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Transgenic over-expression of mammalian heparanase delays prion disease onset and progression

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Abstract

Cellular heparan sulfate (HS) has a dual role in scrapie pathogenesis; it is required for PrP^{Sc} (scrapie prion protein) formation and facilitates infection of cells, mediating cellular uptake of prions. We examined the involvement of heparanase, a mammalian endoglycosidase degrading HS, in scrapie infection. In cultured cells, heparanase treatment or over-expression resulted in a profound decrease in PrP^{Sc}. Moreover, disease onset and progression were dramatically delayed in scrapie infected transgenic mice over-expressing heparanase. Together, our results provide direct *in vivo* evidence for the involvement of intact HS in the pathogenesis of prion disease and the protective role of heparanase both in terms of susceptibility to infection and disease progression.

Keywords

Prion disease; heparanase; heparan sulfate; scrapie prion protein; transgenic mice; survival time

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AUTHOR CONTRIBUTIONS

O.K.B-Z, Y.T., S.T., I.N., and I.S. conducted the experiments. E.Z. and S.M. established the mouse models. I.V. wrote the manuscript. A.T. and I.V. coordinated the research and supervised the project.

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Introduction

Several amyloid diseases exist and their classification is based on the specific protein that makes up the amyloid fibril. Hitherto, more than 28 proteins have been identified to be able to form local or systemic amyloidosis in human [1,2]. Beside the amyloid-specific protein, other components such as serum amyloid P (SAP) and proteoglycans (PGs) are present in amyloid deposits, where both glycosaminoglycans (GAGs) and core proteins have been identified [3,4,5]. Heparan sulfate (HS) dominates as the most frequent encountered GAG in amyloid deposits [6,7,8,9,10], occurring as cell membrane associated syndecan and glypican [11], and perlecan and agrin that constitute major components of the extracellular matrix (ECM) and basement membrane [10]. The function for HS in amyloidogenesis is not entirely understood, but accumulated information points to an important role during initiation of amyloid formation [12]. We have generated heparanase over-expressing transgenic (*Hpa*-tg) mice [13] and demonstrated that in a mouse model of AA-amyloidosis, organ specific differences in human heparanase overexpression coincided with development of amyloids [14]. Thus, liver and kidney with high levels of heparanase showed little or no amyloid depositions whereas the spleen without heparanase expression displayed extensive deposits [14]. The hypothesis behind this finding is that the shorter fragments of HS produced in the liver of *Hpa*-tg mice due to extensive degradation by heparanase [13,14], failed to form complex with serum amyloid A, precluding its aggregation. Heparanase is the sole mammalian endoglycosidase that specifically cleaves HS chains [15,16], leading to reduced length of cell surface-bound and ECM associated HS. It is a major protagonist in pathophysiological settings such as cancer, inflammation, diabetic nephropathy, diabetes, atherosclerosis and other pathologies [15,16,17,18]. The protective role of heparanase in AA-amyloidosis [14] led us to investigate its effect on the onset and progression of prion disease. The transmissible spongiform encephalopathies that comprise infectious, familial and sporadic neurodegenerations such as Creutzfeldt-Jakob disease of humans, scrapie of sheep, and bovine spongiform encephalopathy are caused by prions [19,20]. These proteinaceous agents propagate by refolding the normal cell surface glycoprotein of the host, the cellular prion protein (PrP^C), into an abnormal β -sheet-rich conformer PrP^{Sc} (PrP^{Sc}) [19,21]. We have previously shown that cellular HS serves both as a cofactor in the propagation of PrP^{Sc} and as receptor for purified prion rods [22,23]. Notably, various sulfated glycans reduce the formation of PrP^{Sc} in infected cells and in some cases prolong the incubation time of experimental prion diseases [24,25,26]. In the present study, we investigated whether heparanase can affect PrP^{Sc} *in vitro* and disease progression *in vivo*. Our results indicate that heparanase treatment or over-expression resulted in a profound decrease in PrP^{Sc}. Moreover, disease onset and progression were dramatically delayed in scrapie infected transgenic mice over-expressing heparanase.

Materials and methods

Proteins

Recombinant 50+8 kDa active heparanase was kindly provided by Dr. Hua-Quan Miao (ImClone systems Inc., New York, NY) [27]. Latent 65 kDa heparanase was expressed in transfected CHO cells and purified as described [28].

Cells

Mouse neuroblastoma ScN2a-M are ScN2a cells that stably express the chimeric mouse/Syrian hamster MHM2 PrP that reacts with mAb 3F4 [22,23,29,30]. An uninfected version (N2a-M) was obtained by curing ScN2a-M cells with pentosan polysulfate (PPS, 5 µg/ml, 5 days) [31,32] and subsequently maintaining them without inhibitors for at least 1 month prior to use. ScGT1-1 cells are mouse hypothalamus cells infected with mouse RML prions [33]. Cells were maintained in low glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, as described [22,23,30].

Animals and scrapie infections

Heparanase transgenic (*hpa-Tg*) mice carrying human heparanase under the beta actin promoter have been described [13,34]. Mice were crossed for over 10 generations with C57BL/6J mice to produce pure genetic background [13,34]. All procedures were conducted using facilities and protocols approved by the Animal Care and Use Committee of the Hadassah-Hebrew University School of Medicine. Four- to six-week-old heparanase transgenic (*hpa-Tg*) mice, and age- and sex-matched C57BL mice (The Jackson Laboratory, Bar-Harbour, ME) were inoculated intraperitoneally (i.p) or intracerebrally (i.c.) with 100 µL or 50 µL, respectively, of diluted 1% (w/v) brain homogenate (in 4.5% BSA; w/v) derived from the Rocky Mountain Laboratory - Chandler (RML) strain of mice terminally sick with experimental scrapie [35,36]. All infections in the i.c. and i.p. groups were carried out on the same day using the same aliquot as inoculum. Animals were monitored at least twice a week for the development of clinical signs of the disease, which typically included a poor coat condition, a hunched posture, and development of a hind leg paralysis, weight loss and behavioral changes. Once clinical signs of the disease were noted, the mice were examined daily. The attack rate was 100% and the time when clinical signs first appeared is defined as 'disease onset'. Mice were sacrificed by isoflurane at the terminal stage of the disease reached when the scrapie symptoms indicated that the animals would die within the next 72 h [36]. Brains and spleens were frozen for biochemical analysis. The survival times of i.c. and i.p. inoculated mice were converted to percent survived animals [36]. These values were then compared using the log-rank test (GraphPad Prism). Brains and spleens were kept at -80°C until homogenized in PBS to 10% w/v, using an Ultra Turrax T8 IKA Labor Technik (Germany) homogenizer (10,000 rpm, 1 min, RT). The homogenates were lysed [8 µL of homogenate diluted to 200 µL with 2% N-octyl glucoside (NOG) in HEPES buffer, pH 7.4], insoluble material was removed by a 30s spin in a microcentrifuge, and 200 µL supernatants were brought to 1% Sarkosyl and incubated for 30 min on ice prior to proteolysis with proteinase K, SDS PAGE and Western blotting, as described under PrP analysis below (see Fig. S1) [36].

Antibodies

Rabbit antiserum RO73 recognizes both mouse PrP and MHM2-PrP (36). mAb 3F4 binds to residues Met108 and Met111 of chimeric MHM2-PrP, but does not recognize the endogenous wild-type mouse PrP of N2a cells [29]. Both antibodies were used at a dilution of 1:5000 for Western blot analysis [22,23]. Anti-heparanase pAb 733 which preferentially

recognizes the active 50+8 kDa enzyme was used at a dilution of 1:1000 for Western blotting and 1:200 for immunostaining [37].

Histology and immunohistochemistry

Histological examination and immunostaining of formalin-fixed, paraffin-embedded 5 micron sections were performed essentially as described [37]. Images were acquired by Nikon ECLIPSE microscope and Digital Sight Camera (Nikon) with objectives 20x or 40x.

PrP isoforms and PrP analysis

The PrP isoforms were characterized and separated as described [22,23,38]. Cells were lysed in ice-cold lysis buffer (0.5% Triton X-100, 0.23% Na-deoxycholate, 150 mM NaCl, 10 mM Tris-HCl pH 7.5, 10 mM EDTA) and lysates were immediately centrifuged for 30s at 14000 rpm in a microcentrifuge. All biochemical analyses were performed on this post-nuclear supernatant also referred to as 'cell lysate'. Protease-resistant PrP^{Sc} is defined as the PrP fraction resistant to standard proteolysis by proteinase K (Roche) (20 µg/ml, 37°C, 30 min for cultured cells, and 40 µg/ml, 37°C, 1 h for brain samples) [29]. The same proteolytic conditions were used to assay protease resistance of PrP^C fractions [22,23]. Proteolysis was terminated by 2 mM phenylmethyl sulfonyl fluoride (PMSF). SDS-PAGE and Western immunoblotting of PrP isoforms were carried out as described [22,23,30]. Western blots were developed using an ECL system.

Results

Recombinant heparanase reduces the amount of PrP^{Sc} in ScN2a-M cells

We treated (3 days) chronically scrapie infected ScN2a-M mouse neuroblastoma cells with purified active 50+8 kDa human heparanase or with its latent, 65 kDa proenzyme. The amount of proteinase K-resistant (+PK) PrP^{Sc} was then examined (Fig. 1a). Treatment with active heparanase resulted in a profound decrease in the amount of protease-resistant PrP^{Sc} (Fig. 1a, 50, middle panel), while the latent 65 kDa enzyme had no effect, indicating that heparanase enzymatic activity is required. The same treatment of ScN2a-M cells that were first cured of scrapie by pentosan polysulfate (PPS) [22,23,39] had no effect on the amount PrP^C (Fig. 1a, right panel). Next, we examined the effect of heparanase over-expression on scrapie. Stable transfection of persistently scrapie infected GT1-1 cells [33] with human heparanase resulted in a marked decrease in PrP^{Sc} compared to mock transfected cells (Fig. 1b, bottom).

Heparanase over-expression delays prion disease

In order to examine the involvement of mammalian heparanase in scrapie infection *in vivo*, we applied transgenic mice that over-express human heparanase in most tissues [13], including the brain [40]. Since heparanase expression in the brain tissue of these *hpa*-Tg mice was lower relative to other tissues, we determined the levels of heparanase in different areas of the brain applying a sandwich ELISA [41]. The heparanase protein was markedly elevated in all areas with the highest levels detected in the *hpa*-Tg cerebellum (Fig. 2a). Immunostaining revealed that heparanase expression in the cerebellum of *hpa*-Tg mice was

mainly localized in Purkinje cells and in the molecular layer which comprises the Purkinje cells' dendritic tree (Fig. 2b), areas that preferentially express PrP^C [42].

Both the *hpa*-Tg and their control wild type (*wt*) C57BL mice were inoculated intraperitoneally (i.p.) or intracerebrally (i.c.) with the Rocky Mountain Laboratory-Chandler (RML) strain of mouse adapted scrapie prions [35]. The mean survival time of i.p. inoculated male mice was 274 ± 14 days post infection (dpi) and 230 ± 18 dpi for *hpa*-Tg vs. control C57BL mice, respectively (Fig. 3A; $P = 0.0002$) (Table 1). Similarly, the survival time of i.p. inoculated female mice was 249 ± 7 dpi vs. 225 ± 8 dpi for *hpa*-Tg and control mice, respectively (Fig. 3B; $P < 0.00001$) (Table 1). Video presentation of i.p. infected *hpa*-Tg and control C57BL mice on day 215 of the experiment is presented in figure S2.

In subsequent studies, mice were inoculated with RML intracerebrally. The *hpa*-Tg i.c. inoculated mice succumbed to the disease on average 211 ± 18 days post infection, whereas the control C57BL mice died already on day 178 ± 5 post infection (Table 1; Fig. 3C). Statistical analysis showed a significant difference ($P < 0.0001$) in incubation time (duration from infection until disease onset) between *hpa*-Tg and control mice. The shortest and longest mean survival times for each group of mice are shown in Table 1. As expected, i.c. vs. i.p. inoculation gave rise to shorter incubation times. Mice inoculated i.c. or i.p. with PBS did not show any signs of prion disease for >400 days post injection. Notably, the highly significant difference in survival of *hpa*-Tg vs. control mice is equivalent to infection of the *hpa*-Tg mice with a 1000 fold higher infectious dose, as deduced from dose/scrapie survival time relations previously reported for wild-type C57BL mice [43].

While the disease onset (defined as the time when clinical signs first appeared) was significantly delayed in i.p. RML inoculated *hpa*-Tg mice vs. control mice, no significant difference in disease onset was observed in i.c. inoculated mice. Instead, duration of the symptomatic phase was markedly prolonged in the i.c. inoculated *hpa*-Tg mice, significantly extending their survival compared to control C57BL mice (Table 1). The latter finding was confirmed by measuring weight loss in the i.c. inoculated mice. As expected, weight loss was observed prior to the appearance of any neurological symptoms. In order to obtain an objective comparison of incubation times between *hpa*-Tg and control mice, i.c. inoculated animals were monitored for their weight starting from inoculation until 70 dpi. The *hpa*-Tg mice started to lose weight at about the same time as the control C57BL mice, but at a slower rate (not shown). Clinical signs of the disease were the same in *hpa*-Tg and control mice, in both the i.c. and i.p. routes of infection. Thus, heparanase over-expression led to a delayed death compared with control mice, but did not modify the scrapie symptoms in the final stages of the disease.

Unlike the human enzyme, chicken heparanase is localized primarily at the cell surface and is readily secreted [44]. Moreover, cells transfected with a chimeric construct composed of the human enzyme and the chicken heparanase signal sequence exhibited cell surface localization and secretion of heparanase, similar to cells transfected with the full-length chicken enzyme [44]. We infected male and female mice transgenic for the chimeric construct (*sphpa*-Tg) by i.p. inoculation of experimental scrapie (RML). The mean survival time of the inoculated male mice was 238 ± 7 dpi and 215 ± 6 dpi for *sphpa*-Tg and control

male mice (Fig. 4A; $P = 0.0005$) and 252 ± 26 dpi vs. 222 ± 14 dpi for *sphpa*-Tg and control C57BL female mice, respectively (Fig. 4B; $P = 0.0087$) (Table 2). The survival of scrapie infected *sphpa*-Tg mice was prolonged to a higher extent than that of *hpa*-Tg mice, further supporting the notion that extracellular heparanase is required to exert an anti-scrapie effect.

Discussion

Our results provide the first *in vivo* evidence for the pivotal involvement of HS-degrading mammalian heparanase in scrapie disease. Recombinant heparanase reduced the amount of scrapie protein in infected mouse neuroblastoma cells, while its precursor PrP^C was not affected. Importantly, only the processed active form of heparanase inhibited scrapie disease, while the latent enzymatically inactive precursor had no effect. Similarly, over-expression of heparanase by stable transfection of GT1-1 cells that are persistently infected with scrapie resulted in a marked decrease in PrP^{Sc} compared to mock-transfected cells.

Heparanase over-expression in *hpa* transgenic mice infected with experimental scrapie resulted in a dramatically prolonged survival as compared to control C57BL mice, irrespective of the inoculation route (i.p or i.c). Notably, heparanase over-expression had a more pronounced effect on the incubation time until disease onset in *hpa*-Tg mice that were infected by i.p. vs. i.c. inoculation. This suggests that heparanase over-expression in the peripheral tissues is capable of exerting potent anti-prion effect, taking into account that upon peripheral prion infection, the infectious agent accumulates in lymphoid organs, in particular follicular dendritic cells in spleen [45]. Neuroinvasion is thought to occur as a result of direct uptake of prions by the sympathetic nerve fibers which innervate the spleen or by an as yet unknown cell-mediated delivery system to the nerve termini of the sympathetic nerves [46]. Since HS, primarily syndecan 1, is known to mediate cellular uptake of heparin-binding proteins, including prions [23], it is conceivable that heparanase over-expression in peripheral organs would delay the entry of prions into the CNS and thereby delay disease onset, as in fact observed following i.p. infection. Once the symptoms appeared, disease progression was similar in *hpa*-Tg and control mice. In contrast, the i.c. route of infection prolonged the symptomatic phase in *hpa*-Tg vs. control mice. In both routes of infection, *hpa*-Tg mice eventually died of scrapie disease and revealed similar amounts of PK-resistant PrP in their brains and spleens, as determined by Western blot analysis (Figure S1). Similarly, the clinical signs of the disease were the same in *hpa*-Tg and control mice, further indicating that heparanase over-expression does not modify the scrapie symptoms at the final stage of the infection.

Collectively, our results provide a direct *in vivo* evidence for the role of HS and HS-degrading heparanase in prion disease both in terms of susceptibility to infection and disease progression. A protective effect of heparanase was previously demonstrated by showing that heparanase rich tissues of *hpa*-Tg mice are resistant to experimental amyloid protein A amyloidosis [14]. Notably, overexpression of heparanase also reduces amyloid load in animal model of Alzheimer's disease (47) and formation of islet amyloid *in vitro* (48). It appears that the shorter fragments of HS produced due to extensive degradation by heparanase, fail to form complex with proteins that generate local or systemic amyloidosis,

thereby precluding protein aggregation. Our results provide direct in vivo evidence for the involvement of intact heparan sulfate in the pathogenesis of prion disease and the protective role of mammalian heparanase in terms of disease onset and progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

PrP^C	cellular prion protein
PrP^{Sc}	scrapie prion protein
GAGs	glycosaminoglycans
HS	heparan sulfate
HSPGs	heparan sulfate proteoglycans
PK	proteinase K
<i>hpa</i>-Tg mice	heparanase over-expressing transgenic mice
PPS	pentosan polysulfate
RML	brain homogenate derived from scrapie infected Rocky Mountain Laboratory mice
i.c	intracerebrally
i.p	intraperitoneally
ECM	extracellular matrix
<i>sphpa</i>-Tg mice	mice overexpressing the secreted form of heparanase

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- Intact heparan sulfate is involved in the pathogenesis of prion disease
- Heparanase treatment/over-expression results in profound decrease in cellular PrP^{Sc}
- Over-expression of mammalian heparanase delays prion disease onset and progression

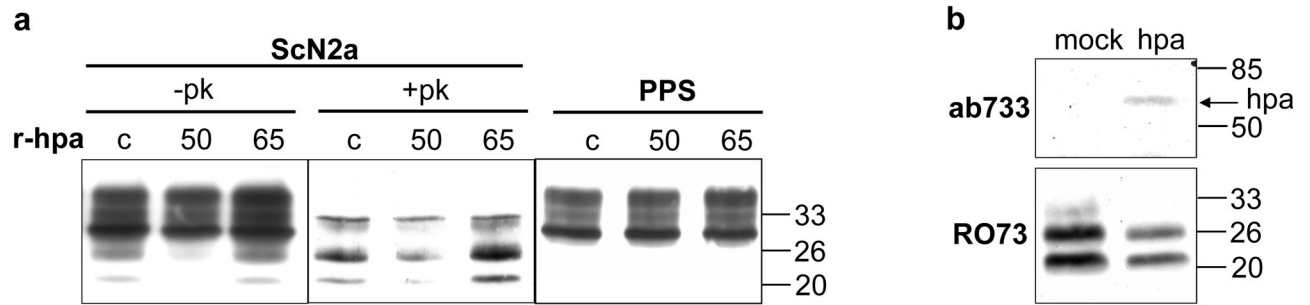


Figure 1. The amount of PrP^{Sc} is reduced following treatment of scrapie-infected cells with recombinant heparanase, or over-expression of heparanase

a ScN2a-M cells (ScN2a) and pentosan polyphosphate treated N2a-M cells (PPS) growing in 6-well plates were left untreated (c) or treated (3 days, 0.1 $\mu\text{g}/\text{ml}$) with either active 50+8 kDa (50) or latent 65 kDa (65) recombinant heparanase (r-hpa). Subsequently, the cells were lysed, incubated (37 $^{\circ}\text{C}$, 30 min) with (+PK) or without (-PK) 20 $\mu\text{g}/\text{ml}$ proteinase K, and immunoblotted with mAb 3F4. **b** Scrapie infected GT1-1 cells were stable transfected with human heparanase or mock-transfected. Cell lysates were subjected to SDS/PAGE and immunoblotting with anti-heparanase pAb 733 [37] (upper panel), or incubated (37 $^{\circ}\text{C}$, 30 min) with 20 $\mu\text{g}/\text{ml}$ PK and immunoblotted with anti PrP^{Sc} pAb RO73 (lower panel). Notably, unlike the 3F4 mAb that recognizes the chimeric MHM2 PrP^{Sc} produced in ScN2a-M cells (Fig. 1a), the RO73 antibody detects the endogenous PrP^{Sc} produced in scrapie infected GT1-1 cells (Fig. 1b).

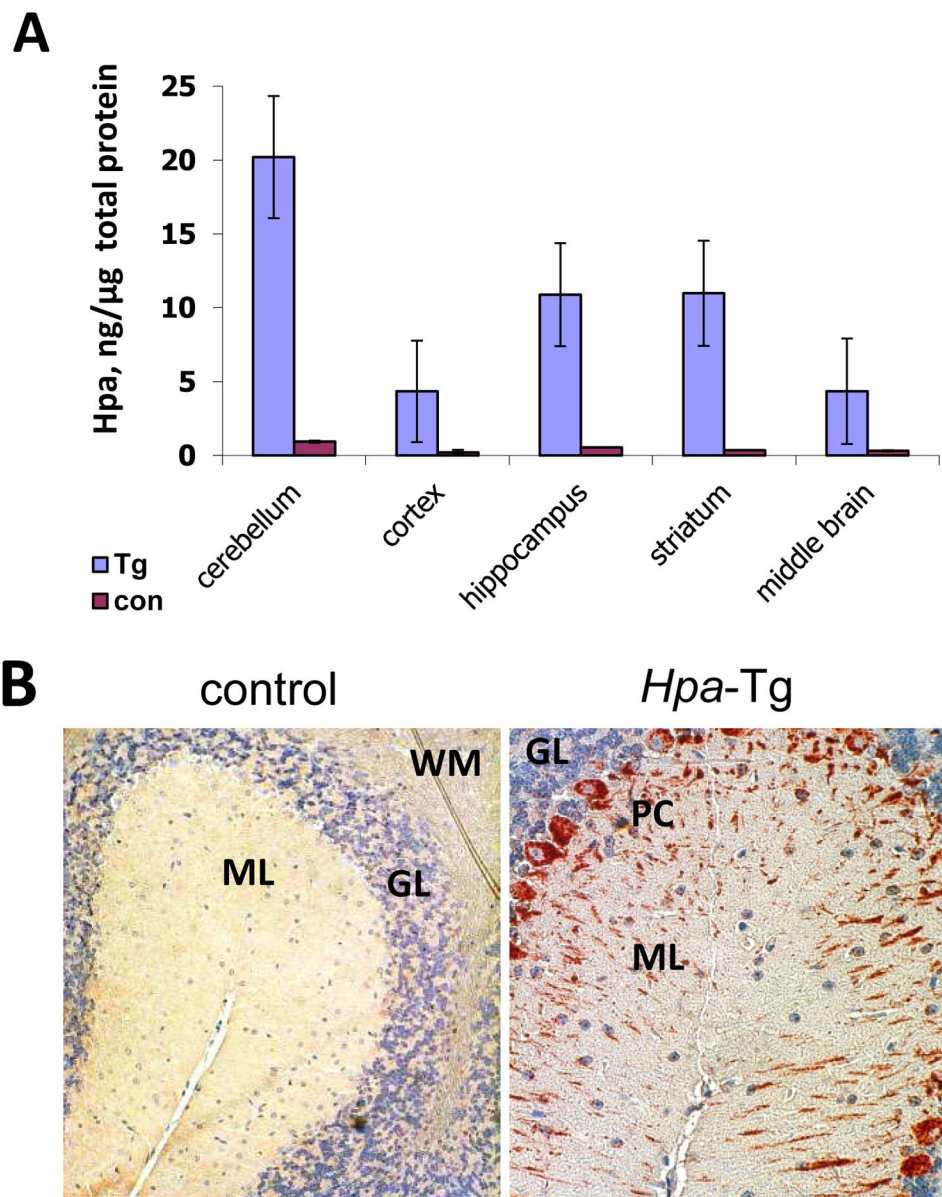


Figure 2. Heparanase protein is over-expressed in the brain of *hpa*-transgenic mice

A) ELISA. Heparanase protein was assayed by sandwich ELISA [41] in extracts of different brain areas of *hpa*-Tg vs. control C57BL mice (control). Each bar graph is the mean + SD of triplicate determinations. **B)** Immunohistochemistry. Immunostaining with anti-heparanase pAb 733 antiserum showing intense heparanase expression primarily in Purkinje cells (PC) of the cerebellum of *hpa*-Tg (right panel; original magnification X20) vs. weak expression in control C57BL mice (left panel; original magnification X10). ML = molecular layer; GL = granular layer; WM = white matter; Purkinje cells = PC.

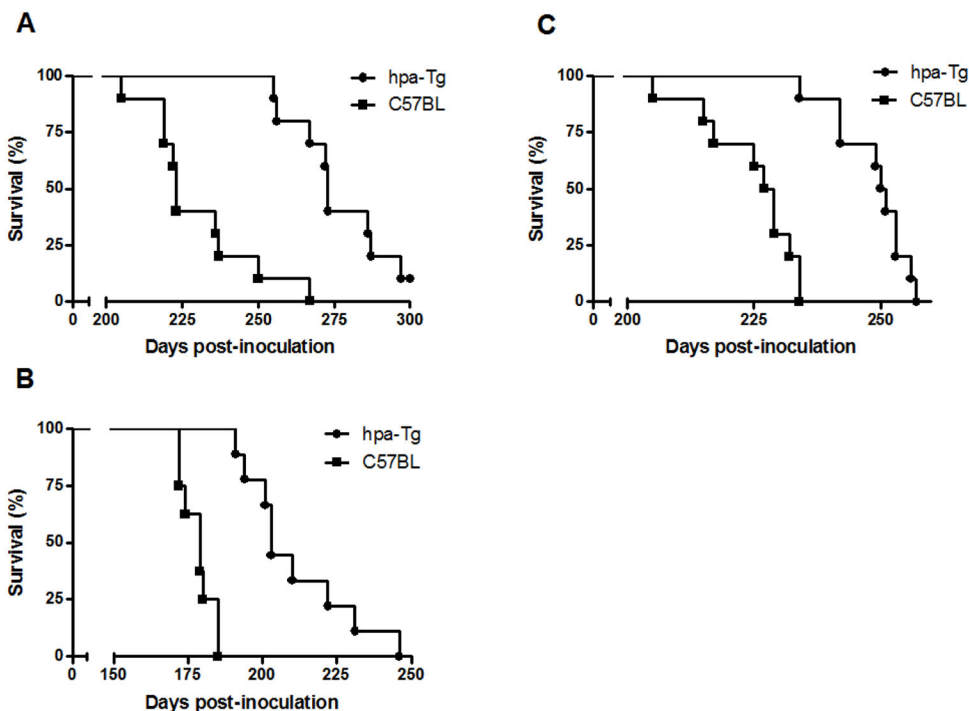


Figure 3. Significantly prolonged survival times of scrapie-infected heparanase transgenic (*hpa-Tg*) mice vs. C57BL control mice

A–C) Survival plots of RML inoculated mice. Survival times are presented as percentage of mice that did not die of scrapie at different time points post-inoculation. Significantly prolonged survival times of scrapie-infected *hpa-Tg* mice as compared to control C57BL mice are noted following intraperitoneal (**A**, males; **B**, females) and intracerebral (**C**, males) inoculation of RML prions. See video presentation of i.p. infected *hpa-TG* and control C57BL mice on day 215 of the experiment (Fig. S2).

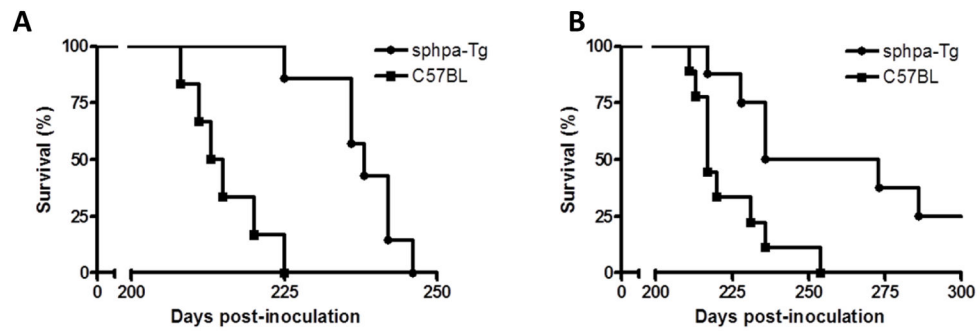


Figure 4. Survival plots of *sphpa*-Tg mice vs. C57BL control mice inoculated i.p. with RML prions

RML was inoculated (i.p.) into transgenic mice (**A**, males; **B**, females) over-expressing a secreted form of human heparanase (*sphpa*-Tg) vs. control C57BL mice. Mice inoculated i.c. or i.p. with PBS did not show any signs of prion disease >400 days post-inoculation.

Table 1Survival times of i.p. and i.c. RML inoculated *hpa-tg* vs. C57BL mice

Mice	i.p. survival time (days \pm SD)						i.c. survival time (days \pm SD)		
	Male			Female			Shortest	Longest	Mean
	Shortest	Longest	Mean	Shortest	Longest	Mean			
<i>hpa-tg</i>	255	297	274 \pm 14 (n = 9)	234	257	249 \pm 7 (n = 10)	191	246	211 \pm 18 (n = 9)
C57BL	205	267	230 \pm 18 (n = 10)	205	234	225 \pm 9 (n = 10)	172	185	178 \pm 5 (n = 9)

i.c., intracerebrally; i.p., intraperitoneally; SD, standard deviation; n, number of mice in each group.

Table 2

Summary of survival times in i.p. *sphpa*-tg inoculated mice

Mice	Survival time (dpi \pm SD)					
	Male			Female		
	Shortest	Longest	Mean	Median	Shortest	Longest
<i>sphpa</i> -tg	225	246	238 \pm 7 (n = 7)	238	211	>370*
C57BL	208	225	215 \pm 6 (n = 6)	214	217	254

SD, standard deviation; n, number of mice inoculated in each group.

* 2 of the female mice didn't show any signs of disease until 370 days post infection (dpi).