The role of *PRNP* codon 129 genotype in defining strain transmission properties of human transmissible spongiform encephalopathy

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DECLARATION

I declare that this thesis has been composed entirely by myself and that the work presented is my own, except where otherwise stated. All experiments were designed by myself, in collaboration with my supervisors. No part of this thesis has been, or will be, submitted for any other degree, diploma, or qualification.

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"Nothing before had ever made me thoroughly realise, though I had read various scientific books, that science consists in grouping facts so that general laws or conclusions may be drawn from them." Charles R Darwin 1809-1882

ABSTRACT

The human prion protein (PrP) gene (*PRNP*) codon 129 (M/V) polymorphism is a susceptibility factor for variant Creutzfeldt-Jakob Disease (vCJD) and a major determinant of clinico-pathological phenotype in sporadic CJD. The role of codon 129 in defining susceptibility and strain transmission properties has been investigated in three lines of transgenic mice that express human PrP. The human *PRNP* gene has directly replaced the murine version, by gene targeting, and variation at codon 129 has given the three genotype lines (HuMM, HuMV, and HuVV). The genetics of these three mouse lines are otherwise identical, and therefore differences in transmission properties can be directly attributable to the codon 129 genotype.

vCJD inoculation has shown that all three codon 129 genotype mice are susceptible with a ranking of transmission efficiency of HuMM>HuMV>HuVV. HuMM mice develop the most widespread neuropathology with features similar to human vCJD. Subclinical infection was noted in each mouse line. These data suggest that the vCJD strain is transmissible to humans of each of the three codon 129 genotypes, implying that non-MM cases of human infection with bovine spongiform encephalopathy (BSE) may exist but with long subclinical incubation periods. Inoculation of material from blood transfusion associated vCJD showed no change in transmission properties suggesting that the threat of a future epidemic of human-to-human vCJD infection has not been increased by adaptation of the vCJD strain. However the route of infection, for example via blood transfusion or surgery, may be more efficient that the original oral route of BSE infection.

sCJD is classified into six subgroups according to clinico-pathological features, and defined by codon 129 genotype and electrophoretic mobility type (1 or 2) of disease associated PrP^{Sc} (MM1, MM2, MV1, MV2, VV1, VV2). Typical cases from each subgroup have shown specific transmission properties suggesting that the subgrouping is defining separate disease strains. The commonest subgroup (MM1) was the most transmissible and the HuVV mouse line the most susceptible host. These data outline the transmission risk from all sCJD types to recipients of each codon 129 genotype should an infection event occur, and show the significant role of recipient codon 129 genotype in defining the clinical or subclinical state and the success or failure of transmission. This is important for determining individual risk following known exposure, and for modelling the potential of iatrogenic infection from sCJD patients.

ABBREVIATIONS

129Ola: wild type mouse line used to generate transgenic lines
bp: base pair
BSE: bovine spongiform encephalopathy
CJD: Creutzfeldt-Jakob Disease
CNS: central nervous system
D178N/128V: D178N mutation on the V codon 129 allele (humans)
D178N/129M: D178N mutation on the M codon 129 allele (humans)
D178N: Aspartic acid (D) to Asparagine (N) mutation at codon 178 (humans)
DM: dura mater
DNA: deoxyribonucleic acid
E200K: Glutamic acid (E) to Lysine (K) mutation at codon 200 (humans)
EEG: electroencephalography
ES: embryonic stem cells
fCJD: Familial CJD
FFI: Fatal Familial Insomnia
FSE: Feline spongiform encephalopathy
GPI: glycosyl-phophatidylinositol anchor
GSS: Gerstmann Sträussler Scheinker Syndrome
GT: gene targeting
hGH: human growth hormone
HLA: Human leukocyte antigen
HuMM: NPD transgenic mouse homozygous for methionine
HuMV: NPD transgenic mouse heterozygous for methionine / valine
HuVV: NPD transgenic mouse homozygous for valine
i.c.: intracerebral
ICC: immunocytochemistry
ICD: intercurrent death
iCJD: iatrogenic CJD
i.p.: intraperitoneal
IP: incubation period
i.v.: intravenous
kb: kilo base pairs
kDa: kilo Dalton
MM: methionine homozygous at codon 129
MM1, MM2, MV1, MV2, VV1, VV2: subgroups of sCJD
MRI: magnetic resonance imaging
mRNA: messenger RNA
MV: methionine / valine heterozygous at codon 129
NIBSC: National Institute for Biological Standards and Control
NCJDSU: National CJD Surveillance Unit
NFTs: neurofibrillary tangles
NMR: nuclear magnetic resonance
NPD: Neuropathogenesis Division, Roslin Institute
ORF: Open Reading Frame
P101L: Proline (P) to Leucine (L) mutation at codon 101 (mice)

P102L: Proline (P) to Leucine (L) mutation at codon 102 (humans)
PCR: polymerase chain reaction
PIPLC: phosphatidylinositol-specific phospholipase C
PNS: peripheral nervous system
PRND: human doppel protein gene
Prnd: mouse doppel protein gene
PRNP: human prion protein gene
Prnp: mouse prion protein gene
Prnp$^{+/+}$: wild-type mouse with two copies of the PrP gene
Prnp$^{0/0}$: wild-type mouse that does not express PrP
Prnp^{a}: wild-type line with leucine at codon 108 and threonine at codon 189
Prnp^{b}: wild-type line with phenylalanine at codon 108 and valine at codon 189
PrP: prion protein
PrP^{C}: normal cellular form of PrP
PrP^{Sc}: abnormal 'scrapie' form of PrP
QTL: quantitative trait loci
RGI: random genomic insertion
RNA: ribonucleic acid
sCJD: sporadic CJD
TME: transmissible mink encephalopathy
TMER: Transfusion Medicine Epidemiology Review
TSE: transmissible spongiform encephalopathy
Type 1: protease resistant core of PrP^{Sc} with 21kDa electrophoretic mobility
Type 2: protease resistant core of PrP^{Sc} with 19kDa electrophoretic mobility
UK: United Kingdom
vCJD (BSE): vCJD associated with BSE infection
vCJD (transfusion): vCJD associated with blood transfusion infection
vCJD: variant Creutzfeldt-Jakob Disease
VV: valine homozygous at codon 129
WHO: World Health Organisation

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CHAPTER 1

INTRODUCTION

1.1 PHD THESIS INVESTIGATIONS

These PhD investigations will provide model data upon which the following hypotheses about human disease can be tested:

- Codon 129 genotype is the key factor in defining susceptibility to human transmissible spongiform encephalopathy (TSE) transmission.
- The agent strain responsible for variant Creutzfeldt-Jakob Disease (vCJD), is transmissible to humans with codon 129 MV and VV genotypes.
- The pathology in the central nervous system following vCJD infection in individuals of codon 129 MV and VV genotype is different from that of MM cases.
- There is an increase in incubation period for vCJD in codon 129 MV and VV individuals.
- Adaptation of the vCJD agent following human passage will produce a more infectious strain.
- The risk of iatrogenic infection from sporadic CJD (sCJD) varies according to the codon 129 genotype of source and recipient.
- The currently defined subgroups of sCJD are unique strains of human disease, with individual transmission properties.

Model data will be generated by the experimental transmission of a number of different forms of human transmissible spongiform encephalopathy (TSE), that vary in clinical features, pathology, codon 129 genotype, and prion protein configuration. The recipient transgenic mice are unique to the field of prion disease transmission research and have been produced by gene targeting so that they express physiological levels of the human prion protein (PrP) as a direct replacement for the original mouse PrP. The inserted full-length human prion gene sequence of these novel lines has been modified at codon 129 to produce mice that are MM, MV, or VV genotype. It will also be possible to examine other biochemical (Western blot type) and histological (plaque development and PrP deposition) phenotypes of human disease that could be mirrored in these mice.

The initial stages of this study will be to assess the transgenic mice to ensure that the replacement human *PRNP* gene is functioning correctly and producing intact prion protein at the correct approximate physiological concentrations and of the normal configuration. Following this the mice will be tested for susceptibility to infection by human TSE agents. Groups of mice will be inoculated with brain material from typical cases of vCJD and sCJD via the intracerebral (i.c.) or intraperitoneal (i.p.) routes, and positive transmissions subsequently subpassaged. This will provide an initial analysis of the susceptibility of these mice to material whose infectivity is well characterised in wild-type mice. This study is important as it will be the first time that it has be possible to study the specific effect of codon 129 genotype on human TSE transmission in a mouse model.

1.2 TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHY (TSE)

TSEs are fatal neurodegenerative disorders that occur in many species of mammal including man. They include the cattle disease bovine spongiform encephalopathy (BSE) (Bradley and Wilesmith, 1993), the human disease Creutzfeldt-Jakob Disease (CJD) (Goldfarb and Brown, 1995), scrapie in sheep and goats (Hunter *et al.*, 2000), and Chronic Wasting Disease (CWD) in deer and elk (Williams, 2005). For a review of animal TSE see reference (Sigurdson and Miller, 2003). Following active surveillance for BSE and scrapie, atypical forms of these diseases have recently been identified with unknown cross-species transmissibility (Benestad *et al.*, 2003, Buschmann *et al.*, 2006, Capobianco *et al.*, 2007, Jacobs *et al.*, 2007, Luhken *et al.*, 2007). There are also experimental TSE diseases where repeated subpassage of disease within one laboratory animal species has lead to characteristic, adapted forms of TSE. Examples of such are mouse-scrapie ‘ME7’, hamster-scrapie ‘236K’, and mouse-BSE ‘301V’ (Bruce, 1993, Bruce, 2003).

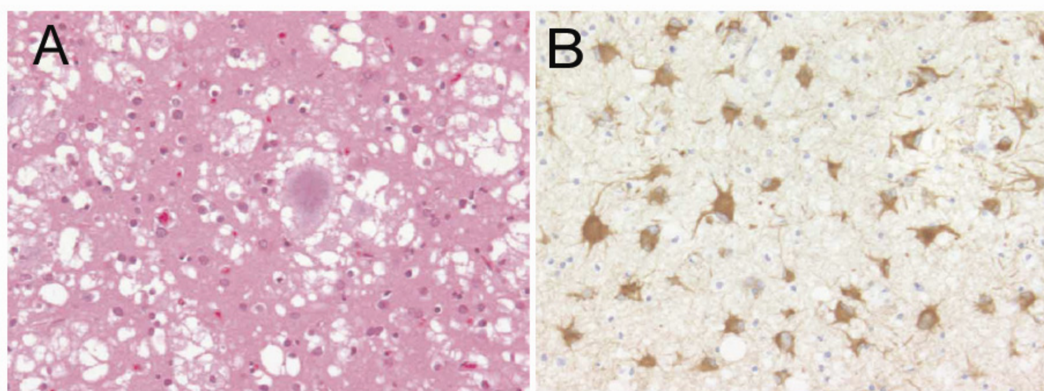
The name TSE is used because these diseases are generally transmissible, within and sometimes between species, and are pathologically defined by appearance of sponge-like vacuolation in the grey matter (location of neuronal cell bodies) of the brain. Pathology of these diseases is also characterised by astrogliosis (proliferation of the neuroglial cells that form a protective scaffold for neurons), neuronal loss, and the deposition of an abnormal form of the host encoded prion protein (PrP) (Rezaie and Lantos, 2001) (Figure 1.F1).

Transmission to a variety of laboratory animal species has shown the presence of distinct strains of TSE, the unique properties which can be maintained on

passage through multiple hosts (Brown *et al.*, 1994b, Bruce *et al.*, 2002, Nonno *et al.*, 2006). Transmission studies in primates and rodents have been performed using brain material from cattle diagnosed with BSE (Lasmezas *et al.*, 2001, Brown *et al.*, 2003, Lasmezas *et al.*, 2005). This has provided significant proof that the acquired form of TSE in humans, variant Creutzfeldt-Jakob disease (vCJD), and in cats (feline spongiform encephalopathy) are attributable to infection by the BSE strain (Will *et al.*, 1996b, Bruce *et al.*, 1997, Hill *et al.*, 1997).

Figure 1.F1: Examples of TSE Histopathology

Example of vacuolation in frontal cortex (A: haematoxylin and eosin stain) and astrocytosis in the pulvinar region (B: staining for glial fibrillary acidic protein – brown colour) in variant CJD, from reference (Ironside *et al.*, 2002b).



1.3 THE PRION HYPOTHESIS

TSE strain properties may be coded for in the mechanism of propagation or structure of the infectious agent. Typical components of this nature that occur in infectious agents such as bacteria or viruses are DNA or RNA molecules in which nucleic acid sequence variation can lead to a characteristic strain appearance. An example of this is the configuration of virus coat proteins that determine how virus molecules are recognised by antibodies in a strain specific immune response. The

coding information for determining a strain must also be propagated by the agent as it multiplies within the host so that the characteristics are replicated in subsequent hosts.

Theories that TSE was a result of virus activity (Diringer, 1991) were tested by attempting to isolate non-host nucleic acid components from fractions of material such as brain tissue known to transmit disease. These fractions were also treated with chemicals known to disrupt nucleic acid. Neither of these methods identified a mechanism of strain identification linked to recognised nucleic acid molecules and it was subsequently proposed that strain identification and infectivity was via a novel disease mechanism involving a protein component only, the prion protein (Prusiner, 1982). This protein component found in TSE diseases has lead to a more common terminology for TSEs, the 'prion diseases'.

The search for additional disease vectors is continuing as it is likely that the prion protein alone does not define all the disease attributes, and the proposal that its involvement is secondary to other truly disease causing factors is still considered by some researchers (Brown, 2001). 'Slow-viruses' were considered during the earlier years of TSE research and have recently been proposed again following the description of 25nm spherical particles in experimental rodent TSE models (Manuelidis, 2007). There is still the possibility that an as yet undescribed mechanism involving a nucleic acid component that is protected from current methods of identification is the true disease agent.

1.3.1 The Prion Protein

Prion protein (PrP) occurs in mammals, birds, and reptiles, as a membrane-bound, glycosyl-phophatidylinositol (GPI) anchored glyco-protein of approximately

20-30 kDa, with possible roles in synaptic transmission and signal transduction in the central nervous system (CNS) (Collinge *et al.*, 1994, Lee *et al.*, 1998, Wopfner *et al.*, 1999, Harris, 2003). It is produced in a wide range of tissues, including lymphoid, heart, lungs, skeletal muscle, salivary gland, and at the highest levels in neurons of the central nervous system (Brown *et al.*, 1990, McLennan *et al.*, 2001). Ablating expression of the protein does not appear to be detrimental to the organism (Bueler *et al.*, 1992, Manson *et al.*, 1994a) although some physiological processes are believed to be affected (Collinge *et al.*, 1994, Sakaguchi *et al.*, 1996, Tobler *et al.*, 1996, Wong *et al.*, 2001).

PrP tertiary structure has been determined through computational analysis of circular dichroism and infrared spectroscopy data (Bazan *et al.*, 1987, Huang *et al.*, 1994) and more recently by crystal structure analysis (Haire *et al.*, 2004). It contains three alpha-helices, two beta-sheets, a single disulphide bridge between helix two and three, and two sites of asparagine (N)-linked glycosylation (Figure 1.F2).

The glycans / sugar molecules attached to the two glycosylation sites confer a considerable degree of variation to this protein (Rudd *et al.*, 2002). Either, both, or neither site can be occupied with sugar chains. The variation in type and overall structure of these chains is an example of one possible mechanism for protein encoded strain variation (Vorberg and Priola, 2002, Lawson *et al.*, 2005, Cancellotti *et al.*, 2006). Strain properties may also be defined by the flexible, unstructured, N-terminal region, as assessed by nuclear magnetic resonance (NMR) imaging of recombinant PrP (Prusiner, 1998). Between the flexible N-terminal and beta-sheet-1 lies the octapeptide repeat sequence that has a copper binding role, and may influence TSE pathogenesis (Hornshaw *et al.*, 1995, Flechsig *et al.*, 2000). This is a

DNA repeat of 24bp coding for the same eight amino-acids, with five repeats present in the human sequence. Genetic expansion of up to an additional ten extra repeats has been linked to familial, inherited forms of human TSE, which will be discussed later in this chapter.

In most types of tissue targeted by TSE disease pathology an over-abundance of the protease resistant form of PrP is found with a modified tertiary structure, one of a predominantly beta-sheet formation (Pan *et al.*, 1993, Prusiner, 1998). The disease associated form of the prion protein is designated PrP^{Sc} (Sc=Scrapie form) to differentiate it from the normal form, designated PrP^{C} (C=Cellular). PrP^{Sc} has remarkable physical properties including an ability to withstand routine autoclave disinfection and incineration procedures up to 600°C (Brown *et al.*, 2000b). Isolation of the two forms has been used to confirm the different structural components, however the true structure of PrP^{Sc} cannot currently be determined because it cannot be crystallized, as it is insoluble in detergents. Other intermediary forms of PrP have been postulated (Review: (Weissmann, 1999)). The differences between the two major prion protein isoforms are summarised in Table 1.T1.

Figure 1.F2: Human PrP Structure

Human prion protein secondary structure components.

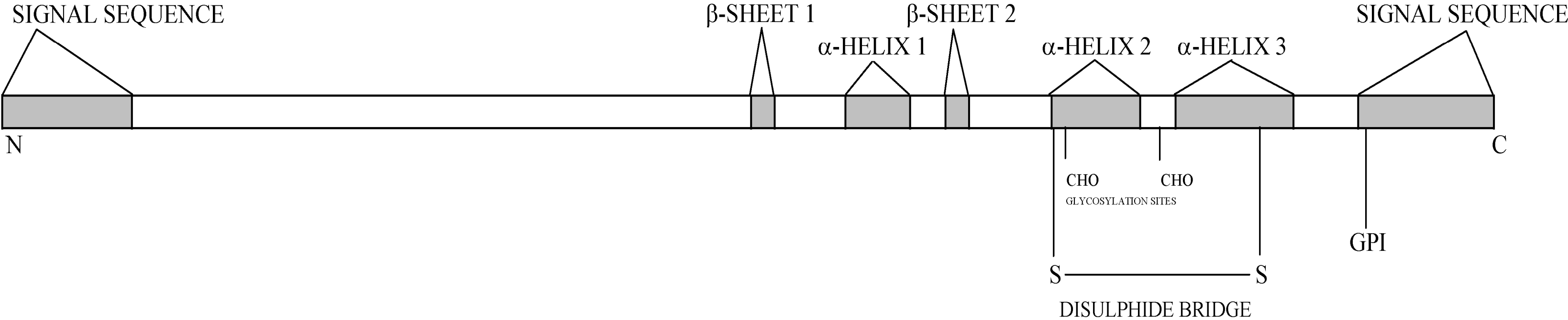


Table 1.T1: Comparison of PrP^C and PrP^{Sc} Properties

A comparison of the two major forms of prion protein.

PrP^C Cellular Form	**PrP^{Sc} Disease Associated Form**
Mainly alpha-helical structure	Mainly beta-sheet structure
Monomeric	Forms aggregates (amyloid)
Soluble under non-denaturing conditions	Insoluble in detergents
Protease sensitive	Partially protease resistant
Rapidly metabolised (T½=5h *in vitro*)	Stable (T½=15h *in vitro*) (Borchelt *et al.*, 1990)
Present in normal tissue	Presence associated with the pathology of prion disease *

* Recent evidence has shown the presence of PrP with attributes of PrP^{Sc} in healthy brain tissue, suggesting that we all may harbour quantities of disease-associated PrP that are routinely maintained at a non-pathological level (Yuan *et al.*, 2006).

In vitro evidence shows that in the presence of PrP^{Sc}, PrP^C undergoes structural conversion to PrP^{Sc} (Raymond *et al.*, 1997, Saborio *et al.*, 2001). In disease this conversion results in an abundance of PrP^{Sc} in the CNS although the precise cellular location and mechanism of conversion is unclear (Harris, 1999). Some TSE such as scrapie, and others acquired via specific infection routes (vCJD), also have an accumulation of PrP^{Sc} in tissues of the peripheral nervous system (PNS) such as the spleen, appendix, and lymph nodes (Brown *et al.*, 1999a, Head *et al.*, 2004b).

Electrophoretic gel analysis of protease treated PrP^{Sc} in human disease has shown the presence of three major conformations commonly designated types 1, 2,

and 2B (Parchi *et al.*, 1996, Parchi *et al.*, 1999, Head *et al.*, 2004a). The difference between type 1 and 2 is a decrease in size of the protease resistant core of PrP due to differential protease action that results from changes in folding properties of the protein. Type 2B shows an increase in the level of PrP present with both glycosylation sites occupied (Figure 1.F3). The type 2B 'glycoform signature' is a common strain characteristic of BSE in cattle and other animal species (Collinge *et al.*, 1996). Humans infected with vCJD have the same glycoform signature (Ironside *et al.*, 2000). An alternative typing nomenclature has been proposed by Prof John Collinge's group but there has been difficulties in replicating this in other laboratories (Collinge *et al.*, 1996).

Examination of sCJD cases in more detail has shown that potentially a significant proportion (36% in one study (Puoti et al., 1999)) have co-occurrence of both PrP^{Sc} types (Parchi *et al.*, 1999, Head *et al.*, 2001, Head *et al.*, 2004a, Puoti *et al.*, 2005). There is also a direct association between type and pathology as shown in remarkable detail by Puoti *et al* where adjacent regions of the cerebral cortex from one brain showed either diffuse or plaque-like PrP^{Sc} deposits which when micro-dissected gave type 1 or type 2 respectively by Western blot (Puoti et al., 1999). In transition fields showing both forms of pathology, mixed PrP^{Sc} type results were found by Western blot. Further to this study the same authors determined that strong microglial activation was associated with the type 1 brain region compared to mild microglial reaction found in those regions with type 2 PrP^{Sc} (Puoti et al., 2005).

Figure 1.F3: Western Blot Analysis of PrP^{Sc}

Western blot analysis of proteinase K treated PrP^{Sc} showing types 1 and 2 (here labelled as 2A) from sporadic Creutzfeldt-Jakob disease, and type 2B from variant Creutzfeldt-Jakob disease associated with BSE infection. (Image from reference (Head *et al.*, 2004a))

1.4 THE PRION PROTEIN GENE

The human prion protein gene region is located on the short arm of chromosome 20 (Band: 20p12-pter). The exon two contains the Open Reading Frame (ORF), the complete DNA sequence required for producing the prion protein, which is 762bp long and translates to a protein of 253 amino acids. The human gene, *PRNP*, was first described in 1986 (Liao *et al.*, 1986) and its DNA sequence is available through the Internet bioinformatics sites (GenBank accession number AL133396), together with bovine *PRNP* (GenBank: AB001468), ovine *PRNP* (GenBank: AJ223072), and murine *Prnp* (GenBank: NM_011170) data. The PrP gene is highly conserved across many different species suggesting an important role for the protein (Lee *et al.*, 1998, Wopfner *et al.*, 1999, Van Rheede *et al.*, 2003, Premzl and Gamulin, 2007). Figure 1.F4 shows a comparison of the PrP amino acid sequence from human beings, chimpanzee, mouse, sheep, goat, elk, cat, bovine. Complete homology across the eight species is widespread (~80% of amino acids) and is shown by the red colour key.

Figure 1.F4: PrP Amino Acid Comparison Between Species

Prion protein amino acid sequence comparison between selected species.
Colour coding for most-to-least homology: red-orange-green-blue

```
+ Majority
Majority  MVKSHIGSWILVLFVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQGSPGGN
                   10        20        30        40        50
Human     M--ANLGCWMLVLFVATWSDLGLCKKRPKPGG-WNTGGSRYPGQGSPGGN
Chimp     M--ANLGCWMLVLFVATWSDLGLCKKRPKPGG-WNTGGSRYPGQGSPGGN
Mouse     M--ANLGYWLLALFVTMWTDVGLCKKRPKPGG-WNTGGSRYPGQGSPGGN
Sheep     MVKSHIGSWILVLFVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQGSPGGN
Goat      MVKSHIGSWILVLFVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQGSPGGN
Elk       MVKSHIGSWILVLFVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQGSPGGN
Cat       MVKSHIGSWILVLFVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQGSPGGN
Bovine    MVKSHIGSWILVLFVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQGSPGGN
```

```
+ Majority
Majority  RYPPQGGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQ-----G
                   60        70        80        90       100
Human     RYPPQGGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQG-----
Chimp     RYPPQGGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQG-----
Mouse     RYPPQGGT-WGQPHGGGWGQPHGGSWGQPHGGSWGQPHGGGWGQG-----
Sheep     RYPPQGGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGG--------G
Goat      RYPPQGGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGG--------G
Elk       RYPPQGGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGG--------G
Cat       RYPPQGGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGG--------G
Bovine    RYPPQGGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGGG
```

```
+ Majority
Majority  WGQGGTHSQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSAMSRPLI
                  110       120       130       140       150
Human     ---GGTHSQWNKPSKPKTNMKHMAGAAAAGAVVGGLGGYMLGSAMSRPII
Chimp     ---GGTHSQWNKPSKPKTNMKHMAGAAAAGAVVGGLGGYMLGSAMSRPII
Mouse     ---GGTHNQWNKPSKPKTNLKHVAGAAAAGAVVGGLGGYMLGSAMSRPMI
Sheep     WGQGGSHSQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSAMSRPLI
Goat      WGQGGSHSQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSAMSRPLI
Elk       WGQGGTHSQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSAMSRPLI
Cat       WGQGGSHSQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSAMSRPLI
Bovine    WGQGGTHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSAMSRPLI
```

```
+ Majority
Majority  HFGNDYEDRYYRENMYRYPNQVYYRPVDQYSNQNNFVHDCVNITVKQHTV
                  160       170       180       190       200
Human     HFGSDYEDRYYRENMHRYPNQVYYRPMDEYSNQNNFVHDCVNITIKQHTV
Chimp     HFGSDYEDRYYRENMHRYPNQVYYRPMDQYSSQNNFVHDCVNITIKQHTV
Mouse     HFGNDWEDRYYRENMYRYPNQVYYRPVDQYSNQNNFVHDCVNITIKQHTV
Sheep     RFGNDYEDRYYRENMYRYPNQVYYRPVDQYSNQNNFVHDCVNITVKQHTV
Goat      HFGNDYEDRYYRENMYRYPNQVYYRPVDQYSNQNNFVHDCVNITVKQHTV
Elk       HFGNDYEDRYYRENMYRYPNQVYYRPVDQYNNQNTFVHDCVNITVKQHTV
Cat       HFGNDYEDRYYRENMYRYPNQVYYRPVDQYSNQNNFVHDCVNITVKQHTV
Bovine    HFGSDYEDRYYRENMHRYPNQVYYRPVDQYSNQNNFVHDCVNITVKEHTV
```

```
+ Majority
Majority  TTTTKGENFTETDIKMMERVVEQMCITQYQRESQAYYQ--RGASVILFSS
                  210       220       230       240       250
Human     TTTTKGENFTETDVKMMERVVEQMCITQYERESQAYYQ--RGSSMVLFSS
Chimp     TTTTKGENFTETDVKMMERVVEQMCITQYERESQAYYQ--RGSSMVLFSS
Mouse     TTTTKGENFTETDVKMMERVVEQMCVTQYQKESQAYYDGRRSSSTVLFSS
Sheep     TTTTKGENFTETDIKIMERVVEQMCITQYQRESQAYYQ--RGASVILFSS
Goat      TTTTKGENFTETDIKIMERVVEQMCITQYQRESQAYYQ--RGASVILFSS
Elk       TTTTKGENFTETDIKMMERVVEQMCITQYQRESEAYYQ--RGASVILFSS
Cat       TTTTKGENFTETDIKIMERVVEQMCITQYQRESEAYYQ--RGASVILFSS
Bovine    TTTTKGENFTETDIKMMKRVVEQMCITQYQRESQAYYQ--RGASVILFSS
```

```
+ Majority
Majority  PPVILLISFLIFLIVG-
                  260
Human     PPVILLISFLIFLIVG.
Chimp     PPVILLISFLIFLIVG.
Mouse     PPVILLISFLIFLIVG.
Sheep     PPVILLISFLIFLIVG.
Goat      PPVILLISFLIFLIVG.
Elk       PPVILLISFLIFLIVG.
Cat       PPVILLISFLIFLIVG.
Bovine    PPVILLISFLIFLIVG.
```

1.4.1 Prion Protein Gene DNA Variation

Polymorphisms fall into two categories those that are directly associated with a disease phenotype (generally producing changes in the amino acid sequence – non-synonymous), and those that may influence clinical features or susceptibility. (For the sake of simplicity in this thesis the former will be referred to as 'mutations' and the latter as 'polymorphisms'.) Twenty-nine mutations, and twenty-one polymorphisms have been found throughout the human gene (Figure 1.F5). There are also polymorphic sites outside of the gene region where variations may determine changes such as gene expression levels (Funke-Kaiser *et al.*, 2001, Mahal *et al.*, 2001, McCormack *et al.*, 2002).

The sheep prion gene has a number of polymorphic sites. Codons 136, 154, and 171 in particular have been associated with varying degrees of susceptibility/ resistance to scrapie infection across many breeds of sheep, however the 136A/154R/171R allele is particularly noted for providing most breeds with a level of resistance to scrapie (Hunter *et al.*, 1994a, Bossers *et al.*, 1996). The UK National Scrapie Plan was instigated to increase the level of resistant genotypes in the National Flock, but the bias towards these genotypes has been brought into question following recent evidence suggesting that new atypical forms of scrapie affect more frequently those 'resistant' genotype sheep (Saunders *et al.*, 2006).

The domestic cattle prion gene is much less variable compared with the sheep gene with no evidence to suggest associations to a disease state (Hunter *et al.*, 1994b, Horiuchi *et al.*, 1998, Hills *et al.*, 2001). There are rare alleles with only five octapeptide repeat elements instead of the usual six that has no known pathological

effect (Hunter *et al.*, 1994b), and four (Seabury *et al.*, 2004) and seven (Schlapfer *et al.*, 1999) repeats that have been found only in healthy Brown Swiss cattle.

The level of genetic variation in the laboratory mouse is limited to amino acids 108 and 189, which are linked to changes in incubation period following experimental inoculation with TSE (Westaway *et al.*, 1994a, Moore *et al.*, 1998, Barron *et al.*, 2005, Cancellotti *et al.*, 2006).

1.4.2 Human Codon 129 Polymorphism

An extensively studied polymorphism of the human prion gene is the methionine to valine change at codon 129. (DNA sequence change ATG (coding M) to GTG (coding V)) The normal population frequencies for the three genotypes are shown in Table 1.T2, showing wide variability in different geographical areas (Soldevila *et al.*, 2003). The frequencies have also been found to vary region-to-region within a country (Plaitakis *et al.*, 2001, Lucotte and Mercier, 2005).

It has been proposed that codon 129 genotype may play a role in or have an effect on the following characteristics of human prion disease:

1. **Susceptibility to disease** (Collinge *et al.*, 1991, Deslys *et al.*, 1994, Zeidler *et al.*, 1997, Deslys *et al.*, 1998, Lee *et al.*, 2001, Brandel *et al.*, 2003)
2. **Survival** (Alperovitch *et al.*, 1999, Pocchiari *et al.*, 2004)
3. **Phenotype of familial forms of CJD** (Goldfarb *et al.*, 1992)
4. **Neuropathology of sCJD** (Parchi *et al.*, 1999, Hauw *et al.*, 2000, Kovacs *et al.*, 2000)
5. **Protease cleavage of** $\mathbf{PrP^{Sc}}$ (Parchi *et al.*, 2000)
6. **Oligomerisation of** $\mathbf{PrP^{Sc}}$ (Tahiri-Alaoui *et al.*, 2004, Lewis *et al.*, 2006)
7. $\mathbf{PrP^{Sc}}$ **amyloid formation** (Baskakov *et al.*, 2005)

Figure 1.F5: Human *PRNP* Gene Variation

Human prion gene variation showing positions of non-pathogenic polymorphisms, and pathogenic mutations. (The codon numbers are shown.)

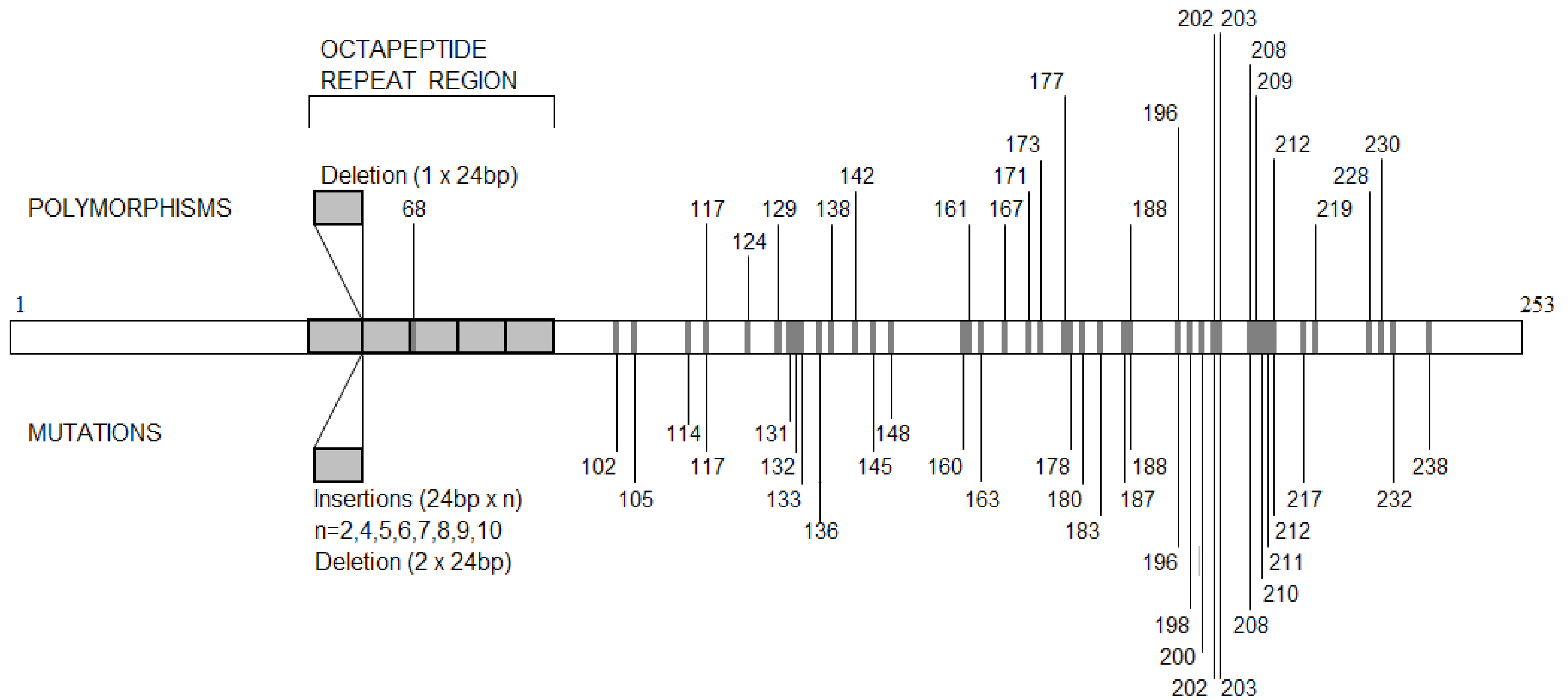


Table 1.T2: Codon 129 Genotype Frequencies Worldwide

Codon 129 genotype frequency variation in human populations worldwide (ordered geographically East to West).

Population	Number Tested	MM	MV	VV
Japan (Doh-ura *et al.*, 1991)	179	165 (92%)	14 (8%)	0 (0%)
Taiwan (Tsai *et al.*, 2001)	100	97 (97%)	3 (3%)	0 (0%)
Turkey (Erginel-Unaltuna *et al.*, 2001)	100	57 (57%)	34 (34%)	9 (9%)
Greece (Crete) (Plaitakis *et al.*, 2001)	205	117 (57%)	77 (38%)	11 (5%)
Greece (mainland) (Saetta *et al.*, 2006)	348	174 (50%)	136 (39%)	38 (11%)
Finland (Nurmi *et al.*, 2003)	1957	969 (49%)	818 (42%)	170 (9%)
Denmark (Dyrbye *et al.*, 2007)	352	131 (37%)	168 (48%)	53 (15%)
Poland (Bratosiewicz *et al.*, 1999)	109	49 (45%)	43 (39%)	17 (16%)
Austria (Zimmermann *et al.*, 1999)	300	129 (43%)	146 (49%)	25 (8%)
Slovenia (Galvani *et al.*, 2005)	97	43 (43%)	46 (46%)	11 (11%)
Germany (Windl *et al.*, 1999)	74	31 (42%)	33 (45%)	10 (13%)
Italy (Medori and Tritschler, 1993, Salvatore *et al.*, 1994)	206	93 (45%)	83 (40%)	30 (15%)
France (Deslys *et al.*, 1994, Laplanche *et al.*, 1994)	161	63 (39%)	82 (51%)	16 (10%)
UK (Collinge *et al.*, 1991, Nurmi *et al.*, 2003)	406	164 (40%)	196 (48%)	46 (11%)
Spain (Combarros *et al.*, 2000)	546	231 (42%)	239 (44%)	76 (14%)
Ireland (Nurmi *et al.*, 2003)	203	69 (34%)	114 (56%)	20 (10%)
Iceland (Georgsson *et al.*, 2006)	208	97 (46%)	93 (45%)	18 (9%)
Brazil (de Paula *et al.*, 2005)	191	87 (46%)	92 (48%)	12 (6%)
USA (Brown *et al.*, 1994a)	86	33 (38%)	44 (51%)	9 (10%)

An association of codon 129 genotype and disease can be seen in the genotype frequencies of the sporadic form of CJD (sCJD) and vCJD cases. In sCJD there is an increase in the homozygote (MM and VV) frequencies with the level of MM at over 70% (Alperovitch *et al.*, 1999, Parchi *et al.*, 1999, Pocchiari *et al.*, 2004), and in vCJD all cases so far analysed have been MM (n=146 of 163) (Zeidler *et al.*, 1997) (See Table 1.T3). This observation indicates a level of susceptibility conferred by homozygosity or a degree of protection by the heterozygote genotype (Prusiner *et al.*, 1990, Cervenakova *et al.*, 1998, Mead *et al.*, 2003). Codon 129 is in the second position of four amino acids that make up beta-sheet-1 (see Figure 1.F2) and therefore could affect elements of the tertiary structure which may be important during conversion of PrP^{C} to PrP^{Sc}, see points 5, 6, and 7 above (Petchanikow *et al.*, 2001).

Table 1.T3: Codon 129 Genotype Frequency Variation in TSE Disease

Codon 129 genotype frequency data from various forms of human TSE disease. (EU data from (Pocchiari *et al.*, 2004), UK control data from (Nurmi *et al.*, 2003), UK data from National CJD Surveillance Unit. (DM: dura mater; hGH: human growth hormone)

	MM (%)	MV (%)	VV (%)	Sample Size
UK Healthy Controls	42	47	11	300
sCJD (UK)	63	19	18	614
sCJD (EU)	68	16	16	1453
vCJD (UK)	100	0	0	146
fCJD-E200K (EU)	78	20	2	113
iCJD-DM (EU)	75	17	8	12
iCJD-hGH (EU)	50	25	25	76

1.5 NON-*PRNP* GENETIC FACTORS

Multi-genic influences are likely to determine the prion disease phenotype. At the present time there are only candidate regions found from murine studies together with analysis of chromosome 20 in the vicinity of the prion gene, as detailed below.

1.5.1 Doppel

Downstream of the prion gene lies the coding sequence for the prion-like doppel protein (Human gene: *PRND*, Genbank: AF106918. Mouse gene: *Prnd*, GenBank: NM_023043). This has a high homology (24% of the coding sequence) to the prion gene with the octapeptide repeat region missing (Makrinou *et al.*, 2002, Premzl and Gamulin, 2007). Generation of PrP knockout mice accidentally generated an overexpression of doppel due to reconfiguration of splicing sites, and this lead to detrimental neurodegenerative consequences (Sakaguchi *et al.*, 1996, Moore *et al.*, 1999, Mastrangelo and Westaway, 2001, Moore *et al.*, 2001a, Rossi *et al.*, 2001). Association studies of doppel polymorphisms and human disease (specifically sCJD) have shown both positive (Schroder *et al.*, 2001, Croes *et al.*, 2004, Jeong *et al.*, 2005) and negative relationships (Mead *et al.*, 2000, Peoc'h *et al.*, 2000). This disparity in results appeared to be dependent on the control population genotype frequencies. A more recent study using larger numbers of samples and controls showed no association between doppel polymorphisms and risk of developing sCJD (Vollmert *et al.*, 2006). Murine studies have added further evidence to dissociate doppel and TSE disease, showing that expression of doppel in the CNS does not affect experimentally induced TSE (Tuzi *et al.*, 2002).

1.5.2 Quantitative Trait Loci Analysis

The most recent technical advances in genetic analysis allow rapid scanning of the whole genome for variations associated with observable phenotypes, quantitative trait loci (QTL), that are linked to a disease state. Such analysis has been carried out to observe differences between lines of mice with short and long incubation periods, those with *Prnp*[a] (leucine at codon 108; threonine at codon 189) and *Prnp*[b] (phenylalanine at codon 108; valine at codon 189) genotypes respectively, when infected with a mouse-adapted form of sheep scrapie (Westaway *et al.*, 1987, Moore *et al.*, 1998, Lloyd *et al.*, 2001). This work identified three highly significantly linked regions on chromosomes 2, 11, and 12 (with the possibility of multiple QTL at each site), and suggestive evidence for linkage on chromosomes 6 and 7. The *Prnp* locus was within the 95% confidence interval for the region on chromosome 2 and is therefore likely to be the candidate for that QTL. A continuation of this work with mouse-adapted BSE incubation time analysis in various lines of mice showed highly significant linkage to regions on chromosomes 2 and 11 (Lloyd *et al.*, 2002).

A further QTL analysis was carried out on two lines of mice (C57BL and RIII) that showed significant difference in incubation periods when challenged with a BSE inoculum but had the same prion gene sequence (*Prnp*[a]) (Manolakou *et al.*, 2001). Four QTL were identified on regions of chromosomes 2, 4, 8, and 15. In this case the *Prnp* locus was outside the 95% confidence interval for the region on chromosome 2. These studies now require more detailed analysis of the candidate regions to try and pin-point the genetic components that are affecting incubation

time. This type of analysis has been made easier since the publication of the mouse genome in 2002.

Finding QTL in human TSE cases is difficult due to the small numbers of case samples available; two of the studies detailed above used over 1000 mice each. This is however under investigation and direct comparisons can be made between homologous regions of the published mouse and human genome (Mead *et al.*, 2001).

1.6 HUMAN PRION DISEASES

Hans Gerhard Creutzfeldt and Alfons Maria Jakob first described prion disease in humans in the 1920s. Since that time the specific clinico-pathological features of Creutzfeldt-Jakob disease (CJD) have been defined in great detail from cases world-wide (Parchi *et al.*, 1999, Zeidler and Ironside, 2000, Van Everbroeck *et al.*, 2001b, Pocchiari *et al.*, 2004). The commonest form of prion disease, sporadic CJD (sCJD), occurs at an incidence rate of approximately one to two cases per million population per year (Will *et al.*, 1996a). Prion disease cases linked to a prion gene mutation contribute 5 – 15% of the total (Windl *et al.*, 1996, Kovacs *et al.*, 2005). The first prion gene mutation associated with a human prion disease was a proline to leucine change at codon 102 (P102L) in a case of Gerstmann Sträussler Scheinker Syndrome (GSS) (Hsiao *et al.*, 1989). (See below for phenotype description.) Since then over 20 mutations have been identified in families with GSS and other inherited forms of human prion disease (Goldfarb and Brown, 1995, Prusiner and Scott, 1997). The high degree of familial association of these mutations with disease, over many generations in some cases, indicate that the mutations are of key pathogenic significance in familial prion disease.

The following list shows the types of human prion disease:

- Sporadic CJD (sCJD)
- Genetic/ inherited prion disease
    - Familial CJD (fCJD)
    - Gerstmann Sträussler Scheinker Syndrome (GSS)
    - Fatal Familial Insomnia (FFI)
- Acquired prion disease
    - Iatrogenic CJD (iCJD) (via medical treatment)
    - Kuru (endo-cannibalism in Papua New Guinea)
    - Variant CJD (vCJD)

1.6.1 Sporadic CJD

Sporadic CJD predominantly occurs in elderly individuals (average age 60 to 70 years) with a rapid duration of illness usually less than 12 months and presenting as a progressive deterioration of intellectual faculties (dementia). There is currently no known cause, or trigger, for the onset of this disease. The onset of sCJD may be controlled by direct genetic influences, such as prion gene promoter regions (Funke-Kaiser *et al.*, 2001, Mahal *et al.*, 2001, Mead *et al.*, 2001, McCormack *et al.*, 2002, Vollmert *et al.*, 2006), or indirect influences such as genes coding for proteins involved in similar cellular processes (Van Everbroeck *et al.*, 2001a, Croes *et al.*, 2004). Spontaneous conformational change from PrP^{C} to PrP^{Sc} due to a chance collection of factors in the biochemical environment, such as a change in pH, could also be a trigger (Zanusso *et al.*, 2001). With increasing age cells become more susceptible to deleterious DNA damage which may cause somatic mutations, a potential explanation for the elderly age of onset for sCJD. It is also possible that an

infection could be passed between individuals, however as sCJD is not seen clustered in families or in healthcare workers this may be unlikely. A link between surgery and sCJD has been suggested but the level of risk is small and the data may be biased because of the way these case/control studies are designed (van Duijn *et al.*, 1998, Ward *et al.*, 2002, Doerr *et al.*, 2003).

Sporadic CJD is a transmissible disease and has been proposed as the likely strain responsible for known cases of iatrogenic (medical transmission) CJD such as those linked to growth hormone therapy, dura mater grafts (replacement of the protective covering of the brain), and neurosurgery (Brown *et al.*, 2000a). It may also have been the initial seed for the kuru disease epidemic in the Fore linguistic group of Papua New Guinea (Gajdusek and Zigas, 1959, Collinge *et al.*, 2006). (See later sections on iatrogenic CJD and kuru in this Introduction.) Monitoring the clinical and pathological transmission characteristics in human iatrogenic cases can only provide a small data-set and the details of the initial contaminating material are likely to be unknown due to pooling of material, and difficulties with traceability. Therefore, experimental animals have been widely used as models for examining the transmission properties of TSE strains (Brown *et al.*, 1994b, Bruce, 2003). (See section 1.7.4 on 'Human TSE Transmission'.)

Statistical analysis of the relationship between codon 129 genotype and the sCJD phenotype, compared with normal, healthy individuals, shows a high level of significance (P value < 0.001) (Parchi *et al.*, 1999, Pocchiari *et al.*, 2004). The increased frequency of codon 129 MM cases in relation to the other genotypes suggests that it is a significant susceptibility factor (Palmer *et al.*, 1991, Alperovitch *et al.*, 1999).

Detailed analysis of clinical and pathological features of 300 sCJD cases has led to classification of the disease according to six different subgroups, differentiated by the PrP^{Sc} type (Figure 1.F3) and the codon 129 genotype (Parchi *et al.*, 1999) (Table 1.T4). This classification has been added to by other groups (Hill *et al.*, 2003) and updated by the original authors (Cali *et al.*, 2006) but the first publication is used by many to define sCJD.

Further subgroup definition provided by the Parchi *et al* paper concerned the detailed pathological analysis of the cases. Table 1.T5 shows the similarities and differences in PrP^{Sc} deposition across the subgroups and Figure 1.F6 compiles data for CNS grey matter pathology profiles, a combined assessment of the severity and location of TSE associated pathological changes (vacuolation, astrogliosis, and neuronal loss).

Data generated for this thesis on transmission of each subgroup of sCJD, to transgenic mice, will provide additional evidence for the classification of this disease.

Table 1.T4: Subgroups of sCJD

sCJD disease subgroups as defined by analysis of 300 cases. (Parchi *et al.*, 1999)
(Typical EEG: electroencephalogram showing periodic sharp-waves complexes typical of sCJD)

Codon 129/PrP^{Sc} type subgroup	Name	% of cases	Onset (yr)	Duration (mo)	Clinical Features	Neuropathological Features
MM1 MV1	Classical CJD	67% 3%	65.5 62.1	3.9 4.9	Rapidly progressive dementia, early and prominent myoclonus, typical EEG, visual impairment or unilateral signs at onset in 40% of cases	'Classic CJD' distribution of pathology; often prominent involvement of occipital cortex; 'synaptic type' PrP staining; one third of cases shows confluent vacuoles and perivacuolar PrP staining
VV2	Ataxic variant	16%	61.3	6.5	Ataxia at onset, late dementia, no typical EEG in most cases	Prominent involvement of subcortical, including brain stem nuclei; in neocortex, spongiosis limited to deep layers; PrP staining shows plaque-like, focal deposits, and prominent perineuronal staining
MV2	Kuru-plaques variant	9%	59.4	17.1	Ataxia in addition to progressive dementia, no typical EEG, long duration (>2 yr) in some cases	Similar to VV2 but with presence of amyloid-kuru plaques in the cerebellum, and more consistent plaque-like, focal deposits
MM2	Thalamic variant	2%	52.3	15.6	Insomnia and psychomotor hyperactivity in most cases, in addition to ataxia and cognitive impairment, no typical EEG	Prominent atrophy of the thalamus and inferior olive (no spongiosis) with little pathology in other areas; spongiosis may be absent or focal, and PrP^{Sc} is detected in lower amounts than in the other variants
MM2	Cortical variant	2%	64.3	15.7	Progressive dementia, no typical EEG	Large confluent vacuoles with perivacuolar PrP staining in all cortical layers; cerebellum is relatively spared
VV1	Rare	1%	39.3	15.3	Progressive dementia, no typical EEG	Severe pathology in the cerebral cortex and striatum with sparing of brain stem nuclei and cerebellum; no large confluent vacuoles, and very faint synaptic PrP staining

Table 1.T5: PrP^{Sc} Deposition in sCJD Subgroups

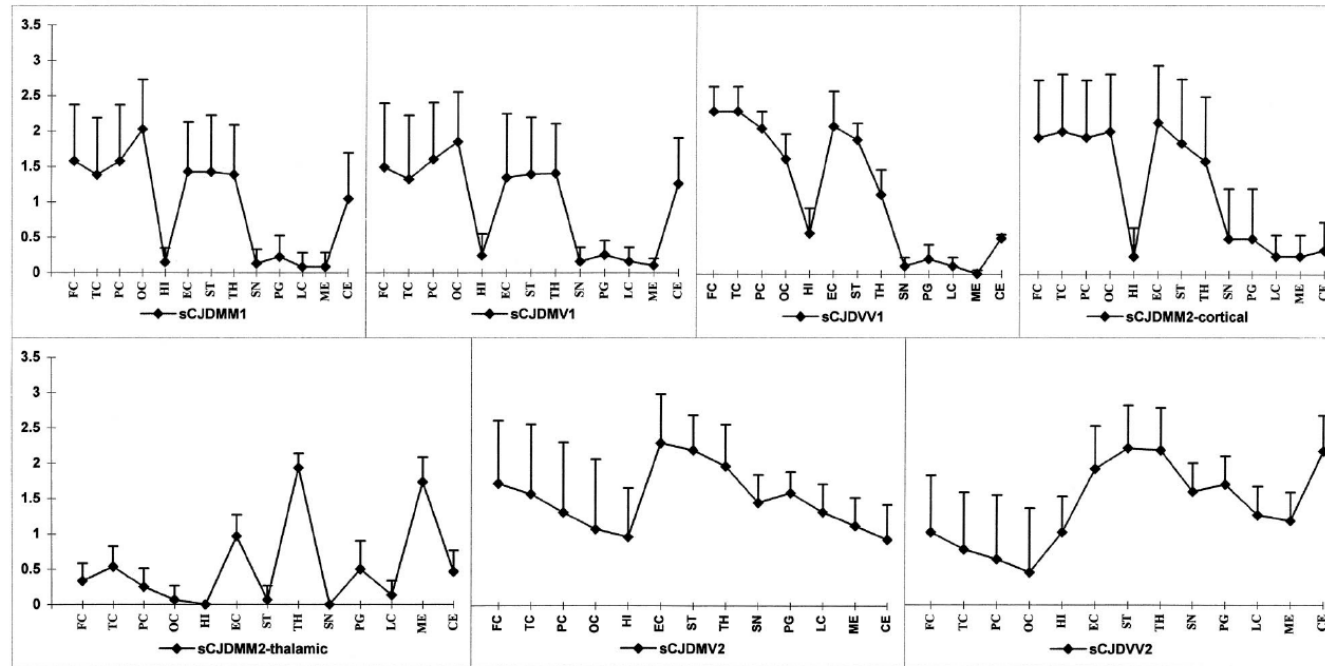
Differences in location and pattern of PrP^{Sc} deposition for subgroups of sCJD. Data from reference: (Parchi *et al.*, 1999)

sCJD Subgroup (Number of cases)	MM1 (111)	MM2 Cortical (5)	MM2 Thalamic (6)	MV1 (5)	MV2 (19)	VV1 (3)	VV2 (30)
Cerebellar or cortical (widespread, diffuse deposition)	✓	✗	✓	✓	✓	✓	✓
Cortical (deposits around spongiform change)	✓	✓	✓	✓	✓	✗	✗
Cerebellar (plaque-like deposits)	✗	✗	✗	✗	✓	✗	✓
Cerebellar kuru plaques (characteristic circular plaques)	✗	✗	✗	✗	✓	✗	✗
Cortical (layered pattern of deposition)	✗	✗	✗	✗	✓	✗	✓

Figure 1.F6: Pathology Profiles for sCJD Subgroups

Comparison of grey matter pathology profiles for sCJD cases grouped by codon 129 and PrP^{Sc} type.

Regions of brain analysed: frontal (FC), temporal (TC), parietal (PC), and occipital (OC) neocortices, hippocampus (HI – CA1 region), parasubiculum and entorhinal cortex (EC), neostriatum (ST – nuclei caudatus and putamen), thalamus (TH – mediodorsal nucleus), substantia nigra (SN), midbrain periventricular grey (PG), locus ceruleus (LC), medulla (ME – periventricular grey and inferior olive), and cerebellum (CE). (Mean + SD for three scores – spongiosis, astrogliosis, and neuronal loss. For number of cases analysed see Table 1.T5.) Data from reference: (Parchi *et al.*, 1999)



1.6.2 Genetic / Inherited Prion Disease

Genetic prion disease, diagnosed through the presence of a mutation in the prion gene (shown in the lower half of Figure 1.F5), is thought to occur because the mutant prion protein may more readily convert to the disease associated form, or may alter the fine balance of turn-over for production and degradation of the protein. This may be due to destabilisation of the tertiary structure following changes in the protein amino acid sequence. Supportive evidence for this comes from that fact that PrP^{Sc} is formed from the mutant allele PrP^{C} in all of the limited number of cases studied. Whether the wild-type PrP^{C} is converted also (most genetic prion disease cases are heterozygous for the mutation), is dependent on which mutation is present (Chen *et al.*, 1997, Silvestrini *et al.*, 1997).

According to the non-prion hypothesis instability of the mutated protein may lead to increased susceptibility to infection by an alternative disease agent or pathway; a proposal given further backing by the fact that some carriers of some *PRNP* mutations can live long healthy lives (low penetrance) (Pocchiari *et al.*, 1993, Spudich *et al.*, 1995, Mitrova and Belay, 2002).

Some of the more common mutations are associated with diseases which show near complete penetrance with increasing age (all carriers of the mutation develop disease), however only a minority of genetic prion disease cases have a family history of neurological disease, suggestive of a prion disease. Mutations at codons 178 and 200 and a six octapeptide repeat insertion have been traced back through the generations to where and when the original mutation events are thought to have occurred (Nicholl *et al.*, 1995, Harder *et al.*, 1999, Lee *et al.*, 1999). Due to the relatively late onset age in these diseases the mutation phenotype may not show

for a number of generations as asymptomatic carriers of the mutation may die of other causes. (For reviews see references: (Prusiner and Scott, 1997, Kovacs *et al.*, 2002, Kovacs *et al.*, 2005))

Some specific mutations are associated with particular clinical and pathological phenotypes and have thus been classified under alternative names including Gerstmann Sträussler Scheinker (GSS) and fatal familial insomnia (FFI) (see below). The following sections describe in more detail of some of the *PRNP* mutations linked to genetic forms of human TSE, grouped by phenotype.

1.6.2.1 Familial CJD (fCJD)

Familial CJD (fCJD) is a common term given to forms of human TSE, associated with *PRNP* mutations, that frequently have a phenotype similar to that of sCJD, and are unlike GSS or FFI. This group is difficult to recognise through clinical features alone as the phenotypes are too varied both within and between family groups. The following describes details of the common mutations associated with fCJD cases.

1.6.2.1.1 Codon 178: Asp (D) to Asn (N)

With valine at codon 129 on the same allele this mutation has been found throughout the world including a large pedigree in Finland (Haltia *et al.*, 1991, Goldfarb *et al.*, 1992). The phenotype differs from that of sCJD in that it affects younger people, has a longer duration, and does not show periodic sharp wave activity by electroencephalography (EEG).

1.6.2.1.2 Codon 200: Glu (E) to Lys (K)

This is the most common fCJD mutation with clinical features and pathology very similar to sCJD. It has been found in clusters in Italy, Slovakia, Chile, and Sephardic Jews. In Jews of Libyan origin the incidence of fCJD is 100 times that of sCJD and accounts for the majority of Israeli CJD cases (Meiner *et al.*, 1997, D'Alessandro *et al.*, 1998, Lee *et al.*, 1999, Colombo, 2000, Mitrova and Belay, 2002).

1.6.2.1.3 Codon 210: Val (V) to Ile (I)

Identified initially in Italian and French families, and now found around the world, the codon 210 mutation presents a phenotype very similar to sCJD (Pocchiari *et al.*, 1993, Ripoll *et al.*, 1993).

1.6.2.1.4 Insertions

Insertion mutations are variable numbers of additional octapeptide repeats (two to ten copies of a 24bp sequence) in the repeat region of the prion gene. Some insertions occur more frequently than others, such as cases with six extra repeats that have been found in UK families (Nicholl *et al.*, 1995, Mead *et al.*, 2006). This type of mutation causes a wide spectrum of phenotypes dependent on the number of additional repeats causing difficulties with diagnosis (Vital *et al.*, 1998, Moore *et al.*, 2001b). The disease duration can be between two months and 18 years, with phenotypes similar to CJD or GSS (Kovacs *et al.*, 2005). As a general rule the CJD-like phenotype occurs with up to six extra repeat elements and a duration of less than one year. The GSS-like phenotype occurs with a larger number of repeats and a longer duration (Parchi *et al.*, 1998b).

1.6.2.2 Gerstmann Sträussler Scheinker Syndrome (GSS)

The original GSS study involved an Austrian family that now encompasses 221 members in nine generations. The clinical features that specifically differentiate GSS from sCJD are earlier age at onset (30-40 years old) and longer duration (~5 years) (Hainfellner *et al.*, 1995). Pathologically GSS is characterised by the presence of multicentric amyloid plaques and a truncated form of PrP^{Sc} by Western blot (Ghetti *et al.*, 1996, Parchi *et al.*, 1998a). This disease phenotype can occur associated with a number of different mutations (Piccardo *et al.*, 1998).

1.6.2.2.1 Codon 102: Pro (P) to Leu (L)

This is the commonest GSS phenotype mutation and is recognised as the ataxic variant of GSS (due to early movement disorders) with age at onset approximately ten years earlier than sCJD (Hainfellner *et al.*, 1995, Young *et al.*, 1997).

1.6.2.2.2 Codon 117: Ala (A) to Val (V)

This mutation has been studied in families from Italy, Hungary, France, and the UK. It is described as the dementia variant of GSS with occurrence of presenile dementia at onset (Mallucci *et al.*, 1999, Kovacs *et al.*, 2001, Tagliavini *et al.*, 2001).

1.6.2.2.3 Codon 198: Phe (F) to Ser (S)

With valine at codon 129, this mutation has been studied in detail for one pedigree referred to as the Indiana kindred. This mutation is recognised by the slow progression of disease, and pathologically by the presence of neurofibrillary tangles (NFTs - accumulation of twisted protein fragments inside nerve cells.) (Dlouhy *et al.*, 1992).

1.6.2.2.4 Codon 217: Gln (Q) to Arg (R)

With valine at codon 129, found in a Swedish family, this mutation shows similar clinical features and pathology to codon 198 cases including the presence of NFTs (Hsiao *et al.*, 1992).

1.6.2.3 Fatal Familial Insomnia (FFI)

This phenotype is clinically characterised by inattention, sleep loss, and dysautonomia (abnormal functioning of the autonomic nervous system), and pathologically characterised by a preferential degeneration of the thalamus (the organ that organizes peripheral sensory information (Reder *et al.*, 1995, Cortelli *et al.*, 1999). It is associated with a single mutation, D178N, as shown above for fCJD but with methionine at codon 129. This disease is clearly distinct from the fCJD phenotype where the mutation occurs with valine at codon 129 (Goldfarb *et al.*, 1992). This mutation has been found in over 20 individual families and has been recognised since 1986 (Lugaresi *et al.*, 1986, Medori and Tritschler, 1993). Variation in this phenotype has been reported in an Australian FFI cohort where individuals were characterised with typical CJD, FFI, and cerebellar ataxia (McLean *et al.*, 1997).

1.6.3 Acquired Prion Disease

This group of TSE diseases arise due to the passage of infectious material from one individual to the next and has so far been limited to the three following causes in humans:

1. Iatrogenic CJD (iCJD): disease acquired from infected human tissues by medical or surgical procedures

2. Kuru: ritualistic cannibalism of infected human tissues

3. Variant CJD (vCJD): disease primarily acquired via diet from infected bovine tissues, and secondarily via blood donated by individuals who went on to develop vCJD

1.6.6.1 Iatrogenic CJD (iCJD)

ICJD has been shown to be transmitted through a variety of means and was first reported in 1974 in the recipient of a corneal graft from a donor who died of unsuspected CJD (Duffy *et al.*, 1974). The current world-wide total for all identified iCJD cases is 405 (Brown *et al.*, 2006). Now that the risk of TSE transmission is known precautions are in place to attempt to avoid further cases in the future. In the mid-1980s the major risk factors for this acquired disease were identified as contaminated human dura mater grafts (the outermost protective membrane of the brain) and human growth hormone (hGH) derived from cadaver pituitaries. The highest incidence of iCJD is in growth hormone patients in France, USA and the UK; and contaminated dura mater graft recipients in Japan.

The pathology and clinical features of iCJD show similarities to vCJD, which might be predicted, as both are prion diseases acquired through an exogenous infectious source. Differences seen may be a result of the alternative routes of infection, oral for vCJD and intravenous or intracerebral for iCJD.

It is of interest to examine the codon 129 genotype of iCJD cases because vCJD case genotypes might follow the same temporal patterns, e.g. incubation time and the appearance of different codon 129 genotypes at different periods from the point of infection. This is of specific relevance to peripherally acquired iCJD such as

for the growth hormone cases, as BSE infection of humans was via the periphery (although the routes are different: intravenous versus oral respectively). As seen in sCJD, homozygosity (MM or VV) is more frequent in iCJD cases and the UK hGH cases show a marked increase in VV genotypes indicating possible contamination by VV genotype infectious material (Collinge *et al.*, 1991, Brown *et al.*, 2000a). It is possible that for this TSE infection the MV genotype individual may be relatively protected to some degree. There is a statistically significant increase in the incubation time for heterozygotes, as found in French growth hormone cases (d'Aignaux *et al.*, 1999). A study of donor/ recipient pairs where material was available for codon 129 analysis showed all donors were MM and the recipients were both MM and MV. As MV individuals were affected, complete genetic homology was not required for disease transmission (Brown *et al.*, 1998).

1.6.6.2 Kuru

Identified in the Fore linguistic group in the Eastern Highlands of Papua New Guinea, kuru was first reported in 1959 (Gajdusek and Zigas, 1959). It has been classified as a form of acquired prion disease as it is proposed that this prion disease spread through ritualistic cannibalistic practices involving exposure to, and probably consumption of, CNS material. The numbers of kuru cases currently stands at approximately 3000 (Goldfarb, 2002). The last cases (mostly MV genotype) appeared over 40 years after cessation of cannibalism, suggesting incubation periods of over 50 years (Collinge *et al.*, 2006). This is of significant relevance to other acquired human prion diseases, such as vCJD, which may have similarly long incubation periods in certain individuals.

A codon 129 study in the Fore group showed the variation in genotype frequencies of the population over the course of the kuru epidemic. The kuru survivor population sampled in the late 1950s was found to have a significantly lower level of MM genotypes, indicating that these individuals were the first to die from kuru. The last group of kuru cases, who would have been infected at a similar time, was subsequently found to have a high frequency of MV. This genotype may have been subject to balancing selection over many generations because of the occurrence of acquired TSE diseases such as kuru that appear because of cannibalism (Hedrick, 2003, Mead *et al.*, 2003). These data suggest variation in incubation time between the genotypes with MM being the most susceptible, and having the shorter incubation period (Cervenakova *et al.*, 1998, Lee *et al.*, 2001, Collinge *et al.*, 2006).

1.6.6.3 Variant CJD (vCJD)

Variant CJD (vCJD) is an acquired form of human prion disease caused by a cross-species transmission of the BSE infectious agent from cattle to humans (Bruce *et al.*, 1997, Hill *et al.*, 1997). Following the identification of vCJD in 1996, (Will *et al.*, 1996b) there have been many attempts to estimate the extent of the UK epidemic for public health reasons (Ghani *et al.*, 2000, Valleron *et al.*, 2001, Ghani *et al.*, 2003). It has been estimated that over 500,000 BSE infected cattle entered the human food chain (Anderson *et al.*, 1996) and therefore a significant number of people may have been exposed to infection through their diet. However, as of July 2008, there have been only 167 cases of the disease in the UK, and a further 43 cases in other countries worldwide. (www.cjd.ed.ac.uk) Annual mortality rates

indicate that the vCJD outbreak is now in decline in the UK following a peak in 1999/2000 (Andrews *et al.*, 2003).

One explanation for the current low number of cases is that there may be genetic determinants, or influences, on susceptibility to infection. DNA sequence analysis of the prion protein gene in vCJD cases has shown that 100% of tested cases are homozygous for methionine (MM) at the codon 129 polymorphism, compared with about 40% of the UK population (Nurmi *et al.*, 2003) and about 70% of sporadic CJD cases (Pocchiari *et al.*, 2004) suggesting that this may be the only susceptible genotype.

Human leukocyte antigen (HLA) typing in vCJD cases (n=50) indicated that there was a reduction in frequency for the class-II type DQ7 compared with sporadic CJD cases (Jackson *et al.*, 2001) however this association was not maintained on testing a larger sample size (Pepys *et al.*, 2003). Detailed analysis of a 35kb sequence flanking the prion gene locus identified 56 single nucleotide polymorphisms (SNPs) and one of these showed strong association with sCJD but not vCJD or iatrogenic CJD (Mead *et al.*, 2001). At this stage no genetic factor has been found to link the few vCJD cases other than MM at codon 129. Identification of such factors may shed light on the reasons why these specific individuals developed vCJD at this time, and thus may allow for treatments to be developed based on correcting the genetic changes that have occurred. Testing for these genetic factors could also be used to determine someone's risk of developing vCJD following a known point of infection.

The clinical and pathological features of vCJD are clearly distinct from those of sCJD. The younger age of affected individuals (median age at death 28

years) and the longer disease duration (median time 13 months) are quite different when compared with sCJD (65 years, and <12 months) (Will *et al.*, 2000). The presenting clinical signs are generally psychiatric, such as depression, followed by ataxia after about six months, then involuntary movements and cognitive impairment. World Health Organisation approved criteria are available to assist with diagnosis (www.cjd.ed.ac.uk/criteria.htm).

There are pathological changes unique to vCJD that assist with confirmation of the cause of death at autopsy. As with most prion diseases, vCJD, has vacuolation, gliosis, and neuronal loss, although the targeting and intensity are not the same as other human TSEs. Some of the unique features of vCJD are listed below:

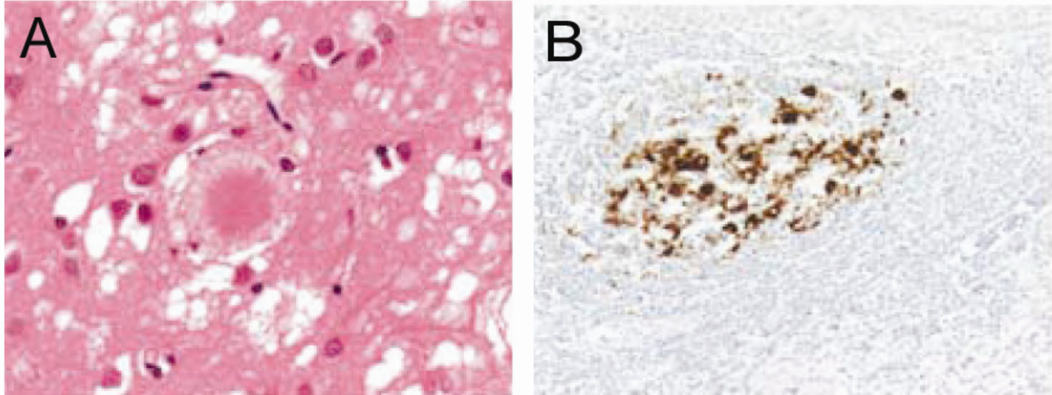
- Presence of 'florid' plaques in the brain (as shown in Figure 1.F7)
- Non-CNS tissue deposition of PrP^{Sc} (Figure 1.F7)
- Type 2B pattern of protease treated PrP^{Sc} by Western blot (Figure 1.F3)
- Hyperintensity of the pulvinar of the thalamus region of the brain by magnetic resonance imaging (MRI)

Figure 1.F7: vCJD Histopathology

Characteristic pathological features of vCJD (images from reference: (Ironside *et al.*, 2002a))

Panel A: typical florid plaque in the cerebral cortex (centre) comprises an eosinophilic core with a pale radial periphery, surrounded by spongiform change (haematoxylin and eosin stain)

Panel B: follicular dendritic cells and tangible body macrophages within the germinal centre of a tonsil (brown stain: anti-PrP antibody KG9)



In 2003 the first case of human-to-human transmission of vCJD via blood transfusion was identified through a collaborative study between the UK National Blood Services, the National CJD Surveillance Unit, and the Office of National Statistics (Transfusion Medicine Epidemiology Review, TMER) (Llewelyn *et al.*, 2004, Hewitt *et al.*, 2006). This patient (MM at codon 129) had received a transfusion of non-leucodepleted red cells that had originated from a donor who three years and four months later developed clinical vCJD, this therefore was the likely source of infection. Statistical analysis indicated that the probability of recording a case of BSE derived vCJD amongst those who received blood products from 'infectious' donors, in the absence of transfusion transmitted infection, was between 1 in 15,000 and 1 in 30,000 (Llewelyn *et al.*, 2004). The blood donor must have been in a pre-clinical phase during which PrP^{Sc} levels were increasing in the periphery. Peripheral tissue amplification of PrP^{Sc} during the preclinical phase of TSE infection is a common finding for mouse TSE strains that target the peripheral nervous system (Mabbott and Bruce, 2001).

Two further cases of vCJD linked to blood transfusion, in MM genotype individuals, have subsequently been identified (Wroe *et al.*, 2006, HPA, 2007). These cases instigated policy changes in relation to blood donation in the UK and elsewhere. The UK Blood Service deferred transfusion recipients from acting as blood donors and began out-sourcing blood products from other countries, including the USA, for specific uses such as treating children.

A fourth case, of asymptomatic infection following blood transfusion, was described in 2004, in a patient that died of a ruptured abdominal aortic aneurysm. As they were on the 'at risk' list held by the TMER study group, autopsy tissues were

examined. These were confirmed as having signs of vCJD infection, including the presence of type 2 PrP^{Sc} in spleen and deposition of PrP^{Sc} in germinal centres within spleen and cervical lymph nodes This individual was heterozygous (MV) at codon 129 (Peden *et al.*, 2004). This was the first indication that non-MM individuals might be susceptible.

All three codon 129 genotypes are now thought to be susceptible to vCJD infection following analysis of two of the three positive appendix tissues discovered in a retrospective immunocytochemical study that aimed to detect disease associated prion protein (PrP^{Sc}) in appendix and tonsil specimens from the UK (Hilton *et al.*, 2004a). These two tissues were homozygous for valine (VV) at codon 129 (Ironside *et al.*, 2006).

Further confirmation has come from successful transmission of vCJD to 'humanised' transgenic mice of each codon 129 genotype (this thesis) (Bishop *et al.*, 2006). The implications of these findings are that a significant number of the UK population, of all codon 129 genotypes, may be carriers of vCJD infectivity, that some of the individuals may be donating blood, and that not only those with an MM genotype may be susceptible to infection from this source. Results from second passage of vCJD in human transgenic mice, will help to model the extent to which non-MM individuals infected with vCJD agent, could efficiently transmit infection (See Chapter 4, section 4.3.2).

For the four known cases of blood transfusion transmission of infectivity the time periods between the donation of blood and development of vCJD in the donors were: 40 months (Llewelyn *et al.*, 2004); 18 months (Peden *et al.*, 2004); 20 months (Wroe *et al.*, 2006); and 17 months (HPA, 2007). This confirms the hypothesis that

the vCJD preclinical phase is lengthy and that during this time host tissues or fluids are able to transmit disease. It is known that the kuru incubation time from infection to clinical onset may be up to 50 years but there is no evidence indicating when during the incubation period levels of infectivity / PrP^{Sc} reach a point at which tissues or fluids would be transmissible.

Evidence that PrP^{Sc} is present during the pre-clinical phase in TSEs has come from studies of vCJD (positive appendix eight months prior to onset of clinical vCJD) (Hilton *et al.*, 1998), BSE (PrP^{Sc} detection six months prior to onset) (Schaller *et al.*, 1999, Schulz-Schaeffer *et al.*, 2000), and scrapie (PrP^{Sc} detection approximately 50% through the incubation period) (Schreuder *et al.*, 1998).

The identification of four instances of secondary transmission of vCJD infection from a group of 66 individuals known to have received blood products from vCJD donors, including only 28 who survived at least five years post transfusion indicates that blood transfusion is a significant risk factor for vCJD. It is unknown whether this is because the route of infection is more efficient, or because the agent involved is more infectious than the original BSE strain. A sheep blood transfusion model used intravenous (i.v.) transfusion of whole blood and blood fractions from clinical and preclinical sheep infected with BSE or scrapie. Preliminary data showed that the i.v. route gave relatively short and consistent incubation periods suggesting an efficient transmission route, with success rates of 60% (n=3 of 5 transfused) for sheep infected with BSE and 40-45% for natural scrapie (Houston *et al.*, 2000, Hunter *et al.*, 2002, McCutcheon *et al.*, 2007). Contrary to this is a publication that indicated the i.v. route was less efficient than the intracerebral (i.c.) route in a cross-species transmission of a human TSE strain to a

mouse (Brown *et al.*, 1999b). However, a less artificial transmission model of BSE to macaques by the i.v. route has been shown i.v. to be more efficient than the oral route (Herzog *et al.*, 2004).

1.7 TRANSMISSION OF TSE TO EXPERIMENTAL ANIMALS

Transmission studies in TSE research have been undertaken for many decades, particularly on scrapie as this was the first TSE disease to be characterised. Sheep and goats were used for such studies, as these were the original host species, and are still used today but the use of mice, hamsters, and other small rodents is more practical. Using experimental rodent lines is more practical as they are easier and more cost effective to house and maintain, and they have relatively short gestation times, incubation periods, and life-spans. Genetic manipulation of mice has been used to model many human diseases. Mouse genes that have homology to human genes can be manipulated to observe the phenotypic effect on the host and therefore allow better understanding of the human response to similar genetic changes. An example of such a model is the mutation of the mouse prion gene at codon 101, modelling the human GSS mutation at codon 102 (proline to leucine change). These mice, when over-expressing the gene, developed a spontaneous disease phenotype with similarities to human GSS. (See section 1.8.5 Transgenic Mice With Mutations Associated With Human Disease)

Non-human primates, such as Squirrel Monkeys (Williams *et al.*, 2007) and Cynomolgus Macaques (Lasmezas *et al.*, 2005), have been extensively used as they are evolutionarily closer organisms to humans and therefore physiologically and neuropathologically may model human disease more accurately.

1.7.1 Scrapie Transmission

The use of mouse lines for assaying scrapie has been underway since the 1960s and has provided some of the core descriptions of TSE transmission, leading to the detailed classification of different scrapie strains (Bruce *et al.*, 1991). One of the uses for mice in investigating scrapie is as a bioassay, an *in vivo* diagnostic test system. Confirmation that a Dorset Down ram had died from scrapie (an unidentified disease in that breed) was determined by inoculation of a panel of mice with brain material (Zlotnik and Stamp, 1965).

Many natural strains of scrapie exist, with the possibility of mixed infections. Many more have been isolated from serial passage of scrapie in different mice and hamster lines, generating a number of experimental TSEs that have now become extremely well characterised. These are used as model TSE systems due to the predictable nature of the disease pathogenesis produced. Examples of such are 263K from Syrian hamsters (Kimberlin and Walker, 1977), and RML scrapie and Chandler (Chandler, 1961), and ME7 and 79A (Bruce *et al.*, 1991) in mice.

To determine a possible scrapie origin for BSE, brain material from a number of affected animals was passaged through mouse lines for strain typing (Fraser *et al.*, 1992, Bruce *et al.*, 1994, Bruce *et al.*, 2002). The results of this work showed that from UK sources of BSE only one strain was detected and this was different from any of the many strains of sheep scrapie. It was important to know whether the BSE agent could infect sheep as this could be an alternative route to the human food chain. This was shown to be experimentally possible by intracerebral injection or oral dosing of sheep and goats (Foster *et al.*, 1993).

Alternatively, it may be that during feed production, the sheep carcass rendering process biased selection of a rare scrapie strain that was infectious to cattle. Scrapie transmission to transgenic mice expressing the bovine prion gene has been successful indicating that the scrapie origin of BSE is a possibility, however there are some clear distinctions between the results for BSE and scrapie inocula. One study showed clear differences in the pathology (Scott *et al.*, 1999) and a second showed an increase in incubation period for scrapie transmission compared with BSE (Castilla *et al.*, 2003). Both these studies used mice that over-express bovine PrP and therefore inocula may more readily transmit than with wild-type PrP expression. This does however, add further weight to the hypothesis that BSE may have been due to a spontaneous disease in cattle. (See section 1.7.2 below.)

Scrapie has been successfully transmitted from sheep-to-sheep by blood transfusion, a result published before the identification of human-to-human transmission of vCJD by the same route (Houston *et al.*, 2000, Hunter *et al.*, 2002, McCutcheon *et al.*, 2007). This model is now being examined further to find evidence as to which blood components carry the highest infectivity levels, in order to assist with determining the risk to humans from contaminated blood.

1.7.2 BSE Transmission

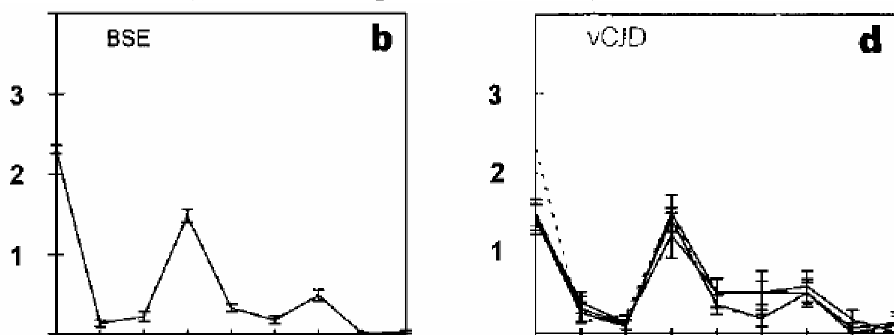
When bovine spongiform encephalopathy (BSE), or ‘mad-cow’ disease, was identified in the late 1980s it was important to ascertain the origins of the infection. The two main theories put forward were that either scrapie had crossed species to become a cattle disease, or it was a spontaneous disease in cattle initiated perhaps by a single mutation event in one animal. Due to the continual recycling of infected

material in cattle feed the disease spread rapidly throughout the UK cattle population (Bradley and Wilesmith, 1993).

An important proof of the hypothesis that BSE had infected humans to produce vCJD was the transmission characteristics of infected brain material from these two diseases to indicative lines of mice (Bruce *et al.*, 1997, Hill *et al.*, 1997). Both sources were characterised as being the same ‘strain’ due to similar lesion profiles (Figure 1.F8) and incubation periods. Further evidence that BSE could infect humans has also been provided from the successful oral inoculation of nonhuman primates (Bons *et al.*, 1999).

Figure 1.F8: Lesion Profiles of Wild-type Mice with BSE and vCJD

Lesion profiles of BSE and vCJD from inoculation of RIII wild-type mice.
Panel ‘b’ from reference (Bruce *et al.*, 1997) shows the pooled grey matter data from four cattle with BSE.
Panel ‘d’ from reference (Bruce *et al.*, 1997) shows the grey matter data from three cases of vCJD (dashed line is pooled BSE data).



1.7.3 Other Non-Human TSE Transmission

Feline spongiform encephalopathy (FSE) has also been transmitted to rodents (Fraser *et al.*, 1994). The comparison of FSE to BSE was important as the disease was only identified in 1990 after the BSE outbreak in the UK. Inoculation of

a mouse typing panel with brain material from three cats provided data on incubation periods, clinical phenotype and neuropathology, that were comparable to BSE data, indicating that these two diseases were likely to have the same source of infectivity (Fraser *et al.*, 1994). Further evidence for BSE infection of other species came from mouse transmissions of Greater Kudu and Nyala (antelopes) suspected of showing signs of TSE (Bruce *et al.*, 1994).

1.7.4 Human TSE Transmission

1.7.4.1 Inoculation of Non-Human Primates

Following the appearance of kuru in the 1950s human material was used to inoculate nonhuman primates (Gajdusek *et al.*, 1966). The clinical disease phenotype seen in these chimpanzees showed similarities to those of kuru and spongiform change was seen on pathological analysis (Beck *et al.*, 1966). One of the largest human TSE transmission data sets available was reported by the United States National Institutes of Health with three hundred experimentally transmitted cases of human prion disease to nonhuman primates: 234 sCJD, 36 fCJD, 8 iCJD, 18 kuru, and 4 with GSS (Brown *et al.*, 1994b). Successful transmission was highest for iCJD (100%), kuru (95%), and sCJD (90%), and lowest for fCJD (68%). Whether the differences were attributable to variation in the codon 129 genotype or PrP type cannot be assessed as these investigations were not in place at that time.

There was some variation found amongst the fCJD group dependent on the specific mutation present in the case material. The E200K cases produced the most efficient transmission rates (85%), similar to sCJD, with which it shares significant similarities in clinico-pathological features. Comparatively less successful were the

P102L GSS cases (39%). None of the three D178N/129M fatal familial insomnia (FFI) cases transmitted disease which was of interest as the fCJD form carrying this mutation (D178N/128V) produced a high transmission rate of 70%. FFI was subsequently been found to be transmissible to wild-type mice (Tateishi *et al.*, 1995).

Even though the familial TSE cases showed variation in transmission rates, as a group, the incubation periods were statistically significantly shorter (in squirrel monkeys) than for sCJD ($p<0.001$; Student's t-test) (Brown *et al.*, 1994b). As familial TSE patients will have expressed the mutated form of PrP^{C} from birth, end-stage disease tissues may have a higher titre of infectivity leading to shorter incubation periods. In contrast, the period from clinical disease onset to death in sCJD in significantly shorter, possibly resulting in tissues with a lower titre of infectivity. Alternatively there may be other characteristics of these TSE strains, such as the PrP^{Sc} conformation, that may effect the transmission incubation period.

Variant CJD and BSE serial passage in non-human primates (macaques) confirmed the BSE origin of vCJD in the UK, Republic of Ireland, and France, that passage of BSE in humans may produce a more human infectious agent transmissible via a peripheral route such as blood transfusion, and that if such a human-to-human infection occurred the clinical and pathological features would be readily recognised by current vCJD diagnostic criteria (Lasmezas *et al.*, 2001). This evidence in an experimental host that is significantly closer to humans than mice, on the evolutionary scale, must remove any doubt that BSE is the origin of vCJD.

1.7.4.2 Sporadic CJD Transmission to Rodents

Sporadic CJD transmissions have been performed on wild-type mice (Bruce *et al.*, 1997), bank voles (Nonno *et al.*, 2006), and an array of transgenic mice

(Telling *et al.*, 1994, Asante *et al.*, 2002, Kitamoto *et al.*, 2002, Korth *et al.*, 2003, Taguchi *et al.*, 2003, Asante *et al.*, 2006, Kobayashi *et al.*, 2007, Beringue *et al.*, 2008). The transgenic mice studies became more frequent following the development of methods to alter the genetic components of the prion protein gene locus; by replacing it with multiple copies, with mutated / chimeric copies, or copies from different species such as humans. Many types of sCJD were found to transmit inefficiently to wild-type mice, without clinical symptoms, (Bruce *et al.*, 1997) and therefore these new mouse models provided the first opportunity to observe more extensive transmission characteristics.

Most of these studies used random genomic insertion to produce over-expressing models, however those from Professor T Kitamoto's group, and the mice used for this study, are based on gene targeting methodology giving physiological (single copy) expression of an introduced 'human' gene. The genetic differences between published transgenic lines will be discussed in a later section of this Introduction.

Sporadic CJD transmission to bank voles has provided some interesting data (Nonno *et al.*, 2006). The classical form of sCJD (subgroups MM1 and MV1) transmits with high efficiency and short incubation times compared to an equally high efficiency but longer incubation times in the less common MM2 subgroup. In contrast, subgroups MV2 and VV2 show no evidence of transmission. As the vole prion gene codes for methionine at the equivalent position to human codon 129, this data may suggests a PrP methionine allele dominance for successful transmission.

1.7.4.3 Genetic TSE Transmission to Rodents

There are variable transmission rates for genetic TSE inocula in wild-type mice which depend on the specific mutation present and the tissue selected for the inoculum, as detailed above in primate studies. An extensive Japanese study of genetic TSE involved inoculation of wild-type mice with a wide variety of source material, although usually only single cases (Tateishi and Kitamoto, 1995). The majority of fCJD cases did not show evidence of transmission. The results for sCJD versus P102L (GSS), where multiple case data are available with successful transmission, show the latter to have a much lower transmission frequency (82% vs. 33% respectively). This is similar to the primate transmission study data discussed above (sCJD 90% vs. P102L 38%) (Brown *et al.*, 1994b).

1.7.4.4 Variant CJD Transmission to Rodents

Variant CJD has been inoculated into both wild-type mice, and a number of different transgenic lines expressing various forms of human and bovine PrP. The results of vCJD transmission to human transgenic mice will be discussed in detail in a later section of this Introduction. Many of these studies were performed to link BSE infection with clinical cases of vCJD. The first of these studies appeared in the subsequent years after the 1996 discovery of vCJD. Wild-type mouse data from NPD (Bruce *et al.*, 1997), wild-type and transgenic mice expressing human *PRNP* (VV at codon 129) data from the Collinge group (Hill *et al.*, 1997), and bovine transgenic mice from the Prusiner group (Scott *et al.*, 1999) all produced compelling evidence for the link between transmission properties of BSE and vCJD suggesting that the human disease was caused by the bovine sourced infectious agent.

Further work on wild-type mice demonstrated that vCJD peripheral tissues (spleen and tonsil) known to contain PrP^{Sc} (unlike sCJD) but not blood fractions, were infectious to mice (Bruce *et al.*, 2001). Also, that detailed neuropathological analysis of mice infected with vCJD and BSE showed close similarities (Brown *et al.*, 2003). An extension of this work was in the use of material from wild-type mouse passaged vCJD. Blood fractions including buffy coat and plasma were found to be infectious using this method (Cervenakova *et al.*, 2003).

The majority of transmission work carried out since these initial studies has involved the use of transgenic mice. VCJD inoculation was to assess new mouse models of human disease (Barron *et al.*, 2001), and in transmission to human transgenic mice to further understand human susceptibility to the bovine origin agent, and the effect of codon 129 genotype on this (Asante *et al.*, 2002, Wadsworth *et al.*, 2004, Asano *et al.*, 2006, Asante *et al.*, 2006, Beringue *et al.*, 2008). All of this work is described in detail in other sections of this Introduction.

1.8 TRANSGENIC MOUSE MODELS OF TSE TRANSMISSION

To investigate species barrier effects on transmission, mouse lines were developed that expressed prion gene sequences from other species. As it is known that transmission is more efficient within species than between species (Bruce *et al.*, 1994), these models have been used to examine this relationship within a controlled host environment. Laboratory methods for the production of transgenic mice have developed significantly over the last two decades, using two distinct methods: random genomic insertion (RGI) and gene targeting (GT) (Manson and Tuzi, 2001).

1.8.1 Random Genomic Insertion (RGI) of Genetic Elements

A section of DNA can be microinjected into a fertilised mouse oocyte. This genetic material integrates randomly into the host's genome, and through genetic screening of the offspring, those mice with the additional gene can be identified. There is no control of the insertion point or how many copies of the transgene have been integrated. The transgene copy number is rarely mentioned in publications. Three lines that express human PrP have this information. One, called Tg110, has 2-4 copies of the gene giving equivalent normal expression levels (Telling *et al.*, 1994), a second, called Tg152, has 30-50 copies giving 4-8x expression levels (Telling *et al.*, 1994), and a third called tg650 has 5 copies giving 6x expression levels (Beringue *et al.*, 2008) (Table 1.T6).

The number of copies present is therefore not directly proportional to expression level, highlighting the effects that insertion point can have on transgene expression. No detailed investigation of the specific location of genomic insertion has been published and therefore any deleterious effects on the surrounding genes are unknown. In addition, regulatory elements such as promoter sequences of host genes in the vicinity of the transgene may determine the location and expression level of PrP. The confirmation that a new over-expressing transgenic line is suitable generally relies on two points: that PrP is expressed in the brain, and that the mice live to normal life-span without any obvious harmful phenotype. This confirmation may be too limited for mice that could have up to 50 lengths of non-murine DNA sequence inserted in the genome, possibly disrupting other genetic elements. Over-expression of even wild-type PrP can have detrimental effects leading to a TSE-like

clinical phenotype and so this method can give data that may be difficult to interpret (Westaway *et al.*, 1994b).

These mice have been used to investigate the relationship between PrP expression levels and susceptibility to disease, and as a result there are mice lines available that develop signs of TSE in less than 100 days post inoculation. In addition, this has made the mouse bioassay system more affordable and with a faster reporting time (Kitamoto *et al.*, 2002). The 'natural' pathogenesis of TSE, and comparisons between mouse lines, however cannot be modelled in such mice, and these are two of the reasons why the gene targeting method was developed for producing mice used in this thesis.

1.8.2 Gene Targeting (GT) for Precise Insertion of Genetic Elements

Predetermined genomic positioning of a transgene allows replacement of murine genes with those of other species. The genetic material to be inserted has flanking DNA homologous to the recipient mouse. This is introduced into embryonic stem (ES) cells where homology between the transgene flanking DNA and the murine DNA ensures precise insertion by homologous replication. The ES cells are then microinjected into mouse blastocysts. Once offspring have been identified as carrying the transgene they are used in the breeding of germline transgenic mice. Continued breeding generates mice homozygous for the transgene. It is this method that has been used successfully in generating the human transgenic mice lines used in this thesis. The benefits of using these gene targeted mice lines are that the inserted human *PRNP* gene is under the direct control of the normal expression modifiers for the equivalent mouse *Prnp* gene, and that following inoculation with human prions there will be homologous human PrP^{Sc} / PrP^{c}

interaction. In addition to this the NPD lines have been generated in an inbred wild-type line (129Ola) therefore the only genetic variation is that of the codon 129 genotype in the inserted human prion gene. The effect of this codon 129 polymorphism can therefore be studied in isolation.

1.8.3 Knockout / Null Transgenic Mice and the Role of PrP

Stopping expression of the prion protein gene in transgenic mice allows analysis of the phenotype attributable to lack of PrP. PrP knockout or null mice (designated *Prnp*$^{0/0}$) have been produced by a few laboratories: NPD (Manson *et al.*, 1994a), Edinburgh (Moore *et al.*, 1999), Zurich (Bueler *et al.*, 1992, Rossi *et al.*, 2001), Nagasaki (Sakaguchi *et al.*, 1996). The usual method is to disrupt the ORF by either insertion or deletion of DNA fragments, however, deletion of the start of the ORF has been found to result in up-regulation of expression of the doppel protein, due to alternative splicing events, producing a deleterious phenotype (Sakaguchi *et al.*, 1996, Moore *et al.*, 1999). The NPD and 'Zurich1' null mice show no adverse symptoms and develop and reproduce normally. However, null mice may show changes to circadian rhythms and sleep patterns (Tobler *et al.*, 1996), electrophysiological defects (Collinge *et al.*, 1994) and alterations in copper binding and superoxide dismutase activity in the CNS (Brown *et al.*, 1997).

Heterozygous null mice (designated *Prnp*$^{+/0}$) with only one active gene have been used to assess the effect of PrP expression levels on TSE pathogenesis. Heterozygous and homozygous null mice were used in mouse scrapie transmission studies to define the requirement for PrP in disease (Bueler *et al.*, 1993, Prusiner *et al.*, 1993, Manson *et al.*, 1994b). Using either mouse inoculum, RML or ME7, the published data-sets show TSE disease occurring in heterozygous null mice at

approximately double the number of days as seen with the *Prnp*$^{+/+}$ wild-type mice. No clinical or pathological signs of disease transmission were seen in homozygous null mice. The heterozygous null mice data suggest that the gene copy number can influence disease onset and pathogenesis, and provides strong evidence to support the central role of PrP.

1.8.4 Chimeric Transgenic Mice and PrP Molecular Interaction

To identify critical components of the prion protein structure, and those required for molecular interaction such as when PrP^{Sc} converts PrP^{C}, chimeric transgenes can be used. Studies using chronically infected mouse neuroblastoma cells (an *ex vivo* method using cells that continually produce mouse derived PrP^{Sc}) were carried out using mouse/hamster PrP chimeras (Scott *et al.*, 1992). The mouse and hamster prion genes were segmented according to restriction enzyme digestion and recombined into a variety of chimeric sequences. Only one of the chimeras tested was found to form PrP^{Sc} in the presence of mouse scrapie prions, indicating that the specific mouse amino acid sequences of that chimera were important for susceptibility to mouse prions.

An *in-vivo* methodology produced a chimeric human/mouse PrP line that expressed normal levels of PrP^{C}, from 2-4 copies of the transgene, with nine different amino acids when compared with mouse PrP (Telling *et al.*, 1994). After inoculation with human TSE material these mice developed signs of neurological disease and deposited PrP^{Sc} formed from the chimeric protein. This indicated that the human component of the chimeric PrP molecule was acting as a recognition site for the infecting agent. This mouse line also expressed mouse PrP^{C} but when this was ablated the incubation periods decreased (Telling *et al.*, 1995b). It was

hypothesized that mouse specific PrP chaperone molecules were preferentially binding mouse PrP^C and so when this was removed they were free to bind to the chimeric protein. This lead to the hypothetical scenario of a 'protein X' chaperone molecule playing an important role in TSE (Telling *et al.*, 1995b).

1.8.5 Transgenic Mice with Mutations Associated with Human Disease

Converting proline to leucine at codon 101 (equivalent to the human P102L GSS mutation – see section 1.6.2.2.1 above) in a transgenic mice line that expressed PrP at levels eight-fold higher than control mice was the first example of a human mutation mouse model (Scott *et al.*, 1989, Telling *et al.*, 1995a). These mice appeared healthy until spontaneous symptoms of muscular incoordination, lethargy, and rigidity occurred at a mean time of ~170 days, a similar ataxic clinical phenotype to that of human GSS (Hsiao *et al.*, 1990). Serial transmission confirmed this disease could be transmitted to hamsters and transgenic mice, but not to wild-type mice (Hsiao *et al.*, 1994).

Mice with single copy P101L transgenes were developed to more closely model the effect of this mutation as it is found in humans (Manson *et al.*, 1999). These mice did not develop a spontaneous TSE, however, the mutation was associated with a dramatic alteration in incubation periods and susceptibility. Inoculation with human GSS material (at 1% concentration) produced clinical disease in only one wild-type mouse (from a group of eight) at 456 days, whereas in transgenic mice homozygous for the mutation 100% (n=15) showed clinical signs at 288 days. Inoculation with mouse passaged scrapie strain ME7 showed 100% transmission for both transgenic and wild-type mice but the incubation period was doubled for the former.

To further investigate this difference in strain transmission for these transgenic mice they were inoculated with TSE material from four different species: human (vCJD), hamster (passaged scrapie), sheep (scrapie), and mouse (passaged scrapie) (Barron *et al.*, 2001). In transgenic mice the human and mouse derived TSE showed an increase in the incubation period, whereas TSE from hamster and sheep decreased the incubation period, all compared with wild-type mice.

These results indicate a significant role for the protein structure of PrP around amino acid position 101, with effects seen across three species barriers, and support the theory that humans carrying the P102L mutation may have altered susceptibility to TSE infection (Manson *et al.*, 1999, Barron *et al.*, 2001).

1.9 TRANSGENIC MICE WITH FULL-LENGTH HUMAN PRION GENE SEQUENCE

There are five research groups that have published data on transgenic mice expressing full-length human PrP with either MM, MV, or VV genotype at codon 129, and an additional group that have published only MM mice. The lines are listed in Table 1.T6 together with the methodology used to create them; either random genomic insertion (RGI) (multiple copy / over-expressing) or gene targeting (GT) (single copy / physiological expression). The data obtained using each method will be discussed below. Much of the data from the other research groups, comparing codon 129 genotype between lines, including the heterozygote genotype, was published since the beginning of this thesis.

Table 1.T6: Transgenic Lines Expressing Human PrP

Summary of transgenic lines expressing human PrP with variation at codon 129. (GT: gene targeting; RGI: random genomic insertion)

Research Group Leader	Transgenic Method	Line Name (Genotype)	Expression Level	Use	References
Manson	GT	HuMM (MM) HuMV (MV) HuVV (VV)	1x 1x 1x	Definition of strain and role of codon 129 genotype in susceptibility	(Bishop *et al.*, 2006, Cancellotti *et al.*, 2006, Bishop *et al.*, 2008)
Kitamoto	GT RGI	Ki-Hu129M/M (MM) Ki-Hu129M/V (MV) Ki-Hu129V/V (VV) Tg-ChM#30 (MM) Tg-ChV#12 (VV) Tg-ChV#21 (VV)	1x 1x 1x 0.7x 2x 4x	Development of bioassay and role of codon 129	(Kitamoto *et al.*, 1996, Kitamoto *et al.*, 2002, Taguchi *et al.*, 2003, Asano *et al.*, 2006)
Prusiner	RGI	Tg440 (MM) Tg152 (VV) Tg110 (VV)	2x 4-8x 1x	Characterisation of PrP structural properties and association with infectivity	(Telling *et al.*, 1994, Telling *et al.*, 1995b, Korth *et al.*, 2003)
Collinge	RGI	Tg35 (MM) Tg45 (MM) Tg45/152 (MV) Tg152 (VV)	1-2x 4x 4-6x (M:V=1:1.5) 4-8x	Definition of strains and human susceptibility	(Collinge *et al.*, 1995, Hill *et al.*, 1997, Asante *et al.*, 2002, Asante *et al.*, 2006)
Laude	RGI	Tg650 (MM)	6x	Definition of strains and human susceptibility	(Beringue *et al.*, 2008)

1.9.1 Random Genomic Insertion (RGI) Method

1.9.1.1 Co-Expression of Human and Mouse Prion Genes

The first transgenic mouse lines with the complete human gene sequence were developed in the mid 1990s as a novel experimental tool for investigating transmission of human strains of TSE (Telling *et al.*, 1994, Telling *et al.*, 1995b). Two such lines were used to study the effect of PrP expression levels on disease transmissibility; Tg110 had between two and four copies of the transgene representing an approximation of normal expression levels, and Tg152 had 30-50 copies and gave a four to eight-fold increase in expression (Telling *et al.*, 1994) (Table 1.T6). The inserted gene coded for valine at codon 129 as this was thought to be the most susceptible genotype in iCJD (Collinge *et al.*, 1991), and the mice still expressed mouse PrP.

Only approximately 10% of these mice were found to be susceptible to infectivity from GSS, sCJD, iCJD, and fCJD cases which was unexpected as transgenic mice expressing chimeric human / mouse PrP were readily infected. $HuPrP^{Sc}$ was however found in the brains of two Tg110 mice that showed clinical signs nearly 600 days after inoculation with a case of iCJD. This suggested that the incubation period was probably greater than the usual life-span.

When six mice of the higher-expressing Tg152 line were inoculated with the same case of iCJD an incubation period ~280 days in three mice. The difference in susceptibility between the Tg110 and Tg152 lines was thought to be due to the level of transgene expression, although it was also stated that the Tg152 mice “may be

homozygous [for the transgene] since both parents carried the transgenes" whereas the Tg110 line was hemizygous (one copy) (Telling *et al.*, 1994).

Compared with the other case material, this iCJD case possibly had a higher titre of infectivity or, as it is likely to have had a VV genotype (from details in a contemporary paper known to be the source of the inocula (Collinge *et al.*, 1991)), it may therefore have transmitted more efficiently to the VV mice (Telling *et al.*, 1995b).

1.9.1.2 Expression of Human Prion Gene in the Absence of Mouse PrP

Valine homozygous Tg152 mice were crossed with PrP null mice to generate a line expressing only human PrP. When these Tg152 / *Prnp*$^{0/0}$ mice were challenged with sCJD or iCJD infectivity there was 100% transmission and short incubation periods (~250 days) (Telling *et al.*, 1995b). This indicated that the presence of mouse PrP^{C} had to some degree inhibited development of a TSE infection from the human inoculum. The mechanism behind this remains unclear but it may be due to host factors, such as chaperones, that had higher specificity for host PrP^{C} during TSE infection, which on removal of host PrP^{C} were then able to interact with the transgenic PrP^{C}.

To predict human response to BSE infection these Tg152 / *Prnp*$^{0/0}$ transgenic mice were inoculated with vCJD and BSE (Hill *et al.*, 1997). These data supported results from wild-type mouse (Bruce *et al.*, 1997) proposing that vCJD cases harboured the same agent strain as BSE. These Tg152 / *Prnp*$^{0/0}$ mice were also inoculated with iCJD and sCJD of different codon 129 genotypes (Table 1.T7).

Table 1.T7: Tg152 (VV) Transgenic Mice with Various TSE Inocula

Inoculation of valine homozygous Tg152 / *Prnp*$^{0/0}$ mice with bovine and human TSE strains. (hGH-growth hormone; DM-dura mater; G-gonadotrophin) (Hill *et al.*, 1997)

Inoculum (# cases)	Codon 129 Genotype	Affected (Success Rate)	Incubation Period (days±SEM)
vCJD (n=6)	MM	25/56 (45%)	228±15
BSE (n=5)	‘MM’	10/26 (39%)	602±50
sCJD (n=9)	MM	66/67 (99%)	210±4
sCJD (n=2)	MV	15/15 (100%)	218±2
sCJD (n=1)	VV	5/5 (100%)	337±11
iCJD(hGH) (n=1)	MM	7/7 (100%)	211±5
iCJD(hGH) (n=1)	MV	4/4 (100%)	195±9
iCJD(hGH) (n=1)	VV	5/5 (100%)	193±4
iCJD(DM) (n=1)	MM	4/4 (100%)	204±6
iCJD(G) (n=1)	VV	8/8 (100%)	187±4

The Hill *et al* 1997 publication identified similar, success rates for transmission, and pathological changes after inoculation with both vCJD and BSE (although the incubation period was longer for BSE inoculation) and differences between these and sCJD and iCJD inocula. This was suggestive of a similar origin for vCJD and BSE. The longer incubation period with BSE may be due to a species barrier effect as the vCJD inoculum is human passaged bovine infectivity compounded by the difference in codon 129 of the host mouse (VV) and the inocula (MM). For sCJD and iCJD, variation in inocula codon 129 genotype did not appear to show a preference for homology to the host, possibly due to the over-expression of PrP.

1.9.1.3 Expression of Human PrP Homozygous (MM or VV) at Codon 129

As the host codon 129 genotype is of importance when considering susceptibility of an individual human being, the next stage in transgenic development was to compare further the VV and MM transgenic lines.

The comparison of host codon 129 homozygous genotypes in HuPrP / *Prnp*$^{0/0}$ transgenic mice has been the subject of five publications (Telling *et al.*, 1995b, Asante *et al.*, 2002, Kitamoto *et al.*, 2002, Korth *et al.*, 2003, Wadsworth *et al.*, 2004). The results from sCJD inoculation, where data are comparable across publications, are summarized in Table 1.T8. The differences in genetic background, expression level, host and inoculum genotype, and the lack of consistency in the source of inoculum used makes overall comparisons difficult.

Table 1.T8: SCJD Inoculation of Various Transgenic Mouse Lines

Summary of sCJD transmission data from, random genomic insertion, transgenic lines expressing homozygous human PrP. (Data shown as incubation period ±SEM, and percentage affected.) (Asante *et al.*, 2002, Kitamoto *et al.*, 2002, Korth *et al.*, 2003)

Research Group	Line Name (Genotype)	Expression Level	Wild-type Genetic Background	sCJD (MM) Type 1 PrP^{Sc}	sCJD (MV) Type 2 PrP^{Sc}	sCJD (VV) Type 2 PrP^{Sc}
Kitamoto	Tg-ChM#30 (MM)	0.7x	All lines: 129Sv	156±14 (100%)	154±20 (100%)	n/d
	Tg-ChV#12 (VV)	2x		175±15 (100%)	171±9 (100%)	n/d
	Tg-ChV#21 (VV)	4x		192±4 (100%)	188±1 (100%)	n/d
Prusiner	Tg440 (MM)	2x	Both lines: FVB	165±4 (100%) 157±3 (100%) 163±2 (100%) 155±3 (100%)	350±38 (50%) 419±13 (100%) 307±27 (100%)	248±12 (43%) 448±34 (43%) 378±7 (43%)
	Tg152 (VV)	4-8x		263±2 (100%) 254±6 (100%)	209±3 (100%) 206±3 (100%) 231±4 (100%)	223±7 (100%) 195±3 (100%) 198±5 (100%)
Collinge	Tg35 (MM)	1-2x	Mixed: FVB/N x 129Sv x C57BL/6	237±10 (100%) 229±5 (100%) 225±7 (100%) 223±1 (100%)	437±31 (100%)	354 (30%)

1.9.1.3.1 Expression Level Effects

To be able to determine the effect of expression level on transmission a comparison can only be made using mice with the same genetic background and codon 129 genotype, and using the same inocula. Therefore, only the data from the Kitamoto group can be used, comparing the two VV genotype lines, Tg-ChV#12 and Tg-ChV#21, that express PrP^C at 2x and 4x respectively. For two sources of sCJD inoculum, of either MM or MV genotype, the 4x expressing line has longer incubation periods contrary to expectations. This underlines the potential problems that can be encountered with this method of transgenic mouse production, as the reasons for the 4x expressing mice having longer incubation period are unclear, and the benefits of gene targeted transgenic production as used for this thesis.

1.9.1.3.2 Genetic Background Effects

To see the effects of the genetic background on transmission, mice are needed to express PrP at the same level, of the same genotype, and be inoculated with the same type of material. In this case a comparison can be made between the MM genotype lines from each research group that express PrP at approximately 1-2x, inoculated with sCJD(MM1). The Kitamoto line Tg-ChM#30 and the Prusiner line Tg440 have similar incubation periods suggesting that the 129Sv and FVB mouse genetics are similar. The Collinge line is of mixed genetics (FVB/N x 129Sv x C57BL/6) and gives incubation periods approximately 70 days longer. This may indicate that the C57BL/6 input into the genetics is lengthening the incubation period. However, there was a near seven-fold increase in the amount of brain homogenate inoculated into the Kitamoto line which could have caused the decrease

in incubation period. (Kitamoto: 20μl of 10% brain homogenate (Taguchi *et al.*, 2003). Collinge: 30μl of 1% brain homogenate (Asante *et al.*, 2002)) From wild-type mouse inoculations performed alongside this thesis, sCJD(MM1) inoculum did not transmit to C57BL/6 mice. This thesis describes the use of gene targeted transgenic lines produced from an in-bred line which removes any effect of background genetics.

1.9.1.3.3 Host Codon 129 Genotype Effects

To see the effect of codon 129 genotype on transmission mice need to express PrP at the same level, have the same genetic background and receive the same inoculum. Due to the random nature of the RGI method of transgenic production only rarely do two lines express the protein at the same level. Only the Kitamoto group data can be used comparing the 0.7x expressing Tg-ChM#30 (MM) line and the 2x expressing Tg-ChV#12 (VV) line. With both sCJD(MM1) and sCJD(MV2) inocula there is a lengthening of the incubation period for the VV mice indicating that this response is due to the host genotype. Gene targeted transgenic mice as used in this thesis are ideal for determining the effect of host codon 129 as the expression level and genetic background are identical.

1.9.1.3.4 SCJD Inoculum Codon 129 Genotype Effects

The Prusiner group data can be used to see the effect of inoculum codon 129 genotype as they have data for sCJD(MM), sCJD(MV),and sCJD(VV) transmission to both MM and VV lines. The data shown in Table 1.T8 show that genotype compatibility will increase the number of clinically affected mice and shorten the incubation period. With either MM or VV mouse lines the sCJD(MV) inoculum

transmits with intermediate efficiency compared with sCJD(MM) and sCJD(VV). Similarities between mouse lines however can only be investigated in detail if they are otherwise identical such as when produced by gene targeting.

1.9.1.4 Expression of Human PrP Heterozygous (MV) at Codon 129

Production of transgenic mice heterozygous (MV) at codon 129 of *PRNP* by the RGI method is problematic as there are difficulties in expressing both alleles at the same level. Only the Collinge group has attempted this by crossing the over-expressing Tg45 (MM) and Tg152 (VV) lines (Asante *et al.*, 2006). These, Tg45/152 (MV), mice express PrP at 4-6x and the levels of the M and V alleles are at a ratio ~1:1.5, due to the effect of the higher expressing VV parent line. Heterozygous MV mice generated for this thesis were generated by cross-breeding the gene targeted homozygous lines, a method that gives equal allele expression at physiological levels and a true representation of human MV individuals.

The Collinge group MV line data covers inoculation with sCJD, vCJD, and BSE and is summarised, together with the group's MM line data for comparison, in Table 1.T9. The sCJD data show that the MV line is equally susceptible as the MM line to all inocula, but develops clinical disease at an earlier time, a difference more pronounced for the valine carrier sCJD(MV1) and sCJD(VV1) inocula. The latter may be due to the greater expression of the valine allele in this line (M:V expression ratio is ~1:1.5) (Asante *et al.*, 2006).

Inoculation of vCJD and BSE show similar properties for both MM and MV lines, with rare occurrence of clinical disease and limited levels of mice affected (defined by positive pathological markers). This suggests that humans with the MV

genotype are likely to be susceptible to this agent. However, pathological signs, e.g. levels and type of PrP^{Sc} deposition, that indicated a less ‘aggressive’ infection. For instance, the MV mice had only moderate spongiform change, and rarely developed the florid plaque structures seen in the MM mice (one such plaque in one mouse) (Asante *et al.*, 2006). This would indicate subclinical BSE infection in MV genotype humans longer than that in MM individuals.

Table 1.T9: Comparison of Collinge Group Transgenic Lines

Comparison of the Tg35 (MM) and Tg45/152 (MV) transgenic mice transmission data from the Collinge group (Asante *et al.*, 2002, Asante *et al.*, 2006). Case number shown as some inoculations are the same between the two lines.

	Tg 35 MM Line 1-2x expression			Tg45/152 MV Line 4-6x expression		
Inoculum	Case ID	Incubation Period ±SEM	Total Affected	Case ID	Incubation Period ±SEM	Total Affected
sCJD(MM1)	I1199 I1202 I1196 I026	237±10 229±5 225±7 223±1	3/3 8/8 8/8 7/7	I1197 I1200 I1203	203±18 189±9 209±3	4/4 6/6 10/10
sCJD(MV1)	I024	241±1	4/4	I024	216±9	8/9
sCJD(VV1)	I022	700,708	4/6	I022	439±20	7/7
sCJD(MV2)	I020	437±31	7/7		n/d	n/d
sCJD(VV2)	I021	354	3/7		n/d	n/d
vCJD	I336 I342 I344	>600 690 >340-720	2/2 5/5 7/7	I336 I344	>284 >518	8/8 7/7
BSE	I038 I060 I062 I064 I066	344,468 >570 338,340 344,492 >500	8/20 1/6 3/7 2/10 0/6	I038 I060 I062 I064 I066	686±97 627,967 346 >546 631±29	6/15 2/8 1/6 0/4 3/8

1.9.2 Gene Targeting (GT) Method

The Kitamoto group are the only research group, in addition to NPD, to have published human TSE transmission data for gene targeted transgenic mice expressing human *PRNP* with all codon 129 genotypes represented (Table 1.T6) (Kitamoto *et al.*, 2002). The differences between the NPD lines and the Kitamoto lines are that following post-translational modification the former produce a complete human amino acid sequence protein whereas the latter produce a chimeric human protein with six amino acids at the C-terminal end that are derived from the mouse sequence (V215, Q219, K220, D227, G228, and R230). In their most recent publication, this group have described a development of their mouse model system into a rapid bioassay where all mice are culled at 75 days post injection and the spleens examined for PrP^{Sc} deposition in the follicular dendritic cells, by immunocytochemistry (Asano *et al.*, 2006). For these transmissions there is therefore no incubation period data, however a previous publication describing only the MM genotype line does have life-span data (Taguchi *et al.*, 2003).

1.9.2.1 Sporadic CJD Transmission to the Kitamoto GT Mice

Data for sCJD transmission to the Kitamoto group mice are only available for the MM line of mice. This showed short incubation periods for sCJD(MM1) and sCJD(MV1) subgroups at ~150 days, longer incubation times and inefficient transmission for sCJD(MM2-thalamic type) at >500 days. and no evidence of transmission for the sCJD(VV2) type inoculum. The sCJD(MM1) and sCJD(MV1) types are classified together as the ‘classical’ form of sCJD and so these data are suggesting that they also share similar transmission properties too. The relative

inefficiency of the PrP type 2 inocula compared to those already mentioned with type 1 suggests that the protein type of the inoculum may have a determining role in the success of transmission. There is also genotype incompatibility between the sCJD(VV2) inoculum and the MM genotype mice which may also impact on the success of transmission for that experiment. Without transmission of these inocula to the MV and VV genotype mouse lines the effect of the host genotype cannot be investigated.

1.9.2.2 Variant CJD and BSE Transmission to the Kitamoto GT Mice

Variant CJD inoculation of the MM genotype Kitamoto line showed rare development of clinical signs at >700 days however there was evidence of TSE pathology in the majority of mice (Taguchi *et al.*, 2003). This suggested a lengthy subclinical phase. No data on incubation times and life-span experiments is available for vCJD inoculation of MV or VV genotype mice, or BSE inoculation.

Data for both vCJD and BSE inoculation in all three codon 129 genotype mouse lines are only available for the bioassay model where all mice were culled at 75 days post inoculation (Asano *et al.*, 2006). This data can only provide comparisons of the relative transmission efficiencies between the three genotype hosts and not data on susceptibility. Some mice may be negative for spleen PrP^{Sc} at 75 days but may go on to develop signs of TSE disease at a later time.

BSE inoculation produced only a single MM genotype mouse that was positive for PrP^{Sc}, and all mice of the MV and VV genotypes were negative. This suggests a significant transmission barrier for BSE to humans. VCJD transmission however was more successful, and showed similar high numbers (mostly >50%) of positive scored mice for both MM and MV lines. The VV line failed to show

evidence of PrP^{Sc} in the spleen. The levels of PrP^{Sc} in spleens of MV mice was lower than that seen for the MM mice. These data suggest that human passage of BSE has removed part of the transmission barrier and yet there is an M allele dominance for infection efficiency. This implies that humans with the MV genotype may be susceptible to BSE or vCJD infection but the progressive spread of TSE pathology is slower than for MM individuals, and may therefore have longer incubation periods. The evidence for lack of transmission to VV mice comes from only three mice. This may not have been a large enough group inoculated to have seen a positive result.

1.9.3 Possible Evidence for Strain Selection

In addition to the complexities in comparing data between multiple lines of mice, of alternative genotypes, and expressing PrP at different levels there is also evidence for a form of strain selection (Asante *et al.*, 2002, Wadsworth *et al.*, 2004, Beringue *et al.*, 2008). The appearance of transmission differences attributable to selection of different strains has been reported previously in hamster inoculation experiments with scrapie (Kimberlin and Walker, 1978) and transmissible mink encephalopathy (TME) (Bartz *et al.*, 2000). The former publication centres on incubation period and susceptibility differences, however the latter also includes PrP^{Sc} typing differences linked to each strain as described below.

The Collinge group compared BSE transmission data between their two MM genotype lines, Tg35 (1-2x expression) and Tg45 (4x) that were independently generated. Tg45 mice gave a long incubation period, no clinical disease but high levels (75%) of sub-clinical disease, with the PrP^{Sc} Western blot type consistent with the BSE strain (type 2B). Tg35 mice however gave very different data: 12% showed

clinical disease, 16% were sub-clinical, and the PrP^{Sc} typing showed that 90% were type 1 (10% type 2B), see Figure 1.F3. Type 1 is the common sCJD type leading to the conclusion that BSE infectivity could also give rise to a sCJD-like molecular strain classification, albeit based solely on PrP typing (Asante *et al.*, 2002). This has significant implications for human disease surveillance as some vCJD cases may be misdiagnosed as sCJD.

The genetic background of these transgenic mice is a mixture of wild-type lines (FVB/N, C57BL/6, and 129Sv) and when these lines were inoculated with BSE or vCJD some produced the sCJD PrP type. Therefore the suggestion that these mice are selecting for an alternate BSE strain could be explained more simply by an alternative host PrP response.

The Laude group have also proposed this theory of strain selection in transgenic mice expressing M allele PrP at 6-fold normal levels (tg650) inoculated with vCJD and sCJD (Beringue *et al.*, 2008). Three French vCJD cases transmitted efficiently with incubation periods near to 500 days and showed the typical vCJD Western blot type in spleen and brain material. This is the first example of transgenic mice expressing human PrP that develop clinical TSE in 100% of mice injected with vCJD. These are the most highly over-expressing MM line of mice published which may be the cause for the 100% transmission rate.

Evidence for strain selection was seen following inoculation with a UK vCJD case (WHO reference case as used for this thesis). With this inoculum only, they found two distinct incubation periods described as 'early' (~300 days) and 'late' (~500 days) which occurred in mice at an equal frequency. The 'late' mice had type 2B PrP^{Sc} in the spleen and brain, however, the 'early' mice had type 2B in the spleen

and sCJD-like type 1 PrP^{Sc} in the brain. This PrP^{Sc} type difference was transmissible on second passage of either brain or spleen material suggesting strain differences.

In the preparation of the WHO reference vCJD material a large amount of tissue was homogenised (100g) compared with the 1-2g that is commonly prepared for an inoculum. It is therefore possible that inoculum prepared from a larger area of vCJD brain may contain more type 1 PrP^{Sc} that could be selected for by this particular over-expressing mouse line. Type 1 PrP^{Sc} has been found at low levels in vCJD brain material through the use of type 1 specific antibodies (Yull *et al.*, 2006). Alternatively these highly over-expressing mice may be causing an adaptation of the properties of the inoculum strain due to the abundance of PrP in the brain.

1.9.4 Summary of Data for Human TSE Transmission to Transgenic Mice

The following table (Table 1.T10) summarises the overall findings from transmission of human TSE material to transgenic mice expressing full-length human PrP, produced by gene targeting or random genomic integration methods.

Table 1.T10: Human TSE Transmission to Various Transgenic Mouse Lines

Summary of sCJD and vCJD transmission data to transgenic lines expressing human PrP. (GT: Gene Targeted; RGI: Random Genomic Integration; * <100% transmission; ** Positive / Negative for PrP^{Sc} in spleen at 75 days;'-': no data; mean incubation time ± SEM; n/t: no transmission)

				Sporadic CJD						
Research Group Leader	Method	Line Name (Genotype)	Expression Level	MM1	MV1	MM2	MV2	VV1	VV2	Variant CJD
Kitamoto	GT	Ki-Hu129M/M (MM)	1x	~140	141±5	-	-	-	n/t	>600
		Ki-Hu129M/V (MV)	1x	-	-	-	-	-	-	Positive**
		Ki-Hu129V/V (VV)	1x	-	-	-	-	-	-	Negative**
	RGI	Tg-ChM#30 (MM)	0.7x	156±14	-	-	154±20	-	-	-
		Tg-ChV#12 (VV)	2x	175±15	-	-	171±9	-	-	-
		Tg-ChV#21 (VV)	4x	192±4	-	-	188±1	-	-	-
Prusiner	RGI	Tg440 (MM)	2x	~160	176±2	232±5	~350	-	~350*	-
		Tg152 (VV)	4-8x	~260	-	368±19	~220	-	~210	228±15*
Collinge	RGI	Tg35 (MM)	1-2x	~230	241±1	-	437±31	~700	354	>600
		Tg45 (MM)	4x	-	155±5	-	-	-	-	~600
		Tg45/152 (MV)	4-6x	~200	216±9	-	-	439±20	-	>500
Laude	RGI	Tg650 (MM)	6x	~160	-	-	-	-	-	~520

1.10 OUTLINE OF THESIS

The contents of this thesis are subsequently divided into six chapters. The next (Chapter 2) details the Methods used and this is followed by a discussion of how the transgenic mice were assessed to ensure that expression of the transgene produced human PrP^C as expected (Chapter 3). Chapters 4 and 5 interpret the findings from transmission of vCJD and blood transfusion associated vCJD respectively, in an attempt to define the role of codon 129 genotype in the host on susceptibility to this BSE-associated strain, and to observe any strain changes following human-to-human passage.

Chapter 6 contains a large data set of results from inoculation of the transgenic mice with material from six typical cases of sCJD, performed to investigate the role of codon genotype in both host and inoculum and the strain-like transmission behaviour of the individual subgroups of sCJD. Following this there is a summary (Chapter 7) of how the data generated during this study has been used to answer the aims set out in the beginning of this Introduction chapter.

CHAPTER 2

METHODS

2.1 GENE TARGETED TRANSGENIC MOUSE LINE PRODUCTION

NPD have developed unique lines of transgenic mice using a methodology called gene targeting. This method allows direct replacement of the mouse prion protein open reading frame for an exogenous version, in this case the human *PRNP* sequence. The non-murine sequence is therefore under the expression control of the host and specific for the prion protein. It will therefore be expressed temporally and spatially as per the host's original prion protein. The methodology of gene targeting used to produce the human transgenic lines is as follows. This work was carried out by the staff at NPD (Dr H Baybutt, Dr N Tuzi, Mrs L Blackford) and not by the author of this thesis. A brief summary of this method is included here to aid understanding of the uniqueness of this specific model system.

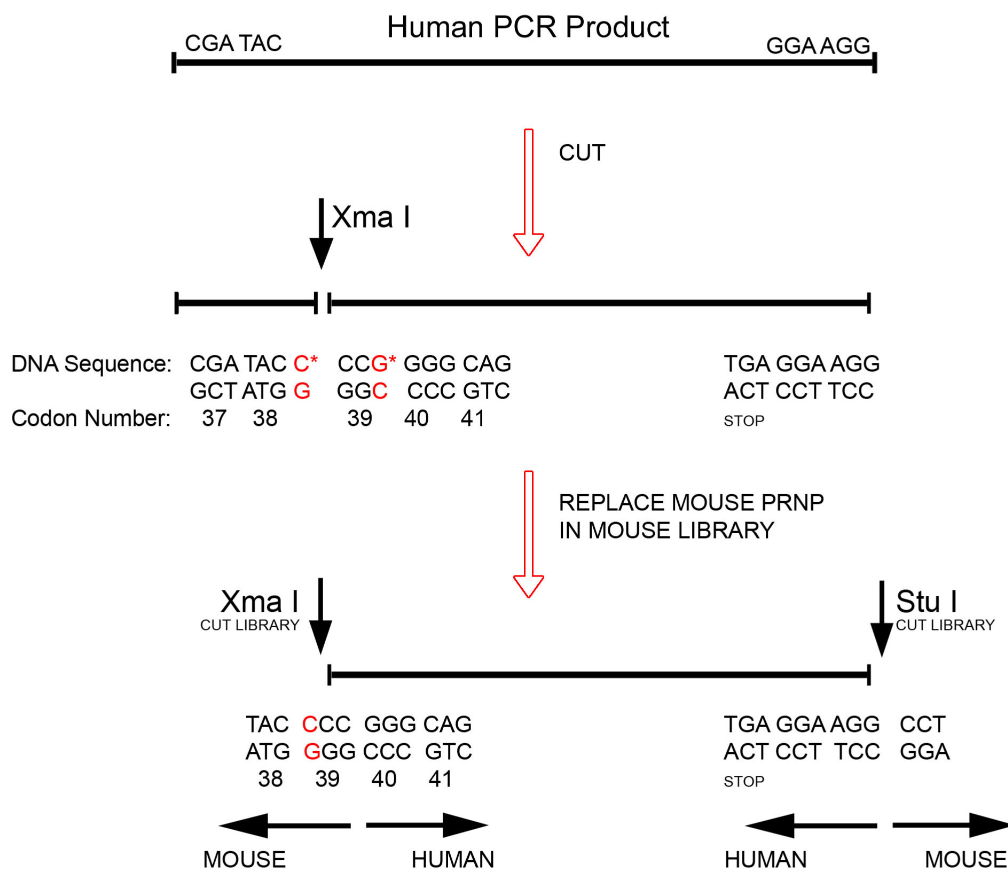
2.1.1 Construction of Gene Targeting Vectors

Prion protein amino acids 40 to 254 of the open reading frame (ORF) were changed from mouse to the human. An amplicon spanning codon 37 to six nucleotides downstream of the stop codon was prepared by polymerase chain reaction (PCR) amplification of DNA from codon 129 methionine and codon 129 valine human samples. PCR products were digested with the restriction enzymes and ligated into a gene targeting vector constructed using 129Ola wild-type DNA so

that the flanking DNA sequence of the human gene was homologous to that of the recipient mice. For an overview see Figure 2.F1.

Figure 2.F1: Gene Vector Flowchart

Flowchart of production of gene vector used to replace the mouse *Prnp* gene with the human version.



* C base added by primer; G base missing from primer

2.1.2 DNA Vector Insertion into Embryonic Stem (ES) Cells

The plasmid vector containing the human gene sequence was inserted into embryonic stem cells by the electroporation method. Successful insertion was confirmed by both antibiotic resistance selection and subsequently by PCR screening of colonies. The ES cells were derived from 129Ola wild-type mice that have an agouti coat colour (light brown) so that if the genetic material is taken up by the recipient black-coated C57BL6 mouse embryo the offspring will be identifiable by a mixed coat colour.

2.1.3 Production of Recipient Blastocysts

Blastocysts are harvested from sacrificed, pseudopregnant, C57BL6 (black coated) mice at day 3.5 following mating with vasectomised males. The blastocysts have to be fully mature so that the blastocoel cavity is clearly visible for ES cell transfer.

2.1.4 Transfer of ES Cells to Blastocysts

Microinjection is used to insert 12-15 gene targeted ES cells into the blastocoel cavity. Only those ES cells that remain intact with an expanded blastocoel can then be used for the following stage.

2.1.5 Embryo Transfer to Female Mice

Pseudopregnant females that were mated with vasectomised males 2.5 days previously are used as host mothers. Unilateral uterine horn transfer of 10-15 microinjected ES cells is carried out on the anaesthetised mice, and then after two weeks it would be clear whether the implantation was successful.

2.1.6 Germline Transmission of Gene Targeted Vector

A chimeric coat colour in the resultant pups indicates an inclusion of the ES cells genetic material into the hosts. Male chimeras are mated to 129Ola females and germline transmission is confirmed by offspring with red eyes and agouti coat colour. These mice will be heterozygous for the transgene and so are then crossed with further such mice to generate a homozygous transgenic line. These are checked by PCR amplification of the gene targeted human prion gene sequence.

2.1.7 PCR Genotyping of Mouse Tail DNA

Mouse tail DNA was extracted using DNeasy Tissue DNA extraction kit (Qiagen, UK) and amplified using a combination of human and mouse specific oligos. The human PCR product is 371bp and the murine 206bp. Codon 129 genotype of the human sequence was determined by digestion with NspI (NEB, UK) (Figure 2.F2).

2.2 DNA EXTRACTION

For confirmation of the codon 129 genotype of transgenic mice that are to be used in breeding programs or experiments, tailsnips are taken and the DNA extracted using the DNeasy DNA extraction protocol for rodent tails (Qiagen, UK). This method can also be used for any other murine tissue such as frozen brain samples.

1. Add 180µl of Qiagen Buffer ATL to tailsnip tissue (~5 mm in length), and 20µl of Qiagen Proteinase K solution. This will breakdown and lyse the cell and nuclear membranes to release the DNA.
2. Mix thoroughly by vortexing, and digest overnight shaking at 55˚C.
3. Vortex for 15 seconds. Add 400µl of 50:50 Qiagen Buffer AL-ethanol mixture and vortex to mix. This will precipitate the DNA allowing it to be captured on the spin column in the next step.
4. Transfer mixture to a labelled spin column. Centrifuge for 1 minute at 7500g.
5. Replace collection tube and add 500µl Qiagen Buffer AW1 to wash off soluble contaminants from the column matrix. Centrifuge for 1 minute at 7500g.
6. Replace collection tube and add 500µl of Qiagen Buffer AW2. Centrifuge for 3 minutes at 12000g.
7. Replace collection tube with 1.5ml tube and add 200µl of Qiagen Buffer AE, incubate at room temperature for 1 minute. This will bring the DNA back into solution allowing it to be eluted from the column matrix. Centrifuge for 1 minute at 7500g. Store DNA at +4˚C.

2.3 GENOTYPING, SEQUENCING, AND SEQUENCE ANALYSIS

2.3.1 Genotyping

To determine the codon genotype of DNA extracted from mouse tissue a part of the open reading frame (approximately from codon 48 to 172) was amplified by PCR then digested with restriction endonuclease NspI (New England Biolabs,

UK). This enzyme cleaves the PCR product at codon 129 when there is a methionine coding sequence (-ATG-) and also at codon 154 (this acts as a digest control).

PCR mix recipe:

	per 50µl reaction
dH$_2$0	36.1 µl
10x PCR buffer (Invitrogen) (final 1x)	5.0 µl
50mM MgCl$_2$ (Invitrogen) (final 1.5mM)	1.5 µl
10mM dNTPs (Invitrogen) (final 0.2mM)	1.0 µl
100 pmol/µl Primer Mix	1.0 µl
[Hum5'>144: CTACCCACCTCAGGGCGGTGGTGG Hum3'<515: TGGTTGCTGTACTCATCCATG]	
5U/µl Taq polymerase(Invitrogen)	0.4 µl
Template DNA	5.0 µl (0.1-0.5µg)

The PCR program was:

- 94°C / 5mins
- 94°C / 30secs ; 62°C / 30secs ; 72°C / 1min ; 30 cycles
- 72°C / 10mins

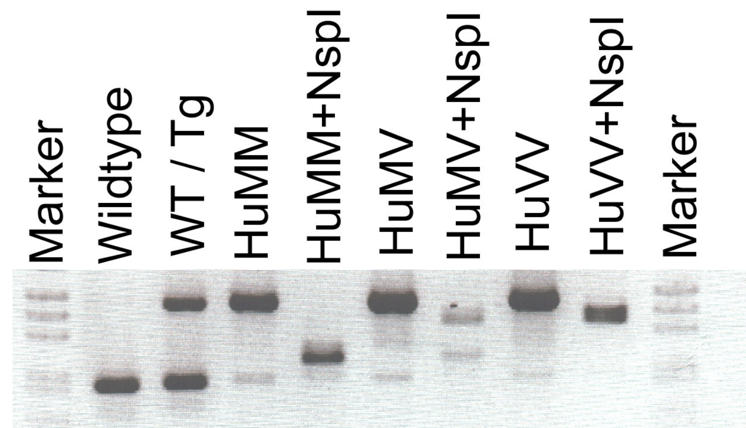
Nsp1 Digest mix:	Per 20µl reaction
NEBuffer 2	2.0 µl
BSA	0.2 µl
dH$_2$0	7.4 µl
Nsp1 (5U/µl)	0.4 µl
PCR Product	10.0 µl

The digest reaction was incubated at 37°C overnight, then the digest products were run on a 1.5% agarose gel and visualised with ethidium bromide under UV light. (Figure 2.F2) The band sizes expected were as follows.

PCR product undigested: 371 bp
MM genotype: 244 bp, 74 bp, 53 bp
MV genotype: 318 bp, 244 bp, 74 bp, 53 bp
VV genotype: 318 bp, 53 bp

Figure 2.F2: Codon 129 Genotyping Gel Image

Agarose gel of genotyping results for mouse tail DNA, showing the respective pattern for each cogon 129 genotype.



2.3.2 Sequencing and Sequence Analysis

The PCR products generated above were purified using the Qiagen QIAquick PCR Purification Kit. This removes remaining primers, dNTPs and Taq, leaving the amplified product ready for sequencing. The kit used for this was the Thermo Sequenase fluorescent labelled primer cycle sequencing kit (GE Healthcare). Two fluorescently labelled primers (Cy5) were used that sequenced the PCR product in both 5' and 3' directions (4L: AGG TGG CAC CCA CAG TCA GT, 6R: CGA TAG TAA CGG TCC TCA TA).

The sequencing program was:

- 95°C / 5mins
- 95°C / 20secs ; 60°C / 10secs ; 68°C / 1min ; 15 cycles
- 95°C / 10secs ; 60°C / 1min 30secs ; 20 cycles

Sequencing products were electrophoresed on an ALFexpress automated sequencer using ReproGel Long Read gel mix (GE Healthcare), and the data checked by comparison to wild-type human and mouse sequence data, using the ALFwin Sequence Analyzer 2.00 software.

2.4 RNA EXTRACTION

RNA from transgenic and wild-type mouse tissues was isolated to characterise the expression of the transgene throughout the mouse.

1. Add 400µl of RNAzol Bee (Tel-Test, USA) to a 1.5ml tube and 1.5ml to a 15ml tube. Keep on ice.
2. Add ~100mg of frozen tissue to the 1.5ml tube and homogenize carefully with a sterile pestle.
3. Immediately transfer the homogenate to the 15ml tube. Mix by vortexing briefly and aliquot the homogenate into two 1.5ml tubes.
4. Add 100µl of chloroform to each tube. Shake vigorously for 15 seconds. (Do not vortex.)
5. Incubate at 4°C for 5 minutes then centrifuge at 12000g for 15 minutes at 4°C.
6. Transfer aqueous phase to a fresh tube. Add equal volume of isopropanol (500µl). Mix by inversion.
7. Incubate at 4°C for 15 minutes then centrifuge at 12000g for 15 minutes at 4°C.
8. Discard supernatant and wash pellet with 1ml of 70% ethanol.
9. Centrifuge at 7500g for 8 minutes at 4°C.

10. Discard supernatant.
11. Tissue-dry with care, and air-dry for 15 minutes at room temperature.
12. Resuspend pellet in 10µl of distilled water and stand at room temperature for 1 hour, then 20 minutes at 4°C.
13. Combine the two aliquots.
14. Measure the OD (optical density) of 1µl of RNA added to 199µl distilled water at 260/280 nm.
15. Calculate the concentration (where 1OD=40µg/ml RNA): 260nm reading x 40(as left) x 200(dilution factor).
16. Calculate purity (260/280 ratio should be >=1.9).
17. Store RNA at -70°C.

2.5 NORTHERN ANALYSIS OF EXTRACTED RNA

This method was used to identify PrP mRNA in various mouse tissues by using a radio-labelled PrP mRNA probe to bind to the separated RNA which had been transferred and fixed to a membrane.

2.5.1 Agarose Gel Separation of RNA Fractions

1. Aliquot required volume of RNA stock to give 20µg for loading.
2. Add 10% of aqueous volume of 3M Sodium oxaloacetate (NaOAc) (pH 5.0).
3. Centrifuge at 12000g for 5 minutes at 4°C.
4. Pour off supernatant with care.
5. Wash pellet in 1ml of 85% ethanol.
6. Centrifuge at 7500g for 8 minutes at 4°C.

7. Pour off supernatant and tissue-dry with care.
8. Allow to dry on ice for 10-15 minutes.
9. Resuspend pellet in 20µl RNA sample buffer (50% deionised formamide, 18% formaldehyde, 1x MOPS)
10. Heat samples to 65°C for 10 minutes, then store on ice.
11. Add 5µl 6x Loading Dye and 1µl ethidium bromide.
12. Prepare a 1% agarose / formaldehyde gel as follows:

    1g Agarose

    10ml 10xMOPS (0.2M 3-Morpholinopropanesulfonic acid, 0.05M sodium acetate, 0.01M EDTA)

    73ml dH_2O

    Melt agarose, cool, then add 17ml formaldehyde and pour gel.
13. Load samples and electrophorese in 1x MOPS buffer.
14. Under ultraviolet light the 18S and 28S RNA bands should be clear.

2.5.2 Northern Transfer

1. Rinse gel in 10x SSC (1.5M sodium chloride / 0.15M sodium citrate) for 2 x 30 minutes to remove the formaldehyde.
2. Cut Hybond-N (GE Healthcare, UK) transfer membrane and 6 x filter papers to size of gel.
3. Clean glass plate with RNAZap (Ambion, UK) and water, and place over tray containing 5x SSC.
4. Soak wick in 10x SSC and lay across plate into 5x SSC.
5. Place gel upside-down on the wick removing bubbles and cover surrounding wick with saran wrap.

6. Wet membrane and 6x filter papers in 10x SSC and place on gel.
7. Add paper towels to top and weigh down.
8. Allow to transfer overnight.
9. View membrane under UV and mark the 18S and 28S bands.
10. Bake membrane in filter paper at 80°C for 2 hours, store at 4°C.

2.5.3 Northern Blot Probe Preparation

1. Dr Herbert Baybutt provided a plasmid containing a 350bp DNA sequence homologous to the 3' UTR of *PRNP* that can be excised using BamHI and EcoRI restriction endonucleases (NEB, UK).
2. Double-digest using the two enzymes above was carried out for 3 hours at 37°C, as follows:

dH_20	20.8µl
Buffer E (10x) (in enzyme kit)	3.0µl
BSA (10µg/µl)	0.2µl
Plasmid (0.675 µg/µl)	5.0µl
BamHI (10U/µl)	0.5µl
EcoRI (12U/µl)	0.5µl
Total:	30.0µl

3. Digest products were separated on a 1.5% agarose gel and the 350bp bands excised and purified using the Qiagen Gel Extraction Kit, as follows.
4. Calculate the weight of the gel slices and add 300µl of Buffer QG per 100µg of gel slice.
5. Incubate at 50°C for 10 minutes until the gel has dissolved.
6. Add 100µl of isopropanol for each 100µg of original gel slice, mix and add to a spin-column.
7. Centrifuge at 12000g for 1 minute and discard eluate.

8. Add 0.5ml Buffer QG and centrifuge at 12000g for 1 minute, discard eluate.
9. Add 0.75ml Buffer PE and centrifuge at 12000g for 1 minute, discard eluate, spin again at 12000g for 1 minute.
10. Place column in 1.5ml tube, add 50µl of Buffer EB, stand for 2-5 minutes, centrifuge at 12000g for 1 minute, and store eluate at 4°C.
11. Spectrophotometer analysis was used to calculate the probe concentration.

2.5.4 Radioactive Probing of Blots

1. Membranes were soaked in prehybridisation solution (ULTRAHYB, Ambion, UK) for 1-2 hours at 42°C,
2. The probe was radiolabelled with ^{32}P using the Amersham Rediprime II kit.
    1. Denature 25ng of probe at 100°C for 5 minutes.
    2. Snap-cool on ice, and pulse down.
    3. Add probe to Rediprime tube.
    4. Add 5µl of ^{32}P d-CTP and mix.
    5. Incubate at 37°C for 30 minutes then stop reaction with 5µl of 0.2M EDTA.
3. The labelled probe was purified using a ProbeQuant G-50 Micro-column (Amersham).
4. The probe was denatured at 100°C for 5 minutes, snap-cooled, then added to membrane for overnight hybridization.
5. In the morning the membranes were washed once in 2x SSC / 0.1% SDS and then twice in 0.1x SSC / 0.1% SDS, and exposed to x-ray film.

2.6 PROTEIN EXTRACTION FROM UNINFECTED MICE

A 10% tissue homogenate can be prepared by homogenising 100mg of tissue, on ice, in 1ml NP40 lysis buffer.

NP40 Lysis Buffer:

1% NP40

0.5% Sodium deoxycholate

150mM NaCl

50mM Tris/HCl pH 7.5

1. Add 100mg of tissue to 200µl of NP40 lysis buffer.
2. Homogenise until uniform and tissue macerated.
3. Transfer 100µl to 1.5ml tube containing 900µl NP40 lysis buffer.
4. Add 900µl NP40 lysis buffer to remaining sample, and mix.
5. Centrifuge at 12000g for 3 minutes, at 4°C.
6. Transfer supernatant to -80°C storage tubes.

2.6.1 Deglycosylation of PrP^C

To clarify that the triplet of bands seen on a Western blot are the alternate glycoforms of PrP the glycans can be removed with PNGaseF (peptide N-glycosidase F, NEB, UK), leaving one unglycosylated band.

1. Add 20µl of 5% tissue homogenate to 3µl of Denaturation Solution (provided in PNGaseF kit).
2. Incubate at 100°C for 10 minutes.

3. Add 8μl dH_2O, 4μl 10x G7 buffer, 4μl 10% NP40, and 500U PNGaseF.
4. Incubate overnight at 37°C, then add 60μl dH_2O to leave 100μl of 1% homogenate ready for gel loading.

2.6.2 GPI Anchor Cleavage of PrP^C

To confirm that the transgenic form of PrP is correctly bound to the plasma membrane by a glycophosphatidylinositol anchor, the GPI anchor can be cleaved by PIPLC (phosphatidylinositol-specific phospholipase C). The resultant two fractions at the end of this method are labelled 'supernatant' and 'pellet'. If the PrP^C seen in the supernatant fraction has an apparent decrease in electrophoretic mobility following Western blot detection then it is concluded that this form of the protein has been released from the membrane anchor.

1. Prepare a 10% tissue homogenate. Half-brain is homogenised in 400μl of cold phosphate buffered saline (PBS) then transferred into 1.6ml of PBS with 20μl each of 100mM phenylmethylsulphonyl fluoride (PMSF) and N-ethyl-maleamide (NEM) (protease inhibitors).
2. Split the homogenate into two 2ml tubes and centrifuge at 900g for 5 minutes at 4°C.
3. Transfer the supernatant to ultracentrifuge tubes.
4. Resuspend and pool the pellets in 1ml of cold PBS (this fraction is the 'cell debris').
5. Top up the ultracentrifuge tube with cold PBS to balance and centrifuge at 16,000g for 12 minutes at 4°C.

6. Discard the supernatant and resuspend the pellet in 500µl of cold PBS. Split this sample into two equal aliquots.
7. To one tube add 0.5U of PIPLC. Mix well and incubate at 4°C overnight or on ice for 4 hours.
8. Centrifuge both treated and non-treated samples at 12000g for 15 minutes at 4°C.
9. Transfer the supernatant to a 1.5ml tube labelled 'S'.
10. Resuspend the pellet in 600µl of cold PBS, labelled 'P'.
11. Store samples at -20°C.

2.7 CLINICAL AND PATHOLOGICAL INVESTIGATION OF INOCULATED TRANSGENIC MICE

Mice were anaesthetised with halothane and then injected either via the intracerebral (20µl volume) or intraperitoneal (100µl volume) routes with an inoculum (at 1% dilution) made from homogenised tissue in sterile saline. Inoculated mice were assessed weekly, from 100 days posts injection until the end of their natural life-span for clinical signs of TSE disease, such as kyphosis (hunching of the back) and movement disorders (ataxia). The time at which mice were culled due to the appearance of a clinical TSE phenotype was recorded as the incubation period. The mean incubation period from the group of mice inoculated gives an indication as to the relative efficiency of disease transmission in different mouse lines, and is one of the transmission characteristics that help to define TSE strains.

Following culling of the mice due to TSE or for animal health reasons (intercurrent deaths) the brains were removed for biochemical and histological analysis.

Histological analysis of brain material is a key component for confirmation of successful transmission, as the detection of TSE associated vacuolation and deposition of PrP^{Sc}, are sensitive markers signifying that TSE disease is present.

2.7.1 Assessment of TSE Associated Vacuolation – Lesion Profiling

One of the standard methods of TSE strain identification in wild-type mice is the use of lesion profiling matched with recording the incubation period. (Fraser and Dickinson, 1967, Dickinson *et al.*, 1968, Fraser and Dickinson, 1968) Lesion profiling is the categorisation of the degree of vacuolation, scored from one to five (Table 2.T1), seen in nine grey matter areas of the brain and three areas of white matter. (Fraser and Dickinson, 1968, Bruce *et al.*, 2004) (Figure 2.F3) The scoring is shown by graphical representation to enable quick, visual comparison of different experiments. Figure 2.F4 shows examples of these lesion profiles for mouse-passaged BSE and scrapie. This method has been developed for use in analysis of pathological material from wild-type mice and was used without alteration to assess the transgenic mice in this thesis.

Table 2.T1: Vacuolation Score Definition

Definition of vacuolation scores. (From reference: (Bruce *et al.*, 2004))

Vacuolation Score	Description
0	No vacuoles
1	A few vacuoles widely and unevenly scattered, not convincingly TSE specific pathology
2	A few vacuoles evenly scattered, more than for score 1 and convincingly diagnostic for TSE
3	Moderate numbers of vacuoles, evenly scattered
4	Many vacuoles with some confluence
5	Dense vacuolation with most of microscopic field confluent, lace-like appearance

Figure 2.F3: Lesion Profiling Scoring Regions

Scoring regions are coloured blue and labelled as below. (Image: W-G Lui, NPD)
Grey matter: 1: dorsal medulla, 2: cerebellar cortex, 3: superior colliculus, 4: hypothalamus, 5: thalamus, 6: hippocampus, 7: septum, 8: retrosplenial and adjacent motor cortex, 9: cingulate and adjacent motor cortex. White matter: WM1: cerebellar white matter, WM2: mesencephalic tegmentum, WM3: pyramidal tract.

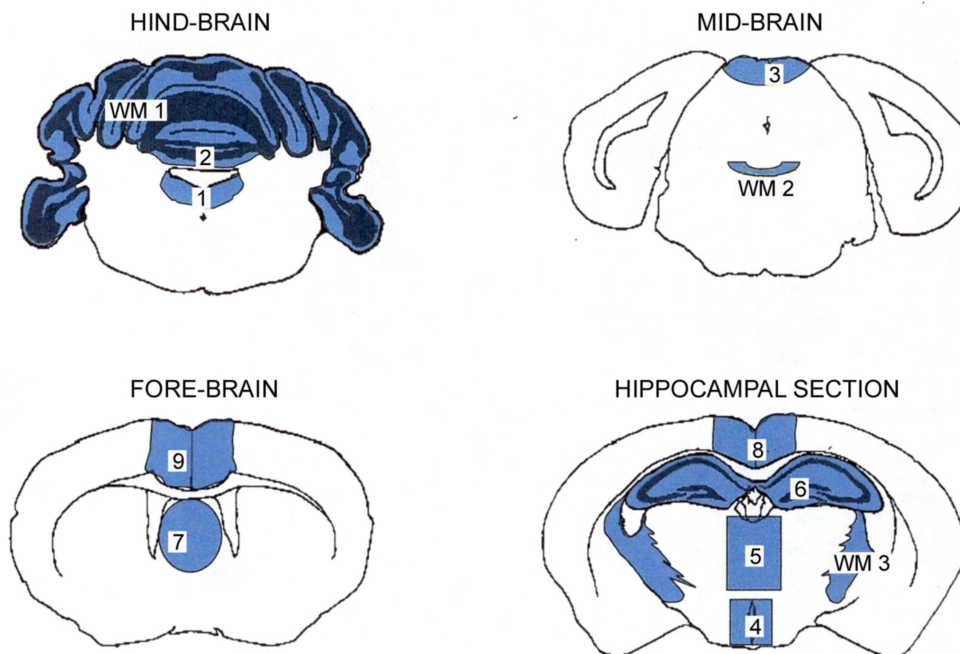
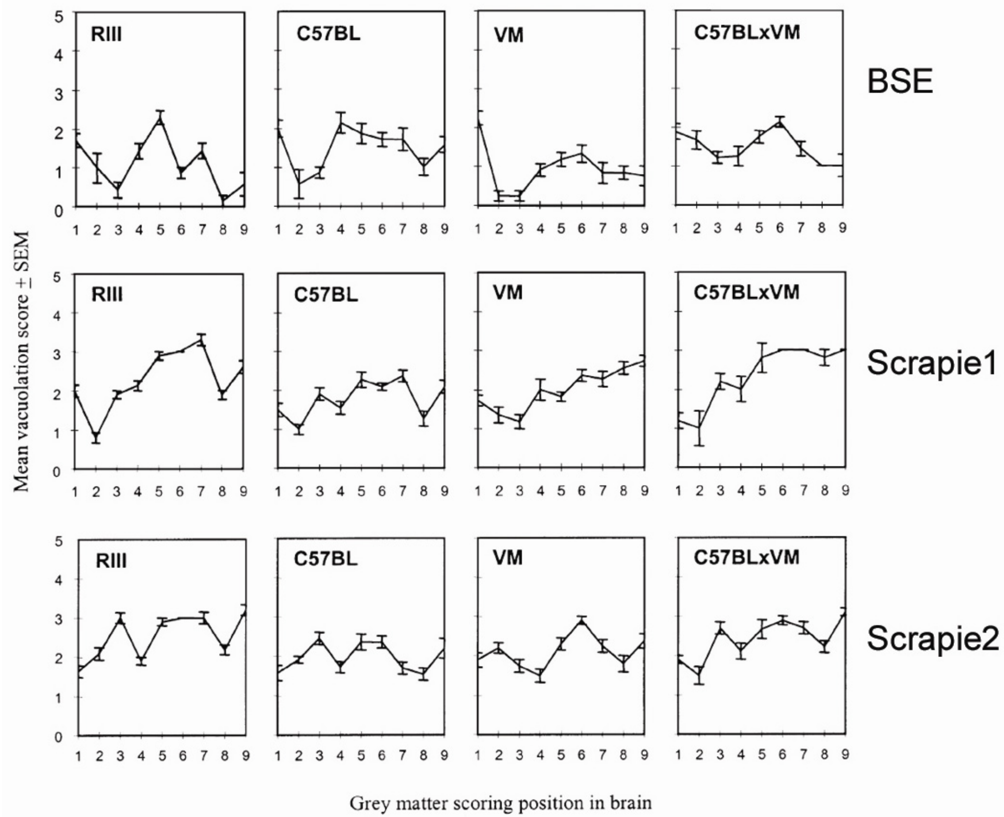


Figure 2.F4: Lesion Profile Examples

Examples of grey matter lesion profiles from the NPD strain typing mice panel (R111, C57BL, VM, and C57BL/VM cross) inoculated with BSE and sheep scrapie that have been sub-passaged in the VM wild-type mouse line. (Reproduced from reference (Bruce *et al.*, 2002))



2.7.2 Assessment of TSE Associated PrP^{Sc} Deposition

Key strain differences may be seen by comparing the type and spread of PrP^{Sc} deposits throughout the brain. Assessment of PrP^{Sc} by immunocytochemistry in mice inoculated for this thesis will hopefully provide evidence for relationships between the different inocula and codon 129 genotype of the inoculated mice.

2.7.2.1 Brain Sectioning

Half-brains that were required for immunocytochemical analysis were primarily fixed in 10% formal saline. Then they were treated for 1.5 hours in 98% formic acid (to reduce the titre of infectivity for safety reasons) and cut in the coronal plane into four sections using a cutting guide to reproducibly section all of the brains for comparative analysis. These brain sections were then embedded in paraffin blocks, cut into 6 μm slices, and dried onto microscope slides for further analysis. All of this work was carried out by staff at NPD and the CJDSU as cutting and slicing mouse brains requires significant training.

2.7.2.2 ICC Detection of PrP

PrP detection used the Vectastain Elite ABC Kit (Vector Labs, UK) with overnight primary antibody incubation, 6H4 at 1:2000 dilution (Prionics, Switzerland; Product number 01-010; 0.5-2mg/ml). Detection of antibody binding was through deposition of DAB (3,3'-diaminobenzidine) chromogen via a horseradish peroxidase reaction.

1. Incubate slides at 60ºC overnight to ensure sections are fixed securely to slides.
2. Remove the paraffin and hydrate the sections as follows:

   Xylene for 5 minutes

Absolute alcohol for 5 minutes

74OP (IMS) for 5 minutes

70% alcohol for 5 minutes

Rinse well in tap water

3. Autoclave under water for 10 minutes at 121ºC. This will open-up more of the antibody epitopes on PrP to improve the signal. Rinse in tap water.
4. Submerse slides in formic acid (98%) for 5 minutes. This will also release more of the PrP antibody epitopes. Rinse carefully in tap water.
5. Submerse slides in block (9ml Hydrogen Peroxide in 300ml Methanol) for 10 minutes. This will inhibit the endogenous peroxidases leaving a clearer background which will allow better detection of the true PrP antibody binding. Rinse carefully in tap water.
6. Wash slides in 1xTBS/BSA for 3 x 5 minutes.

Tris Buffer Saline (TBS):

10x stock of Tris pH 7.6

500ml of 0.5M Tris (30.25g / 500ml)

Add N HCl until pH 7.6 (150ml)

Make up to 2 litres with dH_2O

Saline:

0.85% stock (8.5g NaCl in 1 litre)

1xTBS/BSA:

1 litre 1xTBS (900ml TBS + 100ml Saline)

1g Bovine Serum Albumin

7. Add four drops of Normal Rabbit Serum (NRS) to cover sections and incubate for 20 minutes.

   NRS: 1:5 dilution of stock in TBS/BSA (2 ml NRS + 8 ml TBS/BSA)

8. Tip off NRS.
9. Make up primary antibody.

   6H4 – 4μl in 8ml of TBS/BSA equals a 1:2000 dilution of stock solution.

10. Add 6H4 (3 drops) to cover the sections and incubate overnight at room temperature. Add NRS to negative control.
11. Wash slides three times in TBS/BSA for 5 minutes each.
12. Drain slides then add secondary antibody (Stratech, UK; Product number 315-067-003; 1.1mg/ml) biotinylated rabbit anti-mouse at 1:400 (15μl in 6 ml TBS/BSA)) to cover the sections. Incubate for 1 hour.
13. Make up 'ABC' reagent (at least 30 minutes prior to use) from Vectastain Elite PK6100 kit.

    ABC: 10 ml TBS/BSA + 100μl A + 100μl B (mix and store at 4°C)

14. Wash slides three times in TBS/BSA for 5 minutes each.
15. Add ABC to cover the sections and incubate for 30 minutes.
16. Prepare the DAB chromogen.

    DAB: Add 10 drops of Substrate Buffer Concentrate (Tris-HCl) to 10 ml of dH_2O, then add a Substrate Tablet (10 mg 3,3'-diaminobenzidine tetrahydrochloride (DAB)). Mix.

17. Wash slides three times in TBS/BSA for 5 minutes each.
18. Add 1 drop of hydrogen peroxide (0.8%) per 2ml of DAB (5 drops in 10 ml), then add to slides (4 drops).

19. Leave for 2 minutes or until signal is clear in positive control slide. Rinse in tap water.
20. Counterstain the nuclei in the sections with haematoxylin as follows.

    Submerse in haematoxylin for 12-15 seconds.

    Rinse well in tap water.

    Dip into Acid Alcohol (1%) bath. (99ml 70% alcohol + 1ml conc. HCl)

    Rinse well in tap water.

    Dip into lithium carbonate (1% w/v) six times.

    Rinse well in tap water.

21. The sections are then dehydrated back to Xylene for automated coverslipping.

    Brief dip in the following:

    70% alcohol

    OP74 (IMS)

    Absolute alcohol

    Xylene (x3)

22. Bath in formic acid (98%) for 5 minutes. This allows for handling the slides outside of the laboratory.

2.8 WESTERN BLOT DETECTION OF PRP^{SC}

The same Western blot method was also used for detection of PrP^{C} in uninfected mice following an alternative protein preparation method (see section 2.6).

2.8.1 Purification of PrP^{Sc} from Frozen Brain Tissue

1. All tissue manipulation was carried out in a Class 1 Microbiological Safety Cabinet in the CJDSU High Risk Lab as human derived material and material from these human transgenic mice is Hazard Group 3 classification. Sealed rotors were used for all pulses and longer centrifugations.
2. The following tubes were labelled as shown:
    - 2ml safelock labelled 'I' for 'inoculum'
    - 1.5ml safelock 'P' for 'pellet'
    - 1.5ml safelock 'S' for 'supernatant'
    - 1.5ml screwtop 'PK' for 'PK digested'
3. Weigh the 'I' tube and note the weight.
4. Transfer the tissue sample to the 'I' tube and reweigh. Note the weight and calculate the amount of saline to add which will give a 10^{-1} inoculum. All samples were made up as inoculum so that transmission experiments can be performed if necessary.
5. Add a maximum of 500µl of saline (0.9%, sterile) to the tissue and homogenise using a disposable plastic micropestle. Add the remainder of the saline and vortex to mix.
6. Add 25µl of 10x Extraction Buffer to the 'P' tube and add 225µl of the homogenate. Vortex to mix then centrifuge at 2000 rpm for 5 minutes at 4°C. (Extraction Buffer: 0.5% NP-40 detergent, 0.5% sodium deoxycholate, 1xTBS pH 7.4)

7. Transfer 100µl of the supernatant to the 'PK' tube and the remainder to the 'S' tube. The remaining tissue in the pellet tube can be used for determining the genotype at codon 129.
8. Add 2.5µl of 2mg/ml Proteinase K to 'PK' tube and vortex to mix. (Final PK concentration 50µg/ml.)
9. Incubate at 37ºC for 60 minutes.
10. Add 1µl of Pefabloc SC (Roche, UK) (final concentration 1mM, stored at -20°C) to halt further action of the proteases. Vortex and store at -20°C.
11. For centrifugation concentration of prion protein:
    11.1. Centrifuge the 'PK' tube at 14,000 rpm for 60 minutes at 4°C.
    11.2. Decant and dispose of supernatant.
    11.3. Resuspend pellet in 20µl of 2x Sample Buffer (Invitrogen).
    11.4. Vortex and boil at 100°C for 10 minutes before storing at –20°C.

2.8.2 Polyacrylamide Gel Electrophoresis of Protein Extracts

1. Type 1 and Type 2B human tissue standards are loaded alongside mouse samples for comparison of the mobility and glycoform ratio.
2. Ensure that the stocks are vortexed thoroughly before taking an aliquot. The usual sample and marker mixes for loading are:
    2.1. 5µl sample + 10µl Extraction Buffer + 5µl Sample Buffer (4x)
    2.2. 5µl Benchmark (Prestained Protein Ladder, Invitrogen) + 1µl Magic Marker (Western Protein Standard, Invitrogen) (+ 9µl of Extraction Buffer + 5µl of Sample Buffer (4x)
3. Vortex the samples to mix. Boil at 100ºC for 10 minutes, then pulse down.

4. Clamp 2 gels (or 1 gel and a blank cassette) into the holding cassette. Gels used are 10 or 12% Bis/Tris NuPAGE (Invitrogen).
5. Fill the inner compartment with 1x MES (2-(*N*-morpholino) ethanesulfonic acid) running buffer (50ml 20x MES (Invitrogen), 950ml dH_2O).
6. Load the samples, fill the outer compartment with running buffer (until 3/4 full) and replace gel tank lid.
7. Run the gel for 50-60 minutes at 200V, then proceed with the Western transfer.

2.8.3 Western Transfer

Once the proteins have been separated by the electrophoretic mobility they are then transferred and fixed onto a membrane so that they can then be detected with an anti-PrP antibody.

1. Make up 1x transfer buffer. (Per gel: 375ml dH_20, 100ml of methanol, 25ml of 20x NuPAGE Transfer Buffer (Invitrogen))
2. Soak the PVDF (polyvinylidene fluoride) membrane (9cm x 6cm) in methanol, rinse thoroughly with dH_20 and soak in transfer buffer. Soak the blotting paper and the sponges in transfer buffer.
3. When the electrophoresis is complete place the gel into a transfer cassette sandwiched thus: 3 sponges / blotting paper / gel / PVDF membrane / blotting paper / 3 sponges (from back of cassette to front).
4. Completely fill the transfer cassette with transfer buffer, and the tank 3/4 full with tap water. Run at 30V for 60 minutes, then proceed to antibody detection of the PrP protein.

2.8.4 Antibody Binding to PrP

1. Make up the following reagents:
   1.1. TBST / Milk blocking solution (2.5g milk powder in 50ml TBST)
   1.2. Primary antibody (6H4 1µl in 40ml TBST – 1:40000)
   1.3. Secondary antibody (GE Healthcare; Product number NA9310; Anti-mouse IgG peroxidase-linked 1µl in 40ml TBST – 1:40000)
2. After the Western transfer stage perform the following incubations and washes.
   2.1. 3 x 3 min washes in TBST
   2.2. 45 min-1 hr in Milk / TBST or overnight shaking at 4°C
   2.3. 3 x 3 min washes in TBST
   2.4. 45 min-1 hr in 1° Ab / TBST
   2.5. 3 x 3 min washes in TBST
   2.6. 45 min-1 hr in 2° Ab / TBST
   2.7. 4 x 3 min washes in TBST

2.8.5 Chemiluminescence Detection of Antibody Binding

ECL+ (GE Healthcare, UK) kit was used for the chemiluminescence detection of the secondary antibody.

1. Remove two ECL+ reagents from the fridge to allow to come to room temperature (large brown bottle and small brown bottle).
2. Aliquot 2ml (per gel) of the large brown ECL+ bottle into a 15ml universal tube.
3. Lay out a piece of cling film onto a piece of tissue and cut relevant sized acetate sheet for the size or number of membranes (A4 sheet cut in half per 2 gels).

4. Once the TBST washes are complete, place the membrane onto the cling film (protein side up).
5. Add 50µl (per gel) of the small bottle to the 15ml universal and mix.
6. Distribute 2ml of the solution evenly over each membrane, rocking the membrane to ensure there are no dry areas.
7. Leave to develop for 5 minutes, occasionally rocking the membrane.
8. Using tweezers, lift the membrane from the top and drain the solution against the tissue.
9. Place the membrane onto the acetate sheet and cover with a same sized piece.
10. Rub the acetate firmly with a piece of tissue from the middle outwards to remove all excess solution from the membrane, squeezing the excess to the edges. This will reduce the background noise.
11. Place the membrane into an x-ray cassette and expose the membrane to film for 30 seconds, 3 minutes, and 30 minutes. Films were developed using an automated processor.

CHAPTER 3

CONFIRMATION OF GENETIC, BIOCHEMICAL, AND PHYSIOLOGICAL CONFIGURATION OF TRANSGENIC MICE

3.1 AIMS

This chapter describes the assessment of the novel lines of transgenic mice used in this study, confirming that they are a suitable model system.

The first assessment carried out was to confirm that the transgene introduced into the mice had the correct DNA sequence. This had already been done by Dr H Baybutt during the production stage of the mice, however was repeated for a HuVV mouse as part of this thesis.

The next check was that the transgene was expressed in the correct tissues of the mice, most importantly the brain, and that the levels were comparable with the wild-type 129Ola line which was used to generate the transgenic lines.

Analysis of the mature PrP^C was then required to confirm that it was attached to the cell membrane by a glycophosphatidylinositol (GPI) anchor and that it existed in the three glycosylation states, at relative levels to that found in wild-type mice.

The final check was that the lifespan of the transgenic mice had not been affected by the genetic manipulation, and that there was no evidence of spontaneous TSE related disease pathology.

3.2 INTRODUCTION

The key component of this new mouse model of human TSE transmission is the ability to compare data within and between mouse lines, and to identify the specific role of codon 129 genotype in disease transmission. To this end the three transgenic mice lines used in this study were: inbred, with an identical genetic background, were produced by the gene targeting method involving direct replacement of the mouse *PRNP* gene with the human equivalent, and could be cross bred between the HuMM and HuVV lines to generate the true MV heterozygote line (HuMV).

There have been many transgenic mouse lines produced by other research groups but only one other (Kitamoto Group) has produced the three codon 129 genotype lines by gene targeting. (See Introduction section 1.9) The Kitamoto group culls all their mice at 75 days post injection, and so this thesis is the first description of experimental data for susceptibility to human TSE over the entire mouse lifespan.

3.3 EXPERIMENTAL RESULTS

The following sections describe the data showing confirmation that the transgene, gene expression, and mature PrP^C production, are as expected for these novel lines of transgenic mice, and that the genetic manipulation has not affected the lifespan survival.

3.3.1 Sequence Analysis of the *PRNP* Transgene

One HuVV mouse was positive for transmission of the vCJD agent as described in Chapter 4, and therefore to ensure that there was nothing peculiar about

the genetics of this mouse, *PRNP* sequence analysis was carried out to check the transgene. Previous work by Dr H Baybutt at NPD had confirmed the transgenic lines had been produced correctly, but in case the genotype of this HuVV mouse had been labelled incorrectly it was checked again as part of this thesis.

Figure 3.F1 shows the alignment of amino acid sequence data from this mouse alongside the published human and mouse sequences. These data confirm that the N-terminal signal sequence (up to amino acid 20) was like that of mouse and the remaining sequence was human. The genotype at codon 129 was also confirmed as valine homozygous.

Figure 3.F1: Transgene Sequence Comparison with Wild-type Reference

PRNP amino acid sequence alignment of HuVV mouse (ID # 115, experiment 553A-1A) with published mouse and human sequences. (Top: human, Middle: mouse, Bottom: HuVV mouse. Red shows differences.)

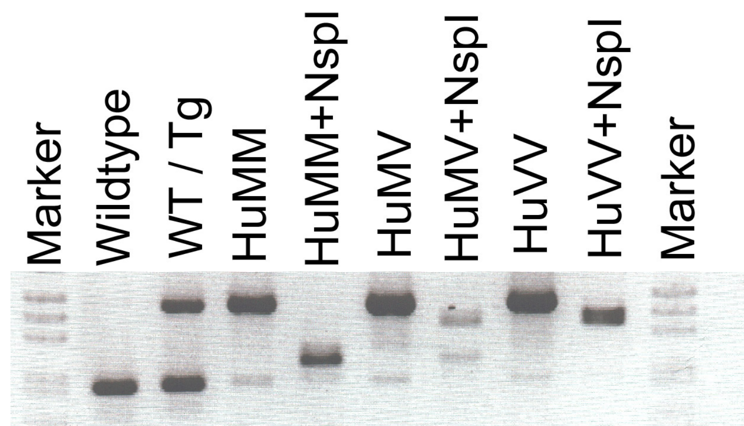
```
MANLGCWMLV LFVATWSDLG LCKKRPKPGG WNTGGSRYPG QGSPGGNRYP PQGGGGWGQP 60

-----Y-L-A ---TM-T-V- ---------- ---------- ---------- ----T*---- 59

-----Y-L-A ---TM-T-V- ---------- ---------- ---------- ---------- 60


HGGGWGQPHG GGWGQPHGGG WGQPHGGGWG QGGGTHSQWN KPSKPKTNMK HMAGAAAAGA 120

---------- -S-------S ---------- ------N--- --------L- -V-------- 119

---------- ---------- ---------- ---------- ---------- ---------- 120


VVGGLGGYML GSAMSRPIIH FGSDYEDRYY RENMHRYPNQ VYYRPMDEYS NQNNFVHDCV 180

---------- -------M-- --N-W----- ----Y----- -----V-Q-- ---------- 179

--------V- ---------- ---------- ---------- ---------- ---------- 180


NITIKQHTVT TTTKGENFTE TDVKMMERVV EQMCITQYER ESQAYYQ--R GSSMVLFSSP 238

---------- ---------- ---------- ----V---QK ------DGR- S--T------ 241

---------- ---------- ---------- ---------- ---------- ---------- 240


PVILLISFLI FLIVG   253

---------- -----   256

---------- -----   255
```

3.3.2 Codon 129 Genotyping of the *PRNP* Transgene

Producing sequence data for the full length of the transgene for a number of transgenic mice is costly and time consuming, and so a more rapid genotyping method was used to check that the human *PRNP* gene was present, and the codon 129 genotype. This PCR / restriction enzyme digest method was used to check the genotype of breeding stocks and also the mice that were selected for use in the experiments. (See Methods, section 2.3, Genotyping, Sequencing, and Sequence Analysis) Figure 3.F2 shows a representative agarose gel of genotyping results.

Figure 3.F2: Codon 129 Genotype Analysis

Agarose gel of codon 129 genotyping tests showing the banding pattern obtained for wild-type mice, wild-type / transgenic cross (WT/Tg), and transgenic mice of each of the three genotypes. (+Nsp1: PCR product digested with restriction enzyme. All other lanes undigested.)



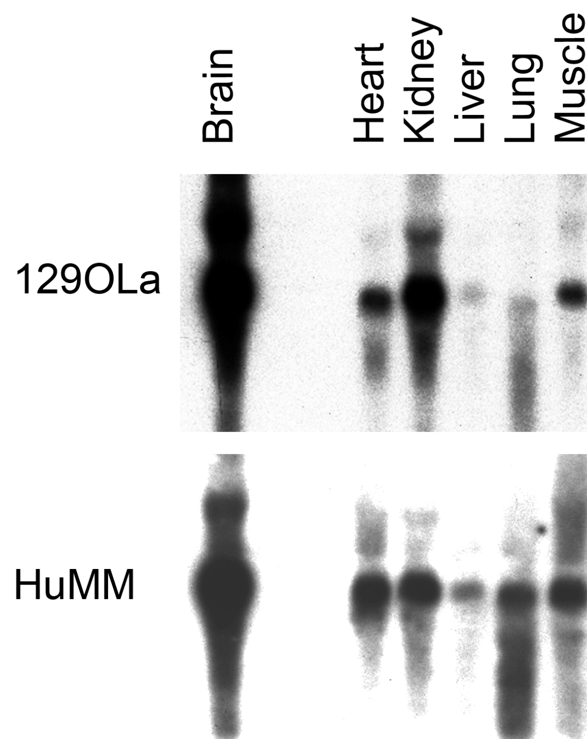
3.3.3 *PRNP* RNA Assessment in the Transgenic Mice

It was proposed to assess the mRNA levels of the transgene in a number of tissues to compare with data from the 129Ola wild-type. The tissues selected were: brain, heart, kidney, liver, lung, and muscle. Due to the difficulties in obtaining reproducible quantity and quality or RNA from these samples only a limited analysis was undertaken of the HuMM and wild-type mice. It was deemed more critical that the mature prion protein was present in the correct configuration in the brain than whether the levels of mRNA were equivalent.

Figure 3.F3 shows the Northern blots obtained from these mice. *PRNP* transgene mRNA was seen at equivalent levels in both transgenic and wild-type mice in all tissues studied.

Figure 3.F3: Northern Blot of RNA Levels Across Various Tissues

Northern blots of HuMM and 129Ola wild-type mice showing similar levels of *PRNP* mRNA in six different tissues.



3.3.4 Transgenic Prion Protein (PrP^C) Characterisation

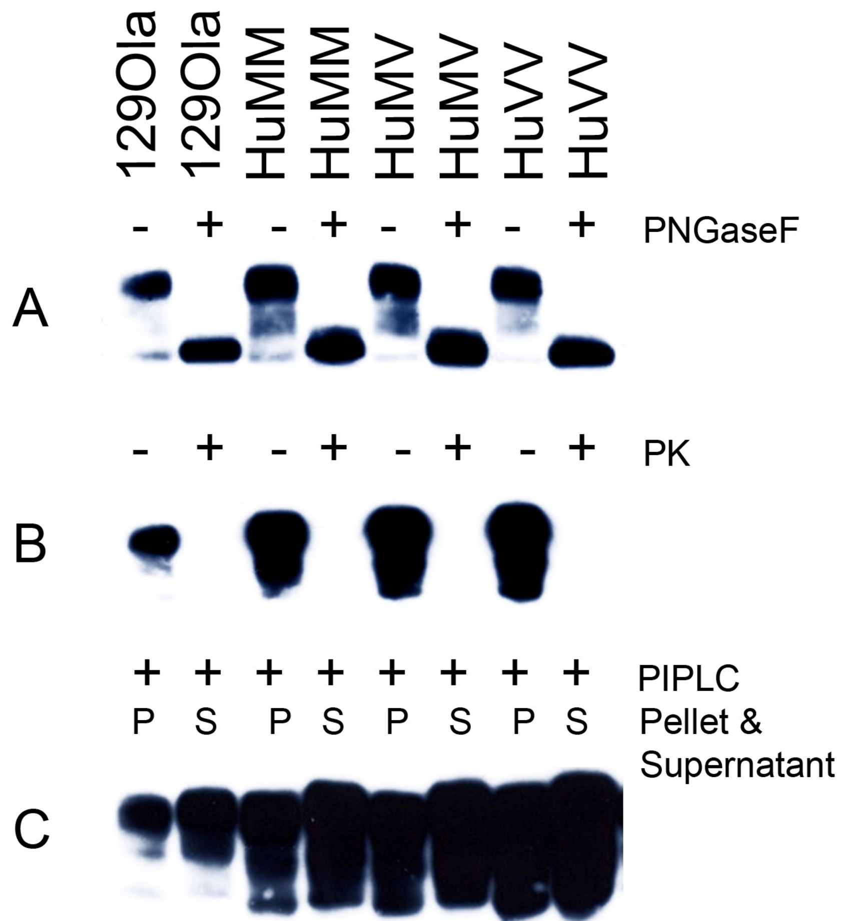
Four properties of the PrP^C produced by the transgenic mice were checked to ensure that the introduced transgene was being expressed by the host mice as if it was the endogenous copy of the murine gene.

1. Similar expression levels were indicated by the relative amounts of PrP^C detected on the Western blots for both the wild-type mice and the transgenics.
2. Glycosylation profiles were correct for the transgenic mice as shown by the presence of the three bands, and confirmation that the bands were attributable to glycans was shown by specific cleavage of these structures by the PNGaseF enzyme (peptide N-glycosidase F) leaving only the unglycosylated fragment.
3. The PrP^C produced was of the protease sensitive form as shown by the disappearance of the blot signal following digestion of the sample with proteinase K. This is important as abnormal forms of PrP tend to be more PK resistant.
4. The PrP^C produced was attached to the surface of the cell membrane by a glycophosphatidylinositol (GPI) anchor, confirmed by the apparent increase in size of the protein bands in the supernatant fraction following GPI cleavage by PIPLC (phosphatidylinositol-specific phospholipase C).

Figure 3.F4 shows the confirmation of all four properties for the transgene expressed human amino acid sequence PrP^C.

Figure 3.F4: Characterisation of PrP^C by Western Blot

Confirmation, for both transgenic and wild-type 129Ola mice, of equivalent PrP^C expression level and glycosylation (A), that the PrP^C is protease sensitive (B), and that the protein is GPI anchored to the membrane (C).



3.3.5 Effect on Survival of Transgenic Modification

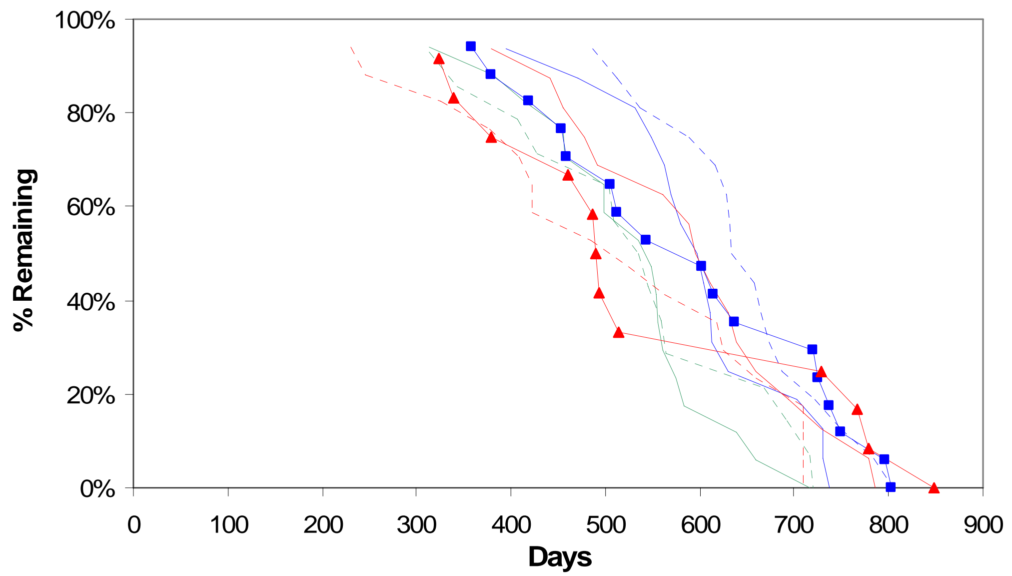
Twenty-four mice of each homozygous genotype line (HuMM and HuVV) were left to age naturally without inoculation or any other experimentation. Six were culled at one year of age and a further six at 500 days old. The remaining twelve were left to live to the end of their natural life.

The mice were scored routinely as per the experimental animals for signs of TSE disease, and when culled they were scored blind for TSE vacuolation. A few of the mice that lived to their natural lifespan were also tested by immunocytochemistry for abnormal PrP deposition in the brain. No evidence was seen of any of these observations, indicating that these mice did not develop spontaneous TSE disease due to the inserted transgene.

Figure 3.F5 shows the survival curve of those mice that were left to live to their natural lifespan. There was no evidence of an adverse effect on survival between either HuMM or HuVV mice and no evidence of a difference compared with the survival curves of 129Ola wild-type mice. The latter had been inoculated with the six sCJD subgroups where only sCJD(MM1) showed high level of transmission (although without clinical disease). As the majority of these mice did not develop clinical or pathological TSE disease their survival curves should be similar to uninfected mice.

Figure 3.F5: Survival Curves for Aged Transgenic Mice

Kaplan-Meier survival curves for aged mice, showing no difference between the two transgenic lines nor between the transgenic lines and 129Ola mice infected with sCJD. (HuMM: blue solid squares, HuVV: red solid triangles, sCJD(MM1): blue solid, sCJD(MM2): blue dashed, sCJD(MV1): green solid, sCJD(MV2): green dashed, sCJD(VV1): red solid, sCJD(VV2): red dashed)



3.4 DISCUSSION

The novel nature of the mouse model used in this thesis centred around generation of three lines of mice that expressed human amino acid sequence PrP^C with variation at the polymorphic codon 129 residue. The three lines would be methionine homozygous (HuMM), valine homozygous (HuVV), and methionine / valine heterozygous (HuMV). Not only would the mice express these three genotype versions of PrP^C but due to the gene targeting method used, the transgenic protein would be expressed in direct replacement of the mouse version. This would mean that in accordance with the original mouse PrP^C, the transgenic human protein would be expressed in the exact temporal and spatial patterns (with variation over the mouse lifespan and in different tissues around the body, respectively). The inbred nature of the mice, on the 129Ola background, also meant that the three codon 129 genotype lines would be genetically identical except for just this specific polymorphism. Together these factors mean that experimental transmission of TSE disease to these mice should follow a more natural physiological course, and the differences between the three lines would be attributable to the variation in codon 129 genotype.

DNA sequence analysis has confirmed the specific genetic changes in the mice. The N-terminal signal sequence is mouse derived to ensure that the immature protein is correctly transported through the endoplasmic reticulum, for post-translational modification, to the cell surface. The C-terminal sequence that codes for the recognition sequence for the GPI anchor is human derived, however as the human amino acid sequence is identical to that of the mouse, there should not be any problems with membrane attachment.

Confirmation that the transgenic PrP^C was membrane bound and glycosylated correctly signified that the human *PRNP* transgene was being transcribed and translated into a mature protein, with post-translational modification and trafficking according the original pathway of the murine prion protein. This means that the resultant TSE disease transmission pathogenesis would not be affected by unusual location or biochemistry of the transgenic prion protein.

The expression level of the transgenic protein was shown to be similar to that of wild-type PrP^C by assessment of mRNA levels in a few tissues, and the amount of protein in the brain by Western blot.

Finally, the survival curve data show that expression of human PrP^C has not lead to spontaneous TSE disease, by clinical and pathological analysis, or a reduction in the usual lifespan of the mice. These mice therefore show no evidence of a detrimental effect due to the genetic manipulation and express human PrP^C as if it were the original murine protein.

CHAPTER 4

IDENTIFYING THE UNIQUE SPECIFICITY OF VARIANT CJD STRAIN TRANSMISSION PROPERTIES DEFINED BY CODON 129 GENOTYPE IN HOSTS OTHERWISE GENETICALLY IDENTICAL

4.1 AIMS

This chapter aims to address two important areas of research concerning the transmission properties of vCJD.

- The role of codon 129 genotype in susceptibility to disease.
- The potential for iatrogenic spread of vCJD in the human population.

The goal of much of the research into vCJD centres around the reasons why only approximately 200 individuals worldwide have so far shown susceptibility to the disease, as understanding this may eventually lead to finding ways to halt or reverse the clinical and pathological symptoms. Presentation of clinical vCJD has so far been confined to only MM genotype individuals at codon 129 of the *PRNP* gene, and therefore being able to predict the presence of disease in non-MM people would enable better estimation of the number of future vCJD cases. Also, the valine allele in non-MM people may have a protective effect and therefore PrP^C with valine at codon 129 could be used as part of a potential treatment program.

Before the NPD transgenic mouse lines were generated there were no mouse models of codon 129 genotype effect, where true comparisons could be made between the three genotypes (MM, MV, and VV). Most transgenic lines expressing human PrP had multiple copies of the gene inserted randomly into the genome and therefore between line comparisons were problematic. There was too much genomic variation, and difference in expression levels of PrP, to be able to directly link changes in transmission characteristics to codon 129 genotype alone. Wild-type mice, although susceptible to the vCJD agent, could not be used to directly model human PrP interaction, and the effects of codon 129.

A more detailed understanding of the strain transmission properties of vCJD, through this study, may allow predictions to be made about whether human-to-human iatrogenic spread of this disease may be more of a public health problem than the original BSE associated infection. Serial passage of vCJD in humans may lead to a more infectious 'humanized' strain of the original bovine disease. The discovery of vCJD infection via blood transfusion has raised the possibility of an additional epidemic of vCJD cases acquired by human-to-human passage of the agent.

Data from this study were published in 2006 in Lancet Neurology (See Appendix) (Bishop *et al.*, 2006).

4.2 INTRODUCTION

4.2.1 The Role of Codon 129 Genotype in vCJD Disease Transmission

All tested patients with clinical vCJD (147/167 in the UK) have been found to be homozygous for methionine (MM) at codon 129, including three cases of vCJD infection via contaminated blood products (Hewitt *et al.*, 2006). One case of subclinical infection via blood transfusion was heterozygous (MV) (Peden *et al.*, 2004). Two of three anonymous appendix samples that were positive for PrP^{Sc} deposition (from a screen of 12,674 samples), which is highly specific for vCJD infection (Hilton *et al.*, 2004b), were valine homozygous at codon 129 (Ironside *et al.*, 2006). It is however unknown whether these individuals would go on to develop vCJD. Clinical and pathological evidence from an extensive surveillance programme over nearly 20 years in the UK clearly shows that the codon 129 genotype plays a significant role in determining susceptibility of individuals to BSE and vCJD infection.

Transgenic mice expressing the human *PRNP* gene sequence have provided laboratory data on the role of codon 129 genotype and its effect on experimental transmission of vCJD (See Introduction). The MM genotype appears to be the most susceptible in terms of shorter incubation periods and more extensive pathological changes. VV homozygous mice when over-expressing the gene, were susceptible, but were observed not to propagate the specific biochemical vCJD strain properties (type 2B by Western blot) (Wadsworth *et al.*, 2004). Physiological expression of PrP in VV mice, by gene targeting methods, imparted resistance to infection (Asano *et al.*, 2006). The MV genotype transgenic mice data indicate an intermediate

response to vCJD transmission with less extensive pathology and longer incubation periods than MM mice (Asano *et al.*, 2006, Asante *et al.*, 2006).

These data are a compilation of information from many lines of mice produced in different ways for different research purposes. Only the Kitamoto group has generated all three codon 129 genotype lines using a gene targeting methodology, but this group sacrifice all the mice at 75 days post inoculation (Asano *et al.*, 2006). Incubation period studies of transmitted disease, with each genotype line, is therefore not possible with these mouse models.

This leaves a significant gap in information concerning lifetime susceptibility to vCJD and the effect of codon 129 genotype. The experimental design of this part of the thesis involves monitoring HuMM, HuMV, and HuVV transgenic mice over lifespan (~750 days) following inoculation with infectious vCJD material. This provides a unique data set with which to assess the specific effect of codon 129 genotype on individual susceptibility to the vCJD agent.

Separate experiments were set-up with either intracerebral (i.c.) or intraperitoneal (i.p.) inoculation routes. As the pathogenesis of vCJD in humans involves PrP^{Sc} deposition targeted to peripheral tissues outside the central nervous system (CNS) (including spleen, tonsils, and appendix (Ironside *et al.*, 2000)) the intraperitoneal injection route may be more relevant to understanding the human disease. The two infection routes could show important differences in the strain properties of vCJD, with pathological targeting specific to different genotype hosts.

4.2.2 Iatrogenic Spread of vCJD Strain in the Human Population

In addition to identifying which codon 129 genotypes are susceptible to infection by the vCJD agent it is of great interest, for public health reasons, to know

whether infected individuals might pass on the disease to others and at what point during the incubation period this is possible. Primary transmission of vCJD to these transgenic lines is a model of iatrogenic spread of vCJD in the UK, and also has the potential to assess the transmission of BSE to MV and VV genotype individuals.

Secondary passage from transgenic mice infected with vCJD was undertaken to confirm the infectious nature of the transmitted disease and to observe propagation of strain differences in the three codon 129 genotype mice lines. The original vCJD inoculum was derived from an individual with an MM genotype at codon 129, but the sub-passage experiments involved material from each genotype of mice. This allowed analysis of the strain transmission properties of the vCJD agent adapted by passage through MV and VV genotype hosts, thus modelling the potential spread of vCJD in the human population.

4.3 EXPERIMENTAL RESULTS

4.3.1 Intracerebral Inoculation of vCJD

Eighteen mice of each transgenic line (HuMM, HuMV, HuVV), and as a control the 129Ola wild-type line (which was used to generate these lines), were inoculated by the intracerebral route (i.c.) with inoculum prepared from the NIBSC vCJD standard (Code NHBY0/0003, see Methods). This route of inoculation is commonly used when new mouse lines or new inocula are to be used. This route gives the best chance of disease transmission as the infectious material is injected directly into the brain. Due to the long duration of these experiments intercurrent illness and death due to non-TSE causes were observed. Where possible these mice

were examined for markers of TSE pathology. Table 4.T1 shows the evaluation of all mice in this study.

Table 4.T1: Data Table for Intracerebral Inoculation with vCJD

Intracerebral inoculation with vCJD with data ordered by Survival Time. (Protocol reference 553A-1A)

Abbreviations: CLIN: clinical TSE signs; VACN: presence of TSE vacuolation; ICC: presence of abnormal PrP by immunocytochemistry; ICD: intercurrent death or culled for animal welfare reasons; n/d: no data available as tissue not testable; n/t: not tested at this time; shaded cell: positive score; End expt: mice that were culled when the experiment was terminated at 700 days

HuMM						HuMV						HuVV						129Ola					
ID	Survival Time	Reason for cull	CLIN	VACN	ICC	ID	Survival Time	Reason for cull	CLIN	VACN	ICC	ID	Survival Time	Reason for cull	CLIN	VACN	ICC	ID	Survival Time	Reason for cull	CLIN	VACN	ICC
103	118	ICD	Neg	Neg	Neg	97	522	ICD	Neg	Neg	n/d	131	462	ICD	Neg	Neg	Neg	189	145	ICD	Neg	Neg	n/t
136	221	ICD	Neg	Neg	Neg	92	542	ICD	Neg	Neg	n/d	132	503	ICD	Neg	Neg	Neg	207	319	ICD	Neg	Neg	n/d
138	333	ICD	Neg	Neg	n/d	139	550	ICD	Neg	Neg	Neg	129	543	ICD	Neg	Neg	n/d	192	403	ICD	Neg	Neg	n/d
106	368	ICD	Neg	Neg	Pos	98	581	ICD	Neg	Neg	Pos	128	559	ICD	Neg	Neg	Neg	225	405	TSE	Pos	Pos	n/d
122	378	ICD	Neg	Neg	Pos	94	595	ICD	Neg	Neg	Pos	119	578	ICD	Neg	Neg	Neg	191	418	ICD	Neg	Neg	n/t
123	424	ICD	Neg	Neg	n/d	140	595	ICD	Neg	Neg	Pos	126	596	ICD	Neg	Neg	Neg	221	438	TSE	Pos	Pos	n/t
110	497	TSE	Pos	Pos	Pos	95	600	ICD	Neg	Neg	Pos	118	622	ICD	Neg	Neg	Neg	205	476	TSE	Pos	Pos	Pos
121	497	ICD	Neg	Neg	Neg	143	608	ICD	Neg	Neg	Pos	112	623	ICD	Neg	Neg	Neg	222	483	TSE	Pos	Pos	Pos
135	504	ICD	Neg	Neg	Pos	100	630	ICD	Neg	Neg	Neg	127	649	ICD	Neg	Neg	Neg	223	483	TSE	Pos	Pos	Pos
105	536	ICD	Neg	Pos	Pos	141	665	TSE	Pos	Neg	Pos	116	651	ICD	Neg	Neg	Neg	193	483	TSE	Pos	Neg	n/t
133	545	ICD	Neg	Neg	Pos	142	686	ICD	Neg	Neg	Neg	130	657	ICD	Neg	Neg	Neg	226	503	TSE	Pos	Pos	Pos
134	567	ICD	Neg	Neg	Pos	144	693	End expt	Neg	Neg	Pos	124	658	ICD	Neg	Neg	Neg	224	508	ICD	Neg	Neg	n/t
137	595	ICD	Neg	Neg	Neg	91	707	End expt	Neg	Neg	Pos	125	686	ICD	Neg	Neg	Neg	206	586	TSE	Pos	Pos	Pos
111	630	TSE	Pos	Pos	Pos	96	707	End expt	Neg	Pos	Pos	113	693	End expt	Neg	Neg	Neg	208	613	ICD	Pos	Pos	Pos
107	651	ICD	Neg	Neg	Pos	101	707	End expt	Neg	Neg	Pos	115	693	End expt	Neg	Pos	Pos						
108	693	ICD	Neg	Pos	Pos	102	707	End expt	Neg	Neg	Pos	120	693	End expt	Neg	Neg	Neg						
109	707	End expt	Neg	Pos	Pos																		
104	707	End expt	Neg	Pos	Pos																		
'Attack Rate'			2/18	6/18	12/16				1/16	1/16	11/14				0/16	1/16	1/15				8/14	8/14	6/6

4.3.1.2 Clinical TSE Findings

Highlighted in grey in Table 4.T1 are the mice positive for clinical signs of TSE, those that were confirmed by pathological analysis (either the presence of TSE associated vacuolation and/or those that had abnormal deposition of PrP by immunocytochemistry (ICC)). Also highlighted are the vacuolation or ICC positive mice that were culled for other reasons (labelled 'ICD' or 'End expt') and were not showing clinical TSE signs. These mice were likely to be in a subclinical phase of the disease which will be discussed later. There were a number of mice culled because of clinical signs that suggested the TSE phenotype but were negative by pathological analysis. These were not classed as positive transmissions.

The data table for i.c. challenge (Table 4.T1) shows that two pathologically confirmed clinically positive mice were seen in the HuMM line (at 497 and 630 days post inoculation), one in the HuMV line (at 665 days post inoculation), and none in the HuVV line. A higher percentage of the 129Ola mice developed to a clinical disease stage (57%, 8/14) with a mean incubation period of 498 days (range 405 to 613).

4.3.1.3. Survival Analysis

A statistical analysis of the survival rates was performed to observe any effects on survival due to the subclinical disease present in many of the mice. With the absence of clinical disease, incubation period comparisons could not be made. Intercurrent deaths may be due in part to an adverse effect of the inoculum and the numbers vary according to the codon 129 genotype of the mouse. To observe effects on overall survival rates between the three transgenic line and the wild-type mice,

survival curves were drawn and a Tarone-Ware test performed (SPSS v14.0) (Figure 4.F1 and Table 4.T2). This test was used, in preference to others, as it has an even bias across the lifespan of the mice. The data show a significant difference between the wild-type 129Ola and the non-HuMM transgenic lines (P<0.001), between the HuMM and HuVV lines (P=0.04), and also between the HuMM and HuMV lines (P=0.012).

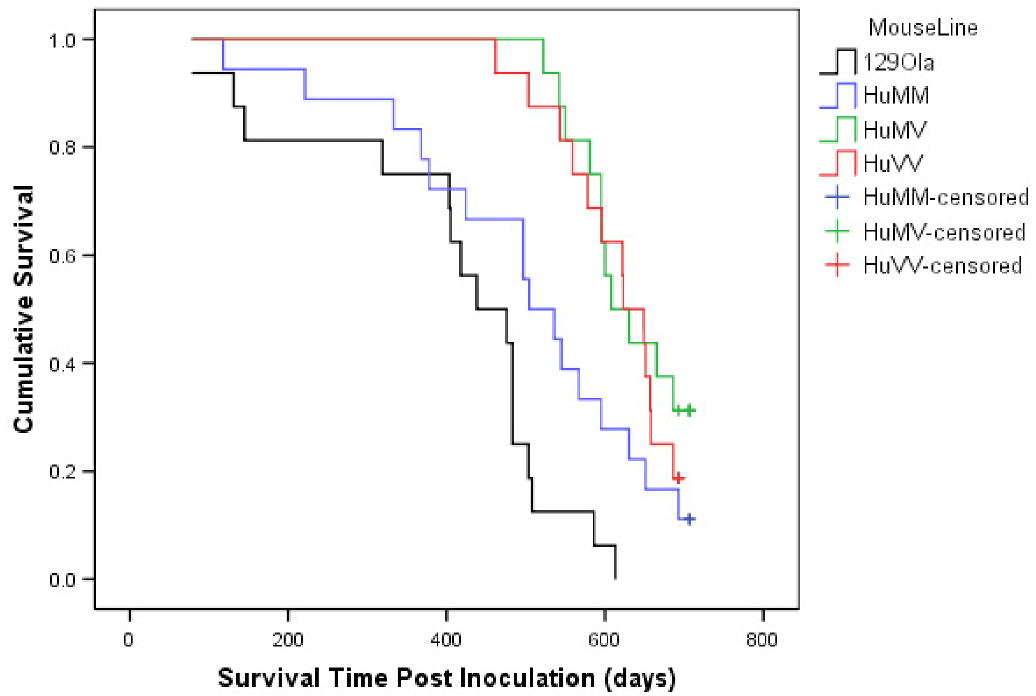
Table 4.T2: Tarone-Ware Test for Survival Analysis of Intracerebral Inoculation with vCJD

Survival analysis by genotype following i.c. inoculation of vCJD shows the difference between the HuMM mice and the rest. (Statistical analysis: Tarone-Ware Test using software SPSS v14) Significance rating for clearer view of differences- *:P<0.5; **:P<0.01; ***:P<0.001; n/s: not significant.

Groups Tested	P value	Significance rating
HuMM vs. HuMV vs. HuVV vs. 129Ola	<0.001	***
HuMM vs. HuMV	=0.012	*
HuMM vs. HuVV	=0.040	*
HuMM vs. 129Ola	=0.036	*
HuMV vs. HuVV	=0.615	n/s
HuMV vs. 129Ola	<0.001	***
HuVV vs. 129Ola	<0.001	***

Figure 4.F1: Kaplan-Meier Survival Curve of Intracerebral Inoculation with vCJD

Kaplan-Meier survival curves following i.c. challenge in HuMM (blue), HuMV (green), HuVV (red), and 129Ola (black) mice. The experiment was terminated at 700 days when the remaining mice were culled (mice alive at the end of an experiment are termed 'censored' in survival analysis and are noted as crosses in the Kaplan-Meier curve). Also see Table 4.T1.



4.3.1.4 Vacuolation Scoring

The second criteria for successful transmission after clinical TSE signs is *post mortem* assessment of the presence, location, and intensity of TSE associated vacuoles in the brain of each mice. This analysis is detailed in the Methods chapter and is undertaken by only a handful of dedicated NPD staff trained to perform this task (See Acknowledgements). They have analysed, blind to the experimental details and genotype of the mice, nine areas of grey matter and three areas of white matter, from haematoxylin and eosin stained paraffin sections. Scores are given as positive / negative for the presence of TSE vacuoles, and numerically one to five for the intensity of the vacuoles at each of the 12 areas (zero: lowest level to five: highest level). When five or six (or more) mice are scored positive means are generated for each region and a chart produced, a lesion profile.

Following i.c. challenge HuMM mice were more likely to show TSE associated vacuolation than the other transgenic lines, beginning at around 500 days post inoculation. Six (of eighteen) were scored positive, and the mean lesion profile for five of the mice is shown in Figure 4.F2. The highest scores were found in the dorsal medulla, thalamus, and cerebellar white matter (areas G1, G5 and W1 respectively). Only five of the six mice gave complete scores that could be used for the lesion profile but this is considered sufficient to observe the overall targeting of the vacuolation (Bruce *et al.*, 2004). By contrast, only a single mouse in each of the HuMV and HuVV groups scored positive for vacuolation at 707 and 693 days respectively post inoculation and therefore lesion profiles could not be generated. It is therefore not possible to compare these single mouse profiles with the HuMM

data. There are typically a number of differences in individual mouse scores with a single inoculum but the mean profiles highlight the strain specificity.

The mean lesion profile for six i.c. challenged 129Ola wild-type mice is shown in Figure 4.F3. Contrasting with the HuMM mean profile which shows a peak in the data at position G5 (thalamus), there is a peak at position G4 (hypothalamus) in the 129Ola mice.

Figure 4.F2: Lesion Profile of vCJD Inoculated HuMM Mice

Lesion profile for HuMM mice i.c. challenged with vCJD. (Mean score ±SEM; G1-G9: grey matter scoring regions, W1-W3: white matter scoring regions)

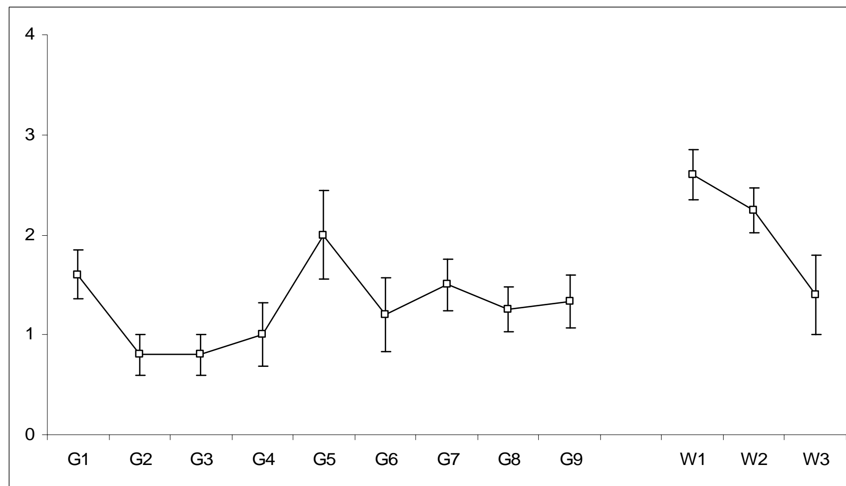
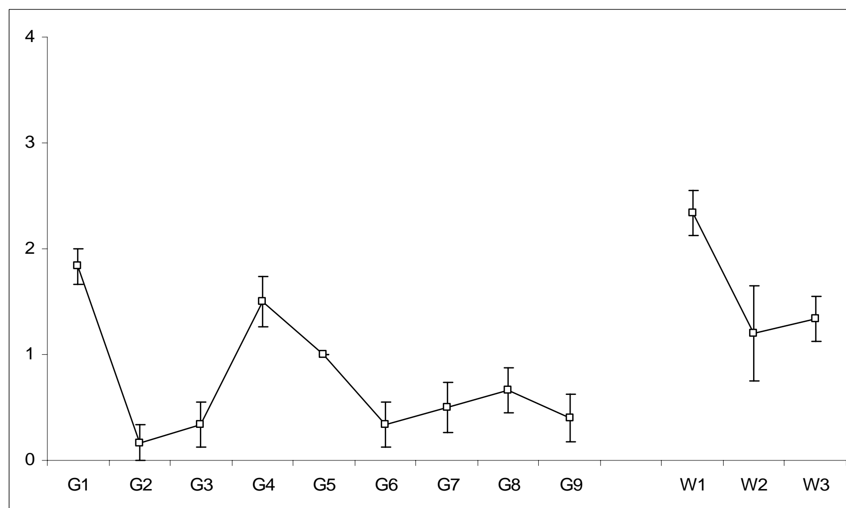


Figure 4.F3: Lesion Profile of vCJD Inoculated 129Ola Mice

Lesion profile for 129Ola mice i.c. challenged with vCJD. (Mean score ±SEM; G1-G9: grey matter scoring regions, W1-W3: white matter scoring regions)



A more detailed analysis of the vacuolation present in each of the transgenic lines (although only single cases in the HuMV and HuVV lines) showed that areas of the thalamus, hippocampus, cerebellar cortex and cerebral cortex were all affected to varying degrees in each line. The HuMV line showed minimal levels in all areas, and the cerebellar cortex was the least affected region across all lines. The HuMM line showed the highest levels in the thalamus (c.f. area G5 in the lesion profile above) a region also targeted to the same degree in the HuVV mouse, although here the cerebral cortex was also involved. The hippocampus showed less severe vacuolation across the three lines.

Plaque structures made up of aggregated PrP^{Sc} molecules, frequently in the form of amyloid, including the florid plaque characteristic of vCJD / BSE infection in humans, were commonly associated with TSE vacuolation (See Introduction, Figure 1.F7). Analysis of the three genotype lines showed florid plaques only in the HuMM mice, in the hippocampus and the cerebral cortex (Figure 4.F4, Panel D). There was no evidence of plaques in the cerebellum which is one of the structures predominantly targeted in human vCJD cases (Ironside *et al.*, 2000). The HuMV genotype line showed no evidence of amyloid plaques in any brain region. The HuVV mouse positive for TSE vacuolation showed clusters of amorphous plaque-like features in the cerebral cortex and the thalamus but these did not have the structured form or the fibrillary nature of the plaques found in the HuMM mice. This evidence therefore points to a specific host genotype response to the vCJD inoculum. This information is summarised in Table 4.T3.

Table 4.T3: Comparison of Neuropathology in Transgenic Mice Inoculated with vCJD

Comparison of TSE-associated neuropathology in transgenic mice inoculated with vCJD. (*Analysed with haematoxylin and eosin staining. †Analysed with immunocytochemical techniques)

	HuMM	HuMV	HuVV
Vacuolation*	Thalamus (severe); cerebral cortex and hippocampus (mild); cerebellar cortex (minimal)	Thalamus, cerebral cortex, hippocampus, and cerebellar cortex (minimal)	Thalamus and cerebral cortex (severe); hippocampus (mild); cerebellar cortex (minimal)
Plaque formation*	Fibrillary amyloid plaques; florid and non-florid plaques in cerebral cortex and hippocampus; no evidence of plaques in cerebellum	No evidence of amyloid plaques	Amorphous non-fibrillary structures often forming into clusters in cerebral cortex and thalamus
PrP deposition†	Intense staining of plaques in hippocampus and cerebral cortex; plaque-like, pericellular, and amorphous deposits in the hippocampus; synaptic, perineuronal, and diffuse perivascular deposits in the thalamus	Occasional small plaque-like deposits and pericellular deposits in the thalamus	Strongly positive large amorphous deposits and clusters of plaques, small plaque-like structures, perivascular aggregates, and sub-pial deposits in the cerebral cortex and thalamus

4.3.1.5 Detection of PrP^{Sc} by Immunocytochemistry

The third criteria for positive transmission is the presence of TSE disease associated PrP (PrP^{Sc}). This is generally regarded as the 'gold standard' for confirmation of TSE disease and is widely used in diagnostic tests for animal TSE. There are however exceptions to this rule where, for example, disease has been found without PrP^{Sc} and also where PrP^{Sc} has been detected without transmissible disease (Barron *et al.*, 2007, Piccardo *et al.*, 2007).

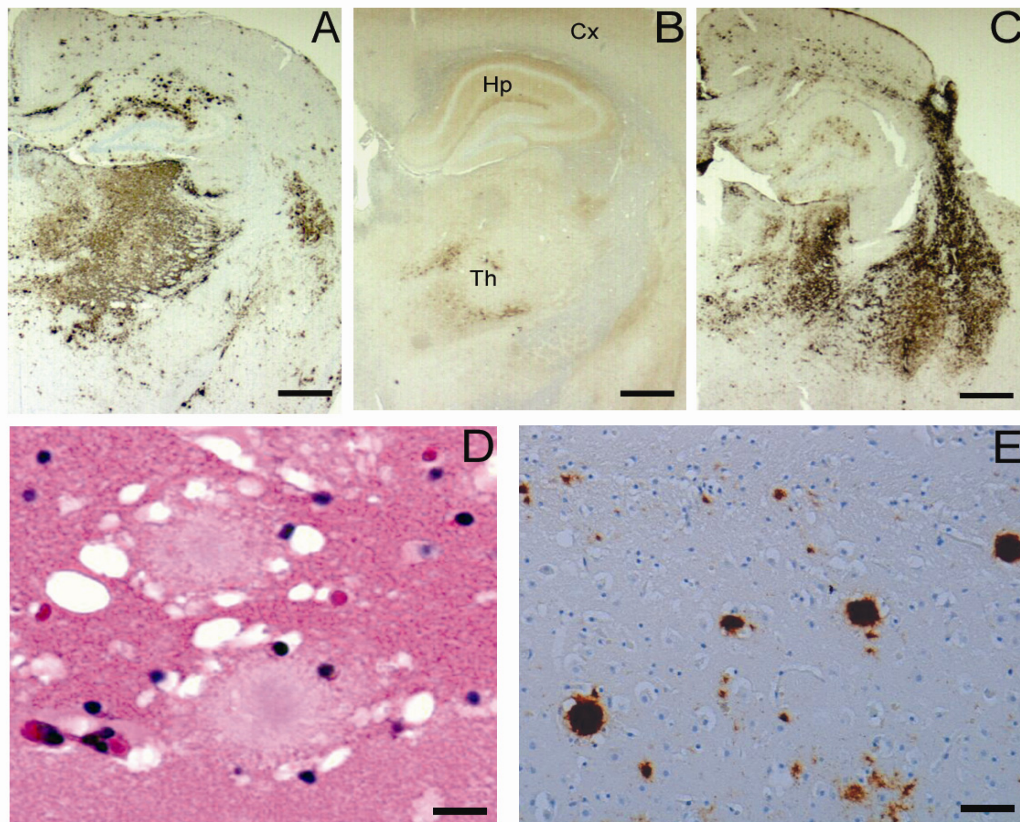
Immunocytochemical (ICC) detection of PrP^{Sc} on paraffin sections gave the positive / negative scoring seen in Table 4.T1 (column ICC) and provided data on the spatial distribution of this form of PrP in the brain which may be host or strain dependent. With i.c. challenge, a majority of the HuMM mice (12/16) were positive for PrP^{Sc} and showed deposition in most areas of the brain at a relatively early stage (from around 370 days post inoculation), before the vacuolar pathology became evident (around 500 days) (Figure 4.F4, Panel A). From 500 days post inoculation the appearance of vacuolation was accompanied by a significant increase in PrP^{Sc} deposition. Hippocampal targeting with high levels of PrP^{Sc} deposition appeared only in the HuMM mice. In this region, these mice showed intense staining of plaques, including the florid plaques mentioned above. The hippocampus has a role in long term memory, spatial memory, and navigation. It is one of the first regions of the brain to be affected in Alzheimer's Disease, causing memory loss and disorientation as initial symptoms. The hippocampus is connected via neurones to many areas of the brain including regions of the cortex, thalamus, and hypothalamus which also showed high levels of PrP^{Sc} deposition.

In contrast, although PrP^{Sc} was detected in many HuMV mice (11/14), deposition was restricted to only a few areas (including the ventrolateral and ventromedial thalamic nuclei and the red nucleus of the mid-brain), even after 700 days post inoculation (Table 4.T1 and Figure 4.F4, Panel B). Although PrP^{Sc} deposition was clearly present at 581 days, the timing of the onset of deposition in the HuMV line could not be established as brain sections were not available from mice at earlier time-points. Further studies using serial culls would be needed to more accurately show the appearance of PrP^{Sc} over the time-course of vCJD infection.

High levels of PrP^{Sc} deposition were seen in the brain of a single HuVV mouse that was also positive for TSE vacuolation, at 693 days post injection (Figure 4.F4, Panel C). The deposition was similar in intensity to that observed in HuMM mice. The HuVV mouse showed intense PrP^{Sc} deposition in the thalamic and cerebral cortex regions where the amorphous plaque-like structures were found suggesting a link between the two pathological changes. As there was only a single positive HuVV mouse it is not possible to conclude that this pathology is typical for HuVV mice that develop TSE from vCJD infection, but the observed distribution was different from both HuMM and HuMV mice. Differences in patterns of PrP^{Sc} deposition among the three genotypes are described in Table 4.T3.

Figure 4.F4: Histopathology of vCJD Transmission to Transgenic Mice

Immunocytochemistry of histological sections with anti-PrP antibody 6H4 showing the cortex, hippocampal, and thalamic regions of the mouse brain with PrP detection (brown). A: HuMM mouse (ID#108) 693 days post inoculation. B: HuMV mouse (ID#101) 707 days post inoculation. C: HuVV mouse (ID#115) 693 days post inoculation (Scale bar 1250μm; Cx: cortex; Hp: hippocampus; Th: thalamus) D: Florid plaques found in the hippocampus of the HuMM mouse in Panel A. Each plaque has an eosinophilic core with a paler halo and is surrounded by a ring of vacuolation (haematoxylin and eosin stain, scale bar 25μm). E: Florid plaques in the cerebral cortex from the Panel A HuMM mouse stained for PrP. (Scale bar 50μm)



4.3.1.6 Detection of PrP^{Sc} by Western Blot

The detection of protease resistant fragments of PrP^{Sc} by Western blotting is a standard method for testing for the presence of such material, in a diagnostic or screening scenario, and for investigating the strain characteristics defined by the physical structure of the protein. In this study the ICC method detected low levels of PrP^{Sc} deposition and was used for positive / negative scoring. The Western blotting technique was therefore used to assess the transmission of strain characteristics defined by the PrP^{Sc} molecule.

Protease treatment of PrP^{Sc} in vCJD brain produces a Western blot profile that is characterised by a 19 kDa non-glycosylated fragment and a predominance of the fragment representing the diglycosylated form (type 2B) (Head *et al.*, 2004a) (See Introduction, section 1.3.1: The Prion Protein and Figure 1.F3). In this study Western blot detection of PrP^{Sc} was performed on frozen brain material set aside during the *post mortem* of each mouse (See Methods). Both the fragment mobility and relative abundance, of mouse derived PrP^{Sc} are maintained when vCJD is transmitted to the human transgenic mice, irrespective of their codon-129 genotype (Figure 4.F5).

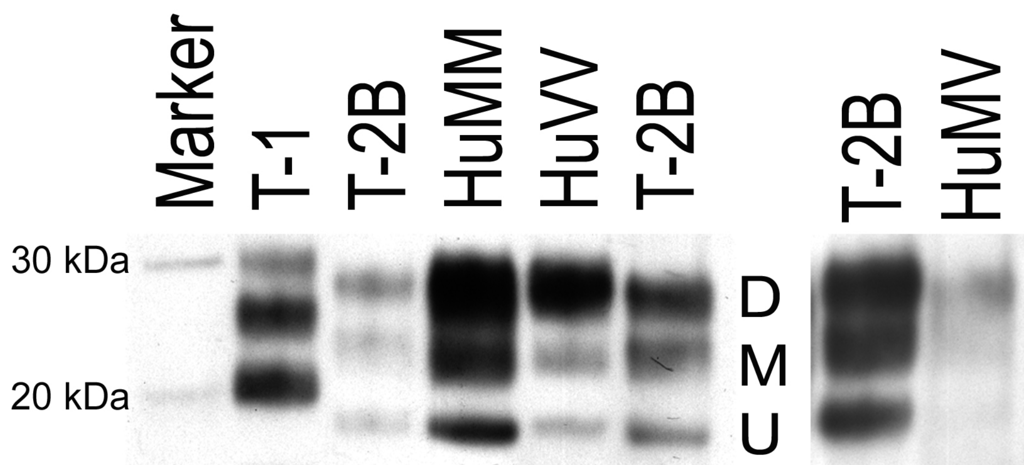
Densitometric analysis, using Quantity One software (Bio-Rad), measured the relative intensity of each of the three bands seen on x-ray film exposures (Head *et al.*, 2004a). Specific exposures were selected so that all three bands fell within the linear range for both the film and the densitometric analysis. These data suggested that there was a relative increase in the diglycosylated form in the HuVV mouse compared with the HuMM mouse. This is preliminary data as there was only one positive mouse tested with the VV genotype, and until more vCJD transmissions in

HuVV mice are achieved it cannot be concluded that this Western blot finding is representative of the VV genotype group.

Lower levels of PrP^{Sc} were seen in brain extracts from the HuMV mice corresponding to the low levels observed by ICC, but the type 2B profile was maintained. Further studies using more sensitive methods of PrP^{Sc} preparation, such as the phosphotungstic acid (NaPTA) method might improve the Western blot sensitivity in these mice (Peden *et al.*, 2007).

Figure 4.F5: Western Blot Analysis of Transgenic Mice Inoculated with vCJD

Western blots of brain extract from three transgenic lines i.c. challenged with vCJD. T-2B corresponds to human vCJD brain homogenate showing the typical PrP^{Sc} type 2B, and T-1 corresponds to human sporadic CJD brain homogenate showing the typical PrP^{Sc} type 1 signature. Type 2B and 1 differ in mobility of the unglycosylated band (~19 kDa and ~20 kDa respectively) and the degree of glycosylation (diglycosylated dominant and mono/unglycosylated dominant respectively). All samples were treated with proteinase K. The anti-PrP detection antibody was 6H4. (D: diglycosylated; M: monoglycosylated; U: unglycosylated)



4.3.2 Secondary Inoculation of Mice with Primary Passage Material

To confirm the infectious nature and strain properties of TSE disease produced in the transgenic mice a series of three subpassage experiments was set up in which brain material from the initial vCJD inoculated transgenic mice was inoculated into further mice of all three genotypes and 129Ola wild-type mice (Figure 4.F6 and Table 4.T4). The specific HuMM mouse was chosen for inoculum as it was clinically and pathologically positive for TSE, the HuMV mouse was one of the oldest surviving and had higher levels of PrP^{Sc} than the others (but no TSE vacuolation), and the HuVV mouse was the only one scored positive for transmission. Mice were injected by the intracerebral route with brain homogenate at a dilution of 1% (10^{-2}). Because of adaptation of the infectious agent within a new host, these subpassage experiments were predicted to show a reduction in the incubation period to clinical disease, and an increase in the prevalence of pathological TSE features. This adaptation occurs because the infectivity, including PrP^{Sc}, has been produced by the new host thereby removing any effects of between host transmission barriers (Lasmezas *et al.*, 1997, Bruce *et al.*, 2002, Kitamoto *et al.*, 2002, Scott *et al.*, 2005).

These experiments are relevant to public health issues. If a subclinical disease state is infectious in humans and is passed on iatrogenically via medical treatment it is important to establish whether the disease strain becomes progressively more infectious after each passage, and also if it retains the original strain phenotype in the host (so that it can be recognised by clinicians and pathologists).

Figure 4.F6: Immunocytochemistry of Mouse Brains Selected for Second Passage

Immunocytochemistry of fixed brain sections from mice inoculated with vCJD, that were selected for second passage inoculum sources. Panel A: HuMM mouse ID# 110; Panel B: HuMV mouse ID# 101; Panel C: HuVV mouse ID# 115. (Antibody: 6H4, scale bar 50µm)

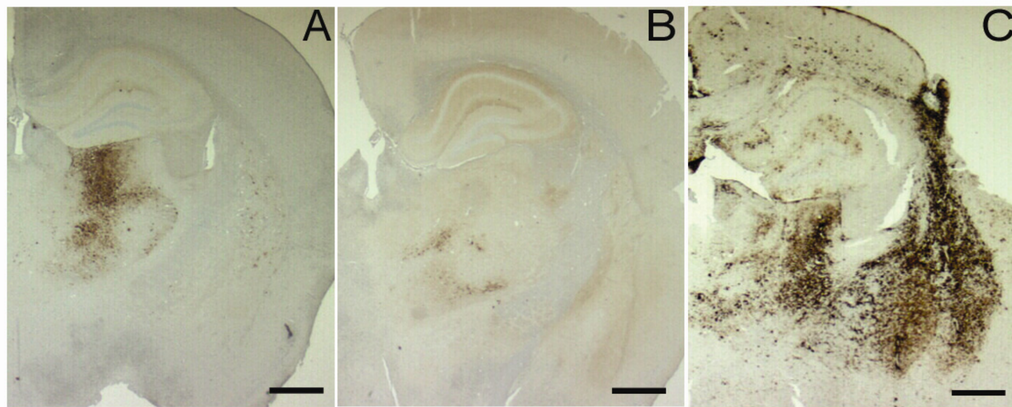


Table 4.T4: vCJD Second Passage Data

Second passage inoculations of brain homogenates, from primary vCJD transmissions (experiment 553A-1A), injected by intracerebral route into transgenic and 129Ola wild-type mice.
Experiment 553A-2A: Brain homogenate from HuMM mouse ID# 110 (Clin: +VE; Vacn: +VE; ICC: +VE; Incubation Period: 497 days)
Experiment 553A-2B: Brain homogenate from HuMV mouse ID# 101 (Clin: -VE; Vacn: -VE; ICC: +VE; Survival Time: 707 days)
Experiment 553A-2C: Brain homogenate from HuVV mouse ID# 115 (Clin: -VE; Vacn: +VE; ICC: +VE; Survival Time: 693 days)
(IP: incubation period; n/d: no data as no clinical disease present)

Inoculum Source	Recipient Mice	No. Mice	Median Survival Time	Mean IP	IP range	CLIN +ve	VACN +ve	ICC +ve
HuMM	HuMM	12	651	624	624	1/11	0/9	7/12
	HuMV	12	481	n/d	n/d	0/11	0/11	2/12
	HuVV	9	569	n/d	n/d	0/9	0/9	0/9
	129Ola	12	547	556	470-617	8/11	6/11	10/12
HuMV	HuMM	13	471	554	554	1/13	0/13	5/13
	HuMV	11	480	n/d	n/d	0/10	0/9	1/11
	HuVV	11	435	n/d	n/d	0/11	0/11	0/11
	129Ola	11	547	519	491-547	2/11	1/11	2/11
HuVV	HuMM	11	397	n/d	n/d	0/11	0/11	2/11
	HuMV	10	554	n/d	n/d	0/11	0/8	0/10
	HuVV	10	560	n/d	n/d	0/10	0/8	0/10
	129Ola	12	441	477	345-596	8/12	10/12	12/12

4.3.2.1 Clinical Assessment

Of the three mice selected as tissue donors for second passage inoculum only the HuMM mouse was positive for clinical symptoms, and this occurred at 497 days post injection. It was hypothesised that the recipient HuMM mice, of this specific inoculum, would develop TSE clinical signs at or before this time period if there was strain adaptation. However, only one HuMM mouse was scored positive for clinical signs (at 624 days) and six of the group of 12 mice survived beyond this time point. The median survival time (the point at which 50% of mice are still alive) was 651 days. One HuMM mouse also developed TSE clinical signs following inoculation with the HuMV derived inoculum, at 554 days. Neither the HuMV nor HuVV mice developed clinical TSE with any of the three inocula, and there were no positive HuMM mice inoculated with the HuVV derived material. There was no evidence of TSE associated vacuolation in the transgenic lines from any of the three second pass experiments.

In contrast to the transgenic results, more clinical positive results were obtained for the wild-type mice inoculations. With the HuMM inoculum clinical TSE was observed, and confirmed by vacuolation scoring or ICC, in 8/11 mice with a mean incubation period of 556 days. For comparison, primary vCJD transmission to these wild-type mice gave an incubation period of 498 days and 8/14 mice were positive (Table 4.T1). The HuMV mouse inoculum produced clinical TSE, confirmed pathologically, but in only two wild-type mice at 491 and 547 days. The response of the wild-type mice to HuVV mouse inoculum showed similarly high level of clinical disease (8/12) but a shorter mean incubation period (477 days) than

the HuMM mouse inoculum. This incubation period is shorter, by about 20 days, than primary transmission of vCJD in wild-type mice.

4.3.2.2 Vacuolation Scoring

As only the 129Ola wild-type mice had TSE vacuolation, their data were compared to observe any changes in the targeting of pathology following subpassage, this may indicate strain adaptation. The number of wild-type mice positive for TSE vacuolation confirmed the clinical findings in that the HuVV inoculum gave the highest score (10/12) followed by the HuMM inoculum (6/11), and lastly the HuMV inoculum (1/11). Lesion profiles could therefore only be generated for 129Ola wild-type mice inoculated with HuMM and HuVV derived material; these showed similar patterns. In wild-type mice inoculated with HuMM derived inoculum, the lesion profile is identical to that of primary vCJD inoculation in wild-type mice and therefore different from the profile in the inoculum donor mouse. (Figure 4.F7, Panel A)

Comparison of the second pass lesion profiles in wild-type mice with those of primary pass vCJD shows close similarities (Figure 4.F7, Panels B and C). Primary pass vCJD at 1% concentration gave a profile with lower scores for grey matter and higher scores for white matter regions, whereas with 10% concentration inoculum the profiles were more similar.

Figure 4.F7: Second Passage Lesion Profile Comparisons for 129Ola Mice

Lesion profile comparison of 129Ola mice following second passage from two transgenic lines and the initial primary passage of vCJD.

Key:

Open Triangles: Primary pass vCJD (1% inoculum) in 129Ola

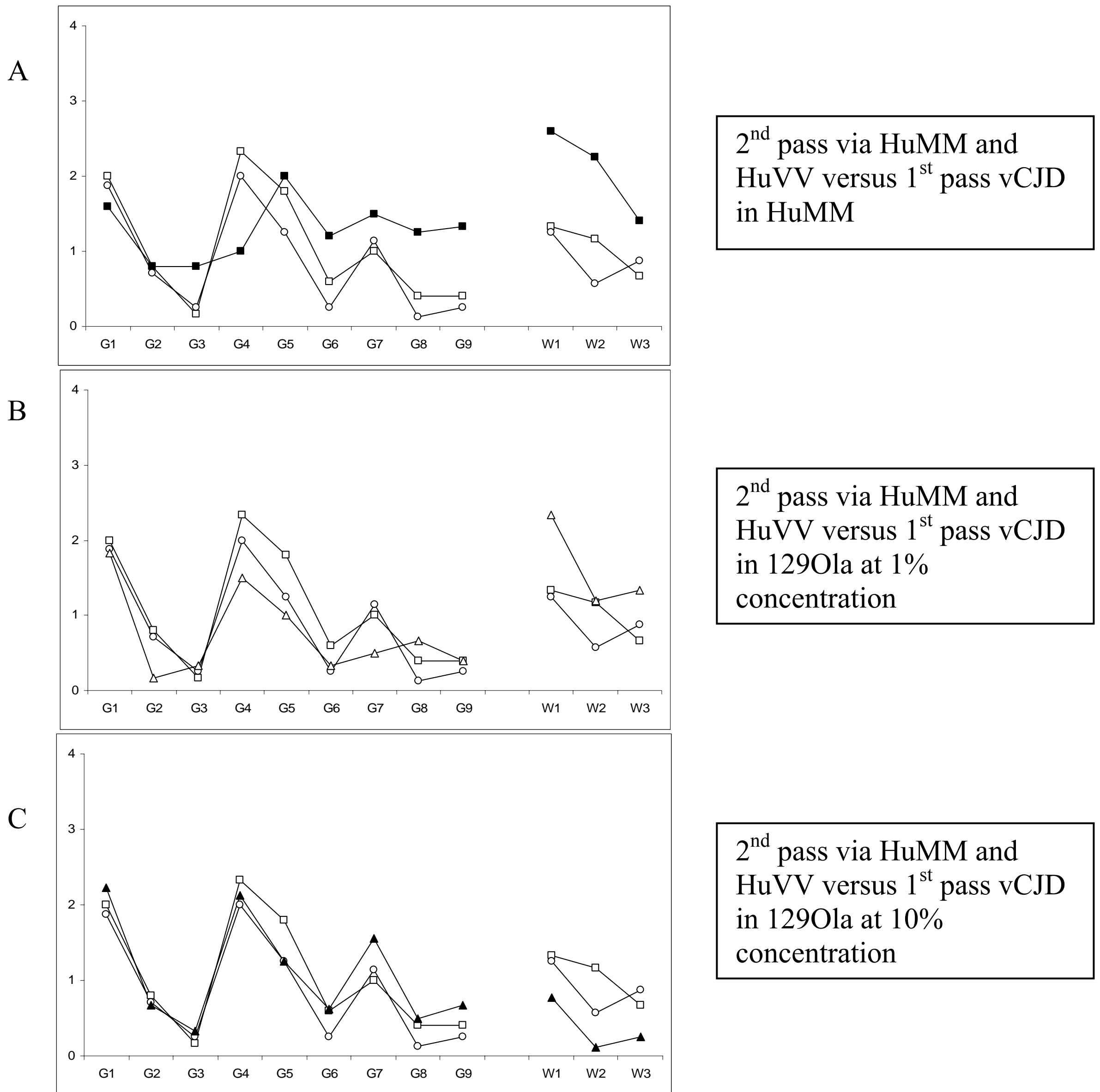
Filled Triangles: Primary pass vCJD (10% inoculum) in 129Ola

Open Squares: Second pass vCJD via HuMM in 129Ola

Filled Squares: Primary pass vCJD in HuMM

Circles: Second pass vCJD via HuVV in 129Ola

G1-G9: Grey matter scoring regions. W1-W3: White matter scoring regions.



4.3.2.3 ICC Detection of PrP^{Sc}

All transgenic mice in the three experiments were negative for vacuolation scoring, but PrP^{Sc} was detected by ICC in a number of mice from each experiment, suggesting a carrier or subclinical disease stage (Table 4.T4). HuMM mice gave positive ICC scores with each of the three inocula, with the number of positives directly proportional to the presence of the methionine allele (HuMM inoculum: 7/12; HuMV: 5/13; HuVV: 2/11). HuMV mice gave positive ICC scores for only the HuMM (2/12) and HuMV (1/11) derived inocula. These scores were lower than seen for the HuMM recipients. No HuVV mice were positive by ICC with any of the three inocula.

It appears that regardless of the codon 129 genotype origin of the inocula the transmission strain characteristics are remarkably similar to that of the primary vCJD source. This is also confirmed by the mice that were ICC positive (HuMM and HuMV), in which the brain region distribution, and type of PrP^{Sc} deposits, were similar to primary pass vCJD in mice that died at early time points, with mainly thalamic targeting, large intensely stained plaques, and no evidence of florid plaques.

Detection of PrP^{Sc} has confirmed positive transmission in wild-type mice that had clinical TSE and positive vacuolation scores. The distribution and type of PrP^{Sc} deposits looked identical to that seen in primary pass vCJD, as suggested by the lesion profile patterns. The number of ICC positive mice reflected the scores for vacuolation and clinical signs in that the codon 129 homozygous inocula resulted in a high proportion of positives (HuMM: 10/12; HuVV: 12/12) while the heterozygous (MV) inoculum had a low number of positives (2/11).

4.3.3 Intraperitoneal Inoculation of vCJD

Twenty-four mice of each line (HuMM, HuMV, HuVV, 129Ola) were inoculated by the intraperitoneal (i.p.) route with 1% inoculum prepared from the NIBSC vCJD standard (Code NHBY0/0003, see Methods). This alternative injection route to the previous intracerebral experiment was chosen as it may be a more relevant route. The BSE agent is believed to have infected humans via the oral route and therefore the agent will have had to spread through the peripheral nervous system to eventually target the CNS. VCJD pathology is different from other forms of human TSE, and non-TSE diseases that involve lymphoreticular tissue (such as human immunodeficiency virus infection), in that PrP^{Sc} deposition is found in peripheral tissues such as spleen and appendix (Hilton *et al.*, 2004b). Oral dosing of mice is an option, however this route can be less efficient, and the inoculated mice may require a thorough investigation of the gut and peripheral nervous tissues to observe differences between the difference mouse lines. The aims of this thesis concentrate on the role of codon 129 genotype rather than modelling the route of BSE infection in humans.

Due to the long duration of these experiments intercurrent illness and death due to non-TSE causes were observed. Where possible these mice were examined for markers of TSE pathology. Table 4.T5 shows the evaluation of all mice in this study.

Table 4.T5: Data Table for Intraperitoneal Inoculation with vCJD

Intraperitoneal inoculation data (protocol reference 553G-1A)

Abbreviations: CLIN: clinical TSE signs; VACN: presence of TSE vacuolation; ICC: presence of abnormal PrP by immunocytochemistry; ICD: intercurrent death or culled for animal welfare reasons; n/d: no data available as tissue not testable; n/t: not tested at this time; shaded cell: positive score

HuMM

ID	Survival Time	Reason for cull	CLIN	VACN	ICC
66	238	ICD	Neg	Neg	Neg
61	285	ICD	Neg	Neg	Neg
29	336	ICD	Neg	Neg	Neg
52	357	ICD	Neg	Neg	Neg
54	364	ICD	Neg	Neg	Neg
51	419	ICD	Neg	Neg	Neg
78	434	ICD	Neg	Neg	Neg
62	476	ICD	Neg	Neg	Neg
74	476	ICD	Neg	Neg	Neg
64	480	ICD	Neg	Neg	Neg
65	487	ICD	Neg	Neg	Neg
63	525	ICD	Neg	Neg	Neg
49	531	ICD	Neg	Neg	Neg
73	545	ICD	Neg	Neg	Neg
76	560	ICD	Neg	Neg	Neg
50	567	ICD	Neg	Neg	Neg
26	630	ICD	Neg	Neg	Neg
25	672	ICD	Neg	Neg	Neg
77	686	ICD	Neg	Neg	Neg
30	698	ICD	Neg	Neg	Neg
53	700	ICD	Neg	Neg	Neg
27	730	ICD	Neg	Neg	Neg
'Attack Rate'			0/22	0/22	0/22

HuMV

ID	Survival Time	Reason for cull	CLIN	VACN	ICC
103	280	ICD	Neg	Neg	Neg
45	308	ICD	Neg	Neg	Neg
20	356	ICD	Neg	Neg	Neg
112	378	ICD	Neg	Neg	Neg
101	418	ICD	Neg	n/d	Neg
105	476	ICD	Neg	Neg	Neg
111	476	ICD	Neg	Neg	Neg
23	481	ICD	Neg	Neg	Neg
114	483	ICD	Neg	Neg	Neg
116	504	ICD	Neg	Neg	Neg
43	539	ICD	Neg	Neg	Neg
21	545	ICD	Neg	Neg	Neg
46	553	ICD	Neg	Neg	Neg
106	553	ICD	Neg	Neg	Neg
24	560	ICD	Neg	Neg	Neg
104	574	ICD	Neg	Neg	Neg
44	637	ICD	Neg	Neg	Neg
115	638	ICD	Neg	Neg	Neg
47	644	ICD	Neg	Neg	Neg
102	679	ICD	Neg	Neg	Neg
19	699	ICD	Neg	Neg	Neg
113	700	ICD	Neg	Neg	Neg
22	735	ICD	Neg	Neg	Neg
'Attack Rate'			0/23	0/22	0/23

HuVV

ID	Survival Time	Reason for cull	CLIN	VACN	ICC
59	160	ICD	Neg	Neg	Neg
42	377	ICD	Neg	Neg	Neg
39	471	ICD	Neg	Neg	Neg
57	511	ICD	Neg	Neg	Neg
37	532	ICD	Neg	Neg	Neg
58	539	ICD	Neg	Neg	Neg
89	567	ICD	Neg	Neg	Neg
90	567	ICD	Neg	Neg	Neg
98	588	ICD	Neg	Neg	Neg
86	589	ICD	Neg	Neg	Neg
60	603	ICD	Neg	Neg	Neg
56	616	ICD	Neg	Neg	Neg
88	620	ICD	Neg	Neg	Neg
38	630	ICD	Neg	Neg	Neg
97	637	ICD	Neg	Neg	Neg
55	651	ICD	Neg	Neg	Neg
85	665	ICD	Neg	Neg	Neg
87	686	ICD	Neg	Neg	Neg
41	714	ICD	Neg	Neg	Neg
100	826	ICD	Neg	Neg	Neg
'Attack Rate'			0/20	0/20	0/20

129Ola

ID	Survival Time	Reason for cull	CLIN	VACN	ICC
94	329	ICD	Neg	n/d	n/t
32	427	ICD	Neg	Neg	n/t
93	432	ICD	Neg	n/d	n/t
36	440	ICD	Neg	Neg	n/t
18	467	ICD	Neg	Neg	n/t
91	476	ICD	Neg	Neg	n/t
95	483	TSE	Pos	Pos	Pos
82	484	TSE	Pos	Pos	Pos
84	496	ICD	Neg	Neg	n/t
79	508	TSE	Pos	Pos	Pos
83	518	TSE	Pos	Pos	Pos
81	532	ICD	Neg	Neg	n/t
31	539	ICD	Neg	Neg	n/t
15	540	ICD	Neg	Pos	Pos
96	540	TSE	Pos	Pos	Pos
80	560	TSE	Pos	Neg	n/t
92	588	TSE	Pos	Pos	Pos
33	601	TSE	Pos	Pos	Pos
35	609	TSE	Pos	Pos	Pos
13	622	ICD	Neg	Pos	Pos
16	630	ICD	Neg	Neg	n/t
14	637	TSE	Pos	Neg	n/t
34	662	ICD	Neg	Neg	n/t
17	735	ICD	Neg	Neg	n/t
'Attack Rate'			8/24	10/22	10/10

4.3.3.1 Clinical TSE Findings

In the i.p. inoculation experiment there was no evidence of clinical disease, confirmed by pathology, in any of the three transgenic lines. A number of mice displayed signs suggestive of clinical TSE, but these were negative by pathological analysis, and were not classed as positive for transmission. Confirmation that the inoculum was infectious came from the 129Ola mice. These mice showed confirmed clinical TSE in eight of the twenty-four mice with a mean incubation period of 541 days (range 483 to 609). Comparing this data to that obtained from the i.c. challenge there is a 43 day lengthening of the incubation period and an overall reduction in clinically positive mice (8/24 i.p. versus 8/15 i.c.).

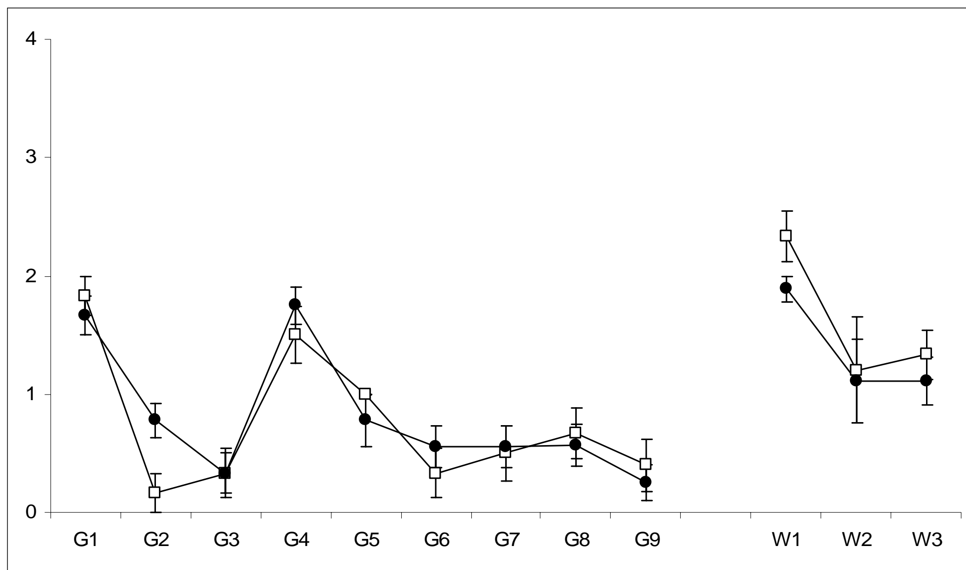
4.3.3.2 Vacuolation Scoring and Detection of PrP^{Sc} by ICC

In addition to finding no clinically positive transgenic mice there was also no evidence of TSE vacuolation, or detectable amounts of PrP^{Sc} by ICC, in the brains of these mice. There may however be peripheral tissue deposition due to the inoculation route used. Spleen material is available for future investigation of these mice.

The wild-type mice that developed clinical disease were scored positive for vacuolation and PrP^{Sc} detection, as expected. The lesion profile for the wild-type vacuolation scores showed an identical pattern to that found for i.c. challenge, with the inoculum at 1% concentration (Figure 4.F8).

Figure 4.F8: Lesion Profile of 129Ola Mice Following Intraperitoneal Inoculation with vCJD

Lesion profile for 129Ola mice i.p. challenged with vCJD at 1% (closed circles), together with data for i.c. challenge at 1% (open squares) concentration. (Mean score ±SEM; G1-G9: grey matter scoring regions, W1-W3: white matter scoring regions)



4.4 DISCUSSION

4.4.1 A New Mouse Model for Human vCJD

The initial goal of this study was to assess the suitability of this transgenic mice model for studying the vCJD strain of TSE.

4.4.1.1 Intracerebral Inoculation Results

With varying efficiency vCJD has been transmitted via the i.c. route to each of the three codon 129 genotype mouse lines, with success rates of 75% for HuMM, 79% for HuMV, and 7% for HuVV mice. The secondary passage of brain material from these mice in further transgenic and wild-type mice has demonstrated the presence of a transmissible agent. As these mice were generated by gene targeting and express the transgenic prion protein at physiological levels these data show that to successfully transmit disease and produce clinical TSE symptoms it is not essential to overexpress PrP, which may itself cause unknown effects on transmission. Gene targeting has produced genetically identical lines varying only at codon 129 of *PRNP* allowing for a direct assessment of the properties of each line.

Transmission of the vCJD agent to these transgenic mice did not produce shorter incubation periods than the 129Ola wild-type mice which readily developed clinical TSE. Replacing the mouse *Prnp* gene with the human version should hypothetically increase susceptibility to a human TSE inoculum as there would be homologous PrP interaction. This is proposed to be an efficient mechanism for PrP^C conversion (Prusiner, 1991, Horiuchi *et al.*, 2000). Data from transgenic mice expressing the methionine allele of human PrP (published since the start of this

thesis) have shown that the clinical phenotype is rarely observed following vCJD inoculation. With three separate sources of vCJD the Kitamoto group's KiChM (1x expression, MM genotype) line showed clinical TSE signs in 0/6, 1/5, and 1/5 mice, and with three other cases the Collinge group's Tg35 (1-2x expression, MM genotype) line showed clinical TSE in 0/2, 1/5, and 0/7 mice. Pathological analysis in these studies however indicated positive transmission in over 90% (27/29) of the mice (Asante *et al.*, 2002, Taguchi *et al.*, 2003). A comparison of the data from this study with that available from the Kitamoto group on vCJD transmission to transgenic mice expressing the human *PRNP* gene at physiological levels, is shown in Table 4.T6. Independent generation of these transgenic lines has lead to near identical transmission properties for the vCJD agent.

Table 4.T6: Comparison with Kitamoto Group Data on vCJD Transmission

Comparison of vCJD transmission in the Kitamoto transgenic lines with data from this study

Name of Line	HuMM	KiChM	HuMV	KiChMV	HuVV	KiChVV
Group (Reference)	This study *(Bishop* et al., *2006)*	Kitamoto (Taguchi et al., 2003)	This study (Bishop et al., 2006)	Kitamoto (Asano et al., 2006)	This study (Bishop et al., 2006)	Kitamoto (Asano et al., 2006)
Codon 129	MM	MM	MV	MV	VV	VV
Expression level	x1	x1	x1	x1	x1	x1
Total Affected	12/16	13/16	11/14	13/17	1/15	0/3
Total Affected (%)	75%	81%	79%	76%	7%	0%

The most over-expressing MM line (6x) published by the Laude group is the only line to show 100% clinical disease with vCJD inoculation (Beringue *et al.*, 2008). This line developed disease at ~500 days, indicating that significant over-expression is required to lower the incubation period to a point shorter than the usual mouse lifespan.

Lack of clinical disease in the transgenic mice may indicate that there was a lengthy subclinical phase during which pathological changes were occurring, for example deposition of PrP^{Sc}. Future transmission experiments aimed at determining the time-scale for the appearance and distribution of TSE pathology may model the extent of subclinical disease, or a carrier state, in humans and the spread of disease through different peripheral tissues.

Data from this study suggest that subclinical pathological changes associated with TSE disease are more severe in the HuMM mice compared with the non-HuMM lines, such as increased numbers of mice positive for TSE vacuolation and more widespread, more intense deposits of PrP^{Sc}. This may manifest as a change in survival rate rather than directly through the appearance of a TSE clinical phenotype as shown by the Kaplan-Meier curves and the Tarone-Ware statistical tests (Section 4.3.1.3 Survival Analysis).

Assessment of TSE vacuolation showed differences between the HuMM transgenic and wild-type lesion profiles, indicating a possible effect on pathological targeting arising from replacing the murine *Prnp* gene with the human equivalent. Differences in the lesion profiles may also be due to data from clinically positive 129Ola wild-type mice being compared with clinically negative HuMM mice.

4.4.1.2 Intraperitoneal Inoculation Results

Intraperitoneal inoculation did not show CNS evidence of TSE disease in any of the transgenic mice, compared with 33% of the wild-type mice. Previous studies of BSE transmission to wild-type mice have shown that the i.p. route may be less efficient than the i.c. route but can produce shorter incubation times (Bruce *et al.*, 1994). This inefficiency of the i.p. route may be the reason for the results seen here. As the occurrence of clinical disease was rare with i.c. route inoculation, and the incubation time for i.p. inoculated wild-type mice was extended, it is perhaps not surprising that there were no transgenic mice with clinical TSE in this experiment. As these experiments were set up concurrently we were not able to predict this potential negative result. Although this suggests that i.p. inoculation of vCJD is not as efficient in these mice analysis of peripheral tissues from these mice may show that transmission has occurred without CNS involvement. Spleen tissue is available from this experiment for such a study.

The similarities between the i.c. and i.p. vacuolation data for the wild-type mice indicate that the vCJD strain targets similar regions of the brain even when infection has occurred by direct injection into the brain or via a peripheral route. It is, however, difficult to explain why the i.p. route in the transgenic mice was dramatically less efficient, with no clinical signs or pathological markers of TSE disease in the brain. Lack of CNS evidence of transmission is important, as the propagation of human PrP^{Sc} through peripheral tissues may occur at a much lower rate to that of mouse PrP^{Sc}. This could suggest one reason for the low numbers of vCJD cases seen. In some individuals the disease would never progress further than

the peripheral nervous tissues. This effect may vary with codon 129 genotype, with MM being the most susceptible to progression of the disease to the CNS.

4.4.1.3 Replication of vCJD Pathological Features

In addition to the successful transmission of vCJD to the transgenic mice, two of the pathological characteristics of vCJD in humans have been replicated.

The first is the appearance of florid plaques. These structures are not unique to vCJD, but are characteristic of this disease in comparison to the majority of other human TSEs (Kretzschmar *et al.*, 2003). They have only been seen in MM genotype vCJD cases. Data from i.c. inoculation of transgenic mice has shown that these structures occur only in the HuMM line (see Figure 4.F4). The formation of florid plaques in the hippocampus and immediate surrounding area of cerebral cortex may be due to the types of cells present or the nature of the cellular environment. Human vCJD cases show florid plaques in most cortical areas and predominantly in the occipital cortex (the rearmost lobe of the brain involved with visual processing) and the cerebellar cortex. (The human cerebellum integrates sensory perception and motor control.) This regional distribution of specific pathological features may be defined by the host and the stage of disease. As clinical symptoms were rare in these HuMM mice, indicating a preclinical stage, the distribution of florid plaques may have been less widespread than in clinical vCJD. The reason why only MM genotype humans and for this study, mice, develop these types of structures is unknown. It appears to be a response of both the infectious strain and the host genotype.

The second pathological feature replicated in these mice is the type 2B Western blot vCJD pattern (19kDa mobility and dominance of the diglycosylated

fragment). This Western blot type was found in transgenic mice of all codon 129 genotypes suggesting that this strain property is dominant regardless of the genotype, and that protein typing might prove useful for the diagnosis of future human cases of vCJD infection in non-MM individuals, should this occur.

An important use of the HuMV mice infected with vCJD is to further understand whether there is dominance of the M allele over the V allele in conversion of PrP^{C} to PrP^{Sc}. If the V allele is protective for this strain then administering V-PrP^{C} to MM genotype individuals could slow the progression of vCJD disease pathology.

4.4.2 Codon 129 Genotype and Transmission Properties of the vCJD Agent

A primary aim of this study was to examine the role of codon 129 genotype in susceptibility to disease.

4.4.2.1 Clinical Disease Associated with Genotype

Clinical symptoms of TSE disease were confirmed in only two HuMM and a single HuMV mice inoculated with vCJD. This appears to reflect the relative rarity of secondary vCJD cases in humans. BSE inoculation of the three transgenic lines showed no transmission. In the human context this may mean that the vCJD agent is more infectious than BSE and may be more efficient in infecting further people. The appearance of clinical TSE in only HuMM and HuMV mice confirms the codon 129 methionine allele dominance and that iatrogenic vCJD may only result in clinical disease in M allele carriers (MM and MV). Survival analysis indicates a deleterious subclinical effect for the MM genotype mice only, further

highlighting this genotype as preferentially targeted by this TSE agent. As the progressive development of CNS TSE pathology is faster in MM than MV or VV mice, there may also be additional early peripheral pathology in the MM mice that may reflect the changes in survival seen in this study.

4.4.2.2 Vacuolation Scoring Associated with Genotype

TSE vacuolation was found almost exclusively in the HuMM mice and its appearance coincided with an increase in deposition of PrP^{Sc}. The appearance of TSE associated vacuolation in similar areas in each of the three lines suggests an agent strain specific effect and the varying levels of vacuolation seen in the different genotypes indicates that the degree of pathological change is determined by the host genotype. If the degree of TSE vacuolation is a predictor of the development of further pathological features then the host genotype may predispose an individual to relatively short or long incubation periods following infection.

4.4.2.3 PrP^{Sc} Properties Associated with Genotype

PrP^{Sc} deposition was the most sensitive test for successful transmission detecting the earliest signs of TSE after inoculation. This was seen in the HuMV mice in which nine of the eleven mice scoring positive had no other signs of TSE except for PrP^{Sc} deposition. Equivalent numbers of HuMM and HuMV mice were positive for PrP^{Sc} but the former had more widespread regions affected and a greater level of deposition. PrP^{Sc} deposition in the HuMV mice never developed further than a few isolated regions (Figure 4.F4), providing further evidence for the protective nature of the valine allele.

To summarise the results of ICC detection of PrP^{Sc}, both the HuMM and HuMV mice were equally likely to show deposits of the abnormal protein in the brain over the lifespan of the mouse. However, the HuMM mice had a significantly more progressive increase in the extent of the deposits over time. The HuVV data suggest an extended delay before the appearance of this disease marker. The appearance of PrP^{Sc} at a stage before the development of TSE vacuolation or clinical disease suggests that this is an important early phase in pathogenesis. If this model is extrapolated to human disease there may be a lengthy subclinical stage, before clinical symptoms appear, following infection with BSE or vCJD. Assuming that the appearance of PrP^{Sc} is associated with infectivity, this early subclinical stage could be a period in which human-to-human infection may occur via blood products or contaminated surgical instruments.

Western blotting has shown that each genotype mouse line has faithfully replicated the type 2B pattern, in terms of electrophoretic mobility and glycosylation, indicating that this is a dominant strain transmission characteristic and not dependent on host genotype. This may aid diagnosis of human cases in non-MM hosts, as in the MV genotype blood transfusion transmission case (Peden *et al.*, 2004). There was a relative increase in the diglycosylated form of PrP^{Sc} in the single HuVV mouse. If this is a true reflection of the VV genotype response then it may be due to the effect the valine amino acid has on the structure of PrP^{Sc}. Potentially if valine at position 129 protects the diglycosylated protein from protease cleavage, to a greater degree than the mono or unglycosylated forms, then the former will be over-represented on the Western blot. Further analysis of the biochemical properties of V-PrP^{Sc} may provide insight into the folding properties of the prion

protein. However, transmission of vCJD to over-expressing VV genotype mice did not show an increase in diglycosylation (Wadsworth *et al.*, 2004) indicating that the abnormal abundance of PrP^{Sc} in these mice may lead to an alternative folding or aggregation state of PrP^{Sc} (Dimcheff *et al.*, 2003).

4.4.3 Modelling Iatrogenic Spread of vCJD

The data provided on vCJD transmissions give an insight into both the infectious nature of the vCJD agent and the potential for iatrogenic spread in humans (already confirmed through blood transfusion), the secondary aim of this study.

Secondary passage of brain material from vCJD infected mice of each codon 129 genotype was used to model more specifically the iatrogenic spread of vCJD originating from each genotype host. Data from this study indicate that transgenic mice primarily infected with vCJD develop a transmissible disease that retains some properties of the original strain. The response of the recipient mice showed that once again the MM genotype is the most susceptible, with the MV mice less so (Table 4.T4). The VV mice showed no evidence of disease and therefore may have a protective genotype. This result was the same for inocula sourced from MM, MV, and VV mice.

Positive transmission scoring in the HuMM and HuMV mice was however restricted to just the deposition of PrP^{Sc} by ICC. There was no evidence of clinical disease or vacuolation. This may be suggestive of an adaptation to a less infectious strain, or to a difference in level of infectivity between the primary and secondary passage inocula.

4.4.3.1 Secondary Passage to Wild-Type Mice

Wild-type mice developed clinical and pathological signs of TSE from all three genotype sources of inocula. Secondary passage of the HuMM source inoculum to wild-type mice produced a lengthening of the incubation period by approximately 50 days. This minor effect could be due to a lower level of infectivity in the mouse brain tissue or due to a minor adaptation of the agent by the mouse host, e.g. the human *PRNP* expressed form of PrP^{Sc} will now have mouse derived N-linked glycosylation compounds which may cause changes in PrP^{C} to PrP^{Sc} conversion efficiency.

The rarity of positive transmission to wild-type mice for the HuMV derived inoculum may also be due to the level of infectivity as the donor mouse was negative for vacuolation and had only relatively small quantities of PrP^{Sc} detectable by ICC (Figure 4.F6). It is also possible that the heterozygous PrP^{Sc} of the inoculum is configured or aggregated in such a way that conversion of the wild-type PrP^{C} is less efficient (c.f. heterozygote 'protection' in kuru and iCJD (Brown *et al.*, 1994a, Mead *et al.*, 2003)).

The HuVV derived inoculum transmitted more efficiently at second passage to wild-type mice, than primary vCJD, with lower incubation period and higher rates of positive scoring for pathological signs. This may be due to removal of the species barrier or a higher level of infectivity in the HuVV mice. Potentially the altered configuration of the HuVV derived PrP^{Sc} (greater abundance of the diglycosylated band by Western blot) may be more efficient in converting the wild-type mouse PrP^{C} which is known to be heavily diglycosylated (Chapter 3, Figure 3.F4).

The lesion profiles generated for 129Ola wild-type mice from HuMM and HuVV derived inocula show close similarities to primary transmission of vCJD in these mice. Together with the incubation period data, this suggests that the original vCJD strain properties have not been altered by passage through different genotype transgenic mice. However, as the transgenic mice show significant differences to the primary transmission there is potential for some form of strain adaptation specific to hosts expressing human PrP.

CHAPTER 5

ADAPTATION OF THE VCJD STRAIN FOLLOWING BLOOD TRANSFUSION ASSOCIATED VARIANT CJD INFECTION

5.1 AIMS

Human-to-human transmission of vCJD, via blood transfusion, may have caused adaptation of the infectious agent to a form that is now more efficiently transmissible to humans, or may cause more rapidly progressive pathological changes. This chapter aims to use data from inoculation of transgenic and wild-type mice, with brain material from the first case of blood transfusion associated vCJD infection, to determine if the vCJD strain has been modified by human-to-human transmission. Comparison with the vCJD (BSE) transmission data described in Chapter 4 will highlight any differences that can be attributable to modification of the BSE agent following human-to-human passage.

Intracerebral inoculation of brain homogenate (at 1% concentration) was performed on the three transgenic lines (HuMM, HuMV, and HuVV) and the 129Ola wild-type control line. These mice were assessed for clinical TSE signs, for TSE associated vacuolation (lesion profiling), and for targeting and biochemistry of the disease associated PrP^{Sc} by ICC and Western blot.

Data from this study were published in 2008 in PLoS ONE (See Appendix) (Bishop *et al.*, 2008).

5.2 INTRODUCTION

Four cases of possible blood transfusion associated infection by the vCJD agent have been identified (Llewelyn *et al.*, 2004, Peden *et al.*, 2004, Hewitt *et al.*, 2006, Wroe *et al.*, 2006, HPA, 2007). Three of these developed clinical signs and were MM at codon 129 of the prion protein gene. The fourth case died of a ruptured abdominal aortic aneurysm but was found by *post mortem* analysis to be positive for evidence of vCJD infection. This individual was heterozygous, MV, at codon 129 (Peden *et al.*, 2004) (See Introduction, section 1.6.6.3 Variant CJD). These four cases were from a small group of 28 individuals, known to have received blood products from vCJD donors, who survived more than five years after the transfusion. This rate of infection appears high compared with the few cases of vCJD that have arisen from a large number of infectious cattle in the food chain, and therefore places those others that are known to have received similar blood products, at significant risk of vCJD infection. The codon 129 genotype of these vCJD blood product recipients is unknown.

It is also important to understand that all the blood donors involved with the above cases were in a subclinical phase of vCJD infection. The longest period between blood donation and onset of clinical symptoms was 3 years and 4 months (the case studied in this thesis) (Llewelyn *et al.*, 2004), suggesting that, although the clinical and pathological changes such as neuronal loss and deposition of PrP^{Sc} may not have developed the blood donor was nevertheless harbouring infectivity.

The incidence of infection among blood recipients in transfusion transmission of vCJD suggests that human derived disease is more infectious or has a greater efficiency of transmission than BSE. The alternative is that the route of infection is more efficient. Blood transfusion infection was by the intravenous (i.v.) route whereas it is likely that BSE infection was by the oral route. The i.v. route may be a more efficient route as, experimentally, a high proportion of orally administered PrP^{Sc} can be destroyed in the gut (Jeffrey *et al.*, 2006). It is also a possibility that a greater infectious dose was given via blood transfusion compared with the dose of BSE taken orally (See Introduction, section 1.6.6.3 Variant CJD).

In comparing vCJD and human passaged vCJD it is also important to note that the infectious material transmitting disease was different and that this may impact on the efficiency of transmission. BSE infection of humans was likely to have involved consumption of meat contaminated with brain and CNS material whereas human-to-human infection was via labile blood products. There is a possibility that PrP^{Sc} molecules present in these two very different cellular environments have different transmission properties. Blood PrP^{Sc} configuration or even its presence is still unknown (Brown, 2005). Blood may be infectious with limited levels of PrP^{Sc}. Evidence for such disparity between low or undetectable levels of PrP^{Sc} and high levels of infectivity has been published for a transgenic mouse model of the human genetic TSE disease, GSS (Barron *et al.*, 2007).

Data from vCJD infection of transgenic mice (Chapter 4) suggests that all three codon 129 genotype individuals are susceptible to the vCJD agent which means that a significant number of the UK population may be carriers of infectivity with some, such as those who are VV, potentially never developing clinical signs. This

may lead to a large pool of infectivity which could spread via medical practice (Garske *et al.*, 2006). There is currently no evidence as yet for vCJD infection via surgery, but, if the incubation period in those exposed is long, e.g. >20 years, then such cases may not yet have occurred. Medical history taken from each vCJD case allows traceability of events such as surgical procedures so that future vCJD transmission cases can be linked to a likely point of infection.

Hypothetically if the vCJD agent was propagated by human-to-human infection within the population, then assuming long incubation periods, the agent could become widespread. A self-sustaining epidemic of a human-adapted form of vCJD is thought unlikely as transmission would only occur in those receiving blood products or undergoing surgery. This is a small fraction of the population and these individuals are unlikely to infect others (Clarke *et al.*, 2007). Deferral of individuals as blood donors if they themselves have had a blood transfusion is a means of preventing the onward spread of infection by this route (Joint UKBTS/NIBSC Professional Advisory Committee, Change Notification 5, 2005: http://www.transfusionguidelines.org.uk/docs/pdfs/change_note_2005_05.pdf).

Serial passage of BSE in sheep using infected sheep brain material showed that secondary transmission occurred, but assessment of clinical onset times and pathology, provided no evidence of strain adaptation (Gonzalez *et al.*, 2007). In contrast serial passage of cattle BSE in non-human primates showed strain adaptation with a 50% reduction in incubation period, although there were no differences in the intensity of pathological changes such a vacuolation (Lasmezas *et al.*, 2001).

This chapter details work carried out to define the transmission properties of the infectious agent found in the first case of blood transfusion associated vCJD. Using data from Chapter 4 (vCJD (BSE) inoculation) a comparison was made of the presence of clinical disease, TSE vacuolation, and PrP^{Sc}, and mean incubation periods and Western blot profiles. These parameters were assessed to determine whether the vCJD agent has been adapted to humans to become more infectious.

5.3 EXPERIMENTAL RESULTS

5.3.1 Intracerebral Inoculation with Transfusion Associated vCJD

Eighteen mice of each transgenic line (HuMM, HuMV, HuVV) and twelve wild-type 129Ola mice, were inoculated by the intracerebral (i.c.) route, with inoculum prepared from a sample of frontal cortex provided by Prof James Ironside (National CJD Surveillance Unit, Edinburgh) from the blood transfusion case (reference RU 03/125), here designated vCJD (transfusion). The concentration of inoculum (1% (10^{-2})) was the same as the vCJD inoculum, here designated vCJD (BSE), used in previous studies. Because of the long duration of these experiments, intercurrent illness and death due to non-TSE causes were observed. Where possible these mice were examined for markers of TSE pathology. Table 5.T1 shows the evaluation of all mice in this study.

Table 5.T1: Data Table for Intracerebral Inoculation with vCJD (transfusion)

Intracerebral inoculation of vCJD (transfusion) with data ordered by Survival Time. (Protocol reference 553K-1A)

Abbreviations: CLIN: presence of clinical signs; VACN: presence of TSE vacuolation; ICC: presence of abnormal PrP by immunocytochemistry; ICD: intercurrent death or culled for animal welfare reasons; n/d: no data available as tissue not testable; shaded cell: positive score.

HuMM						HuMV						HuVV						129Ola					
ID	Survival Time	Reason for cull	CLIN	VACN	ICC	ID	Survival Time	Reason for cull	CLIN	VACN	ICC	ID	Survival Time	Reason for cull	CLIN	VACN	ICC	ID	Survival Time	Reason for cull	CLIN	VACN	ICC
47	365	ICD	Neg	Neg	Neg	21	300	ICD	Neg	Neg	Neg	54	162	ICD	Neg	Neg	Neg	19	383	ICD	Neg	Neg	Neg
42	407	ICD	Neg	Neg	Pos	38	302	ICD	Neg	Neg	Neg	7	244	ICD	Neg	Neg	Neg	16	448	ICD	Neg	Neg	Neg
48	483	ICD	Neg	Neg	Pos	39	368	ICD	Neg	Neg	Neg	12	274	ICD	Neg	Neg	Neg	17	477	ICD	Neg	Neg	Neg
43	538	ICD	Neg	Neg	n/d	22	472	ICD	Neg	Neg	Neg	55	323	ICD	Neg	Neg	Neg	30	547	ICD	Neg	Neg	Pos
44	544	ICD	Neg	Neg	Pos	37	484	ICD	Neg	Neg	Neg	57	477	ICD	Neg	Neg	Neg	35	590	TSE	Pos	Pos	Pos
52	554	ICD	Neg	Neg	Pos	63	519	ICD	Neg	Neg	Neg	58	540	ICD	Neg	Neg	Neg	15	617	ICD	Neg	Neg	Pos
26	618	ICD	Neg	Pos	Pos	40	540	ICD	Neg	Neg	Neg	3	561	ICD	Neg	Neg	Neg	32	617	TSE	Pos	Pos	Pos
45	631	ICD	Neg	Pos	Pos	62	561	ICD	Neg	Neg	Pos	6	614	ICD	Neg	Neg	Neg	31	638	ICD	Neg	Neg	Pos
49	659	TSE	Pos	Pos	Pos	61	593	ICD	Neg	Neg	Neg	53	624	ICD	Neg	Neg	Neg	34	638	ICD	Neg	Neg	Neg
29	673	ICD	Neg	Pos	Pos	64	596	TSE	Pos	Neg	Pos	9	685	ICD	Neg	Neg	Neg	18	666	TSE	Pos	Pos	Pos
51	680	ICD	Neg	Pos	Pos	66	610	ICD	Neg	Neg	Pos	1	693	ICD	Neg	Neg	Neg	33	669	ICD	Neg	Neg	Neg
25	708	ICD	Neg	Pos	Pos	60	638	TSE	Pos	Neg	Pos	10	701	ICD	Neg	Neg	Neg	14	680	TSE	Pos	Pos	Pos
41	708	ICD	Neg	Neg	Pos	59	647	ICD	Neg	Neg	Pos	11	701	ICD	Neg	Neg	Neg						
50	708	ICD	Neg	Pos	Pos	67	659	ICD	Neg	Neg	Neg	5	701	ICD	Neg	Neg	Neg						
24	839	ICD	Neg	Pos	Pos	65	666	ICD	Neg	Neg	Pos	56	750	ICD	Neg	Neg	Neg						
						23	672	ICD	Neg	Neg	Pos	13	764	ICD	Neg	Neg	Neg						
						20	701	ICD	Neg	Neg	Pos	4	869	ICD	Neg	Neg	Pos						
'Attack Rate'			1/15	8/15	13/14				2/17	0/17	8/17				0/17	0/17	1/17				4/12	4/12	7/12

5.3.2 Clinical TSE Findings

Mice scored positive for clinical signs of transmissible spongiform encephalopathy (TSE) were those that had pathological confirmation (either the presence of TSE associated vacuolation and/or the identification of abnormal deposition of PrP by immunocytochemistry (ICC)). The data table shows pathologically confirmed clinical positives for one HuMM mouse (at 659 days post inoculation), two HuMV mice (at 596 and 638 days post inoculation), and none for HuVV mice.

A higher fraction of the 129Ola mice developed to a clinical disease stage (4/12) with a mean incubation period of 638 days (range 590 to 680). This is longer than that seen for vCJD (BSE) inoculation (mean 498 days, in 8/14 mice) and with a reduction in the overall attack rate. This effect could be due to the titre of infectivity in the inoculum and only a titration experiment (inoculating mice with a range of inoculum concentrations) with this material could confirm this. There is still the possibility, however, that this effect is due to an alteration of strain properties. Data are available from this thesis for inoculation of 129Ola mice with vCJD (BSE) material at both 10% and 1% dilution, and together with additional published data (Barron et al., 2001), the lengthening of incubation period following vCJD (transfusion) inoculation is clear (Figure 5.F1). A Kaplan-Meier plot also highlights the differences in survival following vCJD (transfusion) and vCJD (BSE) inoculation (Figure 5.F2).

Figure 5.F1: Incubation Period Plot for 129Ola Mice Inoculated with vCJD

Comparison of mean incubation period (and range) for 129Ola mice inoculated with vCJD (BSE) at two dilutions (10% and 1%) and the vCJD (transfusion) data where the inoculum was prepared at 1% dilution. (* Additional source case of vCJD, data from reference (Barron et al., 2001))

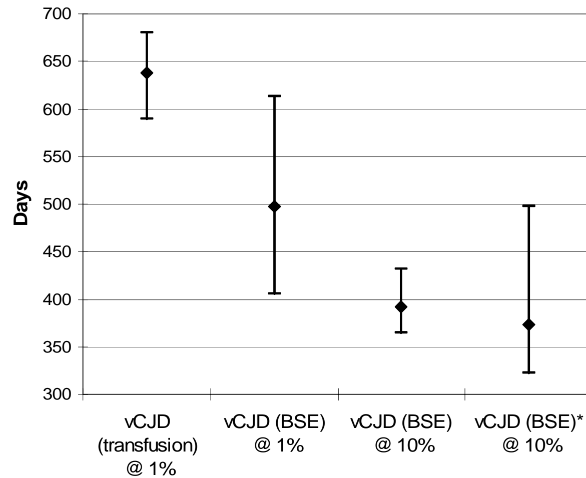
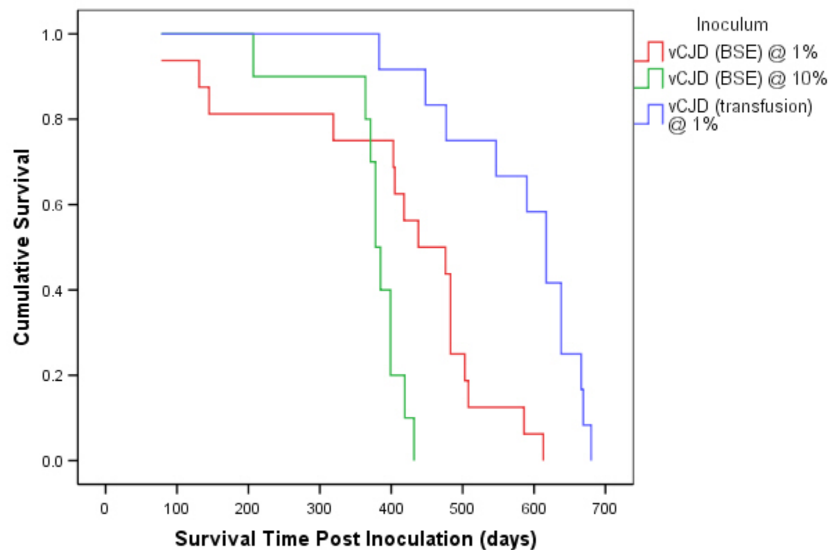


Figure 5.F2: Kaplan-Meier Survival Curve for vCJD Inoculated 129Ola Mice

Survival curves for 129Ola mice inoculated with 10% (green line) and 1% (red line) dilution vCJD (BSE) inoculum and 1% vCJD (transfusion) inoculum (blue line). Statistically significant differences (Tarone-Ware test): vCJD (transfusion) vs. vCJD (BSE) 1%: P=0.001; vCJD (transfusion) vs. vCJD (BSE) 10%: P<0.001. (No significant difference between the vCJD (BSE) inocula.)



5.3.3 Survival Analysis

As seen before with vCJD (BSE) transmission there is little evidence of clinical TSE symptoms and so survival analysis was used to determine whether the variation in rate of intercurrent deaths was specific to the different codon 129 genotypes of the mice. Survival curves were drawn and a Tarone-Ware test performed which has an even bias across the lifespan of the mice, using software SPSS v14.0 (Figure 5.F3 and Table 5.T2). The data show no significant differences between any of the lines, in contrast to vCJD (BSE) inoculation that significantly affected both HuMM and 129Ola mice compared with HuMV and HuVV (See Chapter 4, section 4.3.1.3). The 129Ola mice developed clinical symptoms more readily that the transgenic mice, but, as the incubation time had been extended compared to vCJD (BSE), the curves have become superimposed.

Figure 5.F3: Kaplan-Meier Curve for Inoculation with vCJD (transfusion)

Kaplan-Meier survival curves following i.c. challenge of vCJD (transfusion) in HuMM (blue), HuMV (green), HuVV (red), and 129Ola (black) mice.

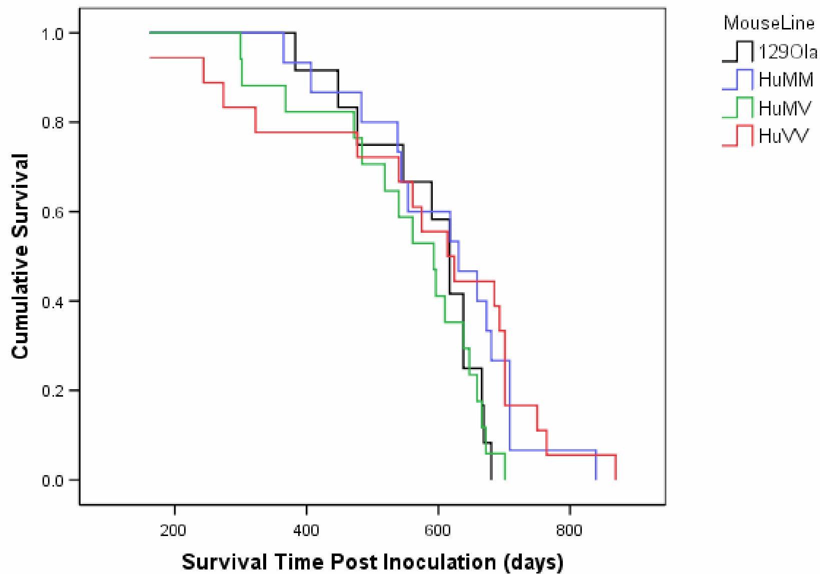


Table 5.T2: Tarone-Ware Test Data for Inoculation with vCJD (transfusion)

Survival analysis by genotype following intracerebral inoculation of vCJD (transfusion). (Statistical analysis: Tarone-Ware Test using software SPSS v14) ns: not significant

Groups Tested	P value (significance rating)
HuMM vs. HuMV vs. HuVV vs. 129Ola	=0.283 (ns)
HuMM vs. HuMV	=0.100 (ns)
HuMM vs. HuVV	=0.962 (ns)
HuMM vs. 129Ola	=0.257 (ns)
HuMV vs. HuVV	=0.168 (ns)
HuMV vs. 129Ola	=0.630 (ns)
HuVV vs. 129Ola	=0.305 (ns)

5.3.4 Vacuolation Scoring

HuMM mice were the only transgenic line to show disease-associated vacuolation, initially detectable at around 600 days post inoculation, approximately 100 days later than HuMM mice challenged with vCJD (BSE) (Chapter 4, Table 4.T1). The number of vacuolation positive mice was higher (8/15 – 53%) than for the vCJD (BSE) inoculum (6/18 – 33%). This may be due to extended survival times for the former mice providing time for development of the vacuolation. The HuMM mice that were scored positive showed a similar distribution of vacuolation in the brain, with the highest levels found in the thalamus and cerebellar white matter, but overall the intensity of vacuolation was less than seen for vCJD (BSE) inoculation, as shown in the lower lesion profile scores. The mean lesion profile from six HuMM mice is shown in Figure 5.F4, compared with the vCJD (BSE) data.

The mean lesion profile for four 129Ola mice is shown in Figure 5.F5 together with data from the vCJD (BSE) experiment. Although routinely five or six mice are required for generating the mean lesion profile these four clinically positive mice gave a profile very similar to that found for vCJD (BSE) transmission and this analysis is therefore included.

Figure 5.F4: Lesion Profile for HuMM Mice Inoculated with vCJD (transfusion)

Vacuolation scoring (mean lesion profile) of HuMM mice i.c. challenged with vCJD (transfusion) (open triangles, ±SEM), and vCJD (BSE) (open squares; ±SEM). (G1-G9: grey matter scoring regions, W1-W3: white matter scoring regions)

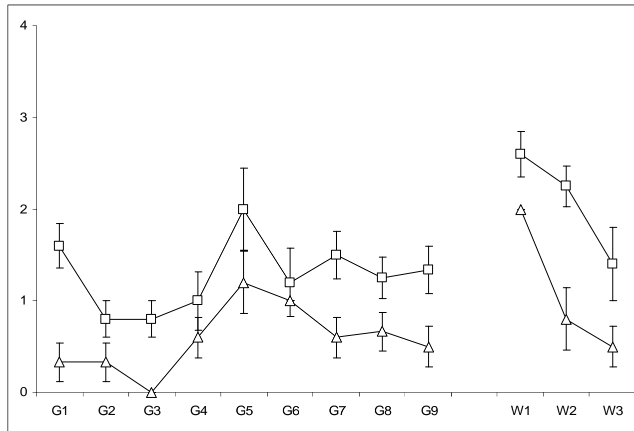
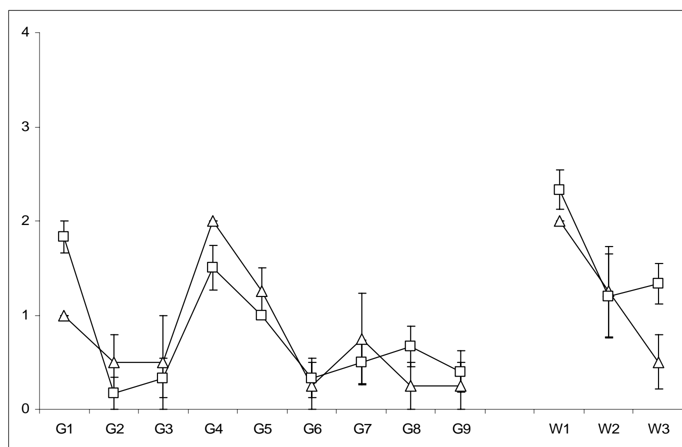


Figure 5.F5: Lesion Profile for 129Ola Mice Inoculated with vCJD (transfusion)

Vacuolation scoring (mean lesion profile) of 129Ola mice i.c. challenge with vCJD (transfusion) (open triangles; ±SEM) and vCJD (BSE) (open squares; ±SEM). (G1-G9: grey matter scoring regions, W1-W3: white matter scoring regions)



5.3.5 Detection of PrP^{Sc} by Immunocytochemistry

Immunocytochemical (ICC) detection of disease associated PrP (PrP^{Sc}) on paraffin sections of mouse brain was found to be a sensitive method of detecting positive transmission for the vCJD (BSE) inoculum. This was seen again in this experiment. The positive / negative transmission scoring is shown in Table 5.T1 (column ICC) and gave a similar attack rate to that found for vCJD (BSE) transmission. A majority of the HuMM mice (13/14) showed PrP^{Sc} deposition in most areas of the brain at a relatively early stage (from around 400 days post inoculation), before the vacuolar pathology became evident around 600 days. The regional distribution of PrP^{Sc} in the brain and the types of deposits seen showed clear similarities to that found for vCJD (BSE) transmission (Figures 5.F6 and 5.F7). Florid plaques were seen only in the HuMM mice confirming the specific vCJD / BSE strain nature of this blood transfusion case (Figure 5.F7). The thalamus was targeted for the most intense deposition (see 'Hippocampus' images in Figure 5.F6, panels 2 and 7) and the 'Forebrain' sections (panels 1 and 6) showed that the cortex contained many intensely stained plaques, many of which were located near the outer surface (sub-pial). The brain stem was only mildly affected, whereas the cerebellum had PrP^{Sc} deposition targeted to the white matter and granular layer regions (panels 4,5,9, and 10).

One HuMM mouse with survival time of 365 days was examined but was negative for PrP^{Sc} deposition. A positive score at 407 days suggested that 350-400 days was the approximate timescale for the onset of PrP^{Sc} deposition for the HuMM mice. The first ICC positive HuMM mouse was found at 368 days for the vCJD (BSE) inoculum.

PrP^{Sc} deposition was identified in approximately half of the HuMV mice (8/17), first appearing at around 550 days, with seven mice negative by ICC between 300 and 540 days. There appears to be a 150 day lengthening of time to onset of PrP^{Sc} deposition in the HuMV mice compared to the HuMM mice. If 550 day survival time was used as a cut-off point for calculating the attack rate then there were 8/10 positives which was similar to the data for vCJD (BSE) inoculation with the same cut-off time (11/13). PrP^{Sc} deposition was targeted to the thalamic nuclei as seen in the vCJD (BSE) data.

Only a single HuVV mouse showed evidence of PrP^{Sc} deposition, at 869 days post inoculation, with very limited deposition found only in the cerebellum. Sixteen mice were negative for deposition at survival times ranging from 162 to 764 days. The single positive HuVV mouse at 869 days suggested an increase in the time period before appearance of this CNS pathology, compared with HuMM and HuMV mice.

ICC positive 129Ola wild-type mice were first found at approximately 550 days concurrent with the appearance of vacuolation. The majority of this group of mice were positive (7/12) as was seen for vCJD (BSE) inoculation (6/6 tested that survived >450 days). A characteristic of 129Ola mice inoculated with vCJD material was the targeting of PrP^{Sc} deposition to the CA2 region of the hippocampus (Brown et al., 2003). This was seen for both vCJD (BSE) and vCJD (transfusion) inoculum (Figure 5.F8).

Figure 5.F6: ICC Comparison in HuMM Mice Inoculated with vCJD (BSE) and vCJD (transfusion)

Immunocytochemistry with anti-PrP antibody 6H4 (brown deposits) on progressive sections through HuMM mouse brains from front to rear. vCJD (BSE) images from HuMM mouse (ID#108) 693 days post inoculation. vCJD (transfusion) images from HuMM mouse (ID#50) 708 days post inoculation. (Images at 1.6x magnification.) Key: Cx: cerebral cortex; Hp: hippocampus; Th: thalamus; CbW: cerebellar white matter; GL: cerebellum granular layer; ML: cerebellum molecular layer; BS: brain stem

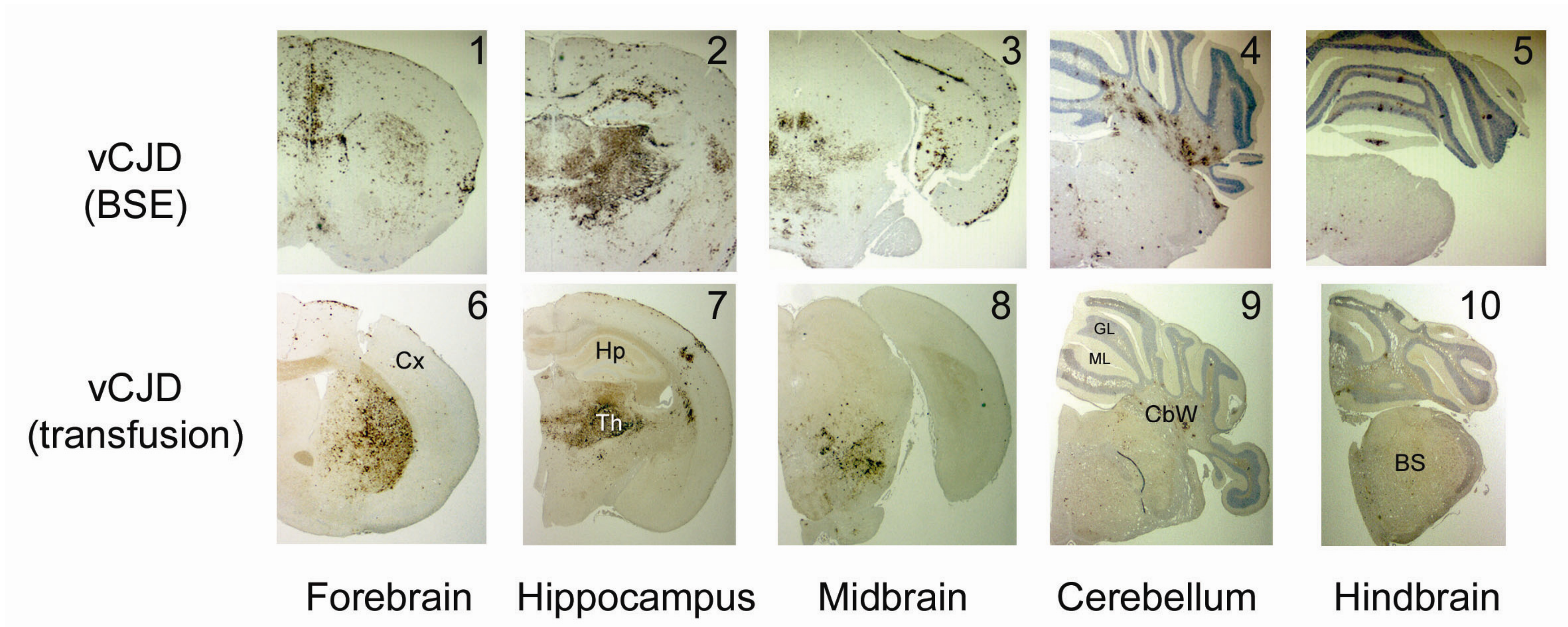


Figure 5.F7: ICC Detection of PrP^{Sc} in Hippocampus of HuMM Mice with vCJD

PrP^{Sc} detection by ICC in the hippocampus and thalamus (lateral posterior nucleus) region of the brain, showing close similarities between the vCJD (BSE) (panel A) and vCJD (transfusion) (panel B) inocula, and the identification of florid plaques (40X magnification of florid plaque structure, see box lower left). (Scale bar 200μm; detection antibody 6H4 (brown stain))

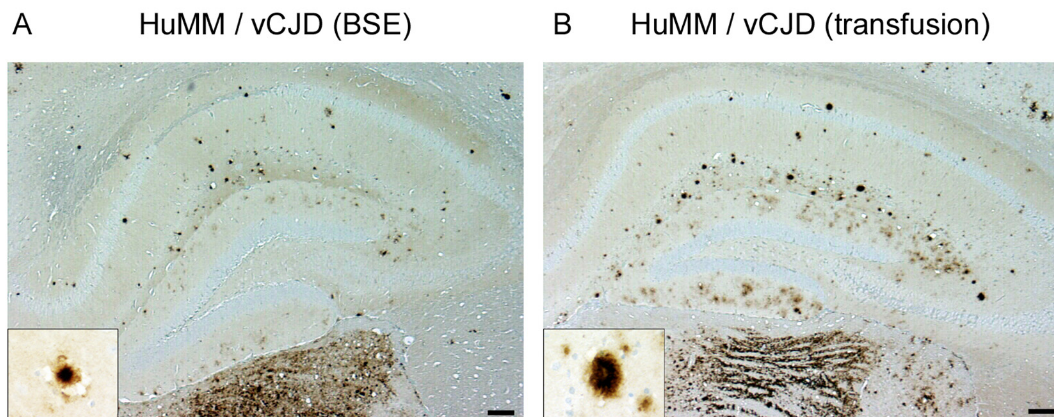
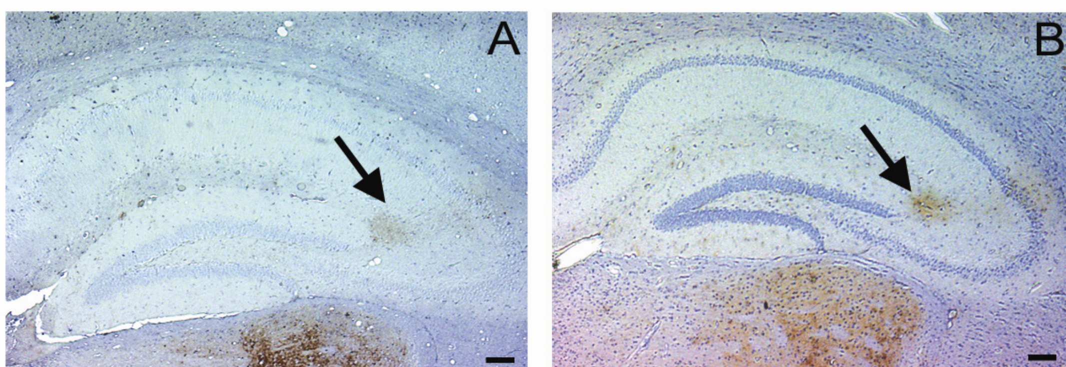


Figure 5.F8: ICC Detection of PrP^{Sc} in Hippocampus of 129Ola Mice with vCJD

PrP^{Sc} targeting to the CA2 region (arrowed) of the hippocampus in 129Ola mice challenged i.c. with A: vCJD (BSE) and B: vCJD (transfusion). (Panel A mouse ID# 208, panel B mouse ID# 14, scale bar 200μm; detection antibody 6H4 (brown stain))



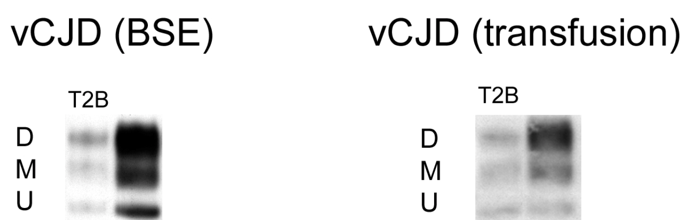
5.3.6 Detection of PrP^{Sc} by Western Blot

The detection and characterisation of protease resistant fragments of PrP^{Sc} by Western blotting can provide further evidence to define strain properties of the vCJD (transfusion) agent. In addition to analysis of incubation periods and vacuolation scoring. PrP^{Sc} found in the brain of this blood transfusion associated vCJD case and the case from which the blood donation originated were both characterised by a 19kDa non-glycosylated fragment and the predominance of the diglycosylated form (type 2B), typical of all vCJD cases (Head *et al.*, 2004a, Llewelyn *et al.*, 2004).

Western blot detection of PrP^{Sc} was performed on frozen brain material set aside during the *post mortem* of each mouse. Both of the biochemical properties of vCJD strain PrP^{Sc}, discussed above, are maintained when vCJD (transfusion) is transmitted to the HuMM transgenic mice (Figure 5.F9). Very low levels of PrP^{Sc} were seen in HuMV and HuVV mice by ICC and therefore future analysis of the frozen tissue will be by high sensitivity methods such a sodium phosphotungstic acid (NaPTA) precipitation.

Figure 5.F9: Western Blot of HuMM Mice Inoculated with vCJD (BSE) and vCJD (transfusion)

Western blot of brain material from HuMM mice i.c. challenged with vCJD (BSE) and vCJD (transfusion) showing the same type 2B profile. ‘T2B’: control vCJD brain homogenate. Anti-PrP detection antibody: 6H4. (D: diglycosylated, M: monoglycosylated, U: unglycosylated)



5.4 DISCUSSION

The aim of this study was to find evidence for adaptation of vCJD transmission properties following human-to-human infection via blood transfusion, as this would have public health implications for the continuing spread of the disease.

5.4.1 Evidence for Strain Adaptation from Clinical Findings and Survival Analysis

The number of transgenic mice scored positive for clinical TSE symptoms, HuMM (1/15) and HuMV (2/17), was as low as found previously with the vCJD (BSE) inoculum (HuMM: 2/18; HuMV: 1/17). Once again there were no HuVV mice positive for clinical signs. The 129Ola mice developed clinical TSE after a longer incubation period (mean 638 days) and in fewer mice (4/12) than seen for vCJD (BSE) (mean 498 days; rate 8/14).

The transgenic data for the presence of clinical signs suggest that there has been no major adaptation of the infectious agent after passage from human-to-human. However, unlike vCJD (BSE) inoculation, survival analysis showed no difference between the HuMM mice and the other transgenic lines. It was proposed that HuMM mice developed deleterious subclinical effects from the vCJD (BSE) inoculation, manifesting in a lower survival rate (See Chapter 4, section 4.4.2.1). As HuMM mice inoculated with vCJD (transfusion) do not show the same effect this may point towards a minor change in the strain transmission properties.

The wild-type mice data also showed that the vCJD (transfusion) inoculum was less efficient in transmission to these mice. This could suggest that either the inoculum is at a lower titre of infectivity, or there has been an adaptation of the agent by human passage. There could have been a form of adaptation of the agent due to the intravenous route of infection (blood transfusion), rather than the oral route of BSE infection. Alternative PrP^{Sc} configuration or binding by chaperone proteins specific to the blood environment may alter the infectivity levels of the agent, although currently there is no evidence for this as disease associated forms of blood PrP have not been isolated and characterised. The presence of PrP^{Sc} in hamster blood has been shown by the protein misfolding cyclic amplification (PMCA) method (Castilla *et al.*, 2005).

If adaptation has occurred due to human passage, making it less transmissible to wild-type mice, then it would be expected that vCJD (BSE) transmission would give longer incubation periods than transmission of BSE derived from cattle. Unpublished data from Dr P Hart (NPD) showed the mean incubation period of BSE inoculation in 129Ola wild-type mice (at 10% concentration) from six

independent experiments to be 405 days. This was very similar to the 392 day mean incubation period from vCJD (BSE) inoculum at 10% concentration, suggesting that the initial cattle-to-human passage had not caused adaptation of the agent. However, 129Ola wild-type transmission data from this study indicated longer incubation periods found with vCJD (transfusion) inoculum. Therefore a form of strain adaptation may have occurred following human-to-human transmission. This effect could also be due to a lower titre of infectivity in the vCJD (transfusion) inoculum (Figure 5.F1).

If a lower titre of infectivity is the reason for the longer incubation period seen in 129Ola mice, and the shifted survival curve for the HuMM mice, inoculated with vCJD (transfusion) material, then possible reasons for this are:

1. The two inocula were prepared differently. The vCJD (BSE) inoculum was sourced from a preparation created as an international standard, in which a large amount of brain tissue (10 pieces of 10g each) was homogenised. The vCJD (transfusion) inoculum was prepared from 1-2g of tissue sourced specifically for this experiment. A 1% homogenate made of these two tissue preparations may therefore contain different amounts of PrP^{Sc} or infectivity dependent on the distribution of cells or tissue types included.
2. Titre is likely to depend on the level of pathological change in the brain tissue selected for the inoculum. Although both the vCJD (BSE) and vCJD (transfusion) cases developed clinical vCJD with similar disease durations (11 and 13 months respectively) they were of very different ages at onset (19 and 68 respectively). The older brain from the vCJD (transfusion) case is likely to have age related degeneration which may have reduced the levels of

infectivity. In addition, this case did not have the characteristic magnetic resonance imaging (MRI) pattern in the brain, which was present in the vCJD (BSE) case. This may also indicate distinct pathological features in this case, which could be associated with a reduced the titre of infectivity.

If the reason for changes in vCJD (transfusion) transmission properties was due to a minor strain adaptation this may have arisen because:

1. The intravenous route of exposure in human-to-human vCJD transmission may produce a less transmissible TSE than the original oral route of exposure to BSE.
2. Infectivity, including PrP^{Sc}, in vCJD (transfusion) material is of human and not bovine origin.
3. Humans may be an inefficient, or 'dead-end' host, for propagating the BSE strain resulting in a TSE disease strain that is less transmissible to further individuals.

5.4.2 Evidence for Strain Adaptation from Vacuolation Scoring

The number of transgenic mice scored positive for TSE vacuolation, HuMM (8/15), HuMV (0/17), and HuVV (0/17) was approximately equal to that found previously for the vCJD (BSE) inoculum (HuMM: 6/18; HuMV: 1/16; HuVV: 1/16). The HuMM mice clearly develop this type of pathological change at an earlier time than the other genotypes suggesting an increased susceptibility with this genotype. The HuMM transgenic line showed a slight difference in attack rate; 8/15 (53%) for vCJD (transfusion) compared with 6/18 (33%) for vCJD (BSE). However, this may be due to the difference in survival rate, as discussed in the previous section. More HuMM mice inoculated with vCJD (transfusion) survived past 600 days, the point at

which vacuolation became more prominent with both inocula. If the positive scoring rates are calculated for those mice that survived past 600 days then the results are: 8/9 (89%) for vCJD (transfusion) and 4/5 (80%) for vCJD (BSE).

In accordance with the variation in levels of clinical positive scoring and mean incubation period, the number of wild-type mice found positive for vacuolation showed a difference between the two inocula. The numbers were equal to those with clinical symptoms as each vacuolation positive mouse was also clinically positive (vCJD (transfusion) 4/12; vCJD (BSE) 8/14) (Table 5.T1).

Vacuolation scoring produced the lesion profiles shown in Figure 5.F4 and Figure 5.F5 for the HuMM and 129Ola wild-type mice. These data suggest the reverse of the findings above, in that there are differences for the HuMM mice and close similarities for the wild-type mice, between each inoculum. As the two lesion profiles for the wild-type mice were both generated from clinically positive animals it can be assumed that these mice had reached a similar pathological end-point. In contrast, both groups of HuMM mice were at an undefined subclinical stage of disease development which may have affected the degree of vacuolation. The difference seen for the HuMM mice (lower scores but similar pattern of targeting for the vCJD (transfusion) inoculum) indicate more strongly a titre effect rather than a strain adaptation as the regional targeting has not changed. Comparing lesion profiles for sCJD and vCJD transmission in wild-type mice shows these regional differences associated with strain (Bruce *et al.*, 1997).

Vacuolar pathology data outlined here shows differences between the vCJD (transfusion) data and that produced for vCJD (BSE) in Chapter 4, with respect to mice scored positive for TSE vacuolation, and the degree of vacuolation seen.

Whether these are attributable to changes in the TSE strain or infectivity titre are difficult to distinguish, but on this evidence it is likely that there has not been a significant adaptation of the infectious agent.

5.4.3 Evidence for Strain Adaptation from Detection of PrP^{Sc}

The number of transgenic mice scored positive for PrP^{Sc} detection by ICC (HuMM: 13/14, HuMV: 8/17, and HuVV: 1/17) was similar to that shown in Chapter 4 for vCJD (BSE) inoculum (HuMM: 12/16; HuMV: 11/14; HuVV: 1/15). This method gave many more positive scores than vacuolation scoring, especially for the HuMV line, and was therefore a good marker for detecting early signs of TSE disease transmission. There were slightly less positive HuMV mice in the vCJD (transfusion) experiment, but this effect appears to be due to the loss of mice due to intercurrent deaths from non-TSE related causes. Survival analysis did not indicate that there was a significant difference in this genotype line between inocula. (Figure 5.F3) The 550 day time-point is when deposition of PrP^{Sc} first becomes detectable by this method and this time-point was similar for both vCJD (BSE) and vCJD (transfusion) in the HuMV mice. The timing for onset of detectable levels of PrP^{Sc} was similar for all three genotype transgenic lines with either inoculum, suggesting no evidence of strain adaptation. The number of 129Ola wild-type mice positive for PrP^{Sc} shows no difference between the two inocula.

Regional distribution of PrP^{Sc} throughout the brain shows no evidence of differences between the two inocula. There are clear similarities including the detection of florid plaques in the hippocampal region of HuMM mice only, the low levels only in the thalamus for HuMV mice, and the specific targeting of the CA2

region of the hippocampus for the 129Ola mice, all of which are indicative properties of typical vCJD / BSE transmissions (Figure 5.F7 and Figure 5.F8).

Western blotting of brain material from HuMM mice infected with vCJD (transfusion) inoculum shows the characteristic type 2B profile, giving further supportive evidence that this material contains the same strain as vCJD (BSE), as defined by the biochemical properties of PrP^{Sc} and its conversion of host PrP^{C}.

The deposition and biochemical properties of PrP^{Sc} in the brains of transgenic mice inoculated with vCJD (transfusion) showed no evidence that human passage of vCJD has caused adaptation of the agent strain.

5.4.4 Summary

In conclusion, this study indicates that the strain transmission characteristics of vCJD have not been significantly altered by secondary transmission through blood transfusion in an individual that was MM at codon 129 of *PRNP*. This suggests that adaptation of the infectious agent to humans following secondary passage has not increased the risk of onward iatrogenic transmission of vCJD through routes such as blood transfusion, although this route of infection may increase the efficiency of transmission. Data in this chapter suggest that human-to-human transmission of vCJD may even produce a strain with longer incubation periods, a lower infectious titre, or one that would not be sustained through serial passage. MM genotype individuals are still likely to be the most susceptible in relation to those that are MV or VV.

The characteristics of the infectious vCJD agent from different genetic backgrounds (MV and VV) has not yet been defined. Data in Chapter 4 (section 4.3.2) indicate that regardless of the inoculum source genotype, sub-passage of the

vCJD strain will still preferentially affect MM over other genotypes. *Post mortem* material from the sub-clinical MV genotype, blood transfusion associated, case has been inoculated into these transgenic mice, however the results were not available for inclusion in this thesis. It is hoped that use of these mice lines will be of significant help in the continuing surveillance and investigation of human forms of vCJD infection.

CHAPTER 6

IDENTIFYING THE UNIQUE TRANSMISSION PROPERTIES OF SPORADIC CJD SUBGROUPS DEFINED BY THE *PRNP* CODON 129 GENOTYPE AND PRP^{SC} TYPE

6.1 AIMS

In sporadic CJD patients the *PRNP* genotype at codon 129 and the PrP^{Sc} Western blot type combine to form six subgroups (MM1, MM2, MV1, MV2, VV1, and VV2) that has formed a basis for defining typical cases of sCJD since the publication in 1999 (Parchi et al., 1999) (See Introduction, section 1.6.1 Sporadic CJD). Should each of the six subtypes show unique transmission properties to the panel of three transgenic lines, then these data can be used as a comparative tool for investigating atypical forms of sCJD, or novel forms of acquired human TSE, for example from atypical BSE or atypical scrapie.

To define the role of codon 129 genotype in transmission of sCJD, each of the three transgenic mouse lines (HuMM, HuMV, and HuVV) were inoculated with infectious material from sCJD sources also from each of the three genotypes. Understanding how the host codon 129 genotype influences susceptibility may

provide evidence for individual risk in humans from iatrogenic infection via sCJD sources.

Intracerebral inoculation of sCJD brain homogenate from six typical cases was performed on the three transgenic lines and the 129Ola wild-type line. These mice were assessed clinically for signs of TSE disease, and pathologically for TSE associated vacuolation (lesion profiling), and targeting and biochemistry of the disease associated PrP^{Sc}. Brain material from HuVV mice that developed TSE after inoculation with sCJD(MV2) was also inoculated into further transgenic and wild-type mice to confirm the infectious nature of the transmitted TSE disease and observe strain adaptation in the transgenic host. This was the only subpassage that could be performed during the timescale of this thesis because of the fast incubation period seen in the HuVV mice with sCJD(MV2).

6.2 INTRODUCTION

6.2.1 Characterisation of sCJD Subgroups as Unique Transmissible Strains

Sporadic CJD is, as the name suggests, the idiopathic form of Creutzfeldt-Jakob disease. There is much that remains to be understood about how and why this disease occurs and the specific biochemical and cellular pathogenesis that leads to the neurodegenerative phenotype. Most frequently sCJD is a rapidly progressive dementia that occurs in elderly people between 60 and 70 years of age, and has a short clinical duration of weeks to a few months. The ‘classical’ clinical presentation of sCJD that is most often observed occurs in individuals who are

homozygous for methionine (MM) at codon 129 of the prion protein gene, and following *post mortem,* biochemical analysis shows the type 1, monoglycosylated dominant, Western blot profile of protease treated prion protein (PrP^{res} or PrP^{Sc}) (See Introduction section 1.3.1, Figure 1.F3.). The clinical presentation and variation in neuropathological features has allowed sCJD cases to be grouped according to the codon 129 genotype and PrP Western blot profile (type 1 or 2) (Parchi et al., 1999, Hill et al., 2003, Cali et al., 2006).

6.2.2 Role of Codon 129 Genotype and PrP^{Sc} Type in Transmission of sCJD

For the purpose of this study of sCJD transmission properties, six cases were selected, each of which showed the typical characteristics of the subgroup: MM1, MM2, MV1, MV2, VV1, and VV2 (See Introduction, section 1.6.1 Sporadic CJD). Inoculation of HuMM, HuMV, and HuVV mice that are genetically identical, except for the polymorphism at codon 129 of their transgene, allowed direct comparisons to be made, to determine the influence of both inoculum and host genotype, and the effect of inoculum PrP^{Sc} type.

6.3 EXPERIMENTAL RESULTS

6.3.1 Primary Intracerebral Inoculation of sCJD

Eighteen mice of each line (HuMM, HuMV, HuVV, 129Ola) were inoculated by the intracerebral (i.c.) route. As these were primary inoculations, the i.c. route was chosen to provide the best chance of infection and the group size was sufficiently large to ensure mice were available up until the end of the usual

transgenic mouse life-span (~700-800 days). Inoculum was prepared from frontal cortex provided by Prof. James Ironside and Ms. Chris-Anne McKenzie (National CJD Surveillance Unit - NCJDSU) from typical sCJD cases of each subgroup as confirmed by clinical investigation and pathological analysis carried out by staff at NCJDSU.

Case references are:

sCJD(MM1): CJDSU reference RU 98/088 (NPD Protocol: 553D-1A)
sCJD(MM2): CJDSU reference RU 98/073 (NPD Protocol: 553L-1A)
sCJD(MV1): CJDSU reference RU 99/072 (NPD Protocol: 553N-1A)
sCJD(MV2): CJDSU reference RU 98/123 (NPD Protocol: 553C-1A)
sCJD(VV1): CJDSU reference RU 96/027 (NPD Protocol: 553M-1A)
sCJD(VV2): CJDSU reference RU 94/083 (NPD Protocol: 553E-1A)

6.3.1.1 Mouse Data Tables

Intercurrent illness and death reduced the total mice available for studying transmission properties over the lifespan of the mice but sufficient mice survived to observe clinical and pathological signs of disease transmission. Tables 6.T1 to 6.T6 show the evaluation of all mice in this study. Highlighted in grey in these Tables are the mice positive for clinical signs of transmissible spongiform encephalopathy (TSE) confirmed by pathology (either the presence of TSE associated vacuolation and/or the identification of abnormal deposition of PrP by immunocytochemistry (ICC)). Also highlighted are the vacuolation or ICC positive mice that were culled for other, non-clinical TSE reasons (intercurrent deaths).

Table 6.T1: Data Table for sCJD(MM1) Inoculation

Intracerebral inoculation data (protocol reference 553D-1A) for sCJD(MM1)
Abbreviations: CLIN: clinical score; VACN: presence of TSE vacuolation; ICC: presence of abnormal PrP by immunocytochemistry; ICD: intercurrent death or culled for animal welfare reasons; n/d: no data available as tissue not testable; n/t: not tested at this time; End expt: mice that were culled when the experiment was terminated at 700 days; shaded cell: positive score

HuMM ID	HuMM Survival Time	HuMM Reason for cull	HuMM CLIN	HuMM VACN	HuMM ICC	HuMV ID	HuMV Survival Time	HuMV Reason for cull	HuMV CLIN	HuMV VACN	HuMV ICC	HuVV ID	HuVV Survival Time	HuVV Reason for cull	HuVV CLIN	HuVV VACN	HuVV ICC	129Ola ID	129Ola Survival Time	129Ola Reason for cull	129Ola CLIN	129Ola VACN	129Ola ICC
59	345	ICD	Neg	Pos	Pos	44	279	ICD	Neg	Pos	Pos	136	233	ICD	Pos	Neg	Neg	122	394	ICD	Neg	Neg	n/t
58	352	ICD	Neg	Pos	Pos	46	366	ICD	Neg	Pos	Pos	41	236	ICD	Neg	Neg	Neg	125	471	ICD	Neg	Neg	n/t
108	401	ICD	Neg	Pos	Pos	42	387	TSE	Pos	Pos	Pos	132	485	ICD	Neg	Pos	Pos	120	531	ICD	Neg	Neg	n/t
109	436	TSE	Pos	Pos	Pos	146	387	ICD	Neg	Pos	Pos	36	513	TSE	Pos	Pos	Pos	98	548	ICD	Neg	Pos	Pos
111	436	TSE	Pos	Pos	Pos	43	447	TSE	Pos	Pos	Pos	133	527	ICD	Neg	Pos	Pos	75	562	TSE	Pos	Pos	Pos
112	436	TSE	Pos	Pos	Pos	147	448	ICD	Neg	Pos	Pos	135	541	TSE	Pos	Pos	Pos	72	569	ICD	Neg	Pos	Pos
171	442	TSE	Pos	Pos	Pos	144	454	TSE	Pos	Pos	Pos	28	548	TSE	Pos	Pos	Pos	99	580	ICD	Neg	n/d	n/t
174	442	TSE	Pos	Pos	Pos	154	454	TSE	Pos	Pos	Pos	134	555	TSE	Pos	Pos	Pos	121	597	ICD	Neg	Neg	n/t
55	447	TSE	Pos	Pos	Pos	159	454	TSE	Pos	Pos	Pos	26	563	ICD	Neg	Pos	Pos	77	604	ICD	Neg	Pos	Pos
110	447	TSE	Pos	Pos	Pos	145	456	TSE	Pos	Pos	Pos	137	583	ICD	Neg	Neg	Pos	73	611	TSE	Pos	Pos	Pos
56	454	TSE	Pos	Pos	Pos	143	478	TSE	Pos	Pos	Pos	30	584	ICD	Pos	Pos	Pos	101	612	ICD	Neg	n/d	n/t
57	454	TSE	Pos	Pos	Pos	158	484	TSE	Pos	Pos	Pos	37	611	ICD	Neg	Pos	Pos	100	630	ICD	Neg	Pos	Pos
170	466	TSE	Pos	Pos	Pos	155	487	ICD	Neg	Pos	Pos	29	625	TSE	Pos	Pos	Pos	74	702	End expt	Neg	Pos	Pos
						47	499	TSE	Pos	Pos	Pos	27	667	TSE	Pos	Pos	Pos	97	730	End expt	Neg	Pos	Pos
												40	667	TSE	Pos	Pos	Pos	102	730	End expt	Neg	Pos	Pos
												25	702	End expt	Neg	Pos	Pos	123	737	End expt	Neg	Pos	Pos
'Attack Rate'			10/13	13/13	13/13				9/14	14/14	14/14				8/16	13/16	14/16				2/16	10/14	10/10

Table 6.T2: Data Table for sCJD(MM2) Inoculation

Intracerebral inoculation data (protocol reference 553L-1A) for sCJD (MM2)
Abbreviations: CLIN: clinical score; VACN: presence of TSE vacuolation; ICC: presence of abnormal PrP by immunocytochemistry; ICD: intercurrent death or culled for animal welfare reasons; n/d: no data available as tissue not testable; n/t: not tested at this time; End expt: mice that were culled when the experiment was terminated at 700 days; shaded cell: positive score

HuMM						HuMV						HuVV						129Ola					
ID	Survival Time	Reason for cull	CLIN	VACN	ICC	ID	Survival Time	Reason for cull	CLIN	VACN	ICC	ID	Survival Time	Reason for cull	CLIN	VACN	ICC	ID	Survival Time	Reason for cull	CLIN	VACN	ICC
153	232	ICD	Neg	Neg	n/t	132	322	ICD	Neg	Neg	n/t	114	147	ICD	Neg	Neg	n/t	188	486	ICD	Neg	Neg	n/t
146	244	ICD	Neg	Neg	n/t	138	358	ICD	Neg	Neg	n/t	62	259	ICD	Neg	Neg	n/t	189	512	TSE	Pos	Neg	n/t
69	280	ICD	Neg	Neg	n/t	79	434	ICD	Neg	Neg	n/t	137	268	ICD	Neg	Neg	n/t	187	537	ICD	Neg	Neg	n/t
64	337	ICD	Neg	Neg	n/t	98	438	ICD	Neg	Neg	n/t	131	364	ICD	Neg	Neg	n/t	186	589	ICD	Neg	Neg	n/t
63	403	ICD	Neg	Neg	n/t	112	466	ICD	Neg	Neg	n/t	147	384	ICD	Neg	Neg	n/t	193	617	ICD	Neg	Neg	n/t
154	504	ICD	Neg	Neg	n/t	130	497	ICD	Neg	Neg	n/t	129	402	ICD	Neg	Neg	n/t	158	629	ICD	Neg	Neg	n/t
72	511	ICD	Neg	Neg	n/t	75	510	ICD	Neg	Neg	n/t	51	423	ICD	Neg	Neg	n/t	195	631	ICD	Neg	Neg	n/t
85	538	ICD	Neg	Neg	n/t	111	522	ICD	Neg	Neg	n/t	66	493	ICD	Neg	Neg	n/t	159	634	ICD	Neg	Neg	n/t
134	546	ICD	Neg	Neg	n/t	60	525	ICD	Neg	Neg	n/t	110	493	ICD	Neg	Neg	n/t	161	658	ICD	Neg	Neg	n/t
117	560	ICD	Neg	Neg	n/t	102	558	ICD	Neg	Neg	n/t	155	536	ICD	Neg	Neg	n/t	81	665	ICD	Neg	Neg	n/t
100	567	ICD	Neg	Neg	n/t	65	560	ICD	Neg	Neg	n/t	61	539	ICD	Neg	Neg	n/t	192	673	ICD	Neg	Neg	n/t
113	593	ICD	Neg	Neg	n/t	68	560	ICD	Neg	Neg	n/t	67	565	ICD	Neg	Neg	n/t	191	687	ICD	Neg	Neg	n/t
71	616	ICD	Neg	Neg	n/t	76	588	ICD	Neg	Neg	n/t	78	627	ICD	Neg	Neg	n/t	160	721	ICD	Neg	Neg	n/t
135	637	ICD	Neg	Neg	n/t	77	602	TSE	Pos	Neg	n/t	74	654	ICD	Neg	Neg	n/t	190	750	ICD	Neg	Neg	n/t
101	651	ICD	Neg	Neg	n/t	80	602	ICD	Neg	Neg	n/t	73	658	ICD	Neg	Neg	n/t	83	784	TSE	Pos	Neg	n/t
84	658	ICD	Neg	Neg	n/t	70	614	ICD	Neg	Neg	n/t	52	678	ICD	Neg	Neg	n/t	82	805	ICD	Neg	Neg	n/t
						136	651	ICD	Neg	Neg	n/t	103	735	ICD	Neg	Neg	n/t						
						53	678	ICD	Neg	Neg	n/t												
‘Attack Rate’			0/16	0/16					0/18	0/18					0/17	0/17					0/16	0/16	

Table 6.T3: Data Table for sCJD(MV1) Inoculation

Intracerebral inoculation data (protocol reference 553N-1A) for sCJD(MV1)
Abbreviations: CLIN: clinical score; VACN: presence of TSE vacuolation; ICC: presence of abnormal PrP by immunocytochemistry; ICD: intercurrent death or culled for animal welfare reasons; n/d: no data available as tissue not testable; n/t: not tested at this time; End expt: mice that were culled when the experiment was terminated at 700 days; shaded cell: positive score

HuMM						HuMV						HuVV						129Ola					
ID	Survival Time	Reason for cull	CLIN	VACN	ICC	ID	Survival Time	Reason for cull	CLIN	VACN	ICC	ID	Survival Time	Reason for cull	CLIN	VACN	ICC	ID	Survival Time	Reason for cull	CLIN	VACN	ICC
20	400	TSE	Pos	Pos	n/t	3	232	ICD	Neg	Pos	n/t	93	162	ICD	Neg	Neg	n/t	82	314	ICD	Neg	Neg	n/t
21	407	ICD	Neg	Pos	n/t	117	298	ICD	Neg	Neg	n/t	140	241	ICD	Neg	Neg	n/t	52	380	ICD	Neg	Neg	n/t
172	429	TSE	Pos	Pos	n/t	60	338	ICD	Neg	Pos	n/t	5	260	ICD	Neg	Neg	n/t	53	413	ICD	Neg	Neg	n/t
174	429	TSE	Pos	Pos	n/t	1	414	TSE	Pos	Pos	n/t	141	303	ICD	Neg	Pos	n/t	51	455	ICD	Neg	Neg	n/t
161	436	TSE	Pos	Pos	n/t	118	436	TSE	Pos	Pos	n/t	8	323	ICD	Neg	Pos	n/t	115	457	TSE	Pos	Neg	n/t
170	436	TSE	Pos	Pos	n/t	119	436	TSE	Pos	Pos	n/t	64	360	ICD	Neg	Pos	n/t	54	498	ICD	Neg	Neg	n/t
171	436	TSE	Pos	Pos	n/t	154	436	TSE	Pos	Neg	n/t	144	435	TSE	Pos	Pos	n/t	113	499	ICD	Neg	Neg	n/t
164	443	TSE	Pos	Pos	n/t	58	443	TSE	Pos	Pos	n/t	92	449	ICD	Neg	Pos	n/t	83	534	ICD	Neg	Neg	n/t
162	450	TSE	Pos	Pos	n/t	4	449	TSE	Pos	Pos	n/t	142	457	ICD	Neg	Pos	n/t	168	548	ICD	Neg	Neg	n/t
163	450	TSE	Pos	Pos	n/t	2	470	TSE	Pos	Pos	n/t	143	497	ICD	Neg	Pos	n/t	84	554	ICD	Neg	Neg	n/t
166	450	TSE	Pos	Pos	n/t	150	478	TSE	Pos	Pos	n/t	94	499	ICD	Neg	Pos	n/t	111	555	ICD	Neg	Neg	n/t
18	456	TSE	Pos	Pos	n/t	151	478	TSE	Pos	Pos	n/t	7	515	TSE	Pos	Pos	n/t	167	561	ICD	Neg	Neg	n/t
16	464	TSE	Pos	Pos	n/t	56	513	TSE	Pos	Pos	n/t	176	583	ICD	Neg	Pos	n/t	55	575	ICD	Neg	Neg	n/t
165	464	TSE	Pos	Pos	n/t	57	513	TSE	Pos	Pos	n/t	62	645	TSE	Pos	Pos	n/t	85	584	ICD	Neg	Neg	n/t
17	470	TSE	Pos	Pos	n/t	59	513	TSE	Pos	Pos	n/t	175	647	ICD	Pos	Pos	n/t	112	639	ICD	Neg	Pos	n/t
19	470	TSE	Pos	Pos	n/t	61	513	TSE	Pos	Pos	n/t	6	659	TSE	Pos	Pos	n/t	114	660	ICD	Neg	Neg	n/t
						153	513	TSE	Pos	Pos	n/t	9	659	TSE	Pos	Pos	n/t	116	716	ICD	Neg	Neg	n/t
						152	520	TSE	Pos	Pos	n/t	63	660	TSE	Pos	Pos	n/t						
'Attack Rate'			15/16	16/16					14/18	16/18					7/18	15/18					0/17	1/17	

Table 6.T4: Data Table for sCJD(MV2) Inoculation

Intracerebral inoculation data (protocol reference 553C-1A) for sCJD(MV2)
Abbreviations: CLIN: clinical score; VACN: presence of TSE vacuolation; ICC: presence of abnormal PrP by immunocytochemistry; ICD: intercurrent death or culled for animal welfare reasons; n/d: no data available as tissue not testable; n/t: not tested at this time; End expt: mice that were culled when the experiment was terminated at 700 days; shaded cell: positive score

HuMM						HuMV						HuVV						129Ola					
ID	Survival Time	Reason for cull	CLIN	VACN	ICC	ID	Survival Time	Reason for cull	CLIN	VACN	ICC	ID	Survival Time	Reason for cull	CLIN	VACN	ICC	ID	Survival Time	Reason for cull	CLIN	VACN	ICC
69	258	TSE	Pos	Neg	Neg	55	223	ICD	Neg	Neg	Neg	106	247	ICD	Neg	Pos	Pos	86	314	TSE	Pos	Neg	n/t
118	295	ICD	Neg	Neg	n/d	174	341	ICD	Neg	Neg	Pos	96	267	TSE	Pos	Pos	Pos	27	342	ICD	Neg	Neg	n/t
119	479	ICD	Neg	Pos	Pos	209	394	ICD	n/s	Neg	Pos	102	271	ICD	Pos	Pos	Pos	28	406	ICD	Neg	n/d	n/t
170	496	ICD	Neg	Neg	n/d	206	398	ICD	Neg	Neg	Neg	214	279	TSE	Pos	Pos	Pos	87	427	ICD	Neg	Neg	n/t
170	496	ICD	Neg	Neg	Pos	159	426	ICD	Neg	Neg	Pos	103	279	ICD	Pos	Pos	n/d	73	503	ICD	Neg	Neg	Neg
171	521	ICD	Neg	Pos	Pos	160	426	ICD	Neg	Neg	Pos	104	279	TSE	Pos	Pos	Pos	25	509	ICD	Neg	Neg	Neg
111	545	ICD	Neg	Pos	Pos	157	479	ICD	Neg	Neg	Pos	107	279	TSE	Pos	Pos	Pos	30	534	ICD	Neg	Neg	Neg
109	559	TSE	Pos	Pos	Pos	176	503	ICD	Neg	Pos	Pos	97	280	TSE	Pos	Pos	Pos	84	545	ICD	Neg	Neg	Neg
70	565	TSE	Pos	Pos	Pos	60	538	ICD	Neg	Neg	Pos	215	290	ICD	Pos	n/d	Pos	88	559	ICD	Neg	Neg	Neg
67	566	TSE	Pos	Pos	Pos	156	538	TSE	Pos	Neg	Pos	101	290	TSE	Pos	Pos	Pos	26	565	ICD	Neg	Neg	Neg
168	577	ICD	Neg	Pos	Pos	207	559	ICD	Neg	Pos	Pos	211	293	ICD	Pos	Pos	n/d	77	668	ICD	Neg	Neg	Neg
169	584	ICD	Neg	Pos	Pos	178	566	ICD	Neg	Neg	Pos	164	293	TSE	Pos	Pos	Pos	89	692	ICD	Neg	Neg	Neg
173	643	ICD	Neg	Pos	Pos	161	612	TSE	Pos	Neg	Pos	167	293	TSE	Pos	Pos	Pos	75	717	End expt	Neg	Neg	Neg
						57	714	End expt	Neg	Pos	Pos	163	299	TSE	Pos	Pos	Pos	76	720	End expt	Neg	Neg	Neg
						59	714	End expt	Neg	Pos	Pos	98	300	TSE	Pos	Pos	Pos						
						205	714	End expt	Neg	Pos	Pos	100	306	TSE	Pos	Pos	Pos						
						208	714	End expt	Neg	Pos	Pos	99	308	TSE	Pos	Pos	Pos						
'Attack Rate'			3/13	9/13	10/11				2/16	6/17	15/17				16/17	16/16	15/15				0/14	0/13	0/10

Table 6.T5: Data Table for sCJD(VV1) Inoculation

Intracerebral inoculation data (protocol reference 553M-1A) for sCJD(VV1)
Abbreviations: CLIN: clinical score; VACN: presence of TSE vacuolation; ICC: presence of abnormal PrP by immunocytochemistry; ICD: intercurrent death or culled for animal welfare reasons; n/d: no data available as tissue not testable; n/t: not tested at this time; End expt: mice that were culled when the experiment was terminated at 700 days; shaded cell: positive score

HuMM						HuMV						HuVV						129Ola					
ID	Survival Time	Reason for cull	CLIN	VACN	ICC	ID	Survival Time	Reason for cull	CLIN	VACN	ICC	ID	Survival Time	Reason for cull	CLIN	VACN	ICC	ID	Survival Time	Reason for cull	CLIN	VACN	ICC
102	260	ICD	Neg	Neg	n/t	3	239	ICD	Neg	Neg	n/t	57	315	ICD	Neg	Neg	n/t	158	379	ICD	Neg	Neg	n/t
67	267	ICD	Neg	Neg	n/t	109	343	ICD	Neg	Neg	n/t	78	337	ICD	Neg	Neg	n/t	153	442	ICD	Neg	Neg	n/t
71	462	ICD	Neg	Neg	n/t	63	379	ICD	Neg	Neg	n/t	112	384	ICD	Neg	Neg	n/t	161	456	ICD	Neg	Neg	n/t
99	481	ICD	Neg	Neg	n/t	5	428	ICD	Neg	Neg	n/t	54	400	ICD	Neg	Neg	n/t	155	477	ICD	Neg	Neg	n/t
103	504	ICD	Neg	Neg	n/t	106	546	TSE	Pos	Pos	n/t	113	430	ICD	Neg	Pos	n/t	30	491	ICD	Neg	Neg	n/t
100	533	ICD	Neg	Neg	n/t	107	568	TSE	Pos	Pos	n/t	76	505	ICD	Neg	Neg	n/t	33	561	ICD	Neg	Neg	n/t
49	536	ICD	Neg	Neg	n/t	110	575	ICD	Neg	Pos	n/t	111	526	ICD	Neg	Pos	n/t	154	588	ICD	Neg	Neg	n/t
51	540	ICD	Neg	Neg	n/t	108	603	ICD	Neg	Neg	n/t	56	540	ICD	Neg	Neg	n/t	157	596	ICD	Neg	Neg	n/t
70	559	ICD	Neg	Neg	n/t	105	610	ICD	Neg	Pos	n/t	59	568	TSE	Pos	Pos	n/t	31	611	ICD	Neg	Neg	n/t
53	603	ICD	Neg	Neg	n/t	60	644	ICD	Neg	Pos	n/t	75	568	TSE	Pos	Pos	n/t	156	630	ICD	Neg	Neg	n/t
52	609	ICD	Neg	Neg	n/t	2	651	ICD	Neg	Pos	n/t	58	575	ICD	Neg	Neg	n/t	32	638	TSE	Pos	Neg	n/t
101	610	ICD	Neg	Neg	n/t	4	651	ICD	Neg	Pos	n/t	74	610	ICD	Neg	Pos	n/t	34	659	ICD	Neg	Neg	n/t
68	618	ICD	Neg	Neg	n/t	104	676	ICD	Neg	Pos	n/t	79	631	ICD	Neg	Pos	n/t	169	694	ICD	Neg	Neg	n/t
66	639	ICD	Neg	Neg	n/t	6	694	ICD	Neg	Pos	n/t	72	635	ICD	Neg	Pos	n/t	171	729	ICD	Neg	Neg	n/t
50	644	ICD	Neg	Neg	n/t													168	778	ICD	Neg	Pos	n/t
69	687	ICD	Neg	Neg	n/t													160	785	ICD	Pos	Neg	n/t
'Attack Rate'			0/16	0/16					2/14	9/14					2/14	7/14					0/16	1/16	

Table 6.T6: Data Table for sCJD(VV2) Inoculation

Intracerebral inoculation data (protocol reference 553E-1A) for sCJD(VV2)
Abbreviations: CLIN: clinical score; VACN: presence of TSE vacuolation; ICC: presence of abnormal PrP by immunocytochemistry; ICD: intercurrent death or culled for animal welfare reasons; n/d: no data available as tissue not testable; n/t: not tested at this time; End expt: mice that were culled when the experiment was terminated at 700 days; shaded cell: positive score

HuMM						HuMV						HuVV						129Ola					
ID	Survival Time	Reason for cull	CLIN	VACN	ICC	ID	Survival Time	Reason for cull	CLIN	VACN	ICC	ID	Survival Time	Reason for cull	CLIN	VACN	ICC	ID	Survival Time	Reason for cull	CLIN	VACN	ICC
157	278	ICD	Neg	Neg	Neg	111	330	ICD	Neg	Neg	Neg	165	258	ICD	Neg	Pos	n/t	22	231	ICD	Neg	Neg	n/t
180	337	ICD	Neg	Neg	Neg	80	416	ICD	Neg	Neg	n/t	46	259	TSE	Pos	Pos	Pos	130	245	ICD	Neg	Neg	n/t
177	355	ICD	Neg	Neg	Pos	106	450	TSE	Pos	Neg	Pos	54	261	TSE	Pos	Pos	Pos	19	325	ICD	Neg	Neg	n/t
181	400	TSE	Pos	Neg	n/t	112	538	ICD	Neg	Neg	Pos	47	261	TSE	Pos	Pos	Pos	127	378	ICD	Neg	Neg	n/t
94	436	ICD	Neg	Neg	Pos	110	562	ICD	Neg	Neg	Neg	167	261	TSE	Pos	Pos	Pos	132	409	ICD	Neg	Neg	n/t
160	436	ICD	Neg	Neg	Pos	104	580	ICD	Neg	Neg	Pos	48	268	TSE	Pos	Pos	Pos	128	422	ICD	Neg	Neg	n/t
179	447	TSE	Pos	Neg	Pos	113	595	ICD	Neg	Neg	Pos	164	268	TSE	Pos	Pos	Pos	134	422	ICD	Neg	Neg	Neg
176	455	ICD	Neg	Neg	Pos	109	599	ICD	Neg	Neg	Pos	166	268	TSE	Pos	Pos	Pos	129	485	ICD	Neg	Neg	Neg
183	462	ICD	Neg	Neg	Pos	81	611	ICD	Neg	Neg	Pos	45	272	TSE	Pos	Pos	Pos	138	524	ICD	Neg	Neg	Neg
96	469	ICD	Neg	Neg	Pos	105	618	ICD	Neg	Pos	Pos	44	279	ICD	Neg	Pos	Pos	131	562	ICD	Neg	Neg	Neg
174	482	ICD	Neg	Neg	Pos	85	629	ICD	Neg	Neg	Pos	162	282	TSE	Pos	Pos	Pos	136	618	ICD	Neg	Neg	Neg
182	491	ICD	Neg	Neg	Pos	82	723	End expt	Neg	Pos	Pos	59	284	ICD	Neg	Pos	Pos	137	625	ICD	Neg	Neg	Neg
159	581	TSE	Pos	Pos	Pos	84	723	End expt	Neg	Pos	Pos	56	288	TSE	Pos	Pos	Pos	133	660	ICD	Neg	Neg	Neg
158	646	TSE	Pos	Pos	Pos	108	729	End expt	Neg	Pos	Pos	57	289	TSE	Pos	Pos	Pos	20	709	End expt	Neg	Neg	Neg
161	653	TSE	Pos	Pos	Pos	115	730	End expt	Neg	Pos	Pos	55	293	TSE	Pos	Pos	Pos	21	709	End expt	Neg	Neg	Neg
92	710	ICD	Neg	Pos	Pos							58	293	TSE	Pos	Pos	Pos	23	709	End expt	Neg	Neg	Neg
93	723	End expt	Neg	Pos	Pos													24	709	End expt	Neg	Neg	n/t
97	723	End expt	Neg	Pos	Pos																		
'Attack Rate'			4/18	6/18	15/17				1/15	5/15	12/14				13/16	16/16	15/15				0/17	0/17	0/10

6.3.1.2 Survival Analysis to Investigate Clinical and Subclinical TSE

An analysis of the survival rates was performed because of the absence of clinical TSE signs in many groups of inoculated mice and the large number of mice that died or were culled on animal welfare grounds (intercurrent deaths), during the course of the experiments. These deaths may be due in part to an underlying effect of the inoculum and the numbers may vary according to the codon 129 genotype of the mouse. To observe the effects on overall survival rates between the different inocula and mice lines Kaplan-Meier survival curves were drawn and Tarone-Ware tests performed (Software: SPSS v14.0). Tarone-Ware tests have even bias across the lifespan of the mice whereas Log Rank (Mantel-Cox) tests apply more weight to late time points and Breslow (Generalised Wilcoxon) tests apply more weight to early time points.

Figure 6.F1 shows Kaplan-Meier survival curves for all the experiments in this study. The majority of mice survived until approximately 300-400 days then were lost gradually, but uniformly over time, with 50% alive at 500-600 days and all dead by 700-800 days (some experiments were terminated at around 700-750 days with the remaining few mice culled). The panel of 'uninfected' mice, that were left to age naturally, showed a similar pattern. Therefore the majority of mice in this study, although inoculated with TSE material followed the normal pattern of aging.

6.3.1.2.1 Effect of Inoculum Source on Survival

Figure 6.F1 shows the Kaplan-Meier curves for each inoculum used and Table 6.T7 highlights the statistically significant comparisons. These data have been

prepared to provide a clearer method of understanding which sCJD source of infectivity has a dominant role in the transmission to the transgenic mice, and whether each sCJD subgroup has specific effects. Those mice groups that developed clinical TSE signs showed near vertical survival curves, compared to other groups whose curves were less steep (For examples, see Figure 6.F1, Panels sCJD(MV2) and sCJD(VV2)). These latter mice may have developed subclinical TSE or may be unaffected, and statistical comparisons can highlight the degree to which subclinical disease is present and in what genotypes of mice. As an example, for sCJD(MM2) inoculation both the wild-type and transgenic mice were all negative clinically and for TSE vacuolation indicating that there did not seen to be an effect attributable to expression of the human prion protein. However, the Tarone-Ware test showed a significant difference between survival in the wild-type line compared with each of the transgenic mice lines ($P<0.01$).

A difference in the response of the HuVV mice to the sCJD(MM1) and sCJD(MV1) inocula was also detected by the Tarone-Ware test, showing a more detrimental effect from the sCJD(MV1) inoculum (no difference between the HuVV mice and the other transgenic lines).

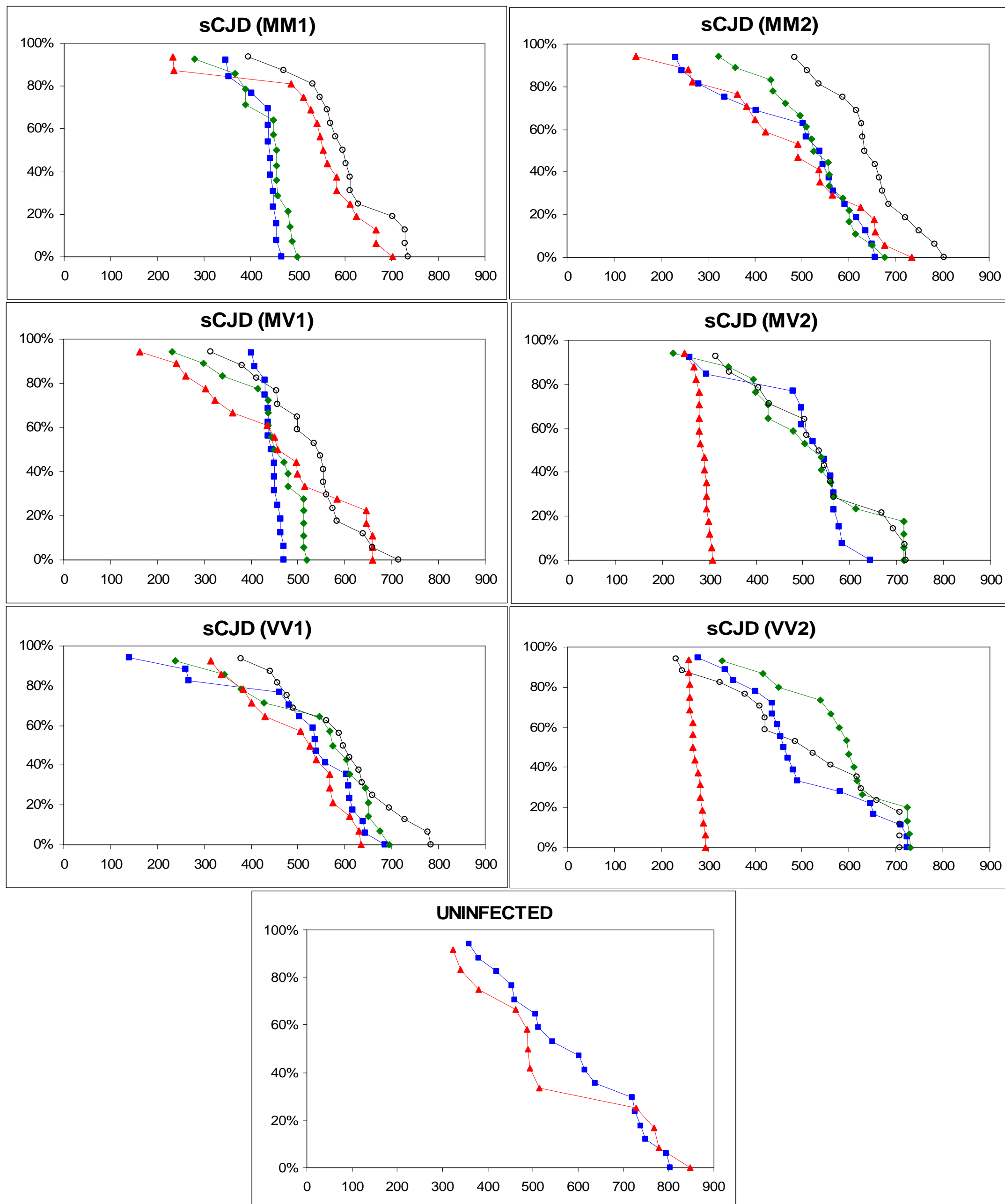
Table 6.T7: Tarone-Ware Analysis by sCJD Inoculum

Statistical analysis of survival data by inoculum group, significant findings only (Tarone-Ware Test; *: P<0.05; **: P<0.01; ***: P<0.001).

Inoculum	Groups Tested	P value (significance rating)
sCJD(MM1)	HuMM and/or HuMV vs. HuVV and/or 129Ola	P<0.001 ***
sCJD(MM2)	129Ola vs. Rest	P<0.01 **
sCJD(MV1)	HuMM vs. 129Ola HuMV vs. 129Ola	P=0.001 ** P=0.004 **
sCJD(MV2)	HuVV vs. Rest	P<0.001 ***
sCJD(VV1)	HuVV vs. 129Ola	P=0.026 *
sCJD(VV2)	HuVV vs. Rest	P<0.001 ***

Figure 6.F1: Kaplan-Meier Survival Curves for Primary sCJD Inoculation

Kaplan-Meier survival curves of uninfected mice, and primary i.c. challenge of sCJD to investigate effects of the inoculum. (HuMM (blue squares), HuMV (green diamonds), HuVV (red triangles), 129Ola (black open circles) mice, x-axis: days post inoculation, y-axis: percentage of mice remaining)



6.3.1.2.2 Effect of Mouse Codon 129 Genotype on Survival

In addition to each individual inoculum having an effect on transmission properties, there could be a more dominant role for the host mouse codon 129 genotype. To test this, survival analysis was performed on data grouped according to codon 129 genotype. If there were effects due to the host genotype then it would be expected that all inocula would produce similar survival curves in one genotype line. Figure 6.F2 shows the Kaplan-Meier curves grouped for each mouse line genotype and Table 6.T8 highlights the statistically significant comparisons.

The data in Table 6.T8 show that there were differences between the inocula (as shown below) but that these were specific to the genotype of the mice. The HuMM data show that there were differences between both sCJD(MM1) and sCJD(MV1) inocula and the others, and uninfected mice. Similarly the HuMV data show these differences for the sCJD(MM1) and sCJD(MV1) inoculations. The HuVV data highlighted the clear differences seen between inocula from sCJD(MV2) and sCJD(VV2) sources, and the rest of the inocula and the uninfected mice.

Figure 6.F2: Kaplan-Meier Survival Curves Grouped by Mouse Line

Kaplan-Meier survival curves following primary i.c. challenge of sCJD grouped by mouse line. (Blue square sCJD(MM1), green diamond sCJD(MM2), red triangle sCJD(MV1), black circle sCJD(MV2), purple cross sCJD(VV1), brown circle sCJD(VV2))

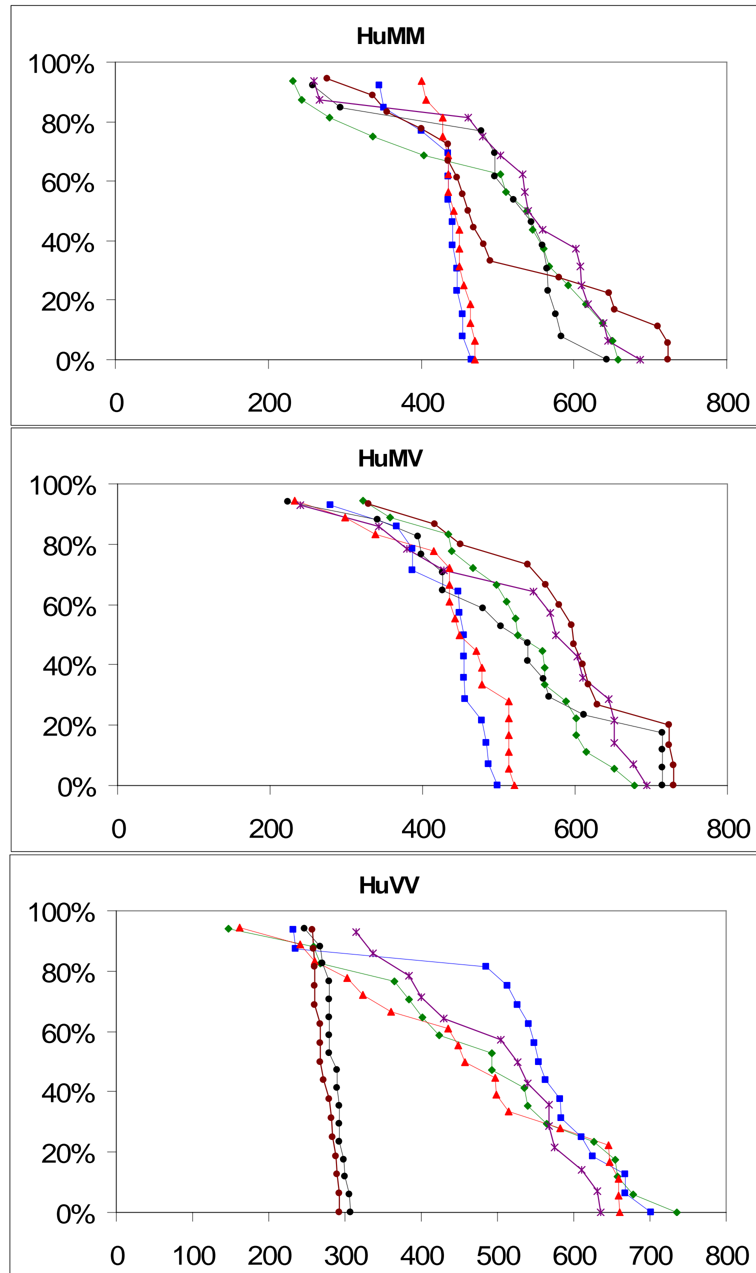


Table 6.T8: Tarone-Ware Analysis Grouped by Mouse Genotype

Primary sCJD transmissions, pairwise survival analysis by genotype. (Tarone-Ware Test - only data with significance P<0.05 shown.)

HuMM	MM1	MM2	MV1
MM2	0.016	/	/
MV1		0.013	/
MV2	<0.001		<0.001
VV1	<0.001		<0.001
VV2	0.022		
Uninfected	<0.001		=0.001

HuMV	MM1	MM2	MV1
MM2	=0.001	/	/
MV1		0.002	/
MV2	0.016		0.037
VV1	0.005		0.003
VV2	<0.001	0.047	<0.001

HuVV	MM1	MM2	MV1	MV2	VV1	VV2
MM1	/	/	/	/	/	/
MM2		/	/	/	/	/
MV1			/	/	/	/
MV2	<0.001	<0.001	<0.001	/	/	/
VV1				<0.001	/	/
VV2	<0.001	<0.001	<0.001	0.016	<0.001	/
Uninfected				<0.001		<0.001

6.3.1.3 Incubation Period Variation Determined by Codon 129 Genotype of Inoculum and Host

Survival analysis can determine an effect of the inoculum or host without clinical signs of TSE being present. There were however, some inoculated groups of transgenic mice that developed clinical TSE signs, and therefore an assessment was made of the mean incubation time across the different inocula and genotype of hosts.

A direct comparison of the incubation time data for the mice showing clinical TSE symptoms is shown in Table 6.T9. The presence / absence, and time of onset, of clinical manifestation of TSE disease was dependent on both the genotype of the host mouse and the inoculum. No two primary transmission experiments gave the same result suggesting that each source of sCJD is a separate strain, although there were some similarities. SCJD types MM1 and MV1 which are combined by the Parchi classification (Parchi *et al.*, 1999), both being the 'classical' form of sCJD, showed very similar incubation time data for the transgenic lines and only differed in that the MM1 type produced clinical TSE in the wild-type mice. The other similar grouping was between the MV2 and VV2 inocula. Both produced clinical disease with relatively short incubation times (<300 days) in the HuVV mice, but long incubation times in a few HuMM and HuMV mice. There was no evidence of a difference between these two inocula. Although the HuMV mice showed different incubation periods between MV2 and VV2 inocula these data were limited to too few mice to compare accurately.

Table 6.T9: Incubation Period Data for sCJD Inoculation

Incubation periods for clinically positive mice shows variation between inocula and mouse lines. (mean (range); ‘attack rate’ and percentage; n/d: no clinical disease)

Inoculum	HuMM	HuMV	HuVV	129Ola
sCJD(MM1)	446 (436-466) 10/13 (77%)	457 (387-499) 9/14 (64%)	588 (513-667) 8/16 (50%)	586 (562-611) 2/16 (12%)
sCJD(MM2)	n/d	n/d	n/d	n/d
sCJD(MV1)	446 (400-470) 15/16 (94%)	478 (414-520) 14/18 (78%)	595 (435-660) 7/18 (39%)	n/d
sCJD(MV2)	563 (559-566) 3/13 (23%)	575 (538-612) 2/16 (12%)	288 (267-308) 16/17 (94%)	n/d
sCJD(VV1)	n/d	557 (546-568) 2/14 (14%)	568 (568-568) 2/14 (14%)	n/d
sCJD(VV2)	582 (447-653) 4/18 (22%)	450 1/15 (7%)	273 (259-293) 13/16 (81%)	n/d

6.3.1.4 Vacuolation Scoring

Examination of the neuropathology of these mice allowed further dissection of the similarities and differences in transmission. All mice in this study were scored blind for the degree and location of TSE vacuolation according to the lesion profiling methods used on all mice at NPD, by their trained staff (Aileen Boyle, Wing-Gee Lui, and Sandra Mack). Presence of TSE associated vacuolation gave the positive scores found in Tables 6.T1 to 6.T6 (column VACN). In general there was a higher rate of positive scores than for clinical positives. Further examination can be made of the differences and similarities between the sCJD inocula, and the effect of host codon 129 genotype. Table 6.T10 shows the comparison of these data.

Wild-type 129Ola mice data have been included as there was a clear difference between the number of positive mice for the sCJD(MM1) and sCJD(MV1) inocula (71% versus 6% respectively). These two inocula were otherwise identical for vacuolation scoring in the transgenic lines, with ~100% of HuMM and HuMV mice, and ~80% of HuVV mice positive. Sporadic CJD cases of the MM1 and MV1 types are group together as the 'classical' form, with similar clinical and pathological features, and so the apparent close similarities in transmission properties in the transgenic mice may be expected.

Inocula from sCJD(MV2) and sCJD(VV2) showed similar responses from the different mice lines. The short incubation periods seen in the HuVV mice were reflected in 100% scores for vacuolation, and the HuMV mice gave equal scores of 33% positive. There was some variation for the HuMM mice with 69% positive with sCJD(MV2) inoculum compared with 33% with the sCJD(VV2) source. 129Ola wild-type mice were negative for both inocula.

The sCJD(VV1) inoculum showed no evidence of vacuolation in the HuMM mice and only a single positive wild-type mouse, and ~50% were positive for the other two transgenic lines. Lastly, the sCJD(MM2) inoculum showed no evidence of TSE vacuolation in any of the four mouse lines.

Table 6.T10: Vacuolation Scoring Data for sCJD Inoculation

Summary and comparison of positive scores (attack rates) for TSE vacuolation assessment.

Inoculum	HuMM		HuMV		HuVV		129Ola	
MM1	13/13	100%	14/14	100%	13/16	81%	10/14	71%
MM2	0/16	-	0/18	-	0/17	-	0/16	-
MV1	16/16	100%	16/18	89%	15/18	83%	1/17	6%
MV2	9/13	69%	6/17	35%	16/16	100%	0/13	-
VV1	0/16	-	9/14	64%	7/14	50%	1/16	6%
VV2	6/18	33%	5/15	33%	16/16	100%	0/17	-

6.3.1.5 Lesion Profile Comparison

In addition to the positive / negative scoring for TSE vacuolation, each mouse was scored for the degree of vacuolation (ranked 1 to 5 with 1 the least affected) found in nine grey matter regions and three white matter regions, combined to produce a mean lesion profile (See Methods). Figure 6.F3 shows the lesion profiles generated. SCJD(MM2) is absent as there were no mice positive for vacuolation.

Comparing the data between mouse lines for each inoculum and also between the separate inocula should provide evidence for the role each is playing with respect to the targeting and intensity of TSE vacuolation. There were close similarities, as found previously, between sCJD(MM1) and sCJD(MV1), and all three transgenic lines appeared to have similar lesion profiles for either inoculum.

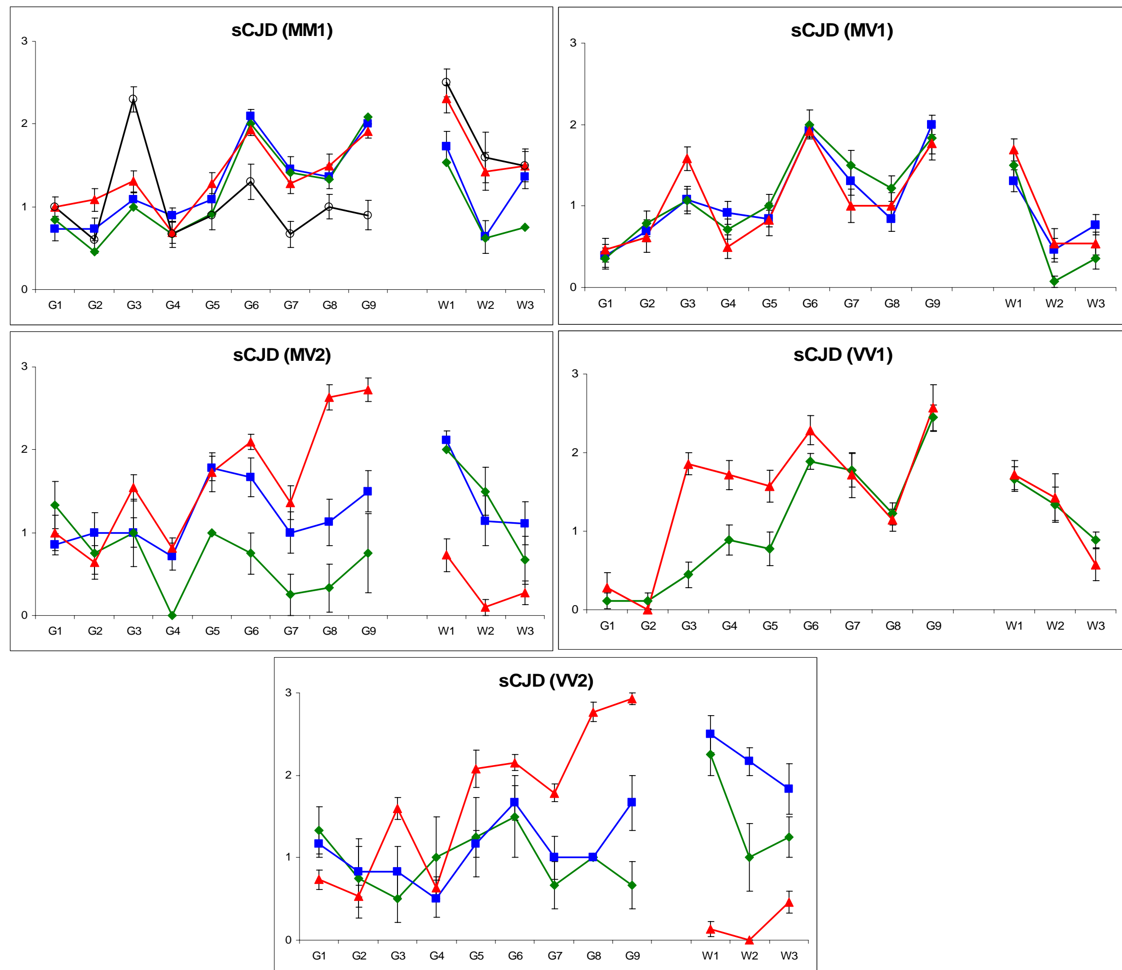
The sCJD(MM1) inoculum was the only one to give positive scores in the wild-type mice. The lesion profile from these mice is included in Figure 6.F3 and

showed clear differences, including a greater intensity of vacuolation at grey matter region three, the superior colliculus. Pathological targeting of this region, associated with processing sensory information and corresponding head and eye movements, may have triggered earlier onset of clinical TSE symptoms in these mice.

Lesion profiles produced for transmission of sCJD(MV2) and sCJD(VV2) showed close similarities for the HuMM and HuVV mice whereas the HuMV data were different. The data for sCJD(VV1) transmission gave profiles for HuMV and HuVV only and these were different from the other inocula. These data provided some evidence that a combination of both inoculum source and host codon 129 genotype can affect the overall transmission properties of each subgroup of sCJD to the transgenic mice. A statistical approach to lesion profile comparison was made to further elucidate these comparisons (see below).

Figure 6.F3: Lesion Profile Data for sCJD Inoculations

Lesion profile variation according to inoculum, in HuMM (blue squares), HuMV (green diamonds), HuVV (red triangles), and 129Ola (black circles) mice. (Error bars: ±SEM, brain regions defined in Methods)



6.3.1.6 Hierarchical Cluster Analysis of Lesion Profile Data

Comparative analysis can be achieved 'by eye' for the lesion profiles that show close similarities, such as the HuVV mice inoculated with sCJD(MV2) and sCJD(VV2). However in assessing the full dataset a statistical approach is more objective, and less subject to operator bias. Hierarchical Cluster Analysis (using software SPSS v14.0) was used to test the lesion profile data. This method is a means of classification of experiments into groups (clusters) that have similarities in the data. The degree of similarity was determined by a distance measure (in this case the Chebyshev interval) that was calculated from the differences between the lesion profile mean scores at each of the twelve brain regions. The hierarchical nature of this analysis builds successive clusters from those already established (containing the most similar data) to form a branching dendrogram. Using Hierarchical Cluster Analysis each of the twelve data points of the lesion profile were tested for similarity between different genotype mice and inocula used, and these similarities in the data were visualised in a dendrogram, the branching tree structure where the most similar lesion profiles are clustered together (Figure 6.F4). The more branches there are between data sets the less similar they are.

Data for lesion profiles from the primary transmissions showed branching of the dendrogram into three main clusters, as follows:

6.3.1.6.1 'Type 1' cluster

All codon 129 genotype mice inoculated with three type 1 PrP^{Sc} subgroups of sCJD (MV1, MM1 and VV1), but not sCJD (MM1) in HuVV mice.

6.3.1.6.2 'Type 2' cluster

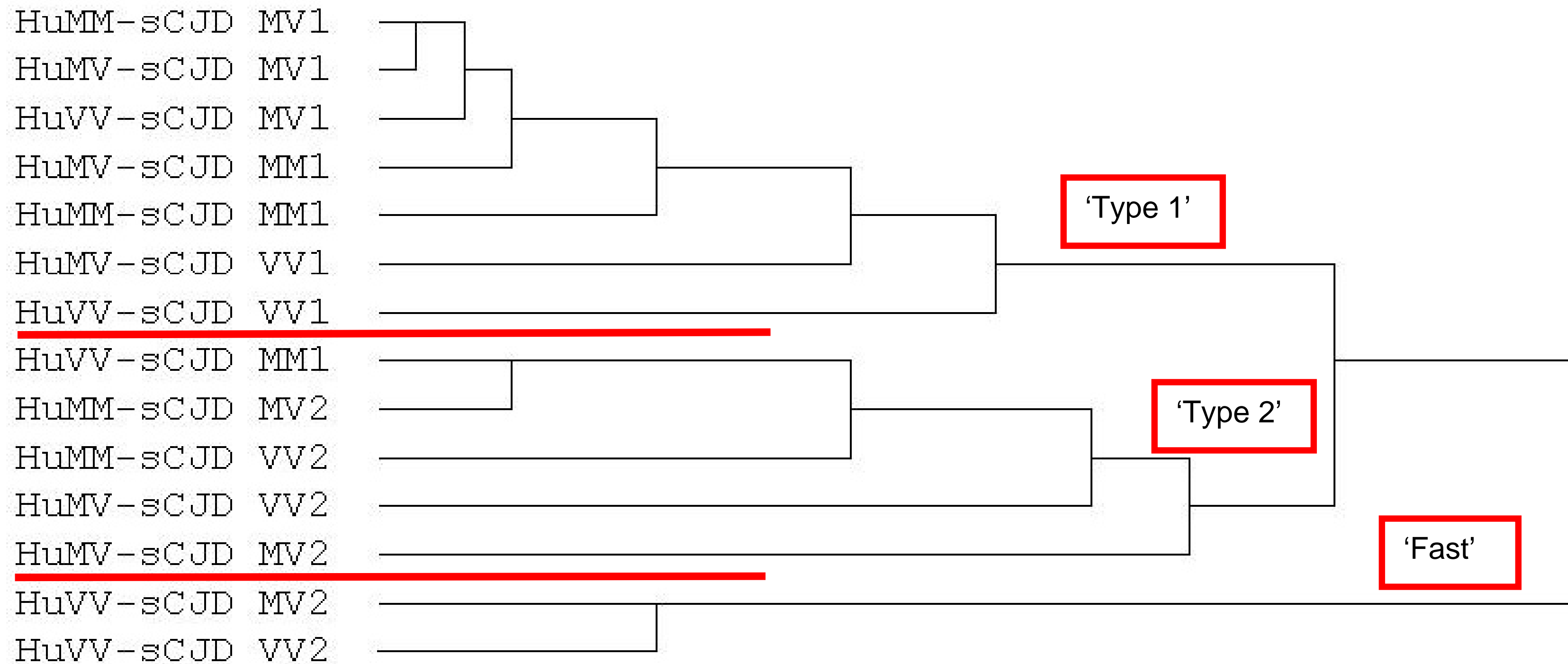
All codon 129 genotype mice inoculated with type 2 PrP^{Sc} sCJD cases (including MV2 and VV2). The exception is HuVV mice inoculated with sCJD (MM1).

6.3.1.6.3 'Fast' cluster

HuVV mice inoculated with type 2 PrP^{Sc} sCJD containing at least one valine allele at codon 129, that show relatively 'fast' incubation periods of <300 days post injection.

Figure 6.F4: Hierarchical Cluster Analysis Dendrogram

Hierarchical Cluster Analysis to assess variation in vacuolation intensity and location. (SPSS v14.0: within-groups linkage and the Chebyshev interval measure (which checks the absolute difference between the values))



6.3.1.7 Detection of PrP^{Sc} by Immunocytochemistry

Immunocytochemical (ICC) detection of disease associated PrP (PrP^{Sc}) in transgenic mouse models is commonly used as the standard method to confirm transmitted TSE disease in transgenic mice (Asano *et al.*, 2006). For this study, ICC gave the positive / negative scoring seen in Tables 6.T1 to T6 (column ICC), and provided a means of demonstrating PrP^{Sc} pathological targeting differences that could be attributable to an effect of the inocula or the codon 129 genotype of the transgenic mice.

The positive scores have been summarised in Table 6.T11. In mice already scored positive for clinical signs and vacuolation, detection of PrP^{Sc} confirmed positive transmission. However, in some mice negative for clinical signs or TSE vacuolation, ICC detected PrP^{Sc} deposition (For example, see Table 6.T4, HuMV data.). This suggested that variation in timing of appearance of pathological signs may be inoculum or host genotype dependent.

The positive scoring obtained by ICC detection of PrP^{Sc} showed that all three transgenic lines were ~100% positive for the three inocula tested (types MM1, MV2, and VV2). It was only wild-type mice with sCJD(MV2) and sCJD(VV2) inocula that showed no evidence of PrP^{Sc}. The remaining experiments will be analysed in the near future as there was insufficient time during this study to complete this work.

Table 6.T11: ICC Scoring Data for sCJD Inoculations

Summary of positive ICC scores compared between inocula and mouse lines. (N/T: not tested)

Inoculum	HuMM		HuMV		HuVV		129Ola	
MM1	13/13	100%	14/14	100%	14/16	87%	10/10	100%
MM2	N/T	-	N/T	-	N/T	-	N/T	-
MV1	N/T	-	N/T	-	N/T	-	N/T	-
MV2	10/11	91%	15/17	88%	15/15	100%	0/10	0%
VV1	N/T	-	N/T	-	N/T	-	N/T	-
VV2	15/17	88%	12/14	86%	15/15	100%	0/10	0%

6.3.1.8 Distribution of PrP^{Sc} as Detected by ICC

Defining the variation of type and location of PrP^{Sc} deposits, by qualitative and quantitative analysis of the ICC slides, in brains of the transgenic mice will hopefully allow further examination of whether it is the individual sCJD inocula, or the codon 129 genotype of the mice, that determines this pathology. This will be the goal of future analysis as there was insufficient time during this thesis to carry out such a detailed study. The differences in PrP^{Sc} deposition for the six subgroups of sCJD are discussed in the Introduction; some of those features may be replicated in the transmission to transgenic mice. Limited analysis of PrP^{Sc} deposition variation has been carried out and some differences and similarities have been observed. (Figure 6.F5) ICC slides from all mice in each inoculated group were checked to give an overall representation of the specific targeting of deposition seen, as discussed below. The degree of within-group variation appeared to depend on the incubation time of the mice, with an increase in deposition over time. The targeted regions did not change over this time period. The specific brain regions mentioned are outlined in diagrammatical form in Figure 6.F6.

6.3.1.8.1 sCJD(MM1) PrP^{Sc} Deposition

The sCJD(MM1) inoculated mice of each genotype showed similar overall patterns of staining: in the thalamic region with discrete, punctate, deposits; hippocampal targeting of the stratum lacunosum moleculare; and occasional larger deposits in deep layers of the cerebral cortex. The cerebellum was spared with only very little evidence of PrP^{Sc} deposition.

6.3.1.8.2 sCJD(MV2) and sCJD(VV2) PrP^{Sc} Deposition

The sCJD(MV2) and sCJD(VV2) inoculated mice showed very similar patterns of PrP^{Sc} deposition, which were different from that found with sCJD(MM1). The same thalamic regions were targeted as for sCJD(MM1), but as well as the punctuate deposits, there was an increase in more diffuse staining. The HuMM and HuMV lines showed a similar degree of staining concentrated in the ventral posteromedial, and posterolateral, thalamic nuclei. For the HuVV line this area extended to include the lateral dorsal nucleus, the posterior thalamic nuclear group, and the medial dorsal and ventromedial thalamic nuclei. Outside the thalamic region the HuVV mice again showed differences to the other lines. The cerebral cortex was heavily stained with distinctive banding associated with the cell type layers present. Band 6 showed the densest deposits. These HuVV mice developed clinical TSE following a significantly shorter incubation time and therefore the more widespread pathological change may be the cause of this phenotype. Intensely stained aggregates were seen in the white matter of the corpus callosum in both HuMM and HuVV mice, and not in the HuMV mice. The cerebellum shows similar staining patterns, targeted to the granular layer, for all mouse lines and both inocula.

Figure 6.F5: ICC Comparison for Three Common sCJD Subgroups

Immunocytochemistry with anti-PrP antibody 6H4 (brown staining) on representative histological sections of transgenic mouse brains, specifically the hippocampus and cerebellum, following i.c. inoculation with three subtypes of sCJD. (Images at 2.5x magnification.)

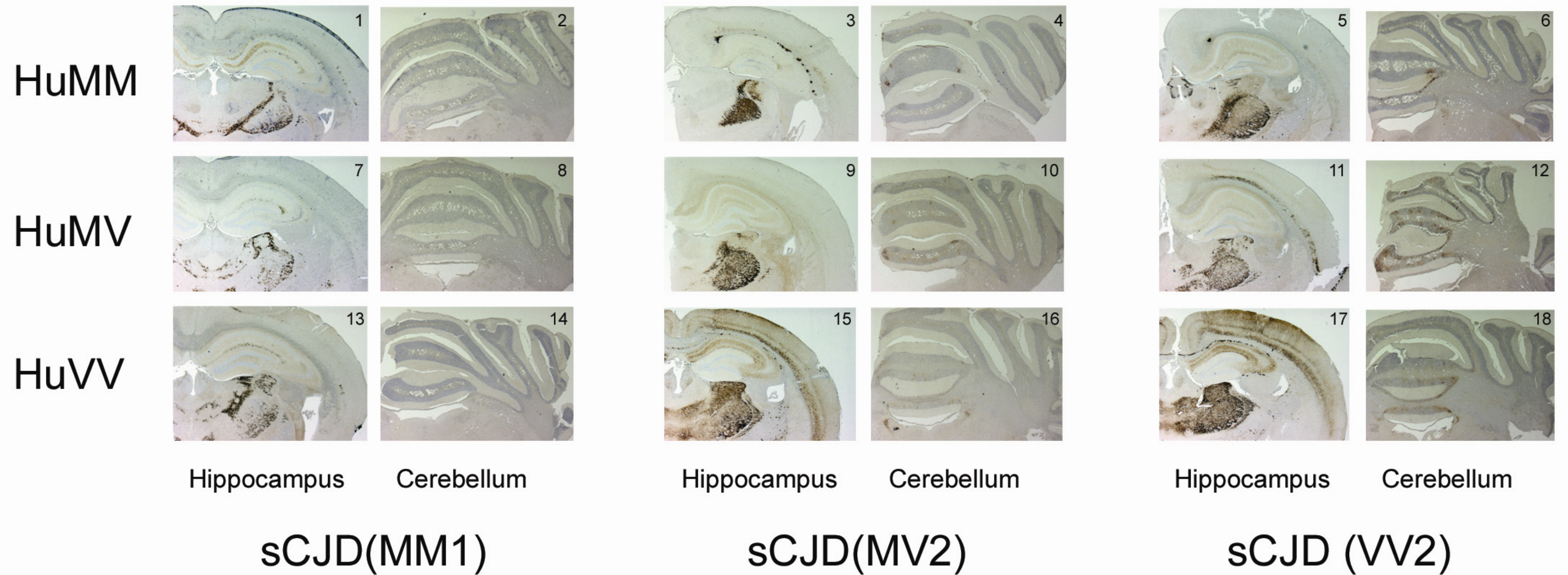
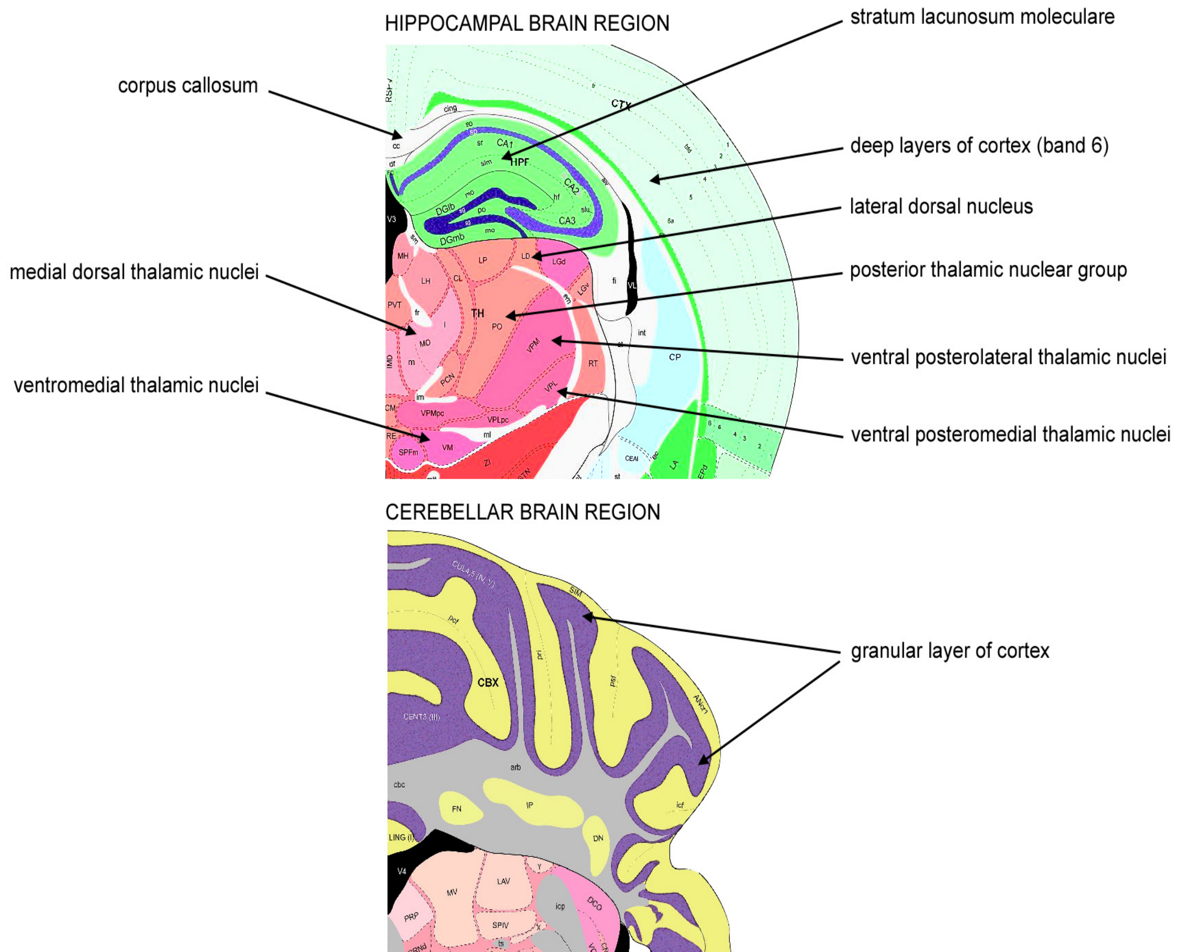


Figure 6.F6: Brain Map Reference Diagrams

Diagrammatic representation of the brain sections used in Figure 6.F5, highlighting the regions mentioned in the text. (Areas within hippocampal section: hippocampus: dark green region, thalamus: pink region, cerebral cortex: light blue) Images courtesy of the Allen Brain Atlas website.



6.3.1.9 Detection of PrP^{Sc} by Western Blot

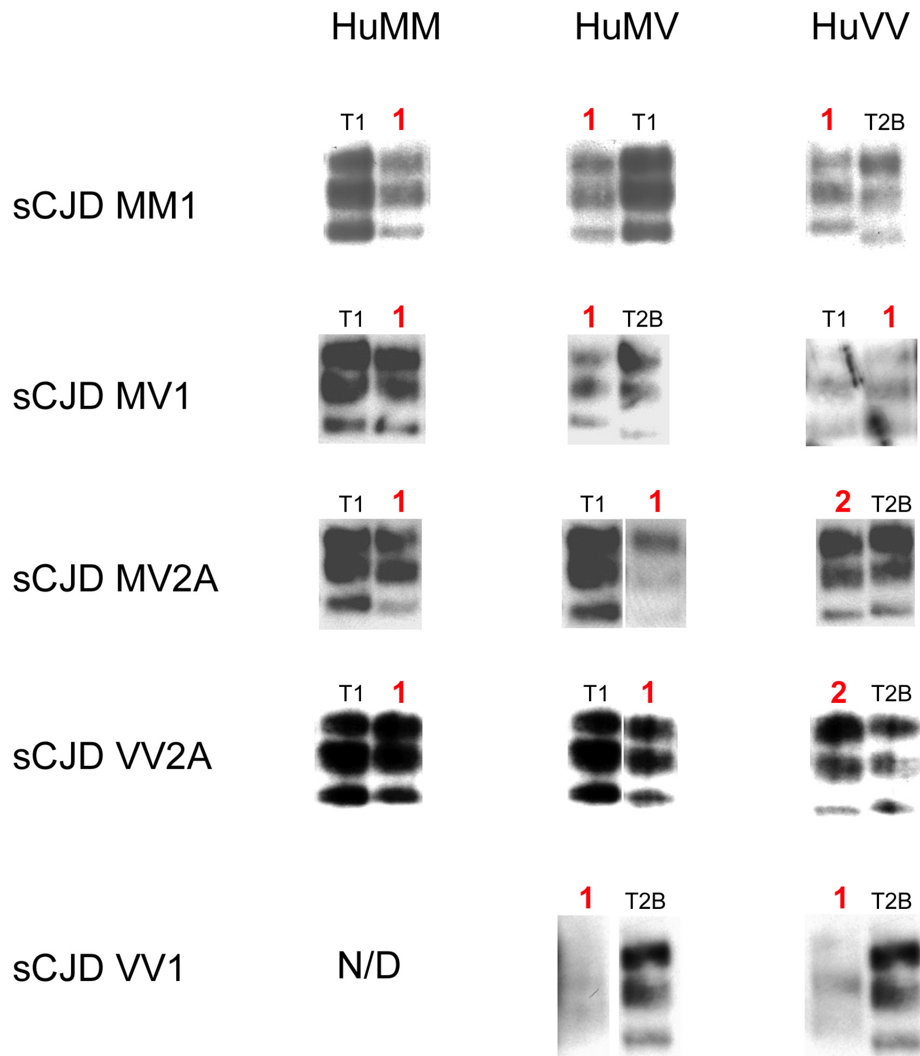
Western blot detection of PrP^{Sc} was performed on frozen brain material set aside during the *post mortem* of each mouse. As there was variation of protein type in the inoculum it could be that the PrP^{Sc} type determined the transmission strain characteristics, as was seen in the Hierarchical Cluster Analysis of the lesion profile data.

The PrP^{Sc} type produced by each of the three genotype host mice as a response to the inoculum in shown in Figure 6.F7. The appearance of type 2 PrP^{Sc} was restricted to the 'Fast' group (defined by the Cluster Analysis) of HuVV mice inoculated with either sCJD(MV2) or sCJD(VV2). These mice also showed a dominance of the diglycosylated fragment, a Western blot profile similar to vCJD (see adjacent lanes in Figure 6.F7). All the other mice / inoculum combinations in which PrP^{Sc} has been detected showed the usual sCJD type 1, monoglycosylated dominant, profile even if the inoculum was type 2. Very low levels of PrP^{Sc} were seen in the sCJD(VV1) experiment and for HuVV mice inoculated with sCJD(MV1).

No variation has been found in human PrP^{Sc} fragment mobility or glycosylation by using addition antibodies and it was therefore not deemed necessary to further analyse the mouse brain extracts (Head *et al.*, 2004a, Yull *et al.*, 2006).

Figure 6.F7: Western Blot Images for sCJD Inoculations

Western blots of brain extract from transgenic mice i.c. challenged with sCJD. (T1 and T2B human control samples of sCJD(MM1) and vCJD respectively; red number is test sample assigned type. Upper of three bands is the diglycosylated form, middle is monoglycosylated and lower is unglycosylated. Antibody: 6H4)



6.3.2 Secondary Inoculation of Mice with Primary Passage Material

Following primary inoculation of sCJD(MV2) all the HuVV mice developed clinical disease after much shorter incubation times than seen for other genotypes of mice and other experiments. This provided an opportunity to perform a secondary passage during the course of this study. Transmission data from secondary passage can provide evidence that the primary passage generated a disease in the mice that is infectious, and also whether there has been any host adaptation of the strain transmission properties. For instance the removal of a species barrier effect, on second passage, is suggested by a decrease in incubation period.

To generate duplicate data for confirmation of the results, two HuVV mice clinically and pathologically positive for transmission of sCJD(MV2) (protocol 553C-1A, see Table 6.T4) were selected for sub-passage of brain homogenate into further groups of 12 transgenic and wild-type 129Ola mice.

Protocol: 553H-1A: HuVV mouse ID# 214 (Incubation period 279 days)
Protocol: 553J-1A: HuVV mouse ID# 97 (Incubation period 280 days)

The number of mice injected was less (12) than the 18 used for primary inoculation as secondary passage material is less likely to be toxic as it is mouse instead of human derived, and should transmit more efficiently. Intercurrent illness and death reduced the total mice available for studying transmission properties over lifespan but sufficient mice survived to observe clinical and pathological signs of disease transmission. Where possible intercurrent deaths were also examined for markers of TSE pathology. Tables 6.T12 to 6.T13 show the evaluation of all mice in this study.

Table 6.T12: Data Table for Second Passage sCJD(MV2) Inoculation via HuVV Mouse

IC inoculation data (protocol reference 553H-1A) for secondary passage of sCJD(MV2) primary pass in HuVV
Abbreviations: CLIN: clinical score; VACN: presence of TSE vacuolation; ICC: presence of abnormal PrP by immunocytochemistry; ICD: intercurrent death or culled for animal welfare reasons; n/d: no data available as tissue not testable; n/t: not tested at this time; End expt: mice that were culled when the experiment was terminated at 700 days; shaded cell: positive score

HuMM						HuMV						HuVV						129Ola					
ID	Survival Time	Reason for cull	CLIN	VACN	ICC	ID	Survival Time	Reason for cull	CLIN	VACN	ICC	ID	Survival Time	Reason for cull	CLIN	VACN	ICC	ID	Survival Time	Reason for cull	CLIN	VACN	ICC
51	127	ICD	Neg	Neg	n/t	29	265	ICD	Neg	Neg	n/t	4	225	TSE	Pos	Pos	Pos	12	358	ICD	Neg	Neg	Neg
54	420	ICD	Neg	Neg	Pos	25	362	ICD	Neg	Neg	Pos	43	225	TSE	Pos	Pos	n/t	7	414	ICD	Neg	Neg	Neg
16	489	ICD	Neg	Pos	Pos	30	442	ICD	Neg	Neg	Pos	44	226	TSE	Pos	Pos	Pos	35	421	ICD	Neg	Neg	Neg
14	529	ICD	Neg	Pos	Pos	58	503	ICD	Neg	Pos	Pos	45	226	TSE	Pos	Pos	Pos	36	526	TSE	Pos	Neg	Neg
17	554	ICD	Neg	Pos	Pos	57	540	ICD	Neg	Pos	Pos	47	226	TSE	Pos	Pos	Pos	31	620	ICD	Neg	Neg	n/t
50	606	ICD	Neg	Pos	Pos	27	559	ICD	Neg	Neg	Pos	3	238	TSE	Pos	Pos	Pos	10	673	ICD	Neg	Neg	Neg
15	610	ICD	Neg	Pos	Pos	59	582	ICD	Neg	Neg	Pos	6	238	ICD	Neg	Pos	n/t	34	680	ICD	Neg	Neg	Neg
52	638	TSE	Pos	Pos	Pos	55	589	ICD	Neg	Pos	Pos	2	239	TSE	Pos	Pos	Pos	32	702	ICD	Neg	Neg	Neg
49	658	TSE	Pos	Pos	Pos	60	670	ICD	Neg	Neg	n/t	46	239	TSE	Pos	Pos	Pos	33	783	ICD	Neg	Neg	n/t
13	673	ICD	Neg	Pos	Pos	56	678	ICD	Neg	Pos	Pos	48	243	TSE	Pos	Pos	Pos	8	812	End expt	Neg	Neg	n/t
18	719	ICD	Neg	Pos	n/t	26	722	ICD	Neg	Neg	Pos	5	246	TSE	Pos	Pos	Pos	9	812	End expt	Neg	Neg	n/t
						28	754	ICD	Neg	Neg	Pos	1	253	TSE	Pos	Pos	Pos						
'Attack Rate'			2/11	9/11	9/9				0/12	4/12	10/10				11/12	12/12	10/10				0/11	0/11	0/7

Table 6.T13: Data Table for Duplicate Second Passage sCJD(MV2) Inoculation via HuVV Mouse

IC inoculation data (protocol reference 553J-1A) for secondary passage of sCJD(MV2) primary pass in HuVV
Abbreviations: CLIN: clinical score; VACN: presence of TSE vacuolation; ICC: presence of abnormal PrP by immunocytochemistry; ICD: intercurrent death or culled for animal welfare reasons; n/d: no data available as tissue not testable; n/t: not tested at this time; End expt: mice that were culled when the experiment was terminated at 700 days; shaded cell: positive score

HuMM						HuMV						HuVV						129Ola					
ID	Survival Time	Reason for cull	CLIN	VACN	ICC	ID	Survival Time	Reason for cull	CLIN	VACN	ICC	ID	Survival Time	Reason for cull	CLIN	VACN	ICC	ID	Survival Time	Reason for cull	CLIN	VACN	ICC
19	264	ICD	Neg	Neg	n/t	4	335	ICD	Neg	Neg	n/t	40	224	TSE	Pos	Pos	n/t	17	426	ICD	Neg	Neg	n/t
33	320	ICD	Neg	Neg	n/t	5	448	ICD	Neg	Neg	n/t	42	225	TSE	Pos	Pos	n/t	58	440	ICD	Neg	Neg	n/t
36	410	ICD	Neg	Neg	n/t	46	455	ICD	Neg	Neg	n/t	38	231	TSE	Pos	Pos	n/t	59	502	ICD	Neg	Neg	n/t
23	473	ICD	Neg	Neg	n/t	44	553	TSE	Pos	Pos	n/t	9	238	TSE	Pos	Pos	n/t	56	567	ICD	Neg	Neg	n/t
35	539	TSE	Pos	Pos	n/t	43	623	ICD	Neg	Pos	n/t	37	238	TSE	Pos	Pos	n/t	15	601	ICD	Neg	Neg	n/t
24	576	ICD	Neg	Pos	n/t	45	655	ICD	Neg	Pos	n/t	39	243	TSE	Pos	Pos	n/t	57	663	ICD	Neg	Neg	n/t
31	595	ICD	Neg	Pos	n/t	47	655	ICD	Neg	Pos	n/t	7	244	TSE	Pos	Pos	n/t	55	670	ICD	Neg	Neg	n/t
22	636	ICD	Neg	Pos	n/t	1	679	ICD	Neg	Pos	n/t	8	245	TSE	Pos	Pos	n/t	14	721	ICD	Neg	Neg	n/t
20	662	ICD	Neg	Pos	n/t	48	686	ICD	Neg	Pos	n/t	11	245	TSE	Pos	Pos	n/t	18	721	ICD	Neg	Neg	n/t
34	679	ICD	Neg	Neg	n/t	3	695	ICD	Neg	Neg	n/t	10	246	TSE	Pos	Pos	n/t	13	791	ICD	Neg	Neg	n/t
32	694	ICD	Neg	Pos	n/t	2	770	ICD	Neg	Neg	n/t	12	246	TSE	Pos	Pos	n/t	16	811	End expt	Neg	Neg	n/t
21	735	TSE	Pos	Pos	n/t																		
'Attack Rate'			2/12	7/12					1/11	6/11					11/11	11/11					0/11	0/11	

6.3.2.1 Summary of Second Passage Data

The results of clinical and pathological scoring of mice from the duplicate secondary passage experiments are presented in Table 6.T14 and show only slight variation between the two data sets. The main conclusion from this work was that the transmission properties of the HuVV brain and original human source inocula showed remarkable similarities in incubation period, targeting of vacuolation, and efficiency of transmission to the three genotype hosts.

6.3.2.1.1 Clinical Features of Transmission

Primary and secondary passage to the HuMM and HuMV mice showed similar low levels of clinical disease, even though the second passage inoculum was derived from a mouse brain. This was remarkable considering that the inocula produced mean incubation periods in HuVV recipient mice of 235 and 239 days for the two experiments. The primary passage gave an incubation time in HuVV mice of 288 days, therefore there had been a reduction for second passage of approximately 50 days, suggesting adaptation of the infectious agent.

6.3.2.1.2 Pathological Features of Secondary Passage Mice

The number of mice scored positive for the presence of TSE vacuolation and PrP^{Sc} by ICC confirmed that the secondary passage transmission properties were identical to that of the primary passage. Approximately one-third of HuMV mice developed vacuolation compared with two-thirds of HuMM mice and 100% of HuVV mice. Almost all mice regardless of codon 129 genotype were positive for PrP^{Sc} deposition in the brain. There was no evidence that the spatial distribution or type of deposits were different from that found in the primary passage mice.

Lesion profile data comparison for vacuolation scoring further confirmed the high degree of similarity between primary and secondary passage, with the distribution and intensity of vacuolation in the latter mirroring that seen in the former (Figure 6.F8). The pathological transmission properties of the original sCJD(MV2) human inoculum strain had therefore been propagated by brain tissue inoculum derived from HuVV mice.

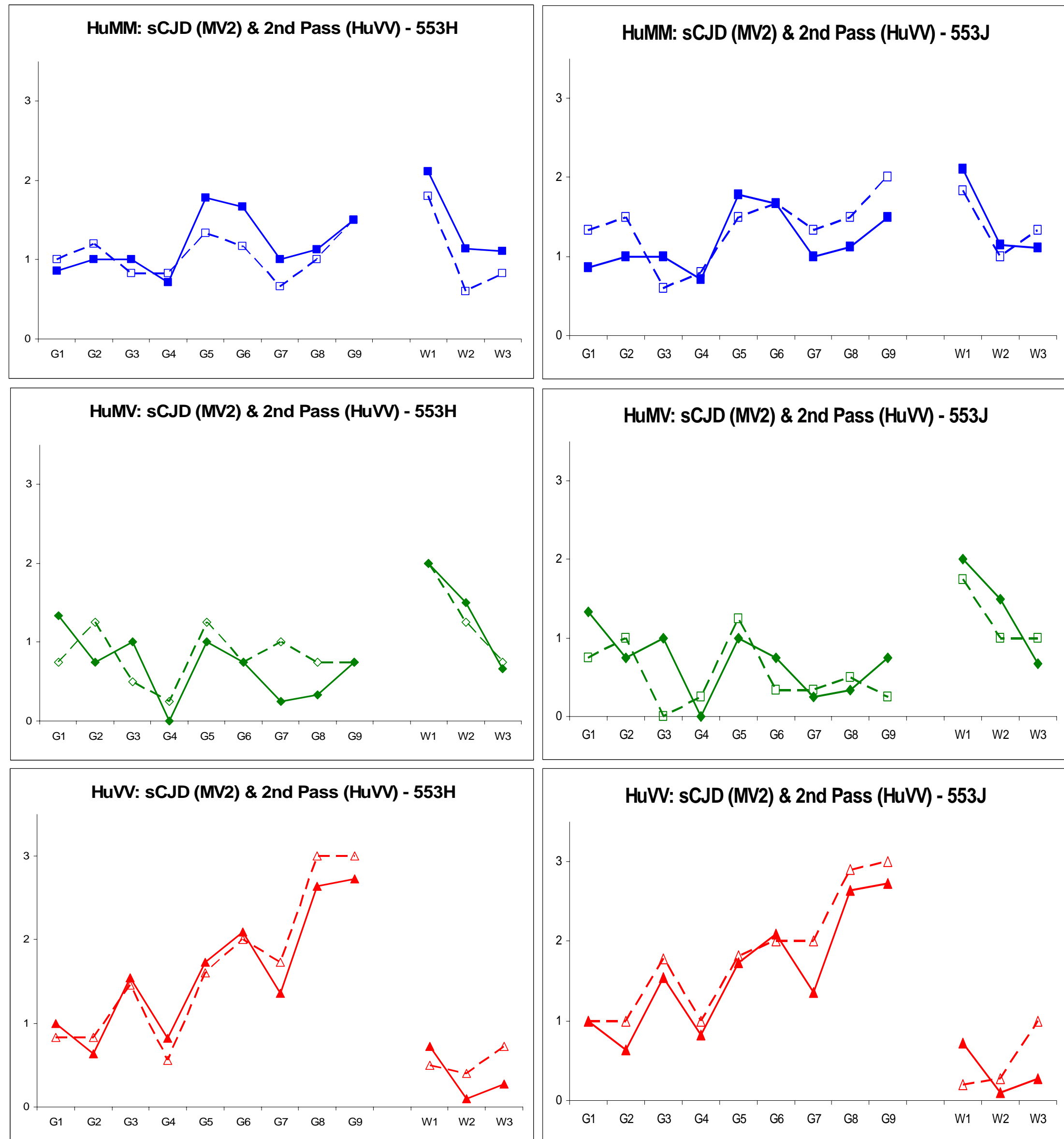
Table 6.T14: Summary of Data for Primary and Secondary sCJD(MV2) Inoculation

Summary of experimental data for primary and secondary inoculation (via HuVV mice) of sCJD(MV2). (Incubation period for clinically positive mice (mean (range)); positive scores out of mice tested; n/t: not tested)

Transgenic Line	sCJD(MV2)	Second Passage (Expt: 553H)	Second Passage (Expt: 553J)
HuMM			
Incubation Period	563 (559-566)	648 (638,658)	637 (539,735)
Clinical Positives	3/13 (23%)	2/11 (18%)	2/12 (17%)
Vacuolation Positives	9/13 (69%)	9/11 (82%)	7/12 (58%)
ICC Positives	10/11 (91%)	9/9 (100%)	n/t
HuMV			
Incubation Period	575 (538,612)	-	553
Clinical Positives	2/16 (12%)	0/12	1/11 (9%)
Vacuolation Positives	6/17 (35%)	4/12 (33%)	6/11 (54%)
ICC Positives	15/17 (88%)	10/10 (100%)	n/t
HuVV			
Incubation Period	288 (267-308)	235 (225-253)	239 (224-246)
Clinical Positives	16/17 (94%)	11/12 (92%)	11/11 (100%)
Vacuolation Positives	16/16 (100%)	12/12 (100%)	11/11 (100%)
ICC Positives	15/15 (100%)	10/10 (100%)	n/t

Figure 6.F8: Lesion Profile Similarities for Primary and Secondary sCJD(MV2) Inoculation

Lesion profiles from sCJD(MV2) primary (solid lines and symbols) and sCJD(MV2)-HuVV secondary (dashed lines and open symbols) transmissions. (HuMM: blue squares; HuMV: green diamonds; HuVV: red triangles; error bars omitted)



6.4 DISCUSSION

Following inoculation of the six subtypes of sCJD, segregated according to codon 129 genotype and PrP type, in the four mouse lines, data for multiple pairwise comparisons determined whether characteristics of the host or the inoculum were the major factors for successful transmission. The significant variability, within and between inocula and mouse lines, in the dataset generated by this study has provided evidence, discussed below, that the subtypes of sCJD behave like separate transmissible strains, and that there are dominant effects attributable to the codon 129 genotype and PrP^{Sc} type of the host and inoculum. Table 6.T15 summarizes the data currently available from this study for the six sCJD subgroups.

Table 6.T15: Summary of sCJD Transmission Data

Overall transmission attack rate from positive scores for vacuolation and ICC (%), mean incubation time if clinical signs present, and PrP^{Sc} type by Western blot. (Green: clinical TSE in majority of mice; Yellow: subclinical TSE disease in majority of mice; Red: no evidence of transmission; * vacuolation scoring only – no ICC data)

sCJD INOCULUM	HuMM	HuMV	HuVV	129Ola
MM1	100% Type 1 446 days	100% Type 1 457 days	87% Type 1 588 days	100% Subclinical
MV1*	94% Type 1 446 days	89% Type 1 475 days	83% Type 1 596 days	6% Subclinical
MV2	91% Type 1 Subclinical	89% Type 1 Subclinical	100% Type 2 288 days	0% >750 days
VV2	88% Type 1 Subclinical	86% Type 1 Subclinical	100% Type 2 273 days	0% >750 days
VV1*	O% >750 days	60% Type 1 Subclinical	50% Type 1 Subclinical	6% Subclinical
MM2*	O% >750 days	O% >750 days	O% >750 days	0% >750 days

6.4.1 Characterisation of sCJD Subgroups as Unique Transmissible Strains

One main aim of this study was to use transmission data from each of the six subgroups of sCJD to understand further the individual strain characteristics. Using survival analysis, incubation times, vacuolation scoring, and detection of PrP^{Sc} by ICC and Western blot, similarities and differences have emerged. Overall the data suggested that each of the subgroups, represented here by a single typical case, had unique transmission properties, although some subgroups were similar. The

following paragraphs discuss each subgroup in turn to highlight their unique features when inoculated into human transgenic mice and the wild-type 129Ola line.

6.4.1.1 SCJD(MM1)

The majority of sCJD cases (~70%) fall into this category (Parchi et al., 1999, Pocchiari et al., 2004); it is the 'classical' / myoclonic form of the disease. Successful transmission to all three transgenic lines and the 129Ola wild-type mice made this the most efficiently transmitted subgroup. If transmission efficiency was comparable to the probability of a human being developing the disease then this is consistent with the prevalence of this subgroup. As the aetiology of sCJD is still a matter of debate there remains a possibility that it is an acquired disease with the MM1 subgroup being the disease caused by the most transmissible / infectious agent.

There was little difference in the number of mice positive for clinical signs, or the incubation periods, for the three transgenic mouse lines inoculated with both sCJD(MM1) and sCJD(MV1). The Tarone-Ware results, however, indicated that the survival rate of the HuVV mice was different between the inocula. The sCJD(MV1) inoculum appeared to have had a more detrimental effect on the HuVV line than the sCJD(MM1) inoculum, with more early intercurrent deaths. This effect may be due to the presence of valine allele associated PrP^{Sc} in the inoculum which may more efficiently convert the valine allele PrP^{C} of the VV genotype mice. A comparison of solely the HuVV mice data between the two inocula did not support this finding (Table 6.T8).

SCJD(MM1) transmission produced clinical TSE symptoms in all three transgenic lines. This was also seen with sCJD(MV1) inoculum but this differed from any of the other four subgroups. There was a methionine allele dominance in

the mean incubation time, with little observable gene dosage effect (i.e. HuMM mice did not have shorter incubation periods than HuMV mice), with HuMM and HuMV mice developing TSE signs at ~450-470 days and HuVV mice at ~600 days post inoculation.

SCJD(MM1) lesion profiles for the transgenic mice showed consistency across the lines, however, Hierarchical Cluster Analysis suggested that the vacuolation distribution in the HuVV mice was different from the others, and more like transmissions from PrP^{Sc} type 2 associated subgroups (Figure 6.F3 and Figure 6.F4). This infers a dominance effect of the VV host genotype over the sCJD(MM1) inoculum strain characteristics.

Detection of PrP^{Sc} by ICC confirmed that when TSE associated vacuolation was present there was also deposition of PrP^{Sc}, with no suggestion of a subclinical disease state where PrP^{Sc} was present without vacuolation. The pattern of PrP^{Sc} distribution in the brain appeared similar for all transgenic lines, with more punctate appearance and less cerebellar involvement than sCJD(MV2) or sCJD(VV2) (Figure 6.F5). This suggested a more dominant role for the sCJD(MM1) inoculum over host genotype in determining the pathological targeting.

100% subclinical disease (presence of TSE vacuolation and / or PrP^{Sc} detection without clinical signs) in 129Ola wild-type mice distinguished this subgroup from all the others. Only the MV1 and VV1 subgroups showed evidence of wild-type transmission with <10% of mice showing subclinical disease. Western blot analysis showed the PrP^{Sc} formed in each transgenic line to be type 1 and monoglycosylation dominant, as per standard sCJD(MM1) brain material.

Together these data indicate that the sCJD(MM1) subgroup strain transmission characteristics were different from the other five studied here.

6.4.1.2 SCJD(MV1)

Approximately 5% of sCJD cases are of this subgroup (Parchi et al., 1999, Pocchiari et al., 2004) and are grouped with the MM1 cases as the ‘classical’ form of the disease. Consistent with these clinical and pathological findings in humans the transmission properties of sCJD(MV1) showed few differences to sCJD(MM1). The main difference found compared to sCJD(MM1) was the much lower transmission efficiently to the 129Ola wild-type mice.

Survival analysis and Tarone-Ware statistical tests confirmed that the HuVV line was more adversely affected by this inoculum compared with sCJD(MM1) (Table 6.T7 and Figure 6.F1). This difference was also seen in the lesion profile pattern of the HuVV line. Hierarchical Cluster Analysis of the lesion profile scores grouped the HuVV data with the other two genotype lines, in contrast to the sCJD(MM1) data (Figure 6.F4). This difference may be attributable to the presence of valine allele PrP^{Sc} in the inoculum and host.

ICC data was not available for mice injected with this inoculum. Western blot analysis showed each transgenic line propagated the type 1 sCJD profile as seen in patients typical of the sCJD(MV1) subgroup.

The sCJD(MV1) strain transmission properties had close similarities to sCJD(MM1) however the differences discussed above indicated that this strain is unique within the 6 subgroups.

6.4.1.3 SCJD(VV2) and SCJD(MV2)

The sCJD(VV2) subgroup is the second most common form of sCJD occurring in ~15% of cases, with sCJD(MV2) the third most common at ~10% (Parchi et al., 1999, Pocchiari et al., 2004). These subgroups shared near identical transmission properties and have therefore been discussed together. For both inocula the HuMM and HuMV mice showed a predominance of subclinical infection, and HuVV mice rapidly developed clinical TSE signs at ~280 days posts inoculation. These data were unlike any of the other 4 sCJD subgroups.

Neither data from survival analysis, presence of clinical symptoms, nor mean incubation periods could distinguish the data sets from sCJD(MV2) and sCJD(VV2) subgroup inocula.

Hierarchical Cluster Analysis of lesion profiling data grouped the sCJD(VV2) data together with the sCJD(MV2) data and therefore there was no evidence to distinguish the two strains. However, the attack rate for positive vacuolation scoring was higher (approximately double) in the HuMM mice with sCJD(MV2) at 69%, compared to the sCJD(VV2) inoculum, at 33% (Table 6.T10). Codon 129 genotype may be the cause of this effect with the MM mouse more rapidly developing TSE pathology with an inoculum containing a methionine allele. This was the only evidence to distinguish between these two sCJD strains.

ICC data for the number of mice positive for PrP^{Sc}, and distribution of PrP^{Sc} in the brain, showed nothing to separate sCJD(MV2) and sCJD(VV2) inocula. The appearance of type 2 PrP^{Sc} by Western blot was only found in HuVV mice challenged with these two inocula, suggesting a specific response from VV hosts to inoculum containing type 2, valine allele PrP^{Sc}. Type 2 PrP^{Sc} from the HuVV mice

had also been shown to be predominantly diglycosylated, similar to the Western blot pattern characteristic of vCJD. This finding highlighted the possibility that if a VV individual was infected by material from a sCJD(MV2) or sCJD(VV2) patient then typing of the PrP^{Sc} present in the brain may suggest vCJD. Two cases of young (<45 years old) VV sCJD patients have been reported with Western blot types indicative of vCJD (Head *et al.*, 2001, Mead *et al.*, 2007). The 2007 publication suggested that patient may represent a novel form of vCJD. No reference was made to a possible iatrogenic cause of infection in those two cases.

Secondary passage of brain material from two HuVV mice, initially inoculated with sCJD(MV2), into the three transgenic lines, produced scores for clinical TSE and vacuolation that were identical to the original transmission (Table 6.T14). Also the lesion profiles when overlaid onto the primary data showed near identical profiles (Figure 6.F8). There was no possibility of residual inoculum being present from the initial injection as it would be significantly diluted by the time the brains were prepared as inocula. The chance of contamination was very small as the primary and secondary experiments were set up approximately one year apart and other tissues had been injected in the intervening time with different transmission results.

Although there were a number of similarities between these two sCJD subgroup strains the relative increase in TSE vacuolation positive HuMM mice with sCJD(MV2) inoculum allowed for discrimination from sCJD(VV2). The rapid transmission and type 2 PrP^{Sc} found in HuVV mice clearly distinguished these two strains from the other 4 subgroups.

6.4.1.4 SCJD(VV1)

This subgroup of sCJD occurs in <5% of cases (Parchi et al., 1999, Pocchiari et al., 2004) and is therefore one of the rarer forms of this disease. There was no evidence of transmission to the HuMM mice and only 50-60% subclinical disease in the other mice lines. This strain was the only one to produce subclinical TSE in the HuVV mice (MM1, MV1, MV2, and VV2 produced a majority of mice with clinical TSE, and MM2 showed no evidence of transmission). The survival curve for these HuVV mice was statistically different from that of the wild-type mice (P=0.026), unlike the other two transgenic lines. Only two of the HuVV and HuMV mice developed clinical TSE at ~550 days, but the survival analysis showed that the former may be more affected subclinically by the inoculum. This may be due to the 100% genotype homology between mouse and inoculum. There was no increase in vacuolation for the HuVV mice and ICC data is not currently available, which may allow further examination of this effect. These data show that this strain had different transmission properties to all other five subgroups.

Lesion profiling and Hierarchical Cluster Analysis placed the HuMV and HuVV data with others in the 'Type 1' group (sCJD(MM1) and sCJD(MV1)) suggesting a common pathological response from all genotype mice to the type 1 configuration of PrP^{Sc} in inocula. These inocula could be of any codon 129 genotype. The presence of a single 129Ola mouse positive for vacuolation indicated some similarity with the sCJD(MV1) inoculum. In fact only type 1 PrP^{Sc} inocula showed evidence of transmission to the wild-type 129Ola mice.

Western blot data indicated low levels of type 1 PrP^{Sc} in the HuMV and HuVV mice. The presence of type 1 PrP^{Sc} in both mouse lines was the same as that

found for the other type 1 inocula, indicating a response to the inocula rather than a host genotype effect.

ICC data is unavailable at present but is likely to improve the attack rate based on the findings of the other inocula. Even without this data there was clear segregation of this subgroup of sCJD, as shown above, compared to all others examined.

6.4.1.5 SCJD(MM2)

Approximately 5% of sCJD cases are in this subgroup. Two types exist described as ‘cortical’ or ‘thalamic’, the former is the more common and was used in this study. No evidence of transmission was observed in either of the four mouse lines, although ICC data is unavailable, the most sensitive test for confirming transmission. There was some evidence however from the survival analysis that this inoculum was more detrimental to the transgenic lines than to the wild-type. The Tarone-Ware test showed that there was a small statistical difference between the wild-type mice and the transgenics ($P<0.01$) suggesting that the inoculum preferentially affected the transgenic lines. Use of additional aliquots of this inoculum in experiments outside this study showed that it was infectious so possibly PrP^{Sc} deposition may be the only indication of successful transmission. The combination of type 2, and MM genotype PrP^{Sc} may also be a barrier to propagation or PrP^{Sc} conversion in the host animals and therefore this ‘negative’ experiment was important. Should an individual become infected with sCJD (MM2) then the risk of developing iCJD is the least for any form of sCJD.

The absence of evidence of transmission in any mouse line showed this subgroup of sCJD to be different from all five others investigated here.

6.4.2 Role of Codon 129 Genotype and PrP^{Sc} Type in Transmission of sCJD

The second aim of this study was to observe the effects on transmission of codon 129 genotype and PrP^{Sc} type, defined in both host and inoculum. Methionine or valine at codon 129 was the only genetic variation between the three lines of transgenic mice and therefore the effect of genotype can be observed in the differences between the lines. Inoculation with sCJD material from patients with each of the three codon 129 genotypes, and either PrP^{Sc} type, allowed for further observation of how each mouse line responded to inocula with different genotypes and type 1 or 2 PrP^{Sc}.

Transmission properties were determined by the genotype of both transgenic mouse line and sCJD inoculum, and by the PrP^{Sc} type. This meant that no generic rules could be defined for the three genotype hosts. The human response to a source of infection is likely therefore to depend on both their own genotype and the genotype and PrP^{Sc} type (the subgroup) of sCJD.

There were some characteristics within the dataset that suggested specific combinations of genotype and PrP^{Sc} type were more dominant, such as the observation that the HuVV genotype line developed clinical TSE features with most inocula. HuVV mice also had the shortest incubation periods, seen for sCJD(MV2) and sCJD(VV2) inocula. These data predict that for human iatrogenic spread of sCJD, this genotype may be the most susceptible or may show shorter incubation periods. An overabundance of iCJD cases in VV genotype recipients of contaminated human growth hormone in the UK would support this hypothesis (Collinge *et al.*, 1991, Brown *et al.*, 2000a). It is also of interest that there is an

increased prevalence of young (<50 years) VV genotype sCJD cases across European countries (UK, Germany, Italy and France) (Alperovitch *et al.*, 1999).

The second common characteristic amongst the data was that HuMM and HuMV mice had similar levels of clinical disease, and mean incubation periods, for four of the six inocula (excluding sCJD(MM2) and sCJD(VV1)). This suggests that the methionine allele PrP^C in the heterozygous HuMV mice may have had a dominant effect over the valine allele PrP^C with regards to the transmission properties.

The most significant effect of the inoculum PrP^{Sc} type was seen in the targeting of vacuolar pathology. Hierarchical Cluster Analysis of the lesion profile data clearly indicated that there was closer similarity between those profiles produced from inoculum with a specific type of PrP^{Sc} than for groups of mice or inocula with the same codon 129 genotype (Figure 6.F4). As PrP^{Sc} type is closely associated with specific pathology in the sCJD patients, these data would indicate that pathological changes in the tissue selected for inocula had potentially the most dominant role in determining transmission properties.

The transgenic mice produced PrP^{Sc} of only two confirmations that matched the type 1 mobility and predominance of monoglycosylation as seen in type 1 sCJD patients, and the type 2 mobility and predominance of diglycosylation as seen in vCJD patients. Figure 6.F7 shows that type 2 PrP^{Sc} was seen in the HuVV mice that had short incubation periods, after inoculation with sCJD(MV2) and sCJD(VV2). All other mice genotype / inoculum combinations produced type 1 PrP^{Sc}. This would indicate a specific link between inocula with valine allele type 2 PrP^{Sc}, and the valine homozygous mouse host.

Type 1 PrP^{Sc} was found in mice of each codon 129 genotype and following inoculation with material of each genotype and either PrP^{Sc} type. The main conclusion that can be drawn from the widespread appearance of type 1 PrP^{Sc}, even if the inoculum is type 2, is that this type is the dominant type produced by these mice following TSE challenge. Finding type 1 PrP^{Sc} in mice inoculated with type 2 sCJD inocula indicated that the PrP^{C}-to-PrP^{Sc} conversion is controlled by the properties of the host mouse PrP.

CHAPTER 7

CONCLUSIONS

7.1 AIMS

The aims of this thesis were to investigate the transmission of a number of different forms of human transmissible spongiform encephalopathy (TSE) to gene targeted transgenic mice that express the human prion protein with variation in genotype at codon 129. This allowed detailed examination of the role of codon 129 genotype in susceptibility and the identification of unique strain properties amongst the types of human TSE. The hypotheses discussed below were tested from the data generated.

7.2 HYPOTHESES

7.2.1 Codon 129 Genotype is the Key Factor in Defining Susceptibility to Human TSE Transmission

Susceptibility is generally defined as whether an infected individual develops any evidence of TSE such as clinical signs, vacuolation, or PrP^{Sc} deposition. However a more common usage is the degree to which an individual is affected, for example the relative progression of pathological changes and the level and distribution of PrP^{Sc} deposition in the brain.

Data from this thesis indicate that both inoculum and recipient codon 129 genotype play significant roles in defining aspects of susceptibility to human TSE such as incubation period and pathological response in the host, and transmission

efficiency of the inocula. The most significant importance of this mouse model is that the specific effects of codon 129 genotype on transmission can be assessed independently of the influence of alternative genetic backgrounds or the location of the transgene. For primary vCJD transmission, variation in codon 129 genotype could not be studied as the source of inoculum, clinical vCJD, has only been detected in MM patients. However, second passage from each genotype transgenic line has shown the influence of non-MM vCJD strains on transmission.

The codon 129 genotype of recipient mice significantly affected the outcome of primary vCJD transmission with the most efficient host being MM followed by MV and then VV. The MM and MV mice had similar frequency of positive scores suggesting equivalent susceptibility, but the MM line more readily developed vacuolar pathology and widespread deposits of abnormal PrP, together with the appearance of florid plaques, which developed only in this line. Pathological changes in the MV mice did not progress further than limited thalamic deposition of PrP^{Sc}, suggesting that the heterozygous genotype may restrict the progression of TSE pathology. That there was only a single HuVV mouse positive for vCJD transmission indicated that this genotype appeared to be the most resistant to infection. Transmission data from the blood transfusion vCJD agent strain showed near identical findings in the three codon 129 genotype hosts indicating that the TSE agent has not been significantly adapted by passage through a second MM genotype human.

Second passage transmission data from the primary vCJD inoculations of each genotype mouse line showed an overall decrease in the pathological changes, but the ranking of codon 129 genotypes in the recipient lines was unchanged.

Regardless of the inoculum source genotype, the MM recipients were the most susceptible to infection followed by the MV mice, with all VV mice negative. The vCJD agent strain transmission characteristics appear to be stable regardless of the genotype of the host.

The robust nature of the vCJD agent transmission properties, seen in this mouse model, with specific targeting of MM hosts and resilience to adaptation by passage is important for assessing individual risk following known exposure and for modelling possible future epidemics of vCJD associated with human-to-human infection. The replication of pathological and biochemical features of human vCJD disease in mice provides an *in-vivo* model system which may allow further investigation into pathogenic mechanisms and therefore may provide an understanding of individual susceptibility and novel targets for therapeutic intervention.

The data from sporadic CJD transmissions showed a complex relationship between the codon 129 genotype of inoculum and recipient mice. However differences in the response between the three mouse lines indicated that the recipient genotype had a major role in determining the transmission properties. An individual's risk of iatrogenic infection from sCJD may therefore be determined primarily by the host codon 129 genotype.

With sCJD the VV genotype mice were the most susceptible overall in terms of the number of mice positive for transmission and had the shortest incubation periods (for transmission of sCJD(MV2) and sCJD(VV2)). One implication is that VV individuals may be the most susceptible to infection following iatrogenic transmission of sCJD, with relatively short incubation times, if the infectious

material originated from sCJD(MV2) or sCJD(VV2) cases. This host targeting is very different from that seen for vCJD transmission. The reason may be that the vCJD agent strain is of bovine origin, is restricted to MM genotype hosts, is characterised by type 2B PrP^{Sc}, and has markedly different neuropathology compared to sCJD.

Sporadic CJD subgroup MM1 is by far the most common form of CJD because of as yet undefined factors (such as non-*PRNP* genetic polymorphisms) that may increase the susceptibility of these individuals. It is therefore likely that this subgroup would represent the major source for secondary infection. All three genotype mouse lines were 100% susceptible to sCJD(MM1) infection, but the HuVV mice had longer incubation periods than HuMM and HuMV mice. The predicted prevalence of MM and MV iatrogenic TSE cases may therefore be higher than VV cases. However, data published in 2000 show that overall, for all types of iCJD combined, there was an increase in MM and VV patients compared with the normal population genotype frequencies (Brown *et al.*, 2000a). As this does not fit the predicted prevalence of cases from the transmission studies, the source of infectivity for the iCJD patients may have included sCJD subgroups that target VV genotype hosts with short incubation periods, such as sCJD(MV2) or sCJD(VV2).

In summary, for human TSE diseases, the codon 129 genotype of both the infectious agent and the host (in this study mice) determine the transmission characteristics. The inoculum genotype acts as one of the determinants of the strain but in addition the infected hosts show specific features dependent on their genotype. Data for the MV genotype recipient mice showed that these responded more like MM than VV mice, suggesting that the methionine allele may have a dominant role

in determining the transmission properties of the heterozygote host. The data from this thesis will hopefully allow for better risk analyses for patients exposed to human TSE by predicting the outcome of infection dependent on the sCJD subgroup and the codon 129 genotype of the patient. The availability of this *in-vivo* model expressing human PrP with codon 129 genotype variation may also allow research into the protein structural effects of codon 129 during pathogenesis and specifically during PrP^C conversion.

7.2.2 The Agent Strain Responsible for vCJD is Transmissible to Humans with Codon 129 MV and VV Genotypes

Data from this thesis provide strong support for this hypothesis and this important finding was published in 2006 in Lancet Neurology (See Appendix) (Bishop *et al.*, 2006). As discussed above the MV genotype was found to be as susceptible as MM to the vCJD agent, with equivalent numbers of mice positive for transmission. However the pathological changes in the brain were significantly less severe than in the MM mice (see the next hypothesis below). HuVV mice appeared relatively resistant to infection with only a single mouse positive at a late stage of normal lifespan. The benefit of a mouse model truly comparable between codon 129 genotypes is clearly shown here, highlighting the *relative* efficiency of transmission.

In the Lancet Neurology article it was reported that BSE did not transmit to the human transgenic mice suggesting a significant species barrier between cattle and humans. This may explain the relatively low number of vCJD cases identified to date. However the vCJD transmission data indicate that this agent can infect MV and VV mice, and suggests that there may be significant subclinical infection present in the human population with these genotypes. This theory has been supported by a

national screening program of UK lymphoreticular tissues (Hilton *et al.*, 2004a, Ironside *et al.*, 2006).

The slow progression of vCJD infection in the MV and VV mice indicated that humans infected with the vCJD agent strain from another human would likely have a long subclinical phase. This phase could last for many years, if not decades, during which time there may be high levels of infectivity present in peripheral tissues and latterly in the CNS. Even in the absence of clinical disease during normal lifespan, the agent could still be transmitted from person-to-person through blood transfusion or surgery, leading to a potentially silent epidemic. Routine screening of blood products, transplant material, or surgical instruments would help to protect public health but currently there are no applicable tests available for this purpose.

In summary, this model of human-to-human vCJD infection predicts successful secondary transmission of this agent strain to non-MM genotype hosts. As transmission data for primary and secondary vCJD material has indicated no significant adaptation of this TSE agent, overcoming the species barrier for BSE infection of MM individuals may have modified the agent into a stable human strain. As BSE material did not transmit to these mice we can only speculate that primary BSE infection of humans might affect non-MM hosts, although it is of note that these experiments all used i.c. inoculation and the current absence of human BSE infection in non-MM hosts may be related to the route of exposure or some other, yet to be identified, factor.

7.2.3 The Pathology in the Central Nervous System Following vCJD Infection in Individuals of Codon 129 MV and VV Genotype is Different from that of MM Cases

The observation that susceptibility to the vCJD agent is different between MM and non-MM genotype mice, is supported by neuropathological findings. The MM and MV mice developed similar early signs of TSE disease with PrP^{Sc} deposition in the thalamus, but pathology never progressed beyond this stage in the MV mice. In contrast, the MM mice developed extensive PrP^{Sc} deposition and TSE vacuolation throughout the brain, and also showed evidence of florid plaques which are characteristic of vCJD (in an MM genetic background). TSE vacuolation was present in the HuMM mice but only in single HuVV and HuMV mice. Because of this no mean lesion profile analysis comparison could be undertaken.

These differences may be important for the identification of vCJD transmission in non-MM individuals. The brain of an infected MV individual may have low levels of detectable PrP^{Sc} concentrated in the thalamus, and therefore a biopsy of the cerebral cortex may be negative. This type of scenario was observed in the MV patient who became infected with vCJD by blood transfusion. The brain was negative for PrP^{Sc} whereas the spleen and lymph nodes were positive (Peden *et al.*, 2004).

The single VV mouse positive for vCJD transmission showed pathological differences, including extensive PrP^{Sc} deposition throughout the brain, unlike the MV line. The type of deposition was also different from the MM mice with no evidence of PrP antibody stained florid plaques and less targeting of the

hippocampus. This comparison is limited by the availability of only the single positive HuVV mouse.

Western blot typing of the PrP^{Sc} present in the MV and VV mice showed the type 2B pattern, as in the HuMM mice. Protein typing may therefore be useful as a diagnostic tool when assessing humans who are suspected of being infected with vCJD.

In summary the prediction for CNS pathology in non-MM human individuals following infection by vCJD, or BSE, is that it will be different in some aspects such as florid plaque development and degree of PrP^{Sc} deposition but similar in the Western blot profile. This may lead to misdiagnosis of such cases as atypical sCJD (see reference (Mead *et al.*, 2007)) and therefore confirms the need for continued active surveillance with extensive *post mortem* analysis of both brain and peripheral tissues. The differences seen here in in-bred lines of mice should provide useful tools for understanding the effect of codon 129 genotype on the progression of pathological changes. If the HuMV and HuVV mice restrict the progression of disease, with this specific strain, then understanding the underlying mechanism may be useful in designing therapies for humans.

7.2.4 There is an Increase in Incubation Period for vCJD in Codon 129 MV and VV Individuals

The pathological differences seen between the three genotype mouse lines predict that the onset of clinical disease following vCJD infection is likely to be delayed for non-MM individuals because of extended incubation periods. If the BSE strain behaves in a similar fashion it may be that non-MM individuals will

develop vCJD after longer incubation periods, leading to a biphasic (MV and VV cases occurring at the same time) or triphasic (MV cases occurring then VV cases at a later stage) epidemic curve. The length of the incubation period in BSE or vCJD infection is unknown, but the evidence from kuru points to a maximum time for onset of disease in some individuals at nearly 50 years from exposure (Collinge *et al.*, 2006). It is difficult to predict incubation times from this mouse model as few mice had clinical signs. However, differences in the pathological features between lines provided clues to how different codon 129 genotype humans may respond to infection.

Clinical TSE disease in the HuMM mice was a rare occurrence (2 out of 18 mice) even though there was extensive PrP^{Sc} deposition in the brains of older mice. This indicates a disparity between overall pathological change and the presence of clinical TSE disease, suggesting that the pathology had not yet targeted those regions responsible for the TSE clinical phenotype, such as the motor cortex neurons or the cerebellum. There was only a single HuMV mouse positive for clinical TSE and no HuVV mice. Understanding the relationship between clinical TSE and pathology may be an important future use of these mouse lines.

The majority of MV genotype mice developed pathological features of TSE disease but this was considerably less extensive than seen in HuMM mice. Only a single VV mouse developed TSE pathology. If the development of pathological TSE is used to predict the development of human clinical vCJD disease, then the incubation period is likely to be ranked in the order shortest to longest: MM, MV, VV.

In summary vCJD infection is likely to result in longer incubation periods for non-MM individuals. This longer incubation period may provide a window of opportunity for potential treatment to halt the progression of disease before the CNS is irrevocably damaged. The ethics of providing a screening test for infection (if this were available) are complex especially as the incubation period may be greater than the normal lifespan of the individual.

7.2.5 Adaptation of the vCJD Agent Following Human Passage will Produce a More Infectious Strain.

The identification of vCJD infection in four individuals via blood transfusion, has lead to the hypothesis that human passage may have resulted in adaptation of the agent. This may in turn lead to an increase in the infectivity of the strain in the human population and to more secondary cases than have arisen from primary BSE infection. Data from this thesis does not support this hypothesis. The transmission properties of material from the first case of blood transfusion associated vCJD (codon 129 genotype MM) were remarkably similar to that of BSE associated vCJD. This finding has now been published in PLoS ONE together with supportive evidence from wild-type mouse studies (Bishop *et al.*, 2008).

The human passaged agent showed a similar preference for the MM mouse host, a decrease in efficiency in the MV host, and rare transmission to the VV host, with evidence of a subclinical phase in methionine carriers. The pathological features (lesion profile and plaque formation) and biochemical PrP^{Sc} profile were very similar to that seen for transmission of BSE associated vCJD.

Although there is little evidence of strain adaptation, the intravenous route of blood transfusion associated vCJD infection is more efficient than the oral route of BSE infection. There is therefore the possibility that even without an adaptation of the vCJD agent the human population may be at greater risk than for BSE because of more efficient human-to-human routes of infection. The predicted long subclinical phase in all genotypes, with associated infectivity, may therefore provide the source of future vCJD cases following secondary infection by blood transfusion, surgery, or organ transplant. These conclusions are based on transmission data from an MM genotype vCJD patient and an MM blood transfusion recipient. It remains unknown whether there could be more substantial strain adaptation in MV or VV genotype hosts. Second passage of vCJD (from BSE) in these mice indicates that the original strain properties are likely to remain the same regardless of host genotype. This knowledge may allow statistical predictions of future human-to-human disease transmissions to use the same modelling criteria associated with codon 129 genotype that have been used for primary vCJD.

7.2.6 The Risk of Iatrogenic Infection from sCJD Varies According to the Codon 129 Genotype of Source and Recipient

With the exception of sCJD(MM2) in all three codon 129 genotype recipients, and sCJD(VV1) in HuMM mice, all other sCJD subgroups can infect each codon 129 genotype host. There are a wide range of clinical and pathological responses which may correlate with the risk of infection for example the sCJD(MV2) and sCJD(VV2) subgroups infect 100% of VV mice with short incubation periods for clinical disease, whereas only subclinical disease is seen in MM and MV mice.

Subclinical disease in the majority of mice was seen in nearly half the transmission experiments suggesting that there may be undetected cases of iatrogenic sCJD transmission. This may not be of major concern as onward human-to-human transmission from these cases of iatrogenic sCJD is likely to be limited to neurosurgical events, as the pathology of sCJD is usually restricted to CNS tissues.

The appearance of clinical TSE signs in mice may have a direct relationship to the risk of iatrogenic infection leading to clinical disease in humans. If this is the case then the sCJD(MM1) and sCJD(MV1) human strains carry the highest risk for human-to-human transmission. Unlike the other inocula these two produced clinical TSE in the majority of mice for each genotype line. It may be that the commonest form of sCJD (MM1 subgroup) is also one of the most infectious, an important consideration for modelling iatrogenic spread of human TSEs.

Looking at the relative risk between each codon 129 genotype mouse line, for all types of inocula, the data suggest that the VV genotype is the most at risk of iatrogenic infection from sCJD. The HuVV line was the only one that had positive scores for the presence of clinical disease with at least four of the six inocula. There is no evidence of heterozygote protection in the HuMV line as seen in kuru (Cervenakova *et al.*, 1998) and French growth hormone associated iCJD (d'Aignaux *et al.*, 1999, Brandel *et al.*, 2003). The HuMV line appears to be more susceptible than the HuMM line as the former shows subclinical signs with sCJD(VV1) and the latter has no evidence of transmission.

The variation in host response to inocula of different codon 129 genotype may predict that similar differences may appear in human iCJD cases infected with sCJD strains. It is assumed that the majority of iCJD cases were due to sCJD

infection as this is the most common human TSE. As tracing the source of contamination is difficult, the available evidence relates to codon 129 genotype frequency and incubation periods for iCJD patients. French human growth hormone associated iCJD patients had a higher incidence of MM (MM: 62%, MV: 22%, VV: 16%, n=77) than UK cases in which there was a higher frequency of VV genotypes (MM: 4%, MV: 52%, VV: 44%, n=27). Amongst the French cases the MV patients had longer incubation periods, with onset for the first case 5 years later than the first homozygous case (Brandel *et al.*, 2003). Production and treatment methods were similar in both countries so the reason for these differences is likely to be the source of infectivity, the sCJD strain. Based on the results of this thesis the UK iCJD cases may have been caused by infection with material from a sCJD subgroup with valine at codon 129 and type two PrP^{Sc} (which produces shorter incubation periods in HuVV mice), and the French cases with MM1 or MV1 subgroup sCJD (that produces clinical TSE in all three genotype transgenic mice).

As cadaveric human growth hormone was produced from large pools of pituitary glands there may have been infectivity from multiple sCJD strains, which may have influenced the host response. This could be tested in this mouse model by infecting with pooled sCJD samples from different subgroups. Additionally, peripheral route challenge rather than the intracerebral inoculation used here may more accurately model susceptibility in human iCJD cases, which developed via ingestion (kuru) or peripheral injection (growth hormone).

7.2.7 The Currently Defined Subgroups of sCJD are Unique Strains of Human Disease, with Individual Transmission Properties

For approximately 40 years research groups have attempted to define sheep scrapie strains. Initially this involved assessing the pathology and clinical features found in the ovine host and this progressed to observing the transmission properties in sheep and goats and then in laboratory mice (Zlotnik and Rennie, 1958, Zlotnik and Rennie, 1962, Dickinson, 1970). Use of mice lead to a definition of strain that was dependent on both agent source and experimental host factors.

The definition of TSE strains was based on incubation period, vacuolation scoring (lesion profile), and serial passage in mouse models, and more recently on genetics, biochemistry and CNS deposition of PrP. Incubation period and vacuolation scoring were the core variables examined in early studies, and are still used today. The original research highlighted some important considerations when using these variables to determine differences between strains. Incubation period is useful only if titre, dose, and inoculation route are constant. Lesion profiles of vacuolation scoring will be the same for clinical disease resulting from variable doses, but will increase in intensity during the course of pathogenesis (Fraser and Dickinson, 1968, Dickinson and Meikle, 1969). Further definition of TSE strains and the response of the host has benefited from the subsequent characterisation of prion protein genetics and biochemistry, and the distribution of disease associated PrP^{Sc} in tissues of an infected animal. Panels of wild-type mice were successfully used to define scrapie strains but have been of very limited use in understanding sCJD. This was due to the absence of clinical disease for most subgroups and the similarities in lesion profiles within mouse lines infected with different sCJD

subgroups. This thesis discusses the generation and use of transgenic mice specifically manufactured to define sCJD strains.

Subgroups of sCJD have been defined by the codon 129 genotype and PrP^{Sc} Western blot type leading to classification into six subgroups (MM1, MM2, MV1, MV2, VV1, and VV2) (Parchi *et al.*, 1999, Hill *et al.*, 2003, Cali *et al.*, 2006). The data in this thesis has distinguished these six subgroups as unique strains as they exhibit differences in transmission properties, such as incubation period to clinical disease, TSE vacuolation, PrP^{Sc} distribution, and PrP^{Sc} type by Western analysis. This thesis has shown that the codon 129 genotype plays a significant role in transmission properties, and that inoculum PrP^{Sc} Western blot type is a significant determinant of TSE vacuolation intensity and distribution (the lesion profile) in the infected host.

Four major patterns have been identified within the subgroups leading to two pairs with similar attributes and two distinct single types. The first pair is sCJD(MM1) and sCJD(MV1), which are usually grouped together in the sCJD sub-classification as the 'classical' form of sCJD, and could be predicted to have shown similar transmission properties. Incubation period data for onset of clinical TSE signs in the transgenic mice cannot distinguish between these two inocula. Comparison of the lesion profile data by Hierarchical Cluster Analysis points to a difference in the HuVV data. The lesion profile from the sCJD(MM1) inoculum is more like those seen with inocula derived from sCJD cases with type 2 PrP^{Sc} whereas the sCJD(MV1) inoculum lesion profile is more like those seen with type 1. This is the only evidence from the transgenic mice that distinguishes these two inocula. However, in the wild-type transmissions ICC detection of PrP^{Sc} was

positive in all wild-type mice inoculated with sCJD(MM1) but in only 6% of mice with sCJD(MV1). These two sCJD subgroups therefore have different transmission properties.

The second pairing of inocula with similar transmission properties are sCJD(VV2) and sCJD(MV2). These two are the second and third most frequent types of sCJD found but, unlike the above pairing, patients show widespread clinical and pathological differences. There are no differences in the appearance of clinical TSE in the mice with both HuMM and HuMV mice showing only subclinical TSE and the HuVV mice developing symptoms after a short incubation period of <300 days. There was also no difference in wild-type mouse transmission, or PrP^{Sc} typing, or lesion profile cluster analysis. The HuMM and HuMV mice produced type 1 PrP^{Sc} whereas the HuVV mice produced type 2 (with both inocula). The latter were the only mice in this sCJD study to show this protein type. This may have been due to a specific biochemical response of PrP only seen in VV genotype mice to inoculum from sCJD patients also with valine allele, type 2 PrP^{Sc}. It may also have been a consequence of the specific pathological targeting to the cerebral cortex in these HuVV mice, which may itself have been a cause of the relatively fast incubation period (as seen in Chapter 6, Figure 6.F5, Panels 15 and 17).

The number of mice positive for TSE vacuolation provided the only evidence of a difference between the inocula as HuMM mice inoculated with sCJD(MV2) were more likely to be positive than those inoculated with sCJD(VV2) (69% versus 33% respectively). This suggests that there was more compatibility between the MV inoculum and the MM mice than for the VV inoculum, leading to a faster development of pathology. This may be due to the methionine allele PrP^{C} in

the host mice. Although the rate may differ, the cluster analysis shows that the final intensity of vacuolation and the distribution was equivalent. Evidence to separate these two sCJD subtypes is limited and could be investigated further, for example, by comparing PrP^{Sc} deposition in different brain regions.

The remaining two inoculum sources, sCJD(VV1) and sCJD(MM2) showed different transmission properties from the two pairings above and to each other. These are the least common forms of sCJD comprising 1-2% of cases. These were the only inocula not to produce significant levels of clinical TSE in any of the transgenic or wild-type mice lines. SCJD(VV1) showed no evidence of transmission to the HuMM mice but showed high levels of subclinical TSE in both the HuMV and HuVV mice, suggesting that with type 1 PrP^{Sc} in a VV genotype inoculum there is a valine dominant codon 129 genotype effect. SCJD(VV2) transmits to the HuMM mice indicating an important effect of the PrP^{Sc} type between these inocula.

There was no evidence of transmission to transgenic or wild-type mice for the sCJD(MM2) inoculum. ICC data is unavailable for these mice, because with no TSE vacuolation, the levels of PrP^{Sc}, if present, are likely to be very low or non-existent (as seen for sCJD(VV1) inoculation of HuMM mice). It may be important for further investigation why sCJD(MM2) material does not transmit whereas sCJD(MM1) transmission is highly efficient as the only difference with respect to PrP is the protein configuration.

These last two sCJD inocula have unique transmission properties and therefore, overall, the six defined subgroups can be distinguished to varying degrees by transmission to this panel of mice. Understanding the reasons for these

differences in strain transmission, and attempting to identify atypical sCJD cases by transmission to this panel, may be key future uses of this mouse model.

7.3 CONCLUDING REMARKS

The overall goal of this PhD thesis was to use a novel panel of gene targeted transgenic mice expressing the human prion protein, in understanding further some of the human TSE strains, and specifically the role of codon 129 genotype in disease transmission and pathogenesis.

7.3.1 The Transgenic Mouse Model

Aside from examining the specific transmission properties of different human TSE this thesis also aimed to set out the benefits of a gene targeted approach to understanding TSE transmission and the effects of codon 129 genotype the most important factor in human *PRNP* genetics.

At the outset of this thesis the three transgenic mouse lines, HuMM, HuMV, and HuVV, were unique in the prion research field. These inbred mice were genetically identical except for the single base change required to code for either methionine or valine at codon 129 of the inserted human *PRNP* gene. The new gene having directly replaced the original mouse copy and therefore under the correct gene expression regulatory processes. Since the start of this thesis Professor Kitamoto's group has also published a similar model however most of their work centres on using the mice as bioassays, rather than lifespan susceptibility, with positive transmission results based on detection of PrP^{Sc} in the spleen at 75 days post injection.

The most significant benefit of this model is the ability to determine the true effect of codon 129 genotype. With other lines of mice developed via the random genomic insertion method there is always the possibility that either the insertion points, or the expression levels, are affecting the data. Gene targeting for insertion of new genetic elements ensures that from birth to death of each mouse the prion gene is expressed in the right place, at the right time, and at the right level. Therefore, if the same inoculum is injected into these three codon 129 genotype lines the relative differences in transmission are due directly to the host genotype.

The uses for this mouse model are varied. This thesis outlines the starting point with transmission of standard cases of human TSE. From this point atypical cases can be analysed to observe how different or how similar they are to specific typical cases. For example, it will be important in the future to be able to classify novel forms of vCJD, such as those with long incubation periods, or those in MV or VV genotype patients, and to differentiate these from atypical forms of sCJD. There may also be novel forms of animal TSE that might pose a threat to humans via the food chain and therefore these mice could be used to understand the potential risks.

As a laboratory tool these mice could be the source of material such as cell lines or tissues with which it may be possible to understand further the pathological processes of prion disease, the biochemistry of PrP^{C} conversion (Jones *et al.*, 2007), or the potential of therapeutic approaches to halt this invariably fatal disease.

7.3.1 Summary of Results

Chapter 4 details the findings of vCJD transmission which were published in 2006 in Lancet Neurology (Bishop *et al.*, 2006). This was the first report of a direct comparison of transmission properties of this strain in hosts of each of the

three codon 129 genotypes. The data indicated that all three genotypes were susceptible with a hierarchy of reducing transmission efficiency from MM to MV to VV, and that there was evidence of a lengthy infectious subclinical phase. The implications of these findings were that MM individuals were not the only susceptible genotype to infection by BSE, and although the number of clinical cases of vCJD has been falling since 2000 there may still be significant numbers of individuals of each genotype harbouring infectivity. A second wave of human-to-human infection via blood products or surgery is therefore a possibility, together with clinical cases occurring in non-MM patients after much longer incubation periods than seen already in MM patients.

Chapter 5 outlines the data obtained from transmission of the first case of blood transfusion associated vCJD infection which were published in 2008 (Bishop *et al.*, 2008). In comparison with the data obtained from vCJD transmission it was shown that material from this patient contained a strain of TSE that had remarkably similar properties. This implied that significant adaptation of the BSE strain to a more highly infectious agent to humans, would not occur via human passage and therefore the individual risk of developing vCJD remained low. This was also confirmed by secondary passage of material from the primary vCJD transmission experiment. Clinical presentation of vCJD infection via human passage may be similarly limited to a low number of MM genotype individuals, but a lengthy subclinical phase in all genotypes would be of concern to future surveillance and risk assessment of disease transmission.

Chapter 6 contains the dataset obtained following transmission of typical cases of each of the six subgroups of sCJD to understand further the strain nature of

the commonest type of human TSE. Although there were a number of similarities between the six experiments, overall it was possible to identify unique properties for each subgroup and therefore they could be described as six individual strains of sCJD. As each sCJD subgroup was shown to be different the inocula clearly had the dominant role in determining the transmission properties. This was confirmed by sub-passage via the HuVV mice where the original human sCJD strain properties were propagated by the mouse host, and by analysis of the mouse lesion profile data which was shown to be primarily affected by the inocula PrP^{Sc} type. The response of the three mouse lines provided much of the data for observing differences between each inoculum. The HuVV mice were the most efficient host overall for transmission and produced the fastest incubation times, with sCJD(MV2) and sCJD(VV2). In contrast, these two inocula produced clinical TSE in only a few HuMM and HuMV mice, with the majority surviving with subclinical infections.

With transmission variation due to the codon 129 genotype of both inocula and host mouse it was difficult to draw an overall conclusion about the role of this polymorphism however this dataset now provides a standard to which atypical sCJD case can be compared, and from which detailed research on the mechanisms of sCJD transmission can be initiated.

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APPENDIX

Data from this thesis were published in the two articles enclosed. Full permission has been granted from the publishing journals to include these reprints in this thesis.

1. Bishop, M., Hart, P., Aitchison, L., Baybutt, H., Plinston, C., Thomson, V., Tuzi, N., Head, M., Ironside, J., Will, R. and Manson, J. (2006) *The Lancet Neurology,* **5,** 393-398.
   Predicting susceptibility and incubation time of human-to-human transmission of vCJD

2. Bishop, M. T., Ritchie, D. L., Will, R. G., Ironside, J. W., Head, M. W., Thomson, V., Bruce, M. and Manson, J. C. (2008) *PLoS ONE,* **3,** e2878.
   No Major Change in vCJD Agent Strain after Secondary Transmission via Blood Transfusion

Predicting susceptibility and incubation time of human-to-human transmission of vCJD

M T Bishop, P Hart, L Aitchison, H N Baybutt, C Plinston, V Thomson, N L Tuzi, M W Head, J W Ironside, R G Will, J C Manson

Summary

Background **Identification of possible transmission of variant Creutzfeldt-Jakob disease (vCJD) via blood transfusion has caused concern over spread of the disease within the human population. We aimed to model iatrogenic spread to enable a comparison of transmission efficiencies of vCJD and bovine spongiform encephalopathy (BSE) and an assessment of the effect of the codon-129 polymorphism on human susceptibility.**

Methods **Mice were produced to express human or bovine prion protein (PrP) by direct replacement of the mouse *PrP* gene. Since the human *PrP* gene has variation at codon 129, with MM, VV, and MV genotypes, three inbred lines with an identical genetic background were produced to express human PrP with the codon-129 MM, MV, and VV genotypes. Mice were inoculated with BSE or vCJD and assessed for clinical and pathological signs of disease.**

Findings **BSE was transmitted to the bovine line but did not transmit to the human lines. By contrast, vCJD was transmitted to all three human lines with different pathological characteristics for each genotype and a gradation of transmission efficiency from MM to MV to VV.**

Interpretation **Transmission of BSE to human beings is probably restricted by the presence of a significant species barrier. However, there seems to be a substantially reduced barrier for human-to-human transmission of vCJD. Moreover, all individuals, irrespective of codon-129 genotype, could be susceptible to secondary transmission of vCJD through routes such as blood transfusion. A lengthy preclinical disease is predicted by these models, which may represent a risk for further disease transmission and thus a significant public-health issue.**

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Introduction

After the identification of variant Creutzfeldt-Jakob disease (vCJD) in 1996,[1] there have been many attempts to estimate the extent of the UK epidemic. Many individuals are likely to have been exposed to bovine spongiform encephalopathy (BSE) material through their diet; however, there have been only 161 cases of the disease in the UK. The predicted total number of future cases has ranged from the low hundreds[2] to hundreds of thousands.[3] However, findings from a retrospective immunocytochemical study that aimed to detect prion protein (PrP) in appendix and tonsil specimens suggested a prevalence of BSE infection of 237 per million people in the UK.[4] DNA sequence analysis of the PrP gene (*PRNP*) in vCJD has shown that 100% of tested cases are homozygous for methionine at the codon-129 polymorphism compared with about 40% of the general white population and about 70% of sporadic CJD cases. The methionine homozygous genotype (MM) has been included as a limiting variable in most mathematical predictions of the size of the epidemic.[2,3] Identification at autopsy of preclinical vCJD infection in a methionine/valine (MV) heterozygous individual who had received a transfusion of red cells from a donor who later died of vCJD, was the first indication that MM might not be the only susceptible genotype.[5]

Polymorphisms and mutations in *PRNP* in various species can affect disease susceptibility, although the precise mechanisms by which these effects are mediated have not been established.[6,7] Codon 129 of the human *PRNP* gene has been shown to affect the clinicopathological phenotype of disease in CJD and fatal familial insomnia.[8–13] Heterozygosity at *PRNP* codon 129, when compared with homozygous individuals, has been reported to lengthen incubation times in iatrogenic CJD cases associated with growth hormone treatment, and in kuru,[9,14] whereas valine homozygosity (VV) has been proposed to be protective for both BSE and vCJD transmission in studies that used murine models overexpressing human PrP.[15] At a molecular level, the biophysical properties of PrP refolding into the disease associated form (PrP^{Sc}) have been shown to be affected by the codon-129 genotype, with the methionine variant having an increased propensity to form PrP^{Sc}-like structures.[16]

We sought to analyse the transmission characteristics of BSE and vCJD to four inbred lines of transgenic mice after intracerebral inoculation with brain homogenate from cases of vCJD and BSE. We then aimed to use these models to address the apparent low level of vCJD in the human population resulting from exposure to BSE and to predict the potential for human-to-human spread of vCJD and the susceptibility of different genotypes in the human population.

Methods

Transgenic mice

Details of how the gene-targeted transgenic lines were created are supplied as supplementary information

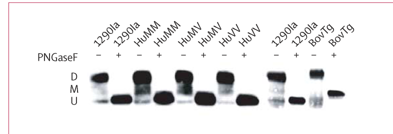

Figure 1: **Western blot of brain extract from uninoculated mice showing that PrP^C is detected with equivalent electrophoretic mobility and glycoform ratio in all three human transgenic lines**

D=diglycosylated PrP^C band; M=monoglycosylated PrP^C band; U=unglycosylated PrP^C band. In the BovTg line, a deglycosylated band is detected of increased molecular weight due to the additional N-terminal octapeptide repeat motif. Protein levels are similar to the wildtype line used in generating the transgenics (129Ola). Glycosylation is confirmed by the reduction to a single band after deglycosylation with the enzyme PNGaseF. The anti-PrP antibody 7A12 was used for the HumTg blot as it will react with both murine and human PrP, and 8H4 was used for the BovTg blot.

See Online for webappendix

(webappendix). Transgenic mice were anaesthetised with halothane and then injected with 0·02 mL of brain homogenate into the right cerebral hemisphere. The vCJD tissue homogenate (at 10^{-2} dilution) was supplied by the UK National Institute for Biological Standards and Control (Code NHBY0/0003). BSE-infected cattle brain (Veterinary Laboratories Agency, reference BBP 12/92) was prepared by maceration of the tissue in sterile saline to a dilution of 10^{-1}. From 100 days they were scored each week for signs of disease.[17] Mice were killed by cervical dislocation whether they had clinical signs of transmissible spongiform encephalopathy (TSE) or another non-specific disorder. The brain was recovered at post mortem. Half the brain was snap-frozen in liquid nitrogen for biochemical analysis and the remaining half was fixed for histology.

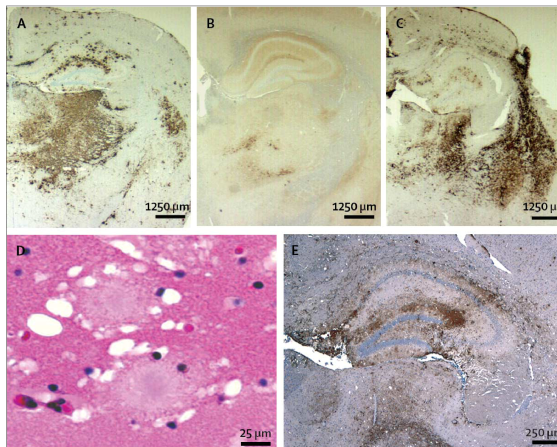

Figure 2: **Immunocytochemistry of histological sections with anti-PrP antibody 6H4 showing the cortex, hippocampal, and thalamic regions of the mouse brain with PrP detection (brown)**

A–D: Human transgenic mice with vCJD inoculum. A: HuMM mouse 693 days post inoculation. B: HuMV mouse 707 days post inoculation. C: HuVV mouse 693 days post inoculation. D: Florid plaques found in the hippocampus of the HuMM mouse in panel A. Each plaque has an eosinophilic core with a paler halo and is surrounded by a ring of vacuolation (haematoxylin and eosin stain). E: Hippocampal region of a BovTg mouse inoculated with BSE. PrP is deposited in a more diffuse/granular form with occasional plaques.

Procedures

Immunocytochemical detection of disease-associated PrP (PrP^{Sc}) deposits in the brain is a key pathological marker of TSE transmission, and variation in location and morphology of PrP^{Sc} deposits can be affected by both the strain of TSE agent and by the host PrP.[7,18] After fixation in 10% formal saline, brains were treated for 1·5 h in 98% formic acid (to reduce the titre of infectivity for safety reasons), cut transversely into four sections, and embedded in paraffin. We used the Vectastain Elite ABC Kit (Vector Labs, UK) with overnight primary antibody incubation (6H4 at 1:2000; Prionics, Switzerland) for PrP detection. Identification of antibody binding was through deposition of 3,3´-diaminobenzidine chromogen via a horseradish peroxidase reaction. The BSE-inoculated human transgenics were also studied using the Catalysed Signal Amplification kit (DAKO K1500). This kit uses the same principles as the Vector Labs kit, but has an additional step, which amplifies the final detected signal and therefore improves sensitivity.

Scoring of the abundance and location of TSE-associated vacuolation in grey and white matter of the brain is routinely used for diagnosis and strain classification in non-transgenic mice[17,19] and was used to assess all the mice in this study. TSE-related vacuolation was assessed at nine grey-matter regions and three white-matter regions to produce a lesion profile, as previously described.[20,21]

Analysis

Frozen brain samples from the human transgenic mice were homogenised in 0·9% saline to give a 10% suspension. This material was cleared by centrifugation and the supernatant treated with 0·05 g/L proteinase K for 1 h at 37ºC, as previously described in detail.[22] The digested product was denatured then loaded onto a 10% Bis/Tris NuPAGE Novex gel (Invitrogen, UK). After electrophoresis the gel was blotted onto polyvinylidine difluoride (PVDF) membrane. We used the ECL+ technique (Amersham Biosciences, UK) with primary antibody 6H4 (Prionics, Switzerland) at 1:40000 and an anti-mouse IgG peroxidase-linked secondary (Amersham Biosciences, UK) at 1:40000 for the detection of PrP. Chemiluminescence was captured on radiographic film. Samples prepared for figure 1 were digested overnight at 37ºC with 500 units of PNGaseF (New England Biolabs, UK) and not with proteinase K; the primary antibody was 7A12.[23]

Frozen brain samples from the bovine transgenic mice were homogenised in an NP40 buffer (0·5% v/v NP40,

0·5% w/v sodium deoxycholate, 0·9% w/v sodium chloride, 50mM Tris-HCl pH 7·5) to give a 10% suspension. This material was cleared by centrifugation and the supernatant digested with PNGaseF. The products were denatured then loaded onto a 12% Novex Tris/Glycine gel (Invitrogen, UK). After electrophoresis the gel was blotted onto PVDF membrane. PrP was identified with the SuperSignal West Dura chemiluminescence detection kit (Pierce, UK) with primary antibody 8H4[24] at 1:20000 and an anti-mouse IgG peroxidase-linked secondary (Jackson Immuno Research Laboratories, UK) at 1:10000. Images were captured on radiographic film and with a Kodak 440CF digital imager (figure 1).

Role of the funding source

The sponsors of this study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

We first investigated the potential effects of the species barrier between BSE and human beings and any alteration in that barrier once BSE had passed through people in the form of vCJD. We then investigated the effect of the codon-129 polymorphism on human-to-human transmission of vCJD using gene-targeted inbred mice developed by direct replacement of the murine *PrP* gene for the human gene. These mice produce PrP under the control of the normal regulatory elements for PrP and thus express physiological concentrations of PrP with the correct tissue distribution (figure 1). Three inbred lines with an identical genetic background were produced to express human PrP with the codon-129 MM, MV, and VV genotypes (designated HuMM, HuMV, and HuVV, respectively). Each line differs by only a single codon in *PRNP* and in all other respects the mice were genetically identical. Additionally, in an identical manner, we produced mice that express bovine PrP to enable direct comparisons to be made not only between transgenic and wild-type mice, but also between each of the transgenic lines.

Typical clinical signs of TSE disease were seen in more than half (15/22) the BovTg mice inoculated with BSE material with a mean incubation period of 551 days (SD 47). These clinical cases were confirmed by a positive test for the presence of TSE vacuolation or PrP^{Sc} deposition by immunocytochemistry. The lesion profiles generated for targeting and degree of vacuolation showed similar patterns for all positive mice. Immunocytochemical data showed PrP^{Sc} deposition mainly in a diffuse and synaptic form, and also as plaque-like structures, frequently associated with areas of spongiform change (figure 2). Deposition was most abundant in the thalamus and hippocampus, but was recorded throughout other regions of the brain. The cerebral cortex showed only occasional plaque-like structures and the cerebellum had only a few areas of PrP^{Sc} deposition limited to the granule cell layer. Further pathological analysis was undertaken on mice that were culled for reasons other than clinical TSE (intercurrent deaths). This analysis showed that all the brains had pathological signs of TSE disease in terms of vacuolation or PrP deposition. Thus, all the bovine transgenic mice (22/22) seemed to be susceptible to BSE infection, although not all developed clinical signs of infection (tables 1 and 2).

HuMM, HuMV, and HuVV mice were inoculated with BSE material and after extensive pathological analysis all were confirmed as negative for TSE transmission (table 1). Mice of each genotype line were inoculated with vCJD material. Two pathologically confirmed clinically positive mice were seen in the HuMM line (at 497 and 630 days post inoculation), one in the HuMV line (at

	Clinically positive	Vacuolation positive	PrP positive*	Negative†
BovTg (n=22)				
0–400	0	3	6	0
401–500	1	1	0	0
501–600	10	11	5	0
>600	4	4	2	0
HuMM (n=18)				
0–400	0	0	0	4
401–500	0	0	0	5
501–600	0	0	0	2
>600	0	0	0	7
HuMV (n=23)				
0–400	0	0	0	3
401–500	0	0	0	6
501–600	0	0	0	4
>600	0	0	0	10
HuVV (n=22)				
0–400	0	0	0	9
401–500	0	0	0	4
501–600	0	0	0	7
>600	0	0	0	2

*Because most mice were positive by both clinical and vacuolation scoring not all mice were tested by immunocytochemistry for PrP deposition. †Negative by clinical or pathological analysis, or positive by clinical scoring but not confirmed by pathology.

Table 1: **Clinical and pathological scoring of BovTg and human transgenic mice, by number of days after BSE inoculation**

	BSE				vCJD		
	BovTg	HuMM	HuMV	HuVV	HuMM	HuMV	HuVV
Susceptibility*	22/22	0/18	0/23	0/22	11/17	11/16	1/16

*Positives confirmed by immunocytochemistry or lesion profile.

Table 2: **Susceptibility to TSE disease comparison of BovTg and human transgenic mice inoculated with BSE or vCJD**

	Clinically positive	Vacuolation positive	PrP positive	Negative*
HuMM (n=17)				
0–400	0	0	2	2
401–500	1	1	1	2
501–600	0	1	3	2
>600	1	4	5	0
HuMV (n=16)				
0–400	0	0	0	0
401–500	0	0	0	0
501–600	0	0	4	3
>600	1	1	7	2
HuVV (n=16)				
0–400	0	0	0	0
401–500	0	0	0	1
501–600	0	0	0	5
>600	0	1	1	9

*Negative by clinical or pathological analysis, or positive by clinical scoring but not confirmed by pathology.

Table 3: **Clinical and pathological scoring of human transgenic mice, by number of days after vCJD inoculation**

Figure 3: **Western blots of brain extract from three transgenic lines inoculated with vCJD**

D=diglycosylated PrP^{Sc} band; M=monoglycosylated PrP^{Sc} band; U=unglycosylated PrP^{Sc} band. T-2B corresponds to human vCJD brain homogenate showing the typical PrP^{Sc} type 2B and T-1 corresponds to human sCJD brain homogenate showing the typical PrP^{Sc} type 1 signature. Type 2B and 1 differ in mobility of the unglycosylated band (~19 kDa and ~20 kDa respectively) and the degree of glycosylation (diglycosylated dominant and mono/unglycosylated dominant respectively). All samples were treated with proteinase K. The anti-PrP detection antibody was 6H4. The HuMV and T2-B control blot had to be overexposed as the signal from the HuMV was weak, due to the low levels of PrP^{Sc} seen by immunocytochemistry.

665 days post inoculation), and none in the HuVV line (table 3). HuMM mice were more likely to show disease-associated vacuolation, beginning at around 500 days post inoculation. Six were scored positive and showed similar distribution of vacuolation in the brain, with the highest levels found in the dorsal medulla, thalamus, and cerebellar white matter. By contrast, only a single mouse in each of the HuMV and HuVV groups scored positive for vacuolation at approximately 700 days post inoculation.

Most of the HuMM mice (11/15) showed PrP^{Sc} deposition in most areas of the brain at a relatively early stage (from around 370 days post inoculation), before the vacuolar pathology became evident. From 500 days post inoculation the appearance of vacuolation was accompanied by a significant increase in PrP^{Sc} deposition. By contrast, although PrP^{Sc} deposition was identified in many HuMV mice (11/13), they had little deposition restricted to only a few areas (including the ventrolateral and ventromedial thalamic nuclei and the red nucleus of the mid-brain), even after 700 days post inoculation (figure 2, table 4). Although PrP^{Sc} deposition was clearly present at 581 days, the timing of initial onset of deposition in this line was not established.

Significant levels of PrP^{Sc} deposition were noted in the brain of the subclinical HuVV case. Indeed, these were similar in intensity to those observed in the clinical HuMM cases. Patterns of PrP deposition and plaque formation show differences among the three genotypes, including the presence of florid plaques only in the HuMM mice (table 4).

PrP^{Sc} found in vCJD brain is characterised by a 19 kDa non-glycosylated fragment and the predominance of the diglycosylated form (type 2B).[22] Both biochemical properties of PrP^{Sc} are maintained when vCJD is transmitted to the human transgenic mice, irrespective of their codon-129 genotype (figure 3). Preliminary densitometric analysis suggested that there was an increase in the diglycosylated form in the HuVV mouse compared with the HuMM mouse. Additionally, comparison of PrP^{Sc} from the BSE inoculum and brain material from BovTg mice also confirmed propagation of the predominantly diglycosylated glycoform signature of PrP^{Sc} associated with the BSE/vCJD agent strain (data not shown).

	HuMM	HuMV	HuVV
Vacuolation*	Thalamus (severe); cerebral cortex and hippocampus (mild); cerebellar cortex (minimal)	Thalamus, cerebral cortex, hippocampus, and cerebellar cortex (minimal)	Thalamus and cerebral cortex (severe); hippocampus (mild); cerebellar cortex (minimal)
Plaque formation*	Fibrillary amyloid plaques; florid and non-florid plaques in cerebral cortex and hippocampus; no evidence of plaques in cerebellum	No evidence of amyloid plaques	Amorphous non-fibrillary structures often forming into clusters in cerebral cortex and thalamus
PrP deposition†	Intense staining of plaques in hippocampus and cerebral cortex; plaque-like, pericellular, and amorphous deposits in the hippocampus; synaptic, peri-neuronal, and diffuse perivascular deposits in the thalamus	Occasional small plaque-like deposits and pericellular deposits in the thalamus	Strongly positive large amorphous deposits and clusters of plaques, small plaque-like structures, perivascular aggregates, and sub-pial deposits in the cerebral cortex and thalamus

*Analysed with haematoxilin and eosin staining. †Analysed with immunocytochemical techniques.

Table 4: **Comparison of TSE-associated neuropathology in human transgenic mice inoculated with vCJD**

Discussion

Although the cattle BSE epidemic in the UK has amounted to more than 180 000 cases since the 1980s, the extent of the human vCJD epidemic has so far remained limited with the total number of cases worldwide currently at 190. One explanation for this apparent discrepancy is that there exists a significant species barrier between cattle and human beings, which limits the susceptibility of the human population to BSE. The data shown here suggest that this could indeed be the case since BSE was readily transmissible to the bovine transgenic mice but not to the human transgenic mice. However, once BSE has passed through human beings in the form of vCJD, the transmissibility of this TSE strain is altered for the human population.

All the human transgenic lines inoculated with BSE were negative for TSE transmission, which suggests that either the human transgenic lines are relatively resistant to transmission of BSE or the incubation time is longer than the length of the experiment (approximately 700 days). BSE transmission previously observed by others, in human transgenic lines overexpressing the human prion protein, could be due to overexpression of the *PrP* gene and may not therefore give a true reflection of the species barrier between BSE and human beings.[15,25,26] This apparent resistance of human transgenic mice to BSE could be explained by a large species barrier and this in turn could explain the low number of vCJD cases in the human population.

vCJD was transmitted to all three human lines with different pathological characteristics for each genotype, and a gradation of transmission efficiency from MM to MV to VV. The greater transmission efficiency in HuMM mice suggests that homozygosity for methionine at codon 129 leads to earlier onset of TSE-related pathological features and clinical disease than for the other two genotypes. The differences in PrP^{Sc} deposition in the HuMM and HuMV lines suggest that the codon-129 polymorphism in human beings is likely to affect the distribution of PrP^{Sc} deposition in the brain. Moreover, the similar numbers that scored positive for PrP deposition in each of the MM and MV groups (11/15 and 11/13 respectively) suggest that the two genotypes might be equally susceptible to vCJD, but with different incubation periods. Titration experiments are needed to fully compare the susceptibility of each line. The single HuVV mouse positive for PrP^{Sc} shows that VV individuals may be susceptible to vCJD with very long incubation times, including a lengthy subclinical phase. Transmission studies from all three genotype mice are now underway to examine the infectious nature of the disease and determine any alterations in the strain characteristics on passage through human transgenic mice. By contrast with published data suggesting that VV individuals cannot propagate the vCJD biochemical phenotype,[15] the data presented here suggest that the PrP^{Sc} type will remain a useful diagnostic feature of secondary vCJD infection irrespective of codon-129 genotype, as has been observed for the two extant cases of transfusion-associated vCJD infection.[5,27]

Transmission of vCJD to the three lines of human transgenic mice indicates that the human population could be at significantly heightened risk of developing disease after iatrogenic exposure to vCJD. Secondary transmission of vCJD has partly removed the cattle-to-human species barrier and has resulted in an agent that can be transmitted from human to human with relative efficiency. Transmission studies in cynomolgus macaques provide further evidence for this agent adaptation as they show reduction in incubation times after serial passage of BSE.[28] Our BSE inoculation at 10^{-1} dilution was compared with vCJD inoculation at 10^{-2} because the latter inoculum was found to be toxic to the mice at 10^{-1}. Use of a higher dose of vCJD inoculum would have maintained or increased the transmission efficiency of vCJD and enhanced the current findings.

Our findings raise concerns relevant to the possibility of secondary transmission of vCJD through blood transfusion, fractionated blood products, or contaminated surgical instruments. For this study mice were injected intracerebrally, whereas the probable human exposure to these agents is by peripheral routes (eg, oral or intravenous), and thus human-to-human exposures might be significantly less efficient. However, it is difficult to know for sure what the practical implications might be in human beings. Peripheral route challenge is in progress; however, BSE transmission studies in primates have shown the intravenous route to be as efficient as the intracerebral route, with an extension of the incubation time.[28]

Although all cases of vCJD up to now have been observed in the MM genotype, this model of human-to-human vCJD transmission suggests that other genotypes are also susceptible. In our experimental setting, all *PRNP* codon-129 genotypes are susceptible to vCJD infection; however, progressive development of pathological TSE features (vacuolation and PrP deposition) is more rapid in the MM-genotype mice. An explanation for this finding might be provided by in-vitro conversion of recombinant human PrP by BSE and vCJD agents, which has shown that PrP with methionine at position 129 is more efficiently converted than PrP with valine, and that conversion by vCJD is significantly more efficient than by BSE.[29] Long incubation periods during which PrP^{Sc} is deposited predicts that, in human beings, infection could be present in all genotypes for a significant period before clinical onset. Incubation periods of more than 30 years have been reported in the human TSE disease kuru.[30]

The possibility that an MV or VV genotype could result in a phenotype distinct from that recognised in vCJD draws attention to the importance of systematic assessment of the clinical, genetic, pathological, and

biochemical features of all human prion diseases. Our findings indicate that for human-to-human vCJD infection it should be assumed that all codon-129 genotype individuals (not just MM) can be infected, that long incubation times can occur, and that a significant level of subclinical disease might be present in the population.

Contributors

MTB, PH, and CP did immunocytochemical and western blot analysis; JCM, NT, HNB, and LA produced the transgenic mouse lines; JWI supplied vCJD case material and reviewed the neuropathology; VT did the mouse inoculations; and MTB, PH, MWH, RGW, JWI, and JCM prepared the manuscript.

Conflicts of interest

We have no conflicts of interest.

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No Major Change in vCJD Agent Strain after Secondary Transmission via Blood Transfusion

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Abstract

Background: The identification of transmission of variant Creutzfeldt-Jakob disease (vCJD) by blood transfusion has prompted investigation to establish whether there has been any alteration in the vCJD agent following this route of secondary transmission. Any increase in virulence or host adaptation would require a reassessment of the risk analyses relating to the possibility of a significant secondary outbreak of vCJD. Since there are likely to be carriers of the vCJD agent in the general population, there is a potential for further infection by routes such as blood transfusion or contaminated surgical instruments.

Methodology: We inoculated both wild-type and transgenic mice with material from the first case of transfusion associated vCJD infection.

Principal Findings: The strain transmission properties of blood transfusion associated vCJD infection show remarkable similarities to the strain of vCJD associated with transmission from bovine spongiform encephalopathy (BSE).

Conclusions: Although it has been hypothesized that adaptation of the BSE agent through secondary passage in humans may result in a greater risk of onward transmission due to an increased virulence of the agent for humans, our data presented here in two murine models suggest no significant alterations to transmission efficiency of the agent following human-to-human transmission of vCJD.

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Introduction

Variant Creutzfeldt-Jakob disease (vCJD) is an acquired form of human transmissible spongiform encephalopathy (TSE) caused by infection by the bovine spongiform encephalopathy (BSE) agent that entered the human food chain in the United Kingdom during the 1980s and early 1990s. [1,2] 164 cases of vCJD have been identified in the United Kingdom and a further 41 cases in other countries worldwide. Annual mortality rates indicate that the vCJD outbreak is now in decline in the UK following a peak in 1999/2000. [3] In 2003 the first case of human-to-human secondary transmission of vCJD via blood transfusion was identified through a collaborative study between the UK National Blood Services, the National CJD Surveillance Unit, and the Office of National Statistics (Transfusion Medicine Epidemiology Review, TMER). [4,5] Statistical analysis showed that the possibility of this case being due to BSE infection was in the order of 1:15,000 to 1:30,000. [4] This patient had received a transfusion of non-leucodepleted red cells that had originated from a donor who 3 years 4 months later developed clinical vCJD. The blood recipient was methionine homozygous at codon 129 of the prion protein (PrP) gene (*PRNP*), the same genotype as all tested vCJD cases. [6]

Two further cases of vCJD linked to blood transfusion, in MM genotype individuals, have subsequently been identified through the TMER study. [7,8] Following the discovery of these cases policy changes were made in relation to blood donation in the UK and elsewhere. In 2004 the UK Blood Service deferred transfusion recipients from acting as blood donors.

A fourth case, of asymptomatic infection following blood transfusion, was described in 2004 and this individual was heterozygous (MV) at codon 129. [9] This case was the first indication that individuals with *PRNP* genotypes other than MM could be infected by the vCJD agent. All three codon 129 genotypes are now thought to be susceptible to vCJD infection following the identification of two VV genotype appendix tissues positive for vCJD associated PrP (PrP^{Sc}) in an anonymous screening study, and the successful transmission of vCJD to 'humanised' transgenic mice of each genotype. [10–12]

The implications of these findings are that a significant number of the UK population may be carriers of vCJD infectivity, that some of the individuals may be donating blood, and that not only

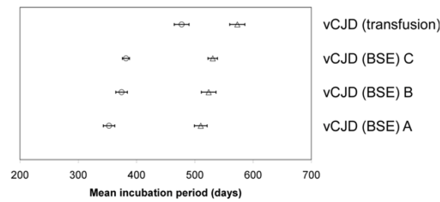

Figure 1. Comparison of incubation periods in wild-type mice. Incubation period plot comparison of vCJD (transfusion) case versus transmissions in wild-type mice of vCJD (BSE) from three sources. (Data shows mean incubation period±standard error of the mean. Open circles RIII line and open triangles VM line.)
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those with an MM genotype may be susceptible to infection from this source. Our research in transgenic models indicates that MV and VV individuals are likely to remain in an infectious preclinical state for a significant period of time with incubation periods potentially longer than average lifespan. [12] The identification of four instances of secondary transmission of vCJD infection from a group of 66 individuals known to have received blood products from vCJD donors, including only 28 who survived at least five years post transfusion indicates that blood transfusion is a significant risk factor for vCJD. This is likely to be due to either the route of transmission being more efficient of the agent being more infectious on human-to-human transmission or a combination of both.

TSE transmission by the blood transfusion route has been investigated in a sheep model. [13,14] These studies used intravenous (i.v.) transfusion of whole blood and blood fractions from clinical and preclinical sheep infected with BSE or scrapie. Preliminary data showed that the i.v. route gave relatively short and consistent incubation periods suggesting an efficient transmission route, with success rates of 60% for sheep infected with BSE and 40–45% for natural scrapie. [14,15]

Strain characterisation using a standard panel of inbred lines of wild-type mice originally demonstrated that BSE and vCJD agents had similar biological properties following transmission. [2,16] Similar work in other murine models has also been undertaken to study other human TSEs (genetic and iatrogenic CJD [17], and sporadic CJD [2]), and has been used to examine emerging TSEs (atypical BSE [18] and chronic wasting disease in deer and elk [19]). [20] The development of transgenic mice expressing human PrP has lead to further dissection of the nature of human TSE strains, including transmission of vCJD to gene targeted human transgenic mice. [12,17,21,22] Extensive data from studies in both wild-type and transgenic models at the Neuropathogenesis Division provide an essential background which will allow us to identify any change in the transmission characteristics of vCJD following secondary transmission. [2,12,23]

To investigate the nature of the transmissible agent following secondary transmission from human-to-human following blood transfusion we have examined the biological properties of brain material from the first case of transfusion-associated vCJD inoculated into panels of both wild-type, and transgenic mice expressing human PrP.

Results

Clinical signs of a TSE in the transgenic mice were rare and occurred after long incubation periods (IP) as found in our previous study. [12] Inoculation of the vCJD (transfusion) case produced one clinically positive HuMM mouse (at 659 days post inoculation), two positive HuMV mice (at 596 and 638 dpi) and no positive HuVV mice. Transmission of the vCJD (transfusion) case to the RIII and VM lines showed extended incubation periods compared to the three vCJD (BSE) cases. However, the hierarchy of incubation periods in the two wild-type lines was identical. (Figure 1 and Table 1) These data also show close similarities to previously published vCJD (BSE) transmission to wild-type mice despite different methodologies. These earlier studies used cerebellar material for the inoculum which was injected by simultaneous intracerebral and intraperitoneal routes. [2,23,24]

The frequency of transgenic mice positive for TSE associated vacuolation was similar between the vCJD (transfusion) case and the published vCJD (BSE) case [12], with positive results in 8/15 HuMM, 0/17 HuMV, and 0/17 HuVV mice and 6/16 HuMM, 1/15 HuMV, and 1/15 HuVV mice respectively. Regional distribution of TSE vacuolation in the brain was assessed through lesion profiling. All wild-type and the HuMM transgenic lines had sufficient positive mice to generate a profile (n≥6 mice). The overall pattern of the lesion profiles was the same in the vCJD (transfusion) and vCJD (BSE) cases for all lines of mice, however,

Table 1. Clinical and pathological assessment of wild-type mice.

Inoculum	Mouse Line	Mice Inoculated[a]	Positive for Clinical TSE Signs	Positive for TSE Vacuolation	Incubation Period (days±SEM)
vCJD(BSE) A	RIII	20	17	17	352.76±9.78
vCJD(BSE) B	RIII	20	18	17	374.35±9.98
vCJD(BSE) C	RIII	21	17	16	381.88±6.07
vCJD (transfusion)	RIII	23	18	18	477.33±12.68
vCJD(BSE) A	VM	22	15	22	510.20±10.97
vCJD(BSE) B	VM	22	20	21	523.75±12.57
vCJD(BSE) C	VM	21	13	18	530.69±8.16
vCJD (transfusion)	VM	22	15	18	572.90±12.96

Wild-type mouse lines RIII and VM, inoculated with vCJD(BSE) and vCJD(transfusion) were assessed clinically and pathologically for signs of TSE and mean incubation periods calculated.
[a]The group of 24 was reduced due to unavailability of some brain material for analysis.
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for the former case the VM and HuMM mice scores were lower. (Figure 2)

Immunocytochemical (ICC) detection of disease associated abnormal PrP in paraffin sections was also used as a method of assessing whether mice were transmission positive. There were 13/14 HuMM, 8/17 HuMV, 1/17 HuVV positive mice in the vCJD (transfusion) case, which was similar to the frequency of positives in the published vCJD (BSE) case: 11/15 HuMM, 11/13 HuMV, 1/15 HuVV mice. ICC data can be used to show variation in targeting of abnormal PrP deposition in the brain and variation in the nature of deposits. The ICC pattern in transgenic mice inoculated with the vCJD (transfusion) case matched that reported for vCJD (BSE) [12]. The thalamus was specifically targeted with deposition of abnormal PrP, and for the HuMM mice the hippocampus contained many intensely stained plaques including vCJD transmission associated florid plaques. ICC pattern in wild-type mice also showed similarities between the data sets with abnormal PrP deposition targeted to the thalamus and hippocampus, and large aggregates in the white matter of the corpus callosum. (Figure 3)

Biochemical analysis of disease-associated PrP by Western blot can discriminate between human cases of vCJD and sporadic CJD. [25] In the vCJD (transfusion) case the HuMM mice had a type 2B gel mobility and glycoform ratio identical to that found in vCJD (BSE) transmission to HuMM mice, and in vCJD itself. (Figure 4) Brain tissue from both vCJD (transfusion) [4] and published vCJD (BSE) [26] patients showed the type 2B pattern. The levels of PrP^{Sc} seen in the HuMV and HuVV were too low to allow typing by this standard Western blot method.

Discussion

Secondary passage of vCJD infection via blood transfusion in an MM codon 129 genotype individual results in a clinical disease phenotype and pathological characteristics that are similar to vCJD derived from BSE. [4] In this paper we confirm that the agent strain properties of primary and secondary vCJD cases are similar in transmission studies in transgenic and wild-type mice. Strain characteristics can be assessed by the frequency of clinical signs in recipient animals, the incubation period, neuropathological features, and PrP typing. All these parameters were similar in the transmission studies of primary and secondary vCJD in transgenic mice, indicating that the strain properties of the vCJD agent have not changed significantly following secondary passage in humans.

There were some differences in the results of the transmission studies which deserve further comment. The incubation period in wild-type mice was relatively extended in the vCJD(transfusion) case. However, the hierarchy of incubation periods in different inbred mouse strains was unchanged and the most plausible explanation for these findings is that, rather than implicating a change in agent characteristics, the titre of infectivity was less in the brain sample from the vCJD(transfusion) case. The distribution and degree of vacuolation was identical in the RIII mice. (Figure 2) While the distribution was identical in the VM and HuMM mice the degree of vacuolation intensity was lower for the vCJD(transfusion) case. This variability could be due to the much longer incubation times observed in these lines of mice or due to minor changes of the strain properties.

Preliminary investigation of the individuals diagnosed with vCJD following blood transfusion does not indicate a change in the neuropathological characteristics of vCJD following secondary transmission, although further studies are required to confirm this observation.

The level of infectivity in peripheral tissues in secondary cases of vCJD is unknown, although spleen and a lymph node were PrP positive in the sub-clinical case linked to blood transfusion. Evidence from BSE inoculation of primates indicates similar peripheral distribution of disease associated PrP following either oral or intravenous infection. [27] Further studies are required to assess the anatomical distribution, strain properties and level of infectivity in peripheral tissues in secondary vCJD infection. This may be important for accurate assessment of the public health risks associated with the potential for iatrogenic transmission of vCJD, which are not solely defined by the agent characteristics in brain.

Blood transfusion appears to be a relatively efficient means of secondary transmission of vCJD. To date, there have been four such transmissions in a cohort of 28 individuals who survived at least five years following transfusion of blood derived from individuals incubating vCJD. Despite extensive exposure of the UK population to the BSE agent in the food chain, there have been a relatively limited number of primary cases of vCJD (164 in the UK) and the outbreak has been in decline since 1999/2000. An important question is why there should be a disparity in the apparent efficiency of infection between primary and secondary vCJD. Transmission is generally more efficient within species than between species which may explain this observation. [28,29] Inoculation of wild-type mice with material from primary and secondary BSE passage in macaques showed that the BSE agent retained a characteristic lesion profile even though the second passage incubation period in the macaques was reduced by 50%. [30] This suggests that efficiency of transmission may increase without obvious changes to the agent strain.

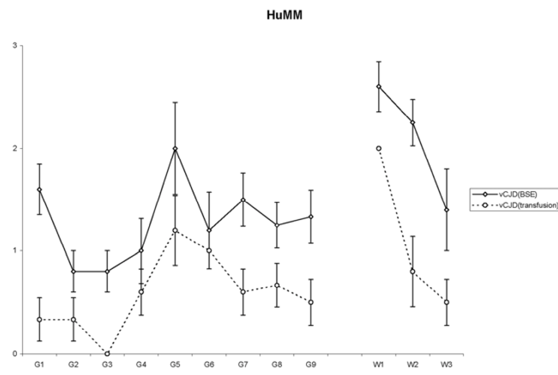
Another factor is that the intravenous route of infection is very much more efficient than the oral route, as shown in experimental models. [27,31,32] Results from this study suggest the major factor here is likely to be the route of infection rather than any changes in the strain of agent. Future studies, including those using experimental oral exposure to infectivity in transgenic mice, will further address this issue.

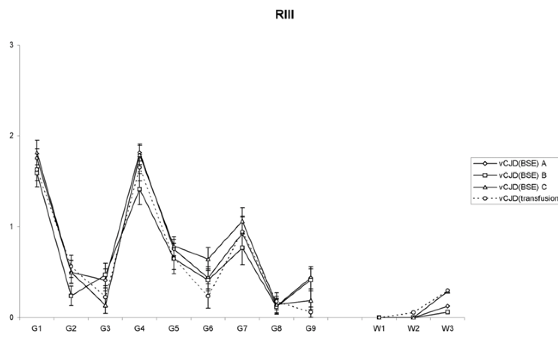
All the primary and secondary clinical cases of vCJD have occurred in individuals with a MM genotype. The sub- or pre-clinical transfusion related infection was in a codon 129 heterozygote and genotyping of positive appendix samples identified in a screening study confirmed valine homozygosity in 2 of 3 samples tested. [10] This indicates that individuals with all codon 129 genotypes may be infected with the vCJD agent and the effect of the MV or VV background on the characteristics of the vCJD agent have not been addressed by the data in this paper.

In conclusion, transmission studies indicate that the strain characteristics of vCJD have not been significantly altered by secondary transmission through blood transfusion. This suggests that the risk of onward transmission of vCJD through other routes, for example contaminated surgical instruments, have not been increased by adaptation of the infectious agent to humans following secondary passage. However the characteristics of the infectious agent in different genetic backgrounds has not yet been defined and the prevalence of vCJD infection in the general population remains uncertain. There is need to continue to implement appropriate policies to protect against the risk of secondary transmission of vCJD until many of the remaining uncertainties are resolved.

Materials and Methods

The transgenic mice (HuMM, HuMV, HuVV) used in these experiments have been described previously. [12] These mice express human PrP under the regulation of the murine promoter


HuMM
vCJD(BSE)
vCJD(transfusion)
G1 G2 G3 G4 G5 G6 G7 G8 G9 W1 W2 W3


RIII
vCJD(BSE) A
vCJD(BSE) B
vCJD(BSE) C
vCJD(transfusion)
G1 G2 G3 G4 G5 G6 G7 G8 G9 W1 W2 W3

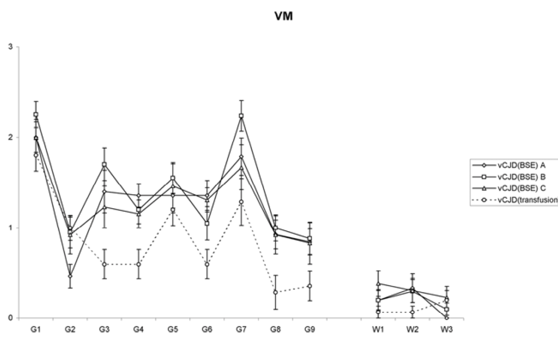

VM
vCJD(BSE) A
vCJD(BSE) B
vCJD(BSE) C
vCJD(transfusion)
G1 G2 G3 G4 G5 G6 G7 G8 G9 W1 W2 W3

Figure 2. Vacuolation scoring in the mouse brain. Lesion profile comparison of vCJD (transfusion) case versus vCJD (BSE) transmissions to identify similarities in vacuolar pathology levels and regional distribution in mouse brains. (mean score ±SEM; dashed line - vCJD (transfusion) case; solid lines – 3x vCJD (BSE) cases for wild-type mice (diamonds – vCJD(BSE) A; squares – vCJD(BSE) B; triangles – vCJD(BSE) C) and published vCJD (BSE) for HuMM transgenic; G1–G9 grey matter scoring regions; W1–W3 white matter scoring regions)
doi:10.1371/journal.pone.0002878.g002

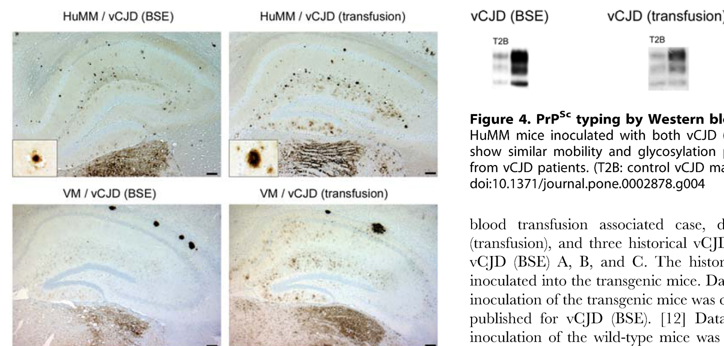


Figure 3. Detection of abnormal PrP in the mouse brain. Immunocytochemical detection of abnormal PrP deposition in hippocampus and thalamus (lateral posterior nucleus) of HuMM transgenic (with additional 40× magnification of florid plaque structure, see box lower left) and VM wild-type mice following inoculation with vCJD (BSE) and vCJD (transfusion) material. (Scale bar 200 µm, anti-PrP antibody 6H4)
doi:10.1371/journal.pone.0002878.g003

Figure 4. PrP^{Sc} typing by Western blot. Brain homogenates from HuMM mice inoculated with both vCJD (BSE) and vCJD (transfusion) show similar mobility and glycosylation profile (type 2B) as material from vCJD patients. (T2B: control vCJD material; antibody: 6H4)
doi:10.1371/journal.pone.0002878.g004

sequences, and survive for the same lifespan as non-transgenic mice of the same genetic background (129Ola) with no adverse effects and no features of spontaneous TSE disease. Wild-type mice (lines VM and RIII) are inbred lines used routinely for strain typing of TSEs. RIII is a *Prnp*-a genotype line and VM is a *Prnp*-b genotype line. [33] Use of mice for this work was reviewed and approved by the Neuropathogenesis Division Ethics Committee for Animal Experimentation.

Mice were inoculated as described previously. Groups of 24 wild-type mice received a 0.02 ml dose at 10^{-1} dilution by the intracerebral route, for vCJD (transfusion) and vCJD (BSE). Groups of 18 transgenic mice were injected with inoculum at a higher dilution of 10^{-2} as in previous experiments more concentrated inocula had been found to be toxic to the mice. Inoculum was prepared as a homogenate in sterile saline from frozen frontal cortex (with full consent from the patient's relatives, and approved by the Lothian NHS Board Research Ethics Committee (Reference: 2000/4/157)) to allow accurate comparison with previous data. Cases used for transmission were: the first blood transfusion associated case, designated here as vCJD (transfusion), and three historical vCJD cases designated here as vCJD (BSE) A, B, and C. The historical vCJD cases were not inoculated into the transgenic mice. Data from vCJD (transfusion) inoculation of the transgenic mice was compared with that already published for vCJD (BSE). [12] Data from vCJD (transfusion) inoculation of the wild-type mice was compared with data from the three historical vCJD cases.

Mice were housed in independently ventilated cages in a Category 3 facility, monitored daily and scored for signs of TSE disease weekly from 100 days post inoculation. Mice were culled, when clinical TSE was evident or for animal welfare reasons, by cervical dislocation and the brain bisected sagittally; one half frozen for biochemical analysis of disease-associated prion protein and the other half fixed in formalin for histology.

Vacuolation scoring was performed according to published protocols and lesion profiles generated. [34,35] Immunocytochemical detection of abnormal PrP deposition was performed as published and Western blotting of disease-associated PrP from the frozen half-brain carried out according to Head *et al.* [12,25]

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Author Contributions

Conceived and designed the experiments: MTB RGW MB JCM. Performed the experiments: MTB DLR VT. Analyzed the data: MTB DLR MWH. Contributed reagents/materials/analysis tools: JWI MWH. Wrote the paper: MTB RGW JWI JCM.

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