of early spongiform change supports the concept that the early stage of disease represents a window when prion diseased neurons can be rescued. The early stage of synaptic dysfunction may be the 'window' here, which is further supported by the findings of White and co-workers, discussed below.

Using RNAi for Therapeutic Gene Knockdown

Transgene-mediated reduction of PrP^C expression does not offer direct therapeutic possibilities in human patients. Potential treatments aimed at removing PrP^C must be achievable using extrinsic means. Recent developments in the field of RNA interference (RNAi) offer new opportunities to achieve such therapeutic gene silencing *in vivo*.

RNAi is a naturally occurring, highly conserved, sequence-specific mechanism for post-transcriptional gene silencing in eukaryotes. It is initiated by the presence of double-stranded RNA (dsRNA), which is exogenously introduced to the cell such as viral RNA, or endogenously encoded such as microRNAs (miRNAs) that regulate gene expression. Exogenously introduced dsRNA is recognized by Dicer, a cytoplasmic ribonuclease that cleaves it into 21–23 nt sequences called short interfering RNAs (siRNAs).³⁰ Both siRNAs and miRNAs interact with a multi-protein RNA-induced silencing complex (RISC) that unwinds the RNA duplex and destroys one of the strands, known as the "passenger" strand.³¹ The remaining "guide" strand is used as a template to localize cellular mRNAs containing a homologous sequence. The degree of homology between the guide strand and the mRNA determines whether RISC initiates endonucleolytic cleavage or translational arrest of the target mRNA, thereby silencing expression of that gene. Generally, siRNAs mediate destruction of target mRNAs whereas miRNAs silence

gene expression through translational repression due to their imperfect complementarity to the target mRNA.³²⁻³⁴

When using RNAi as a biological tool, interfering RNA sequences can be designed to enter the RNAi pathway at various points. siRNA duplexes can be synthesized for direct loading into RISC without requiring further processing.³⁵ This approach has the advantage of bypassing cellular defence mechanisms for recognition of long viral dsRNA, but unmodified siRNAs are typically unstable *in vivo* and cannot cross the blood-brain-barrier unaided. Targeting of brain structures has been achieved successfully with infusion of naked siRNA duplexes alone^{36,37} in conjunction with transfection reagents³⁸⁻⁴² and conjugated to a peptide derived from the rabies virus glycoprotein.⁴³ Whilst promising results have been attained, current technologies mean clinical translation for treatment of many neurodegenerative diseases would require continuous or repeated long-term infusion of the interfering RNA directly to the CNS.

Alternatively, stable, long-term expression of interfering RNA sequences can be achieved through the use of recombinant viral vectors (see schematic in Fig. 1). In this approach, siRNAs are expressed as short hairpin RNA (shRNA) stem-loop structures usually driven by RNA polymerase III promoters.^{44,45} As the shRNAs are expressed in the nucleus, they mimic pre-miRNAs ready for processing by Dicer.

For efficient delivery of shRNA-expressing vectors to neuronal cells *in vivo*, recombinant viruses, including retroviruses, adenoviruses, adeno-associated viruses and herpes-simplex viruses have all been harnessed. Retroviruses are commonly used due to their relatively low immunogenic potential and their ability to integrate into the host genome to facilitate stable expression. Lentiviruses are of particular use in the CNS as they are able to transduce and integrate into the genome of post-mitotic cells such as neurons and yield long-term expression of shRNAs in neurons when delivered intracerebrally.^{46,47} Virally mediated RNAi has been used successfully as a therapeutic treatment in models of a number of different neurodegenerative diseases, including

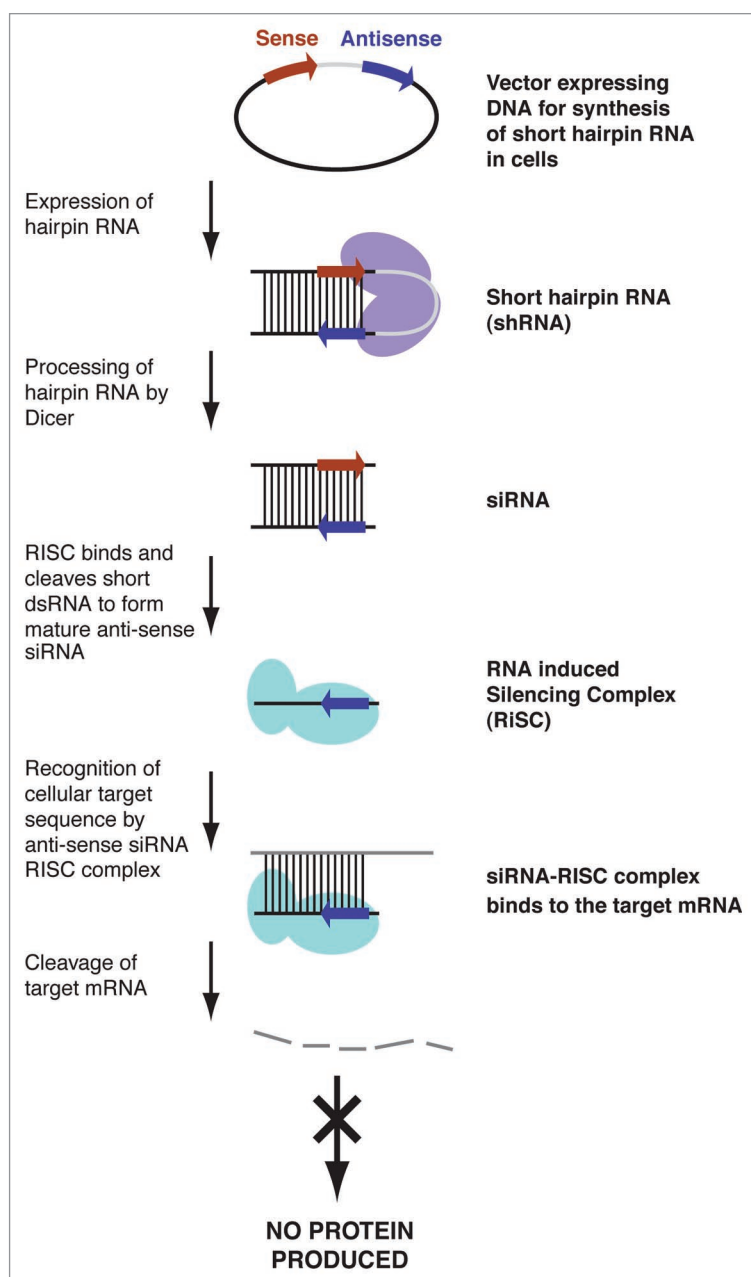


Figure 1. Schematic representing virally mediated RNAi. A viral vector expresses shRNA sequences. These are processed by Dicer and the anti-sense or guide strand is loaded into RISC for targeting of a specific mRNA, which is then degraded.

mouse models of spinocerebellar ataxia,⁴⁸ Huntington disease⁴⁹⁻⁵¹ and Amyotrophic lateral sclerosis (ALS).^{52,53}

Therapeutic Use of RNAi in Prion Disease

The first therapeutic use of lentivirally mediated RNAi against prion protein was demonstrated in mice with established prion infection by White and co-workers.¹

Knockdown of PrP by RNAi,⁵⁴ and resultant inhibition of PrP^{Sc} replication in cell culture, have been described⁵⁵ and RNAi of PrP has been achieved *in vivo*: virally expressed RNAi has been used to reduce the levels of PrP^C in goats, cattle⁵⁶ and mice.⁵⁷ Transgenic mice generated by lentiviral transduction of embryos stably express anti-PrP shRNAs and have increased resistance to prion infection due to resultant RNAi of endogenous PrP.⁵⁷ Here, RNAi

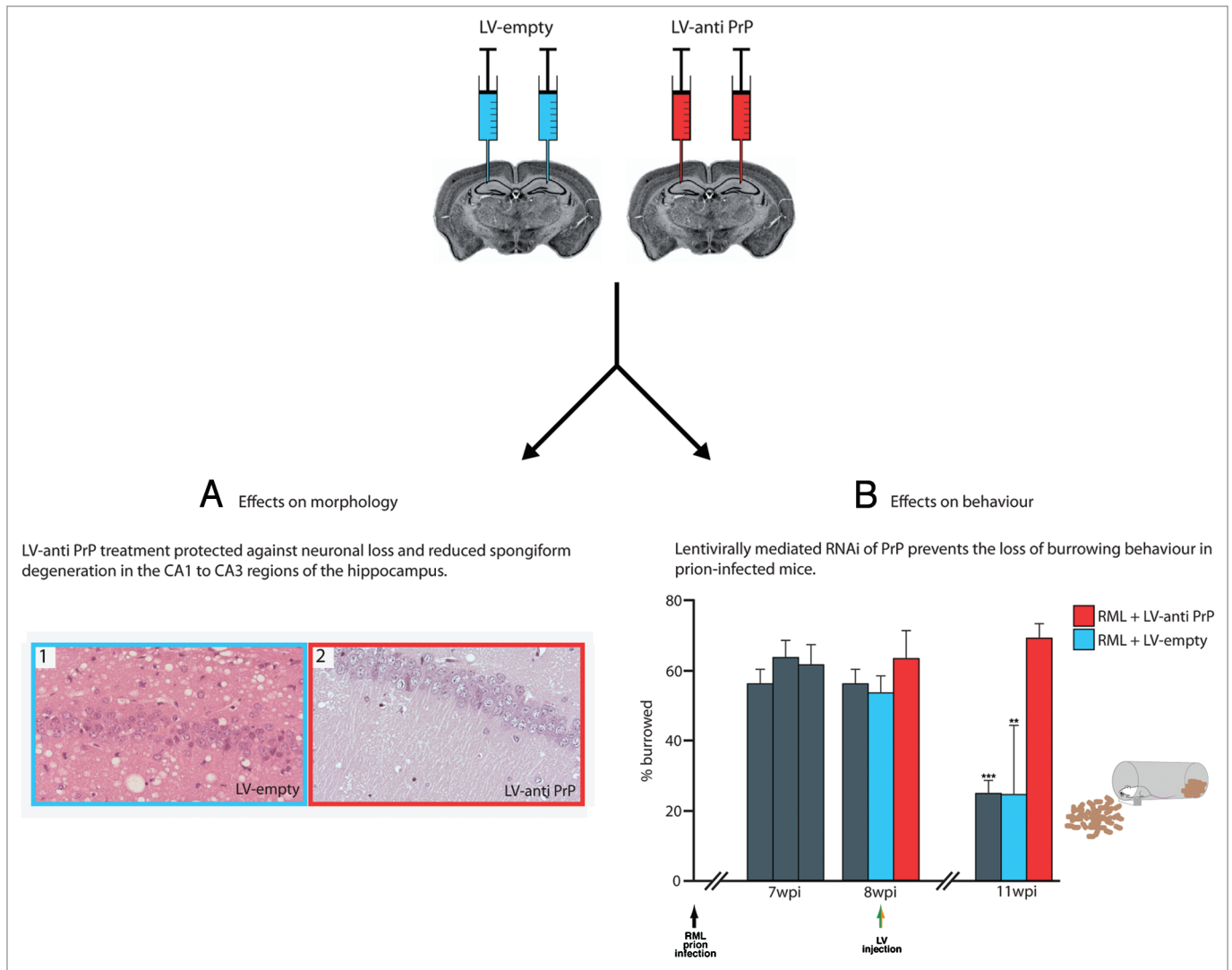


Figure 2. Lentivirally mediated RNAi of PrP expression protects against prion neurodegeneration in vivo. Prion infected mice are treated either with bilateral hippocampal injections of LV-empty (blue) or LV-anti PrP (red) eight weeks post infection. Representative images of neuroprotective effects of virally mediated PrP knockdown: (A) LV-anti PrP treatment protected against neuronal loss and reduced spongiform degeneration in CA1-3 regions (right hand) as compared to LV-empty treatment (left hand), which did not. (The right hippocampus is shown for all mice). (B) Mice were infected with RML prions at one week of age (black arrow) and tested for their ability to actively displace (burrow) food pellets from a tube. Mice were treated with either LV-anti PrP or LV-empty at eight weeks post infection (colored arrows). LV-anti PrP treated mice (red bars) continued to actively burrow, whereas both untreated (grey bars) and LV-empty treated mice (blue bars) lost burrowing behavior, characteristic of prion infection at this stage.

of PrP is due to genetic engineering and is not a treatment. Importantly, however, this model confirmed the safety and longevity of stably expressed shRNAs against PrP in vivo, and the increased resistance of the chimeric animals generated to prion infection give additional insights into targeting prion therapies, which is further discussed below.

White and colleagues have now used lentivirally mediated RNAi of PrP as an actual treatment. They essentially ‘replicated’ their earlier experiments with the transgene mediated PrP depletion,

allowing comparison of therapeutic efficacy of lentiviral mediated RNAi with the effects of adult onset Cre-mediated PrP^C depletion in prion infection. Transgenic mice overexpressing PrP^C were inoculated with mouse-adapted scrapie and allowed to incubate the disease for 8 weeks until early neuropathology was established. They then received a single injection of lentivirus expressing an shRNA targeting PrP^C into each hippocampus. This treatment prevented the onset of early behavioral and cognitive deficits associated with this stage of prion infection, protected hippocampal

neurons from degeneration, reduced PrP^{Sc} deposition and spongiosis (Fig. 2), and resulted in significantly increased survival of the animals (Fig. 3). Thus RNAi-treated animals were protected against developing the first behavioral deficits associated with early pathology of the CA1 region: loss of burrowing activity and object recognition memory. Morphologically, there was significantly less spongiform degeneration and neuronal loss where anti-PrP lentivirus was delivered compared to mock treated animals, suggesting sustained focal protection against neurotoxicity where

PrP knockdown occurred. Interestingly, spongiosis was also reduced beyond the site of lentiviral injection—in thalamus and cortex (reviewed in ref. 1). PrP^{Sc} accumulation was lower in animals with virally mediated RNAi of PrP in the hippocampus than in mock treated animals, and again this reduction was seen beyond the hippocampus in thalamus and cortex. These more widespread changes throughout the brain are likely to reflect altered spread of prion infection after hippocampal PrP knockdown. Of note, PrP^{Sc} accumulation—albeit at lower levels than in control animals—did not appear to affect neuronal function or cell survival, as reflected in preservation of hippocampal behaviors and structural neuronal integrity. This is consistent with observations in mice with transgene mediated PrP depletion, and has implications for the level of knockdown required for therapeutic effect. Thus simply slowing the rate of prion replication—here by reducing PrP^C levels—may be effective for prevention of neurotoxic effects.

Additional Insights: Timing of Treatment and Strain-Driven Targets?

The critical advance is the effect of this treatment on survival of prion-infected mice (Fig. 3). A single treatment with focal injection of virus resulted in significantly prolonged survival time of treated

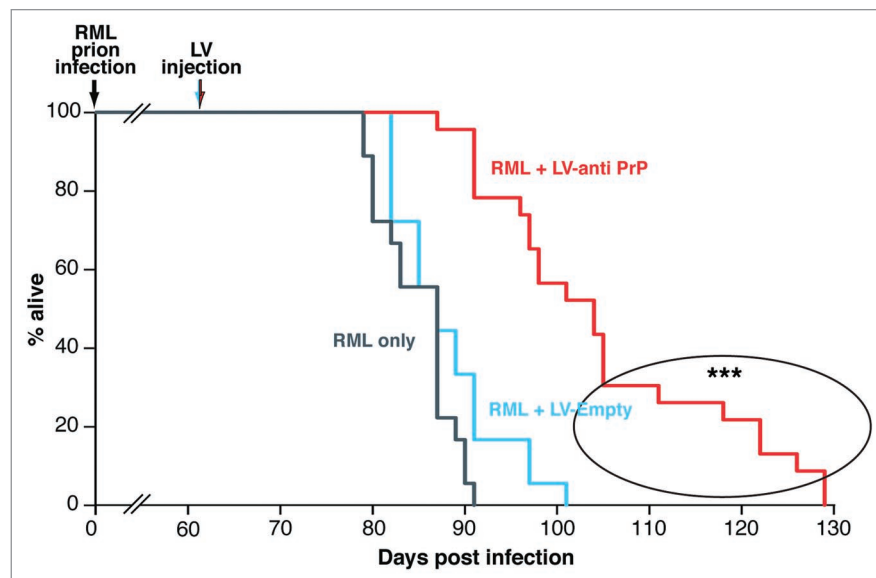


Figure 3. Treatment with anti-PrP shRNA expressing lentivirus prolongs survival in mice with established prion disease. Mice were infected with RML prions at 1 week of age and were treated with bilateral hippocampal injections of either LV-anti PrP (n = 22) or LV-Empty (n = 18) at 8 wpi or with no virus (n = 18). RML-infected mice treated with no virus or with LV-Empty died within 91 and 101 days post infection (dpi) respectively; mice treated with LV-anti PrP survived longer, living up to 129 dpi. (p < 0.0001 Student's t test, 2 tails, compared to both LV-Empty and untreated mice). The difference in survival among LV-anti PrP treated mice may be the result of timing of lentiviral injection. The earliest treated mice survived up to 50% longer (circled) than those injected later. (Reprinted with permission: White et al. PNAS USA 2008; 105:10238–43).

animals, compared to mock- or untreated mice, with a mean increase in lifespan of 23.5% compared to untreated animals.

The increased survival in RNAi treated mice is strikingly large with respect to the very small volume of brain targeted. Prion incubation times are inversely proportional to overall levels of PrP expression,^{4,11}

and it is likely that regional variations also affect prion replication rates and incubation periods. Further, prion diseases exist in different strains, which differ in their clinical and pathological profiles, as well as in the biochemical conformation and glycosylation pattern of associated PrP^{Sc}. The pattern of PrP^{Sc} deposition throughout the

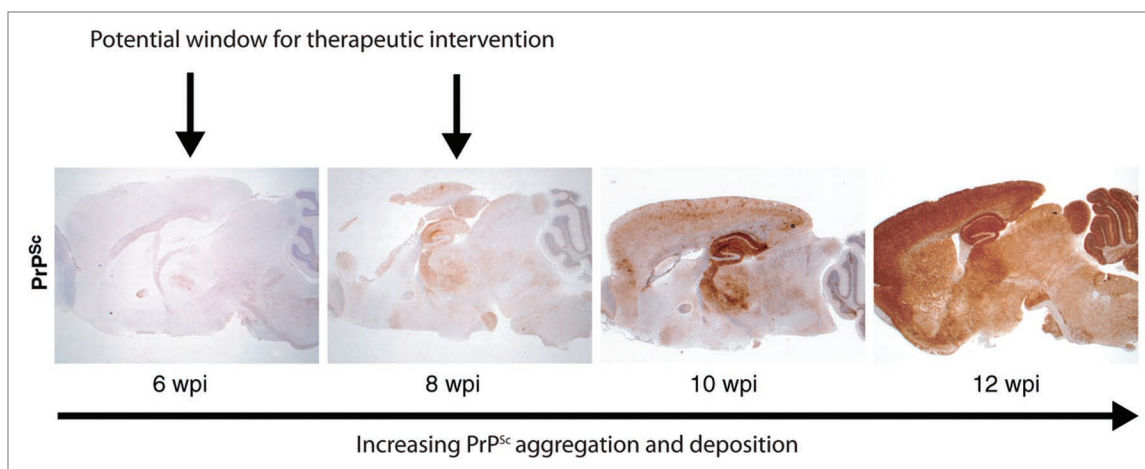


Figure 4. Distribution of PrP^{Sc} over time in RML-infected tg37 mice. PrP^{Sc} staining of RML infected tg37 mice shows that the hippocampus is the main focus of accumulation of PrP^{Sc} prion replication at early and mid-stage prion infection. There is widespread deposition of PrP throughout the brain when mice are terminally ill at 12 wpi. (Reprinted with permission: Suppl. data, White et al. PNAS USA 2008; 105:10238–43).

brain is generally strain specific.^{58,59} The hippocampus is a focus of early prion replication and PrP^{Sc} deposition for the RML prion strain used here (Fig. 4): this localized knockdown may therefore eliminate a key area for early prion replication in this model.

Further, if the survival curve for LV anti-PrP treated mice is examined closely, it is clear the effect of treatment on survival prolongation ranges from 0 to ~50%. This may in part be due to variation in neuronal transduction by virus in individual mice, but likely also reflects differences in timing of therapy: mice treated earlier survived longest. Analysis of survival times showed that animals surviving longest (survival time up by nearly 50% compared to controls, up to 129 days post inoculation, (dpi)) had been treated a mean of 52 dpi, whereas animals surviving least after treatment (as little as 87 dpi) had been injected a mean of 58 days post inoculation. Anticipating treatment by a few days here has a dramatic effect on survival. This may reflect a critical window for neuronal rescue in terms of a neurons' own program of death, or be dependent on the kinetics of prion spread, reflecting the fact that intervention needs to be accurately timed. This introduces the concept that treatment of prion diseases is likely to be most effective if we understand in detail the patterns of prion spread and replication for particular strains. This idea is supported by the observation that the degree of chimerism in mice transgenic for an shRNA targeting PrP^C expression did not always correlate with the survival time following prion infection. Some mice with only 20% chimerism survived as long as others that were 90–95% chimeric (see Pfeifer et al. Suppl. data).⁵⁷ It would be interesting to correlate the regions in which PrP expression is reduced in the different chimeric models with the effect on incubation time in the shRNA transgenic mice also. If this were to correlate with hippocampal knockdown (Pfeifer and co workers also used RML prions) the concept of therapy targeted to key replication areas would be further strengthened. Further the resistance of different chimeric lines to other prion strains would be interesting to compare.

'Taking out' hippocampal prion replication in this model may have disproportionate effects on survival as the focus of replication is removed, or significantly reduced. Interestingly, targeting other brain regions by lentiviral injection in the same model at the same time point had no significant effect on survival, unless the hippocampus was also targeted. Thus thalamic or striatal injections of anti-PrP lentivirus had no therapeutic benefit on survival, but targeting either of these regions along with the hippocampus showed similar increases on survival as targeting the hippocampus alone (data not shown). The findings again speak to the concept of critical areas of prion replication, which likely vary with different prion strains, but may be key in guiding future therapy.

Either earlier intervention is exerting its effects through interference with the kinetics of prion replication, or it is acting at a critical period in neuronal death 'routines', or both. Clearly, all animals succumb eventually, presumably due to prion-mediated neurodegeneration in other, critical, brain regions, but the neuroprotective effects seen within the hippocampus and beyond are undoubtedly a desirable effect of therapy. If transduction were to be more widespread, for example by pseudotyping lentiviruses with coat proteins that allow retrograde transport,^{60,61} or by using evolving mechanical techniques for enhanced delivery,^{62–64} more extensive neuroprotection and longer survival might ensue. Yet given the size of the human brain, perhaps a more focused, localized approach to key areas is a more realistic goal for therapy.

Finally, it appears that the concept of containing 'spread' of disease may well apply in protein folding neurodegenerative disorders beyond prion diseases. A recent paper from Clavaguera et al. shows transmission and spread of tauopathy in transgenic mouse brains.⁶⁵ Mutant tau mouse brain extract injected into wild-type tau expressing mouse brains resulted in a spreading tauopathy in recipient animals. The authors further suggest that different tau mutations and isoforms might represent different strains of tauopathy, with diverse clinical and pathological manifestations. Indeed the evolution or

'spread' of tau or Alzheimer pathology in human brains over time and brain region is well recognized.⁶⁶ Stopping protein propagation in its tracks with localized treatments may be an option worth considering in other human neurodegenerative proteinopathies.

Implications for Therapy in Human Prion Diseases

A significant advantage of the RNAi therapeutic approach in prion disease is its applicability to all known strains of prion disease. Within any species the primary sequence of PrP^C, and PrP^{Sc}, is the same for all strains: thus RNAi should be an effective treatment for all variants. This is in contrast to many previous candidate treatments for prion disease, which have suffered from inconsistent results dependant upon the prion strain involved. This should also apply to familial prion diseases that arise from a coding mutation in the gene encoding PrP^C, *PRNP*. It is likely that allele-specific silencing strategies to reduce expression of the mutant would be effective here. Genetic testing can identify these patients during the pre-clinical phase so successful treatment of this category may be possible through preventative silencing of the mutant *PRNP* allele expression prior to development of pathology.

A further category of prion disease patients that may benefit from RNAi-mediated silencing of PrP^C are those individuals known to have been peripherally infected, such as recipients of contaminated blood products. Reduction of PrP^C expression in organs that are crucial for prion replication and spread, such as the spleen and bone marrow (reviewed in ref. 68), may significantly prolong the incubation period. This could be achieved through regular systemic infusions of modified siRNA duplexes to lower PrP^C levels sufficiently to prevent accumulation of PrP^{Sc} above the cellular threshold for clearance, or for longer-term reduction of PrP^C, through systemic delivery of VSV-G pseudotyped lentiviruses which can efficiently transduce these organs.^{69,70} Selective targeting of routes of neuroinvasion such as the sympathetic nerves within the vertebral column may also significantly

extend incubation time and perhaps even avert neuroinvasion.

Lowering the amount of PrP^C available for conversion may enhance the ability of other agents to inhibit the disease process when administered in combination. For example, by combining RNAi against the prion protein with a drug to increase endogenous clearance of PrP^{Sc} it may be possible to delay disease progression indefinitely.

The major obstacle to the use of therapeutic RNAi in neurodegenerative disease remains the problem of delivery to the brain. The blood-brain-barrier (BBB) restricts passive entry of molecules from the peripheral circulation meaning active transport across this barrier, transient disruption of the BBB's impermeability, or direct injection into the brain are currently required for delivery to the CNS. New technologies recently developed offer hope by targeting receptors in the BBB to mediate active transport of interfering RNA^{42,71} or utilising viruses capable of transverseing the BBB unaided.⁷²

Another caveat to the use of RNAi in vivo is the need to avoid both off-target effects and interference with processing of endogenous miRNAs due to over-loading of the RNAi pathway. Off-target effects can include silencing of unintended genes with limited sequence complementarity to the siRNA guide strand, cytotoxicity or activation of interferon responses. Careful design, in vitro screening and selection of the most potent sequences for RNAi reduces off-target effects and cytotoxicity in vivo,⁷³ and minimizes competition for endogenous miRNA machinery.⁷⁴ In prion disease, since only partial reduction of PrP^C expression is likely to be required for a therapeutic benefit, low doses of RNAi should be sufficient, minimising the potential for unintended side effects.

One drawback of this approach from a public health perspective is that it aims to eliminate neurotoxicity rather than abolish prion replication altogether. While PrP^{Sc} continues to be produced, infectivity remains, so the problem of potential transmissibility persists.

Also, whilst the ablation of PrP^C expression in adult mice is well tolerated, the consequences of reducing PrP^C in humans remain unknown. Initial attempts should

proceed with caution and the use of transient silencing through infusion of siRNA duplexes or expression of shRNAs from inducible viral vectors should be considered so that treatment can be halted if unforeseen adverse effects develop. In the end, the balance of possible adverse effects of PrP loss against the benefits of improved survival and protection against neuronal loss in key brain areas, will determine future therapies for prion and other neurodegenerative disorders.

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