Shadoo (*Sprn*) and prion disease incubation time in mice

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Abstract Prion diseases are transmissible neurodegenerative disorders of mammalian species and include scrapie, bovine spongiform encephalopathy (BSE), and variant Creutzfeldt-Jakob disease (vCJD). The prion protein (PrP) plays a key role in the disease, with coding polymorphism in both human and mouse influencing disease susceptibility and incubation time, respectively. Other genes are also thought to be important and a plausible candidate is *Sprn*, which encodes the PrP-like protein Shadoo (Sho). Sho is expressed in the adult central nervous system and exhibits neuroprotective activity reminiscent of PrP in an in vitro assay. To investigate the role of *Sprn* in prion disease incubation time we sequenced the open reading frame (ORF) in a diverse panel of mice and saw little variation except in strains derived from wild-trapped mice. Sequencing the untranslated regions revealed polymorphisms that allowed us to carry out an association study of incubation period in the Northport heterogeneous stock of mice inoculated with Chandler/RML prions. We also examined the expression level of *Sprn* mRNA in the brains of normal and prion-infected mice and saw no correlation with either genotype or incubation time. We therefore conclude that *Sprn* does not play a major role in prion disease incubation time in these strains of mice.

Introduction

Prion diseases or transmissible spongiform encephalopathies are fatal neurodegenerative disorders and include

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scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, and variant Creutzfeldt-Jakob disease (vCJD) in humans. They exhibit a long, clinically silent incubation period and share similar neuropathological signs such as vacuolation, gliosis, neuronal loss, and the accumulation of abnormal forms of the prion protein (PrP^{Sc}).

The prion protein (PrP) plays a central role in prion disease yet its normal function remains elusive. Mice lacking PrP are grossly normal with only subtle abnormalities but are completely resistant to prion infection, thus confirming the central importance of PrP in prion propagation and disease (Bueler et al. 1992, 1993). Mutations in the human prion gene (*PRNP*) are linked to over 30 inherited forms of human prion disease (Collinge 2001; Mead 2006). Variation in human PrP (M129V) is associated with susceptibility to prion disease (Collinge et al. 1991; Mead et al. 2003; Palmer et al. 1991), and polymorphisms in the mouse prion gene, *Prnp*^{a} (Leu-108, Thr-189) and *Prnp*^{b} (Phe-108, Val-189), are associated with short and long incubation times, respectively (Moore et al. 1998; Westaway et al. 1987). *Prnp* is the main genetic determinant of prion disease incubation time in mice; however, several quantitative trait locus (QTL) mapping studies have demonstrated that other genes are also important (Lloyd et al. 2001, 2002; Manolakou et al. 2001; Moreno et al. 2003; Stephenson et al. 2000). While these studies have been very successful, the genomic regions identified were large and the quantitative trait genes themselves are yet to be identified.

Two paralogs of PrP that share the structural characteristics of PrP have been identified. The Doppel protein (Dpl) is encoded by the *Prnd* gene and maps adjacent to *Prnp* on Mmu2. Dpl is normally expressed in testis and plays an important role in spermatogenesis and is unlikely to be relevant in prion disease pathogenesis (Behrens et al. 2002). However, aberrant expression of Dpl in the

cerebellum of $Prnp^{0/0}$ mice results in a neurodegenerative phenotype that is rescued by the presence of PrP (Moore et al. 2001; Nishida et al. 1999; Rossi et al. 2001). This is very similar to the results obtained with transgenic mice expressing N-terminal deletion mutants of PrP (ΔPrP) (Shmerling et al. 1998). These studies suggested a model that proposes that PrP initiates a cell survival signal through interaction with a ligand (L_{PrP}) that is also able to bind Dpl and ΔPrP, albeit with lower affinity. This also led to the suggestion that a PrP-like molecule named π could substitute for PrP in the knockout mice, thus explaining their developmentally normal phenotype.

Based on comparative gene analysis, a new PrP-like molecule named Shadoo (Sho, shadow of prion protein) has been identified (Premzl et al. 2003). Sho is encoded by the *Sprn* gene and maps to Mmu7 (HSA10). Like *Prnp*, it is predicted to have a glycophosphotidylinositol (GPI) anchor and a conserved hydrophobic region. It has been demonstrated that PrP and Sho have an overlapping, although not identical, pattern of expression in the central nervous system, and like PrP, Sho was able to rescue the neurotoxic phenotype of both Dpl and ΔPrP, suggesting that Sho could be a candidate for π (Watts et al. 2007). It was also demonstrated that prion-infected mice have a significant reduction in Sho.

Sho has been further implicated in prion disease by the demonstration that a frameshift mutation at codon 46 was found in two vCJD patients but not in controls, and an additional coding polymorphism (T7M) was associated with sporadic CJD (Beck et al. 2008).

Three QTL studies have identified loci on Mmu7 as being linked to prion disease incubation time with suggestive levels of linkage (Lloyd et al. 2001; Moreno et al. 2003; Stephenson et al. 2000). These studies used different mouse and prion strains and the loci were not overlapping. The study of Stephenson et al. (2000) showed a peak of linkage (LOD = 2.2) at marker *D7Mit259* which maps within 5 Mb of *Sprn*.

Taking all these data together, we considered that *Sprn* was a good candidate gene for prion disease incubation time in mice. In this study we sequenced *Sprn* to identify polymorphisms and used a heterogeneous stock (HS) of mice to look for an association of *Sprn* with incubation time. We further examined the level of mRNA expression in the HS parental strains and compared expression in both normal and prion-infected mice.

Materials and methods

Mice

DNA was obtained from The Jackson Laboratory (Bar Harbor, ME, USA) or as a gift from Jean-Louis Guénet

(Institute Pasteur, Paris). Live MAI/Pas and PWK/Pas mice were obtained from Jean-Louis Guénet; JU/FaCt (gift of Bruce Cattanach) and CAST/Ei were obtained from the Medical Research Council Mammalian Genetics Unit (Harwell, UK). CD-1 mice were obtained from Charles River and all other inbred lines of mice were obtained from Harlan UK Ltd. The Northport Heterogeneous Stock (HS) (gift of R. Hitzemann, Portland, OR, USA) was received as 28 breeding pairs; offspring from these were randomly mated to produce a total of 49 pairs. One thousand fifty-two offspring (generation 37) were used for inoculation. Mice were identified by individual transponder tags (Trovan) and 0.5-cm tail biopsies were obtained for DNA extraction.

Inoculation and phenotyping

Mice were anaesthetized with isofluorane/O_2 and inoculated intracerebrally into the right parietal lobe with 30 μl Chandler/RML prions as previously described (Lloyd et al. 2001). Incubation time was calculated retrospectively after a definite diagnosis of scrapie had been made; it was defined as the number of days from inoculation to the onset of clinical signs (Carlson et al. 1986). This was assessed by daily examination for neurological signs of disease. Animals were culled as soon as clinical scrapie was confirmed or if they showed signs of distress. All procedures were conducted in accordance with UK regulations (Local Ethics approval and Home Office regulation) and international standards on animal welfare.

Statistical analysis

Incubation time data were collected for 1052 Northport HS mice. The range in incubation time was 103-229 days (mean ± SD = 145 ± 15 days). The normality of the incubation time distribution was established using the Anderson-Darling normality test ($P = 1$). To look for an association between incubation time and genotype, an ANOVA was used with pairwise comparisons carried out using Fisher's protected least significant difference (PLSD) test (Statview, SAS Institute Inc., Cary, NC, USA).

PCR and sequencing

DNA was extracted from 0.5-cm tail biopsies using a DNA extraction kit (Promega, Madison, WI, USA) and resuspended in 50 μl TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). A 1:10 dilution of this stock was used as template for subsequent PCRs. Oligonucleotides were synthesized by Sigma-Genosys (Sigma-Aldrich, St. Louis, MO, USA). PCR reactions for subsequent sequencing were all carried out in 25 μl on 96-well plates using MegaMix Blue (Microzone Ltd, West Sussex, UK) according to the

manufacturer's instructions and 10 pmol of each primer. PCR conditions were determined empirically but, in general, cycling conditions using a PTC-225 thermal cycler (MJ Research, Waltham, MA, USA) were as follows: 94°C for 10 min; 94°C for 30 sec, 60°C for 45 sec, 72°C for 60 sec for 40 cycles; 72°C for 5 min. PCR products were cleaned using Microclean (Microzone Ltd) according to the manufacturer's instructions and resuspended in H_2O. PCR product (100-200 ng) was added to a 15-μl sequencing reaction that included 5 pmol of either the forward or the reverse primer, 1 μl BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), and 5 μl Better Buffer (Microzone Ltd). Cycling conditions were 95°C for 30 sec, 50°C for 15 sec, 60°C for 120 sec for 30 cycles. Reactions were ethanol precipitated, washed in 70% ethanol, and resuspended in 10 μl MegaBACE loading solution (GE Healthcare BioSciences, Piscataway, NJ, USA). Products were detected on a MegaBACE1000 capillary sequencer (GE Healthcare). Samples were injected at 3 kV for 40 sec and run at 9 kV for 100 min.

Allele discrimination

Primers and probes for *Sprn* genotyping were designed using criteria defined by Applied Biosystems and Primer-Express software (Applied Biosystems). MGB probes labeled with either Vic or Fam were purchased from Applied Biosystems and primers for amplification were obtained from Sigma-Genosys. Oligonucleotide sequences were as follows: F-5′-GCCGCACCTTTCCGCT-3′; R-5′-CCTCCCCTACTTACTGGTCACAAG-3′; G-specific probe 5′-Vic-TAGGGGCC**G**TGACC-MGB-3′; T-specific probe 5′-Fam-TAGGGGCC**T**TGACC-MGB-3′. One picomole of each probe and 2.5 pmol of each primer were used in a 5-μl reaction on a 7500 Fast Real-time PCR System (Applied Biosystems) using RoxMegaMix Gold (Microzone Ltd). Cycling conditions were 95°C for 5 min; 95°C for 15 sec and 60°C for 60 sec for 40 cycles.

RNA extraction and quantitative RT-PCR

RNA was extracted from whole brains from either uninfected or RML terminally sick mice. Six- to eight-week-old adult male mice were used for all normal brains. Tissue was homogenized using a Ribolyser according to the manufacturer's instructions (Hybaid Ltd, Ashford, UK). RNA was prepared using either a RNeasy Maxi kit (Qiagen, Valencia, CA, USA) or a TRIreagent kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Samples were treated with DNaseI (Qiagen) and purified further using RNeasy Mini columns (Qiagen) according to the manufacturer's instructions. Four micrograms of total RNA was

reverse transcribed with AMV reverse transcriptase and random primers from the Reverse Transcription System (Promega) according to the manufacturer's instructions. Reactions with no reverse transcription were also carried out for each sample to ensure no genomic DNA contamination. *Sprn* real-time PCR was carried out on a 7500 Fast Real-time PCR System (Applied Biosystems) in a total volume of 15 μl using 1 μl cDNA (200-300 ng) and Rox-MegaMix Gold (Microzone Ltd) according to the manufacturer's instructions. Primers (6 pmol) SPRNF-5′-TGG GCCCGTCTGATTCTG-3′ and SPRNR-5′-TCAACTA-TAAGTCCCTCTGGTTCCA-3′ and probe (3 pmol) 5′-Fa m-ACTAAAGCCCCCAAAGACCTTGAGCCTC-Tamra-3′ were designed using PrimerExpress software (Applied Biosystems) and supplied by Sigma-Genosys. Endogenous controls (*GAPDH* and *β-actin*) were VIC-labeled and supplied as a kit by Applied Biosystems. Primers Thy-F-5′-CA GGCACCCTTGGGATACC-3′ and Thy-R-5′-TGGAACT ATATCCCGACCAACCT-3′and probe 5′-Vic-ACGTAC CGCTCCCGCGTCACC-Tamra-3′ (Applied Biosystems) for an additional endogenous control, *Thy-1*, were designed and used as above. Endogenous controls were duplexed with the *Sprn* reaction according to the manufacturer's instructions or for *Thy-1* using the same primer and probe concentrations as for *Sprn*. All reactions were carried out in triplicate using the following cycling conditions: 95°C for 5 min; 95°C for 15 s and 60°C for 60 sec for 40 cycles. Each sample was quantified using all three endogenous controls. The geometric mean of these data was used for statistical analyses and display in Fig. 1.

Results

Sprn sequencing

To investigate whether coding changes in the mouse Sho protein are likely to influence prion disease incubation time, we sequenced the open reading frame (ORF) of *Sprn*. The 441-bp (147 amino acids) ORF of *Sprn* is encoded entirely within exon 2 and is characterised by an N-terminal region containing tetrarepeats rich in arginine and glycine residues, a hydrophobic domain, and an N-glycosylation site (Premzl et al. 2003). We sequenced one mouse DNA sample from each of 24 lines of mice chosen to reflect the three alleles of *Prnp* (Table 1). The most commonly used inbred lines are all *Prnp*[a]; however, *Prnp*[b] is seen in JU/FaCt and VM/Dk mice, while *Prnp*[c] has been recorded only in MAI/Pas mice (Carlson et al. 1988; Lloyd et al. 2004). The *Prnp*[a] inbred lines have been demonstrated by us and others to show variation in prion disease incubation time and have been widely used in prion research (Lloyd and Collinge 2005). We also chose lines
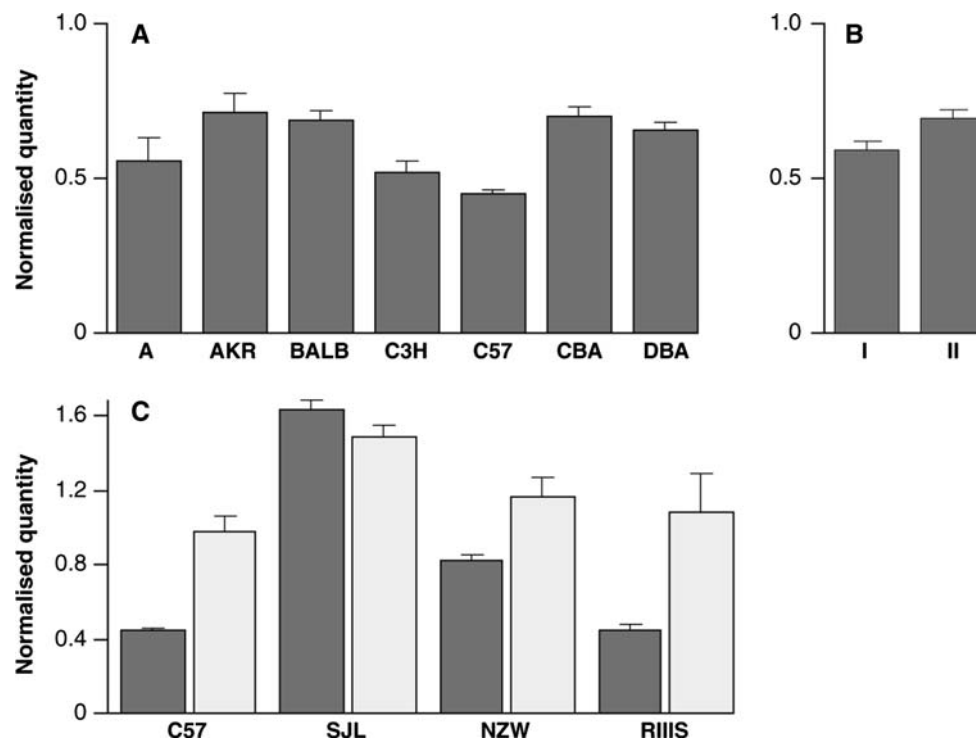
Fig. 1 *Sprn* mRNA expression. cDNA was prepared from the whole brains of uninfected 6-8-week-old male mice or mice at the terminal stages of disease following intracerebral inoculation with Chandler/RML mouse-adapted scrapie prions. All samples were duplexed for *Sprn* (Fam-label) and an endogenous control, *GAPDH*, *β-actin*, or *Thy-1* (Vic-label). Samples were run in triplicate with $n = 6$ for each mouse strain/group (except CBA where $n = 3$ and NZW-RML where $n = 5$). Mean ± SEM. *Sprn* mRNA expression level is expressed in arbitrary units as normalised by the geometric mean of the quantity of the endogenous controls (y axis). **a** Individual parental strains of the HS mice. **b** Mouse strains are grouped according to the strain distribution pattern seen in *Sprn* (Group I = A, AKR, BALB, C3H, C57, DBA; Group II = CBA). **c** Comparison of *Sprn* mRNA expression in normal and RML infected mice. Dark grey and light grey bars represent uninfected and RML infected mice, respectively

derived from trapped wild mice, including *M. m. musculus* (PWK/Pas, MBT/Pas, and MAI/Pas), *M. m. domesticus* (WLA/Pas and WMP/PAs), *M. spretus* (SEG/Pas), and *M. m. castaneus* (CAST/Ei), to capture as much sequence diversity as possible.

Across all 24 strains we detected five polymorphisms in the ORF, only two of which were coding changes (Table 1). Each coding change was seen only in a single line, with G95V and L134F present in CAST/Ei and SEG/Pas, respectively. SEG/Pas was also the only strain for which we detected noncoding polymorphisms at codons 11 (CTG → CTA) and 136 (GGC → GGT). An additional synonymous polymorphism occurred at codon 108 (GGA → GGG), which was seen in four of the wild-trapped mice (MAI/Pas, PWK/Pas, CAST/Ei, and SEG/Pas). No variation was seen across the ORF of the other classical inbred strains. This is of particular interest as this group contains two *Prnp*[b] allele mice. There is considerable variation in incubation time between the inbred *Prnp*[a] mice, ranging from 108 ± 1 days (NZW) to 143 ± 1 days (C57BL/6) (Kingsbury et al. 1983; Lloyd and Collinge 2005; Westaway et al. 1987) even though

there are no coding polymorphisms in the Sho ORF. This suggests that at least in these strains, variations in the primary sequence of Sho are unlikely to be associated with prion disease incubation time.

The mouse *Sprn* gene contains a short 5′ UTR (126 bp) spanning exons 1 and 2 and a long 3′ UTR (2586 bp) encoded by exon 2. The promoter and other regulatory regions have yet to be defined. As it is possible that regulatory regions within the UTRs may influence prion disease incubation time, we extended our sequencing to cover these regions although only in a subset of inbred lines (A/J, AKR/J, BALB/cJ, C3H/HeJ, C57BL/6 J, CBA/J, DBA/2 J, LP/J) that are the parental strains for the Northport Heterogeneous Stock (HS) (Hitzemann et al. 1994).

Two polymorphisms were seen in the 5′ UTR and one in the 3′ UTR. In addition, four SNPs were seen in the intron 1 sequence flanking the 5′ UTR (Table 2). Only one strain distribution pattern (SDP) was seen with Group I (A, AKR, BALB, C3H, C57BL6, DBA, and LP) having one allele and Group II (CBA) possessing the other variant. These polymorphisms do not occur within any known splicing

Table 1 *Sprn* open reading frame polymorphisms

	L11L CTG → CTA	G95V GGC → GTC	G108G GGA → GGG	L134F CTT → TTT	G136G GGC → GGT
A/J	G	G	A	C	C
AKR/J	G	G	A	C	C
BALB/cJ	G	G	A	C	C
C3H/HeJ	G	G	A	C	C
C57BL6/J	G	G	A	C	C
CBA/J	G	G	A	C	C
DBA/2J	G	G	A	C	C
LP/J	G	G	A	C	C
129/Sv	G	G	A	C	C
FVB/NHsd	G	G	A	C	C
SWR/OlaHsd	G	G	A	C	C
NZW/OlaHsd	G	G	A	C	C
RIIIS/J	G	G	A	C	C
SJL/OlaHsd	G	G	A	C	C
JU/FaCt (*Prnp*[b])	G	G	A	C	C
VM/Dk (*Prnp*[b])	G	G	A	C	C
MAI/Pas(*Prnp*[c])	G	G	**G**	C	C
WLA/Pas	G	G	A	C	C
PWK/Pas	G	G	**G**	C	C
WMP/Pas	G	G	A	C	C
MBT/Pas	G	G	A	C	C
CD-1	G	G	A	C	C
CAST/Ei	G	**T**	**G**	C	C
SEG/Pas	**A**	G	**G**	**T**	**T**

All strains are *Prnp*[a] unless otherwise indicated (Carlson et al. 1988; Lloyd et al. 2004)

sites, protein, or miRNA binding sites; therefore, it is difficult to predict whether they are likely to be functional.

Sprn genotyping of HS mice

Because the observed SDP is conserved across the gene, we chose one of the 5′ UTR SNPs (5′ UTR #1 in Table 2) to look for an association with prion disease incubation time in the Northport HS. One thousand fifty-two HS mice at generation 37 were inoculated intracerebrally with mouse-adapted scrapie prions (Chandler/RML) and incubation times (in days) were determined as previously described (Carlson et al. 1986; Lloyd et al. 2001). It is recognised that individuals from the ends of a distribution are more likely to share genotypes affecting the phenotype; therefore, selective genotyping from these ends has comparable power to genotyping all individuals (Darvasi and Soller 1992; Lander and Botstein 1989). Therefore, approximately 20% of animals from each extreme of the incubation time distribution were genotyped. The SNP was genotyped by allelic discrimination using a 7500 Real-Time PCR instrument (Applied Biosystems). Genotypes were collected for 348 animals where the genotypes CC

Table 2 HS parental strains *Sprn* polymorphisms

	Position (bp)	A	AKR	BALB	C3H	C57	CBA	DBA	LP
5′ UTR #1	73	C	C	C	C	C	A	C	C
5′ UTR #2	89	C	C	C	C	C	del	C	C
Intron 1	111	A	A	A	A	A	G	A	A
Intron 1	143	C	C	C	C	C	A	C	C
Intron 1	149	G	G	G	G	G	C	G	G
Intron 1	155	G	G	G	G	G	A	G	G
3′ UTR	2024	G	G	G	G	G	G	G	G

SNP positions for the transcript are numbered from the start of the 5′ UTR and the intronic SNPs are shown from the start of intron 1 (genomic sequence from the UCSC genome browser, release July 2007)

del single base deletion, *UTR* untranslated region

($n = 340$) and CA ($n = 8$) had mean incubation times of 144.1 ± 1.2 and 158.9 ± 8.4 days, respectively. Although this suggests that an increase in incubation time may be associated with the presence of the A allele, this is not statistically significant (ANOVA, $P = 0.064$).

Sprn mRNA expression

Transgenic and knockout mouse models have demonstrated that PrP expression levels correlate directly with prion disease incubation time (Büeler et al. 1994; Carlson et al. 1994; Collinge et al. 1995; Prusiner et al. 1990). Although we did not detect any polymorphisms that are predicted to affect *Sprn* expression, our sequencing was not exhaustive, and particularly since the promoter and other regulatory regions have not been defined, it is possible that we have not identified the key sites. Therefore, we compared the *Sprn* mRNA expression level in the parental lines of the HS mice. RNA was extracted from whole brains of 8-week-old males from the parental strains of the HS (except LP). Samples were analysed by real-time RT-PCR and normalised using the geometric mean of the quantity obtained from three endogenous controls (*GAPDH*, *β-actin*, and *Thy-1*). Expression levels for individual strains are shown in Fig. 1a. Although there are differences between the individual strains (ANOVA, $P = 0.0007$), these differences do not correlate with the known *Sprn* genotype. This is shown in Fig. 1b where the strains are grouped according to the *Sprn* SDP (Group I = A, AKR, BALB, C3H, C57, DBA, LP; Group II = CBA). However, if grouped by expression levels, the inbred lines fall broadly into two groups (Group A = A, AKR, BALB, CBA, DBA; Group B = C3H, C57) that are significantly different ($P = 6.5 \times 10^{-5}$). If a *cis*-acting factor were responsible for this, we would expect to see the above grouping reflected in a SDP; however, this has not been observed in our sequencing. Partial low-coverage genome sequence is available for the strains A/J, AKR/J, BALB/cByJ, C3H/HeJ, C57BL/6 J, and DBA/2J (http://www.informatics.jax.org/); therefore, we searched for SNPs 100 kb upstream and downstream of *Sprn* that might correspond to this SDP and did not find any. The strain differences in expression may therefore be explained by a *trans*-acting factor. We also compared *Sprn* mRNA expression levels with published incubation times; however, no correlation was observed ($R^2 = 0.21$) (Carlson et al. 1994; Kingsbury et al. 1983; Westaway et al. 1987).

It has also been shown that Sho levels are dramatically reduced in prion disease in both C3H × C57 F1 and C57BL6 mice infected with RML prions (Watts et al. 2007). We therefore compared the level of *Sprn* mRNA expression in the brains of uninfected and terminally sick RML-prion-infected mice from four inbred strains (C57BL/6OlaHsd, SJL/OlaHsd, NZW/OlaHsd, and RIIIS/J) (Fig. 1c). In contrast to the results obtained by Watts et al. who looked at the Sho protein, the mRNA level was increased in RML infection in three strains (C57BL6, $P = 0.0001$; NZW, $P = 0.006$; RIIIS, $P = 0.013$) and showed no significant difference in the remaining strain

(SJL, $P = 0.1$). We also compared the incubation times (Lloyd et al. 2004) with *Sprn* expression levels in the RML-infected brains and found that no correlation was observed ($R^2 = 0.26$).

Discussion

Our sequencing of the Sho ORF did not identify polymorphisms in the commonly used inbred strains. All variants were found in the strains derived from wild-trapped mice, particularly CAST/Ei and SEG/Pas, which are expected to be more diverse because they belong to the subspecies *M. m. castaneus* and *M. spretus*, respectively. It is unclear whether these changes would be functionally significant as the amino acid changes do not occur within domains known to be functionally important (Premzl et al. 2003; Watts et al. 2007). Our data suggest that unlike PrP, there is no association of Sho amino acid polymorphisms and prion disease incubation time in mice. This also contrasts with the association of two *SPRN* frameshift mutations in vCJD and the T7 M polymorphisms with sporadic CJD (Beck et al. 2008).

Although there are no significant coding changes in Sho, it is possible that it exerts its effect on prion disease through variation in expression level either at the level of the mRNA or the protein. The regulation of *Sprn* is not well characterised; therefore, we were able to look only at the UTRs. We did not sequence our entire mouse panel but focused on the inbred strains that are the parental strains of the Northport HS so that we could use any polymorphism found for an association study. The UTRs were well conserved between the strains, with the only variants seen in the CBA/J strain. Because our sequencing was not exhaustive, it is possible that we did not see the functional SNPs and that these represent a different SDP. We genotyped only one marker in the HS mice; however, this represents the only SDP found in the UTRs. No significant association with prion disease incubation time was seen with this SNP although the mean incubation time was 14.8 days longer. This may be due to underrepresentation of the minor allele (A) in the HS. Although we cannot rule out an association of this haplotype with prion disease incubation time in other mouse strains and crosses, we find no genetic evidence of a role for *Sprn* in our HS mice.

Previous work has shown that Sho levels are reduced by the clinical stage of RML prion infection (Watts et al. 2007). Our data do not replicate this finding. It is possible that this is a reflection of the mouse strains used; however, C57BL/6 was used in both studies, although it should be noted that while we used C57BL/6JOlaHsd (supplied by Harlan, UK), the substrain designation was not specified by Watts et al. An alternative explanation may be that the previous study looked at the protein level while we

measured the mRNA. Sho-specific antibodies were not available to us; therefore, we considered that measuring the mRNA was a reasonable substitute, particularly as we were interested mainly in measuring *cis* effects on transcriptional regulation. However, this may not hold true in this case as post-transcriptional mechanisms may inhibit translation or the protein itself may be targeted for degradation.

In conclusion, although Sho is an evolutionary member of the prion protein family, is reported to exhibit PrP-like neuroprotective activity, and is a candidate for the hypothetical π protein, our data do not support a major role for *Sprn* in influencing the natural variation in prion disease incubation time in these strains of mice.

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