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A gene-targeted mouse model of P102L Gerstmann-Sträussler-Scheinker syndrome

Rona M. Barron, PhD, Jean C. Manson, PhD*

Institute for Animal Health, Neuropathogenesis Unit, Ogston Building, KB, West Mains Road, Edinburgh EH9 3JF, UK

Transgenic models of disease

Limitations on studying many human diseases and genetic defects are caused by a lack of suitable animal models containing the specified genes and corresponding disease-associated mutations. This problem has been overcome, in part, by the development of transgenic technology, which makes it possible to remove genes or insert foreign, defective, or mutated copies of genes into mice, to create an animal model of disease that can be analyzed in the laboratory.

Gene-targeted transgenic models

Gene targeting allows for the production of lines of transgenic mice that possess either one or two copies of the desired transgene in the correct location in the mouse genome under the correct transcriptional controls [1]. Homologous recombination is used to replace the endogenous murine gene in embryonic stem (ES) cells with a mutated gene or the equivalent gene from another species. These stem cells then can be injected into mouse blastocyst embryos, and contribute toward the developing pup. Any pups born that are derived from both embryo and stem cell line are called chimeric mice, and are identified by the presence of two coat colors (one from the stem cell line, the other from the blastocyst into which the ES cells were injected). If the stem cells contribute to the germ line of the chimera, this animal can be bred to produce a transgenic line that differs from the wild

type only by the inserted mutation. All lines produced by this method will express the mutated gene under the correct transcriptional controls, allowing for the direct comparison of transgenic lines that encode different mutations in the same gene. Although the work described in this article concentrates on the development of a gene-targeted model for human Gerstmann-Sträussler-Scheinker syndrome (GSS), we have used the technology to introduce a number of different mutations into the murine prion protein (PrP) gene to investigate the precise role of PrP in transmissible spongiform encephalopathy (TSE) disease [2].

TSE diseases of humans

The TSEs of humans can occur as acquired, sporadic, or familial diseases. Acquired TSEs include kuru, a disease unique to the Fore people of New Guinea, and, more recently, variant Creutzfeldt-Jakob disease (vCJD), which is thought to have been spread by the ingestion of BSE-contaminated meat. Iatrogenic transmission also has occurred via contaminated neurosurgic instruments and the administration of contaminated human growth hormone [3]. The human TSEs that are less well understood, however, are the sporadic and familial forms of the disease. Most cases of human TSEs are sporadic, and are not associated with any specific source of infectivity or mutation in the PrP gene. Familial forms of TSE disease, which include fatal familial insomnia (FFI) and GSS, appear to be inherited in an autosomal-dominant manner and account for approximately 15% of human TSEs. These diseases are diagnosed by differing clinical presentations and pathologic profiles, and often are associated with specific mutations in the host PrP gene.

PrP is known to be central to the TSE diseases, and its abnormal isoform (PrP^{Sc}) is thought to be the infectious agent [4]. Mutations in PrP are hypothesized to destabilize the structure of the host PrP protein, and promote the conversion of the normal α-helical protein (PrP^{C}) to the abnormal β-sheet conformation (PrP^{Sc}), thus causing a spontaneous TSE disease in the absence of any acquired infectious agent [5].

P102L GSS

One specific mutation, which has been shown to be directly linked to the development of GSS, is a single base mutation in the human PrP gene that results in the substitution of leucine for proline at amino acid 102 (P102L GSS). This mutation has been shown to display 100% penetrance [6], and many geographically distinct families have been identified that carry this mutation and show a family history of neurologic disease [7–9].

A mouse model of the P102L mutation has been produced by the introduction of multiple copies of a transgene containing the equivalent mutation at amino acid 101 in murine PrP (P101L) [10]. Transgenic mice

overexpressing 101L PrP were shown to succumb to a spontaneous neurologic disease between 150 to 300 days of age, depending on the transgene copy number [10–13]. Low copy number transgenic mice expressing approximately wild-type levels of mutant 101L PrP did not show signs of central nervous system disorder until after 600 days of age [14]. Approximately 40% of these low copy number transgenic mice developed neurologic disease before 600 days, however, when inoculated with brain material from sick, high copy number transgenic mice [11,13]. Hence, in this particular transgenic model, overexpression of the P101L transgene appeared to lead to a spontaneous neurologic disease that could be transmitted to 101L transgenic mice but not to wild-type mice [11,13]. Previous transgenic experiments have demonstrated that overexpression of the wild-type PrP gene can cause neurologic disease in mice [15]. We therefore introduced the 101L mutation into endogenous murine PrP by gene targeting, in order to study the precise effect of the 101L mutation in the absence of any possible phenotype caused by overexpression of PrP. By using this method of transgenesis, we aimed to determine whether the 101L mutation would destabilize PrP and lead to the development of spontaneous neurologic disease.

Gene targeting the 101L mutation

Introduction of the P101L mutation

The production of P101L transgenic mice using the double-replacement gene targeting strategy has been described previously [16–18]. The technique involves the replacement of the endogenous PrP gene in an ES cell line with a PrP transgene encoding the 101L mutation ($Prnp^{a101L}$). ES cell clones carrying the transgene were injected into 3.5-day blastocysts to produce chimeric mice, which were bred with 129/Ola mice to produce progeny heterozygous for the $Prnp^{a101L}$ allele. These animals were then inbred to produce the homozygous $Prnp^{a101L}$ line.

The resultant transgenic mice (101LL) were viable, and shown by polymerase chain reaction and sequence analysis to contain the DNA polymorphism responsible for the proline to leucine substitution. Southern blot analysis confirmed that no other alterations had been made to the mice during gene targeting. Northern blot analysis detected similar levels of PrP mRNA in both 101LL and wild-type (101PP) mice; however, Western analysis showed that the steady-state protein levels were marginally lower in 101LL mice [16]. The small differences observed in protein expression levels may have been due to changes in antibody affinity caused by structural differences between 101L and 101P PrP or to altered processing of the mutant protein. Current analysis of the gene-targeted 101LL transgenic mice, however, has indicated that the 101L PrP protein is expressed and processed in the same way as is wild-type 101P PrP (RM Barron, unpublished results, 2003).

Does the 101L mutation cause TSE disease in mice?

A group of approximately 400 homozygous (101LL) and heterozygous (101PL) inbred mice were produced and allowed to age; 110 of these mice were successfully aged beyond 700 days, with the oldest animal reaching 899 days. No clinical signs of TSE disease were observed in any of these animals. The brains of all mice were analyzed for early TSE pathology; no vacuolation or PrP deposition was detected. In addition, no protease-resistant PrP^{Sc} was detected by Western analysis of brain tissue. Brain homogenates from 101LL mice over 600 days old were bioassayed by inoculation into 101LL and 101PP mice. No disease was transmissible from these aged brains to wild-type or transgenic mice. Homogenates of spleen from aged 101LL mice also have been inoculated into 101LL and 101PP mice, but no disease was transmitted from any of the spleens (RM Barron, unpublished results, 2003).

In contrast to the microinjected 101LL transgenic mice [12,13], the gene-targeted 101LL mice did not develop spontaneous TSE disease in their life span, and could not transmit neurologic disease to other mice expressing the transgene. These two conflicting observations simply may reflect the different expression levels of PrP in each model. Human P102L GSS can take over 40 years to develop, and the life span of a mouse expressing normal levels of 101L PrP (600–800 days) may not be sufficient for the specific event that triggers the conversion and accumulation of PrP in the brain to occur. The overexpression of 101L PrP may, therefore, allow this event to occur more rapidly, and cause disease. Neurologic disease was observed in some low copy number P101L microinjected transgenic mice over 600 days old [14], however, suggesting that the wild-type expression levels in the gene-targeted 101LL transgenic mice should have been sufficient to allow for the development of disease.

The two P101L mouse models, therefore, differed in more ways than simply construction, because equivalent protein levels appeared to give different phenotypes in each line of mice. We hope to resolve these issues in the future by performing experiments that will allow the direct comparison of the gene-targeted 101LL mice with the mice overexpressing 101L PrP, and establish exactly what effect the 101L mutation is having in each line. With respect to the P101L gene-targeted model, however, the lack of spontaneous disease led us to investigate whether these mice would display altered susceptibility to TSE disease. If the 101L mutation did, as suggested, make the PrP protein unstable and prone to forming PrP^{Sc}, then these mice might be more susceptible to TSE infectivity instead.

Transmission of TSE disease to P101L transgenic mice

Several different TSE agents were inoculated into the P101L gene-targeted transgenic mice [16,26] to assess whether the line was indeed more

susceptible to TSE disease (Table 1). In complete contrast to the predicted results, the 101LL mice did not show a general increase in susceptibility due to the supposedly unstable PrP molecule. The observed alterations in incubation time, compared with wild-type (101PP) mice, were unpredictable and appeared to be strain dependent.

Transmission of murine scrapie

Inoculation of 101LL mice with the mouse-passaged scrapie strains ME7 and 22A resulted in a prolongation of the incubation times when compared with wild-type (101PP) mice (Table 1). A similar effect was observed previously in transgenic mice overexpressing PrP transgenes that contained two separate mutations in the same region of PrP [19]. The mutations at amino acids 108 and 111 were introduced to create a nonmurine monoclonal antibody epitope in PrP, but their presence in the molecule appeared to result in a nonspecific increase in incubation times [19]. The effect of the 101L mutation did not appear to be nonspecific, because the incubation time of ME7 was doubled (160 days to 338 days) in 101LL mice [16], whereas the incubation time of 22A was only increased by approximately 34 days [26], suggesting that these effects are strain specific. Transmissions to P101L mice with the 79A and 139A strains of scrapie have been performed also. All

Table 1
Incubation times of transmissible spongiform encephalopathy (TSE) strains in P101L mice

TSE strain	Species	Mouse strain	Incubation time (days ± SEM)	TSE disease
ME7	Mouse	101PP	161 ± 2	10/10
		101LL	338 ± 8	18/18
22A	Mouse	101PP	493 ± 6	18/18
		101LL	527 ± 28	9/16
139A	Mouse	101PP	147 ± 2	21/21
		101LL	>200	0/24
79A	Mouse	101PP	139 ± 2	22/22
		101LL	>200	0/23
P102L GSS	Human	101PP	456	1/6
		101LL	226 ± 3	15/15
vCJD	Human	101PP	373 ± 8	24/24
		101LL	556 ± 12	8/18
263K (#1)	Hamster	101PP	707	1/4
		101LL	374 ± 6	4/4
263K (#2)	Hamster	101PP	>600	0/20
		101LL	464 ±18	14/16
SSBP/1	Sheep	101PP	>400	0/18
		101LL	346 ± 5	14/16

Data from Manson JC, Jamieson E, Baybutt H, et al. A single amino acid alteration (101L) introduced into murine PrP dramatically alters incubation time of transmissible spongiform encephalopathy. EMBO J 1999;18:6855–64; and Barron RM, Thomson V, Jamieson E, et al. Changing a single amino acid in the N-terminus of murine PrP alters TSE incubation time across three species barriers. EMBO J 2001;20:5070–8.

wild-type animals succumbed to disease with the expected incubation times (139 days and 147 days, respectively; see Table 1), but no 101LL mice showed signs of disease after 200 days (RM Barron, unpublished results, 2003). These results, therefore, do not agree with the prediction that the 101L mutant protein is unstable and more prone to convert to PrP[Sc]. In contrast, the presence of the 101L mutation extended the incubation times of mouse-passaged scrapie strains in a strain-specific manner.

PrP sequence identity and transmission of disease

Transmission of TSE disease is most efficient when the donor and recipient of infectivity are of the same PrP genotype [20]. Transmission occurs rapidly, with tight, predictable incubation times that can be reproduced on subsequent subpassage in the same species. When attempting to transmit disease between different species, incubation times usually are prolonged and occur over a wide range. Often, these incubation times exceed the life span of the animal, and no clinical signs of disease are detected. This phenomenon is known as the species barrier, and is thought to be due to differences in PrP sequence between species. In vitro experiments have shown that the efficiency of conversion of PrP[C] to PrP[Sc] is greatest when both proteins share identical PrP sequence [21,22]. The presence of heterologous PrP from another species appears to block this conversion reaction, causing extended incubation times [21]. Because the sequences of 101LL and 101PP PrP are identical with the exception of amino acid 101, these data led us to speculate that sequence identity specifically at amino acid 101 was more important than was overall sequence identity when transmitting TSE disease. To further investigate this hypothesis, we performed a series of cross-species transmissions, one of which contained the corresponding mutation at amino acid 102.

Cross-species transmissions

101LL mice were inoculated with infectivity derived from human (P102L GSS and vCJD), hamster (263K), and sheep (SSBP/1) sources (See Table 1). All of these agents, with the exception of vCJD, previously have proved difficult to transmit to wild-type (101PP) mice. A small number of clinical cases are usually detected in wild-type mice after a period of over 600 days, in keeping with the presence of a species barrier. If sequence identity at amino acid 101 in mouse, and the equivalent amino acid 105 in sheep, and amino acid 102 in human and hamster PrP is important in TSE disease transmission, we predicted that incubation times in 101LL mice would be shortened with P102L GSS, which contains the leucine mutation, but unchanged with the other sources of infectivity that all contain proline at this critical position.

Contrary to our predictions, decreased incubation times were observed in all cross-species transmissions, with the exception of vCJD (see Table 1). Transmission of P102L GSS could have been attributed to the presence of the 102L mutation in the inoculum; however, 263K and SSBP/1 both were shown to transmit efficiently to 101LL mice despite the overall sequence incompatibility and the nonidentity at amino acid 101. Transmission characteristics with vCJD were reversed. Wild-type mice showed 100% incidence of disease with vCJD at approximately 370 days (see Table 1), whereas 101LL mice showed increased incubation times of 556 days and only 45% incidence of disease. These results were totally unexpected, and showed that identity at amino acid 101 was not critical for the transmission of disease, but that substitutions at that position could have a major influence on incubation time of disease. This phenomenon also appeared to be strain specific rather than species specific, because two human sources of infectivity (GSS and vCJD) showed the ability to decrease and increase incubation times, respectively, despite the fact that both strains of agent were propagated in the same species.

Alteration of disease pathology in 101LL mice

The altered incubation times observed on transmission of several different TSE strains to 101LL mice could reflect variations in the efficiency of conversion of PrP^C to PrP^{Sc} due to the presence of a slightly different conformer of PrP^C in the host. This single point mutation in host PrP also was shown to be capable of altering the disease pathology [26], suggesting that host PrP plays a major role in the disease process.

Inoculation with vCJD is known to produce disease in wild-type mice [23], which allowed the comparison of disease pathology between 101PP and 101LL mice that could not have been obtained with P102L GSS and 263K. Differences were observed in the lesion profile (Fig. 1, Fig. 2B, 2D) and the distribution of PrP deposition, especially in the hippocampal region (Fig. 2A, C). PrP deposition was evident throughout the entire hippocampus of the 101LL mice, in contrast to the CA2 targeting seen in 101PP mice (Fig. 2A, C), suggesting that targeting is not defined solely by the strain of agent. A major characteristic of the strain of agent responsible for vCJD and BSE is the specific pattern of vacuolation in mice that is retained after experimental passage through a variety of intermediate species [23–25]. The ability of the 101L mutation to alter the pattern of vacuolation and incubation time of vCJD demonstrates the potential for different PrP alleles to determine different phenotypes and incubation times of vCJD in the human population.

Is PrP^{Sc} associated with infectivity?

The prion hypothesis predicts that PrP^{Sc} is the agent responsible for transmission of TSE disease, and levels of PrP accumulation correlate with
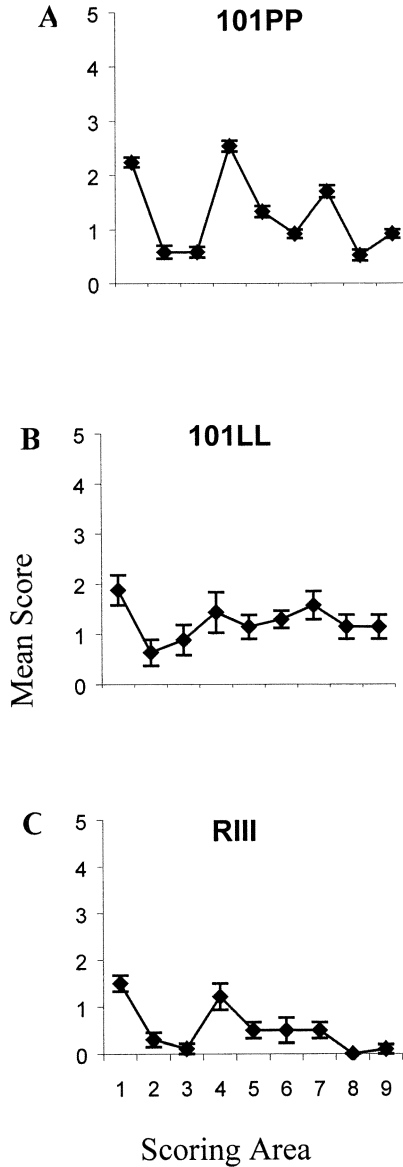
Fig. 1. The extent of the vacuolar change in the brain was assessed in nine areas of gray matter on a scale of 0 to 5 in each specific area, and mean scores (calculated from a minimum of six animals) are shown graphically (error bars ± SEM). Gray matter scoring areas: 1, dorsal medulla; 2, cerebellar cortex; 3, superior colliculus; 4, hypothalamus; 5, medial thalamus; 6, hippocampus; 7, septum; 8, cerebral cortex; 9, forebrain cerebral cortex. (A) 101PP mice infected with vCJD. (B) 101LL mice infected with vCJD. (C) RIII mice infected with vCJD.
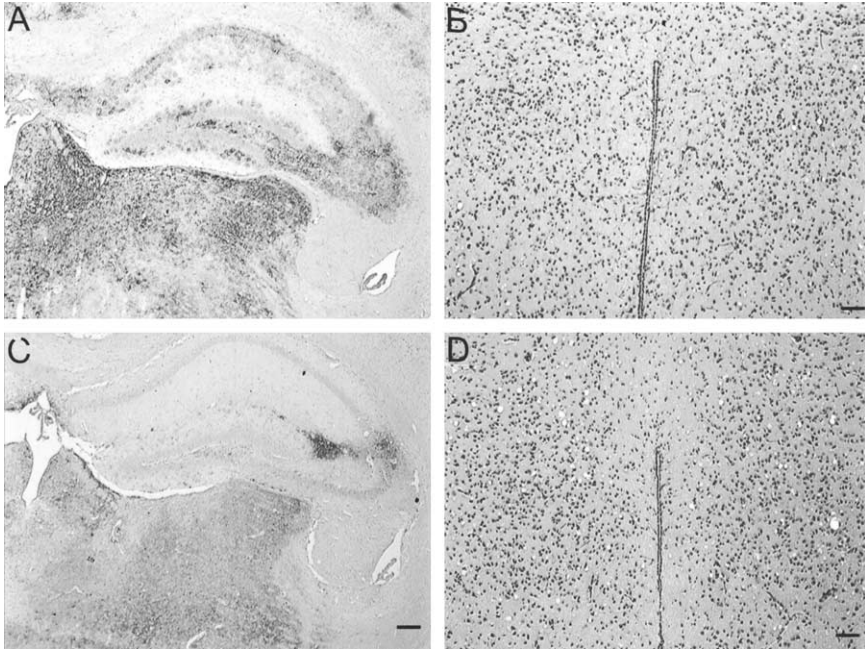
Fig. 2. Differences in disease pathology were observed in 101LL and 101PP mice infected with vCJD. A more widespread pattern of PrP deposition was seen in the 101LL hippocampus (A) than in the 101PP hippocampus (C). Reduced levels of vacuolation also were observed in the hypothalamus of 101LL mice (B) when compared with 101PP mice (D). Sections (A) and (C) were probed with MAb 6H4 and counterstained with haematoxylin, original magnification × 4. Sections (B) and (D) were haematoxylin-eosin stained, original magnification × 10. Bar is 100 μm in (A) and (C), 40 μm in (B) and (D). (See also Color Plate 14).

levels of infectivity. The amount of PrP deposited in the brains of 101LL mice was found to vary greatly, however, depending on the strain of agent inoculated [16,26]. Primary transmissions of both P102L GSS and 263K hamster scrapie to 101LL mice resulted in clinically and pathologically confirmed TSE disease, but little or no PrP deposition could be detected by immunocytochemical analysis [16,26]. A very small amount of proteinase K–resistant material was detected by Western analysis of these tissues, but this was only after long overexposure of the chemiluminescent blots to x-ray film [16,26]. Therefore, contrary to the theory that 101L PrP would be less stable and more prone to forming PrP^{Sc}, its presence in diseased tissue was difficult to confirm. The 101L PrP protein was not incapable of forming PrP^{Sc}, however, because 101L PrP^{Sc} was detected by Western analysis on primary transmission of vCJD [26] and ME7 [16], although the degree of deposition with vCJD was variable and did not correlate with incubation time.

If PrP^{Sc} alone is the infectious agent, then it can be predicted that these models, which contain extremely low levels of PrP^{Sc}, also will contain very low levels of infectivity. Contrary to this prediction, however, both

101LL/GSS and 101LL/263K were shown to transmit not only to 101LL mice, but also to wild-type (101PP) mice, with shortened incubation times (Table 2). Preliminary analysis indicated that the 101LL/GSS-infected tissue contained reasonable titres of infectivity associated with very low levels of PrP^{Sc}, and thus raised the question of whether PrP^{Sc} is always an accurate diagnostic marker for the presence of TSE disease. It also gives cause to reconsider whether PrP^{Sc} is the infectious agent, or a pathologic by-product of the disease process.

What is the role of PrP in TSE disease?

The 101L mutation lies in a region of the molecule shown by nuclear magnetic resonance spectroscopy to be an unstructured "flexible tail," which was previously thought to have little involvement in the disease process. The area of PrP predicted to be involved in species specificity lies in the structured C-terminal region of the protein, specifically involving α-helix 2, and a nearby loop of residues that show some structural differences between species [27–29]. The presence of the 101L mutation has been shown to allow transmission of strains from sheep, hamster, and human sources to mice without altering any of this critical area in the C-terminus. This indicates that structural compatibility and cofactor binding in the C-terminus is not the only key to transmissibility of TSE disease. Interactions in the unstructured N-terminal region appear to be as important.

Recent evidence has suggested that under physiologic conditions, the N-terminal region of PrP may contain some degree of secondary structure [30,31], and it is possible that the 101L mutation alters this structure to more closely resemble the corresponding region in PrP^{Sc} of P102L GSS, 263K, and SSBP/1. This structural identity may be more important in the transmission of TSE disease than is the overall sequence identity, and may

Table 2
Incubation times on second passage of 101LL transmissible spongiform encephalopathy (TSE) strains

Strain	Species	Mouse strain	Incubation period (days ± SEM)	TSE disease
101LL/263K	101LL mouse	101PP	262 ± 3	10/10
		101PL	251 ± 15	8/8
		101LL	131 ± 3	7/7
101LL/GSS	101LL mouse	101PP	226 ± 3	16/16
		101PL	201 ± 3	20/20
		101LL	148 ± 2	18/18

Data from Manson JC, Jamieson E, Baybutt H, et al. A single amino acid alteration (101L) introduced into murine PrP dramatically alters incubation time of transmissible spongiform encephalopathy. EMBO J 1999;18:6855–64; and Barron RM, Thomson V, Jamieson E, et al. Changing a single amino acid in the N-terminus of murine PrP alters TSE incubation time across three species barriers. EMBO J 2001;20:5070–8.

allow for the direct interaction of the N-terminus with the infectious agent. Alternatively, the N-terminal region of host PrP may interact directly with the C-terminal region of PrP that is thought to be involved in species specificity, and contribute toward the stability of this critical structure. Mutations in the N-terminal region then would have the potential of altering incubation times by affecting the critical structure and thus affecting the efficiency of conversion of PrP^C to PrP^{Sc}. The key to transmission of TSE disease may therefore lie in the interaction of the "flexible" N-terminal region of host PrP with the C-terminal region of the same, or other, PrP molecules.

Summary

Transgenic mice that contain a proline to leucine mutation at amino acid 101 in the endogenous murine PrP gene have been produced by gene targeting. This line of mice was generated to model the mutation thought to be responsible for P102L GSS, a familial TSE disease in humans. Gene-targeted 101LL mice showed no evidence of spontaneous TSE disease in their lifetime and were unable to transmit any neurologic disease to other 101LL transgenic mice. 101LL mice have, however, been shown to demonstrate altered susceptibility to several TSE strains, and have shown reduced incubation times with TSE agents that do not readily transmit to wild-type mice. The 101L mutation does not appear to destabilize PrP and promote conversion to PrP^{Sc}, because incubation times are increased with mouse-passaged TSE strains and vCJD. PrP^{Sc} also can be difficult to detect in 101LL mice infected with some TSE strains. We, therefore, have been unable to substantiate the existence of either genetic disease or infectious PrP with the P101L transgenic model, but have provided evidence of altered incubation times of TSE disease in mice carrying the 101L mutation in their PrP protein. We also have shown that mutations in the N-terminal region of PrP can have a major influence over both incubation time and targeting of TSE disease.

Acknowledgments

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