Gene expression analysis in distinct regions of the central nervous system during the development of SSBP/1 sheep scrapie

Elsevier Editorial System(tm) for Veterinary Microbiology
Manuscript Draft

Manuscript Number: VETMIC-D-10-4357R1

Title: Gene expression analysis in distinct regions of the central nervous system during the development of SSBP/1 sheep scrapie

Article Type: Research Paper

Keywords: scrapie; prion; gene expression; CNS; real-time quantitative RT-PCR.

Corresponding Author: Dr Anton Gossner,

Corresponding Author's Institution: University of Edinburgh

First Author: Anton Gossner

Order of Authors: Anton Gossner; Foster Jim; John Fazakerley; Nora Hunter; John Hopkins

Cover Letter

The Editor
Veterinary Microbiology

Dr Anton Gossner
THE ROSLIN INSTITUTE
R(D)SVS
University of Edinburgh
Summerhall
Edinburgh, EH9 1QH
UK

Telephone +44 (0)131 650 6280
Fax +44 (0)131 650 6511
anton.gossner@ed.ac.uk

www.roslin.ed.ac.uk

06 Dec 2009

Dear Dr Gaastra,

I would be grateful if you would consider the manuscript entitled "Gene expression analysis in distinct regions of the central nervous system during the development of SSBP/1 sheep scrapie" for publication in *Veterinary Microbiology*.

Very many thanks,

***Revision Note**

Editor-in-Chief (virology)
Veterinary Microbiology

Dr Anton Gossner
THE ROSLIN INSTITUTE
R(D)SVS
University of Edinburgh
Summerhall
Edinburgh, EH9 1QH
UK

Telephone +44 (0)131 650 6280
Fax +44 (0)131 650 6511
anton.gossner@ed.ac.uk

25 May 2010

Dear Prof. Uwe Truyen,

RE: Manuscript VETMIC-D-10-4357

Thank you for considering the manuscript VETMIC-D-10-4357 entitled "Gene expression analysis in distinct regions of the central nervous system during the development of SSBP/1 sheep scrapie**"** for publication in *Veterinary Microbiology*. I have submit a revised manuscript correcting the reference list on the main manuscript and the typo in Table 4, column 5, row 6 to "<0.03" as per the referees comments. I trust that these revisions are acceptable. The changes in the reference list have not been highlighted are they were deletions of duplications.

Kind regards,

Anton Gossner

Gene expression analysis in distinct regions of the central nervous system during the development of SSBP/1 sheep scrapie

Anton G. Gossner[a,*], Jim D Foster[b], John K. Fazakerley[a], Nora Hunter[b], John Hopkins[a]

[a] *Division of Infection & Immunity, The Roslin Institute & R(D)SVS, University of Edinburgh, Summerhall, Edinburgh EH9 1QH, U.K.*

[b] *Neuropathogenesis Division, The Roslin Institute & R(D)SVS, University of Edinburgh, Roslin, Midlothian, EH25 9PS. U.K.*

* Corresponding author: Tel: +44 131 650 7943; Fax: +44 131 650 6511;
E-mail: anton.gossner@ed.ac.uk

Correspondence address: Division of Infection & Immunity, The Roslin Institute & R(D)SVS, University of Edinburgh, Summerhall, Edinburgh EH9 1QH, U.K.

Keywords: scrapie, prion, gene expression, CNS, real-time quantitative RT-PCR

Abstract

Rodent scrapie models have been exploited to define the molecular basis for the progression of neuropathological changes in TSE diseases. We aim to assess whether CNS gene expression changes consistently observed in mouse models are of generic relevance, for example to natural TSE diseases, or are TSE strain, host species or brain region specific. Six genes, representing distinct physiological pathways and showing consistent changes in expression levels with disease progression in murine scrapie models were analysed for expression (RT-qPCR) in defined regions of the sheep brain at various times after SSBP/1 scrapie infection. Gene expression was examined in relation to the development of neuropathological changes including PrP^{Sc} deposition and vacuolation. Peripheral infection of sheep with SSBP/1 showed consistent progression of neuropathology as assessed by the temporal course of PrP^{Sc} deposition and neuropil vacuolation. The first region affected was the medulla (obex), then the thalamus and finally the cerebellum and frontal cortex. In contrast to mouse scrapie, there were few significant changes in transcript expression for any of the six genes and no consistent changes in patterns of expression in relation to brain region, time after infection or neuropathology in sheep SSBP/1. Gene expression changes in mouse TSE models, even changes consistent with the neuropathology, cannot necessarily be extrapolated to species in which disease naturally occurs. This may represent differences in pathological processes of different scrapie strains or across species; and highlights the difficulties in identifying generic molecular pathways associated to the pathogenesis of TSE disease.

1. Introduction

Scrapie is a sheep transmissible spongiform encephalopathy (TSE). TSEs are fatal neurodegenerative diseases that also include bovine spongiform encephalopathy (BSE) and human Creutzfeldt-Jakob disease (CJD). A characteristic feature of TSEs is the conversion of the membrane glycoprotein PrP (PrP^{C}) to disease-associated, insoluble PrP^{Sc}. The central role for PrP in TSE pathogenesis is illustrated by the resistance to disease of PrP-null mice (Bueler et al., 1993), by the inverse association of incubation period with PrP gene (*PRNP*) copy number (Bueler et al., 1993; Manson et al., 1994) and by the fact that susceptibility and resistance to sheep scrapie infection is largely controlled by polymorphisms of *PRNP* at codons 136 (V or A), 154 (R or H) and 171 (R or Q,). With the scrapie strain SSBP/1, VRQ homozygous sheep have a short incubation period; ARR homozygotes are resistant and heterozygotes have intermediate incubation periods (Houston et al., 2002). Different TSE diseases and scrapie strains can be differentiated by their distinct and reproducible incubation period lengths and characteristic patterns of PrP^{Sc} deposition and pathology, including astrocytosis and neuropil vacuolation (Jeffrey and González, 2007).

Gene expression profiling, largely of murine scrapie (Xiang et al., 2004; Riemer et al., 2004; Brown et al., 2004; Brown et al., 2005; Xiang et al., 2007; Hwang et al., 2009) or terminal human CJD (Xiang et al., 2005) has been used to elucidate the molecular basis for TSE diseases and to identify possible therapeutic targets (Hwang et al., 2009). Studies on mouse scrapie, largely using whole brain preparations, have identified genes that change in expression level in the brain with disease progression (Hwang et al., 2009). However, it is not known if these changes are of generic relevance and occur in relation to diseases progression in scrapie in its natural sheep host or if they are only relevant for the individual model.

Six genes showing consistent changes in mouse models of scrapie and chosen to represent disparate physiological pathways were analysed for expression in sheep infected with SSBP/1 scrapie. The genes chosen for this study were *C1QB*, *CCL5* (*SCYA5* or *RANTES*), *CCR5*, *NCKAP1*, *EGR1* and *FDFT1*. The progressive increase in brain-expressed transcripts for the first component of the classical complement pathway - C1q during the development of murine and hamster scrapie, has been a common finding in several studies (Dandoy-Dron et al., 1998; Riemer et al., 2000; Brown et al., 2004; Brown et al., 2005; Skinner et al., 2006; Hwang et al., 2009). C1qβ is one of three C1q subunits and has been implicated in the localization of PrP^{Sc}, from the site of infection to splenic follicular dendritic cells (Mabbott et al., 2001). Transcripts for the chemokine/receptor pair CCL5 and CCR5 are significantly increased in the hippocampus at late stage disease in ME7 scrapie -infected mice; it is postulated that they exacerbate neurodegeneration by amplifying proinflammatory responses (Lee et al., 2005). NCKAP1 (Nck-associated protein 1) and EGR1 (early growth response gene 1) are both significantly reduced in mouse scrapie (Booth et al., 2004); however NCKAP1 is pro-apoptotic and is repressed in human Alzheimer's disease (Yamamoto et al., 2001), while EGR1 is growth promoting and anti-apoptotic (Virolle et al., 2003) and is increased in Alzheimer's disease (Marella et al., 2005). FDFT1 (farnesyl-diphosphate farnesyltransferase 1 or squalene synthetase) is an important enzyme in cholesterol metabolism and its repression in mouse scrapie (Riemer et al., 2004; Xiang et al., 2007; Hwang et al., 2009) is thought to indicate a link between age-related and scrapie-associated neurodegeneration.

SSBP/1 scrapie (Wilson et al., 1950) is a commonly-used scrapie isolate in sheep (Foster et al., 2001; Houston et al., 2002) because of the well defined link between incubation period and *PRNP* genotype (Hunter, 2007) and is the parent of many commonly used rodent strains including 22C, 139A, RML and 263K (Kimberlin et al., 1989). This project investigated the progression of disease, as defined by PrP^{Sc} deposition and neuropil vacuolation, induced by SSBP/1 in four defined regions of the CNS in sheep of defined *PRNP* genotypes (VRQ/VRQ, VRQ/ARR and

ARR/ARR) with differential susceptibility to scrapie disease. To determine if TSE - associated molecular signatures for the progression of disease in mice are of generic relevance to TSE pathogenesis, gene expression levels of the six putative disease-associated genes from mouse studies were correlated to neuropathology in susceptible VRQ/VRQ sheep.

2. Materials and methods

2.1. Scrapie infection, histology and immunohistology

New Zealand Cheviot sheep of the *PRNP* genotypes VRQ/VRQ, VRQ/ARR and ARR/ARR were from the DEFRA breeding flock (Houston et al., 2002). All were inoculated subcutaneously with 2 ml of 10% w/v brain homogenate; for each genotype at each time point three were infected with SSBP/1scrapie and two mock-infected with normal brain. VRQ/VRQ animals were killed by exsanguination under terminal anaesthesia at 10, 25, 50, 75, 100 and 125 days post-infection (dpi) and at clinical stage. The same protocol was followed for sheep of the other *PRNP* genotypes with additional time points at 150 and 230 dpi (VRQ/ARR and ARR/ARR) and 1200 dpi (only ARR/ARR). ARR/ARR animals are resistant and there was no clinical group for this genotype. Brains were removed immediately post mortem, four brain regions were dissected (medulla (obex), thalamus, cerebellum and frontal cortex) and tissue blocks placed in RNA*later* (Qiagen, Crawley, UK) prior to storage at -80ºC, or fixed in neutral buffered formalin. Animal experiments were approved by BBSRC Institute for Animal Health Ethical Review Committee and conducted under an Animals (Scientific Procedures) Act 1986 Project Licence. Sections were stained using hematoxylin and eosin or the anti-PrP antibody BG4 (epitope 46-54, N terminus; TSE Resource Centre, The Roslin Institute) (Jeffrey et al., 2001) using ABC peroxidase/Vector Nova Red by the hydrated autoclaving method for disease-related PrP (Foster et al., 2001).

123

124 *2.2. Gene expression analysis*

125

126 Frozen brain tissue was converted to powder using a Mikro-Dismembrator U (Sartorius,
127 Aubagne Cedex, Fr) and total RNA prepared using RNeasy Lipid Tissue Mini Kit (Qiagen)
128 including DNase I digestion; RNA quality was assessed using a RNA Nano 6000 kit on the
129 Agilent 2100 Bioanalyser and quantified using a NanoDrop ND-1000 Spectrophotometer. RT
130 reactions were performed with 1 µg of total RNA from each sample with an anchored oligo$(dT)_{20}$
131 primer (Invitrogen, Paisley, UK) and M-MLV reverse transcriptase (Promega, Southampton,
132 UK). A sample without RT was included as control.
133 Gene-specific primers (Table 1) were designed using Primer3 (Rozen and Skaletsky,
134 2000) and Net Primer (http://www.premierbiosoft.com/ netprimer/index.html). BLAST searches
135 were performed for all primer sequences to confirm gene specificity prior to synthesis (Sigma-
136 Aldrich, Poole, UK.). Quantitative real-time RT-PCR (RT-qPCR) was performed in a Rotor-
137 Gene™ 3000 (Qiagen) using FastStart Taq DNA Polymerase (Roche Diagnostics Ltd., Lewes,
138 UK) with SYBR green detection in a final volume of 20 µl. Amplification conditions used were
139 the same for all genes; 5 min at 94ºC, followed by 40 cycles of 20 s at 94ºC, 20 s at 62ºC and 20 s
140 at 72ºC. All reactions were performed in triplicate and 'no template' controls included for each
141 gene. The cycle threshold value (Cq) was determined using the Rotorgene Software 6.0.34.
142 Agarose gel electrophoresis and melt curve analysis confirmed single products, sequence analysis
143 confirmed specificity. The linearity and efficiency of RT-qPCR amplification was determined for
144 each primer pair using a standard curve generated by a dilution series of a pool of sample cDNAs
145 for each tissue. Several genes were evaluated for expression stability and suitability as
146 endogenous reference genes for each of the different tissues using GeNorm v3.4 (Vandesompele
147 et al., 2002) and NormFinder v 0.953 (Andersen et al., 2004). Gene expression levels were
148 calculated using a modified ΔΔ-Cq method implemented in qBase analysis software (Hellemans

et al., 2007). Relative quantities of each of the six target gene transcripts were calculated using the normalized quantities rescaled relative to the same calibrator (the same mock-infected control sheep for each brain area but with different control sheep for each time point). Statistical analyses were performed on data from individual animals using Kolmogorov-Smirnov to test for normality of distribution; the mean normalized expression values from the infected and mock infected groups were compared using unpaired t-tests, with Welch's correction. Data are presented as mean fold change, mock-infected versus infected.

3. Results

3.1. Histopathology of the central nervous system

Vacuolar degeneration and PrP^{Sc} deposition were determined in the medulla, thalamus, cerebellum and frontal cortex of scrapie susceptible and scrapie resistant sheep at time points after infection with SSBP/1 scrapie; incubation periods were 193 ± 12 dpi for VRQ/VRQ and 328 ± 36 dpi for VRQ/ARR sheep. Evidence of low grade vacuolation was detected only in the susceptible VRQ/VRQ and VRQ/ARR genotypes and only at terminal disease time points (data not shown). The vacuolation that did occur was in the medullary and thalamic nuclei and was less conspicuous in VRQ/VRQ than in VRQ/ARR sheep. In contrast, PrP^{Sc} accumulation was detected in the susceptible animals at preclinical stages. In VRQ/VRQ sheep, PrP^{Sc} was first seen by 125 dpi (Table 2) in the medulla in 3 of 3 infected animals and in the thalamus in 1 of 3; and in the medulla of VRQ/ARR sheep by 230 dpi in 1 of 3 infected animals. By the time of onset of clinical disease PrP^{Sc} was detected in all four brain areas of all infected, susceptible animals (Fig. 1). Vacuolation and PrP^{Sc} deposition were not observed in mock-infected sheep or in sheep of the ARR/ARR genotype.

175

176 *3.2. Gene expression analysis in four brain regions*

177

178 Based on consistent changes in gene expression in mouse TSE models, the levels of
179 transcripts of six genes, *C1QB, CCL5, CCR5, EGR1, NCKAP1* and *FDFT1*, were determined in
180 each of the four brain areas in which pathological changes had been observed in SSBP/1 infected
181 VRQ/VRQ sheep; the medulla, thalamus, cerebellum and frontal cortex. The quantification of
182 gene expression in tissues requires the use of reference genes to normalize transcript numbers
183 between different samples. Since brain regions could show considerable difference in gene
184 expression, the stability of expression of several commonly used reference genes was first
185 investigated across the four brain regions. Two reference genes from different functional classes
186 were selected for each brain region, taking into account both intra- and inter-group variations.
187 *SDHA* and *YWHAZ* were used for the medulla, cerebellum and frontal cortex, with *SDHA* and
188 *GAPDH* for the thalamus (Table 3).

189 Analysis of expression levels of transcripts for the six target genes revealed that most
190 significant changes occurred after PrP^{Sc} deposition (Tables 2 and 4). *C1QB* showed no consistent
191 alterations in transcript expression between brain regions or within any one region over time; the
192 only significant changes were a 7-fold increase in the thalamus and an 8-fold reduction in the
193 cerebellum at the clinical disease time point. *CCL5* transcript levels were generally raised (1.27 -
194 2.94 fold) in medulla, thalamus and cerebellum at the earliest three time points after infection ($P \leq 0.05$ only at 125 dpi in the thalamus and 75 dpi in the cerebellum), but reduced at the clinical
196 time point in all four brain areas ($P \leq 0.05$ only in medulla and frontal cortex). *CCR5* expression
197 levels were variable at different time points and between different brain regions; showing a small,
198 but significant increase (1.6-fold, $P \leq 0.03$) at 75 dpi in the medulla and 1.9-fold ($P \leq 0.01$) at 125
199 dpi in the thalamus. Similarly variable and inconsistent over time within each region and
200 between regions at each time point were the expression levels of *EGR1*, *FDFT1* and *NCKAP1*,

which showed no obvious pattern of expression changes in relation to the progression of the neuropathology. The anti-apoptotic *EGR1* was largely unchanged or repressed throughout the course of infection in all four brain areas, but $P \leq 0.05$ only at 25 dpi in the cerebellum; *EGR1* was also strongly repressed at the clinical time point (except cerebellum) but $P \geq 0.2$ in each case. The pro-apoptotic *NCKAP1* was largely unchanged or increased through the course of infection, but $P \leq 0.05$ only at 125 dpi in the thalamus and at clinical time points in the medulla and cerebellum. The gene associated with cholesterol metabolism *FDFT1*, which was significantly raised (2.57-fold) only at 25 dpi in the cerebellum was significantly reduced (–2.63-fold) at the clinical time point in the medulla; it was also repressed (–5.14-fold) at the clinical time point in the cerebellum but $P = 0.17$.

4. Discussion

The unifying feature of the TSE family of diseases is that they are transmissible neurodegenerative diseases which are generally associated with PrP^{Sc} deposition. Other neuropathological changes include gliosis, spongiosis, neuropil vacuolation and neuronal loss. However, not all TSEs are identical; each has a variable and characteristic combination of these different features (DeArmond and Ironside, 1999). Furthermore, within each susceptible species there are different strains of TSEs that have unique incubation periods and distinctive neuropathological profiles. Recently, a mouse/scrapie strain combination has been identified that has little quantitative association with PrP^{Sc} deposition (Barron et al., 2007). Nevertheless, pathologically distinct prion strains give rise to similar profiles of behavioural deficits (Cunningham et al., 2005).

High throughput gene expression profiling of scrapie-infected brains has been used by several laboratories to quantify differentially expressed genes to try and identify a generic TSE profile in order to: (1) understand the molecular basis of TSE pathogenesis (Riemer et al., 2004;

Brown et al., 2005; Xiang et al., 2007; Tamgüney et al., 2008; Hwang et al., 2009); (2) identify novel risk genes and therapeutic targets (Xiang et al., 2004); (3) identify potential biomarkers of infection (Booth et al., 2004). All these studies have used rodent scrapie models with the presumption that results can be extrapolated to natural TSEs in target species (e.g. sheep, cattle and humans). The most extensive of these studies (Hwang et al., 2009) identified 333 transcripts that were commonly differentially expressed in three scrapie strains, in at least one of six mouse strains and at different time points during the course of disease. *C1QB* was represented in this group; and like the other genes that encode the C1q molecule, *C1QA* and *C1QG*, it showed a consistent and progressive increase in expression through the course of disease. An *NCKAP1*-like gene was also identified in this group and showed significantly reduced expression during scrapie disease.

Our data show that in SSBP/1 scrapie in sheep there is a distinct progression of pathology within the brain; PrP^{Sc} accumulation is detected earliest in the medulla and thalamus and this eventually progresses to the cerebellum and frontal cortex. However, unlike murine ME7, RML or 139A and many human TSEs (DeArmond and Ironside, 1999), SSBP/1 shows few signs of astrocytosis and little and variable vacuolation (Foster et al., 1996; Begara-McGorum et al., 2002). These differences in pathological features could explain the variation in gene profiles of the mouse and sheep diseases.

In mice, transcripts for the chemokine *CCL5* and its receptor *CCR5* are up-regulated during terminal ME7-disease (Lee et al., 2005) and expression of *C1QB* increases in an approximate linear manner in the hippocampus during the progressive development of ME7 scrapie (Brown et al., 2004). These three transcripts are probably products of astrocyte and microglial activation and their progressive increase in rodent ME7 infection is probably a result of the progressive gliosis which occurs in this system (Outram et al., 1973). Significant changes were seen in sheep SSBP/1 with both *CCL5* and *CCR5* although there was no consistent pattern to these changes, either between regions or within any one region in relation to progression of

disease. CCL5 might also be a neuronal product (Patterson et al., 2003) and the down-regulation at the clinical time point could be a result of the large-scale neuronal cell loss seen in terminal SSBP/1 scrapie (Foster et al., 2001). The variability is consistent with previous observations that progressive gliosis is a variable part of SSBP/1 pathology (Begara-McGorum et al., 2002) in sheep.

Murine scrapie induces reduced expression of both the pro-apoptotic gene *NCKAP1* and the anti-apoptotic *EGR1* (Booth et al., 2004). *NCKAP1* is expressed predominantly in neuronal cells (Suzuki et al., 2000) and is markedly reduced in human Alzheimer's disease. *EGR1* is a zinc finger transcription factor induced in neurons after extracellular stimulation with neurotransmitters or trophic substances; indeed amyloidosis in Alzheimer's disease has been shown to increase *EGR1* expression (MacGibbon et al., 1997) leading to the up-regulation of *CCL5*, possibly by neurons, explaining recruitment of microglia (Marella et al., 2005). Again, there was no consistent significant change or consistent trend in the expression of these two genes in sheep across either brain regions or with time, possibly because spongiosis and neuropil vacuolation are highly variable in SSBP/1 scrapie and correlate poorly with PrP^{Sc} deposition (Foster et al., 1996; Begara-McGorum et al., 2002).

Cholesterol has been shown to be important in the conversion of PrP^{C} to PrP^{Sc}. Several enzymes of the cholesterol biosynthesis pathway have been shown to be differentially-regulated during both preclinical (Brown et al., 2005) and terminal murine scrapie (Riemer et al., 2004) and in Alzheimer's disease (Ehehalt et al., 2003) and this has been taken as evidence that alterations in cholesterol metabolism may be a common consequence of amyloidogenic processes in both diseases (Brown et al., 2004). The results reported here are inconsistent with this hypothesis as expression levels of *FDFT1* (an important enzyme in the cholesterol biosynthesis pathway) did not correlate with the development of SSBP/1 scrapie and were only significantly reduced at the clinical time point in the medulla. *FDFT1* levels were significantly raised at 25 dpi in the

cerebellum, but this is unlikely to be of biological significance as PrP^{Sc} deposition was not detectable in the CNS at this time point.

Although only six selected genes were used in this study it is clear that results of transcriptome analysis in scrapie must be interpreted and extrapolated with care and each model must be studied separately to find commonalities that may truly define the fundamental disease process.

Acknowledgements

We acknowledge the staff of the Greenfield Sheep Unit, BBSRC Institute for Animal Health, Compton for their care of the animals. We thank Mr Martin Hulme for excellent technical support, Dr Lisa Murphy, Ms M. Baxter and Ms E. Cartwright for tissue collection and preparation and Mr David Parnham for the immunohistology. This work was supported by BBSRC grant 15/BS516875.

References

Andersen, C.L., Jensen, J.L., Orntoft, T.F., 2004. Normalization of real-rime quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 64, 5245-5250.

Barron, R.M., Campbell, S.L., King, D., Bellon, A., Chapman, K.E., Williamson, R.A., Manson, J.C., 2007. High titers of transmissible spongiform encephalopathy infectivity associated with extremely low levels of PrP^{Sc} in vivo. J Biol Chem 282, 35878-35886.

Begara-McGorum, I., Gonzalez, L., Simmons, M., Hunter, N., Houston, F., Jeffrey, M., 2002. Vacuolar lesion profile in sheep scrapie: factors influencing its variation and relationship to disease-specific PrP accumulation. J Comp Pathol 127, 59-68.

Booth, S., Bowman, C., Baumgartner, R., Sorensen, G., Robertson, C., Coulthart, M., Phillipson, C., Somorjai, R.L., 2004. Identification of central nervous system genes involved in the host response to the scrapie agent during preclinical and clinical infection. J Gen Virol 85, 3459-3471.

Brown, A.R., Rebus, S., McKimmie, C.S., Robertson, K., Williams, A., Fazakerley, J.K., 2005. Gene expression profiling of the preclinical scrapie-infected hippocampus. Biochem Biophys Res Commun 334, 86-95.

Brown, A.R., Webb, J., Rebus, S., Williams, A., Fazakerley, J.K., 2004. Identification of up-regulated genes by array analysis in scrapie-infected mouse brains. Neuropathol Appl Neurobiol 30, 555-567.

Bueler, H., Aguzzi, A., Sailer, A., Greiner, R.A., Autenried, P., Aguet, M., Weissmann, C., 1993. Mice devoid of PrP are resistant to scrapie. Cell 73, 1339-1347.

Cunningham, C., Deacon, R.M.J., Chan, K., Boche, D., Rawlins, J.N.P., Perry, V.H., 2005. Neuropathologically distinct prion strains give rise to similar temporal profiles of behavioral deficits. Neurobiol Dis 18, 258-269.

Dandoy-Dron, F., Guillo, F., Benboudjema, L., Deslys, J.P., Lasmezas, C., Dormont, D., Tovey, M.G., Dron, M., 1998. Gene expression in scrapie. Cloning of a new scrapie-responsive

322 gene and the identification of increased levels of seven other mRNA transcripts. J Biol
323 Chem 273, 7691-7697.

324 DeArmond, S., Ironside, J., 1999. Neuropathology of prion diseases. In: Prusiner, S. (Ed.), Prion
325 biology and diseases. Cold Spring Harbor Press, New York, pp. 585-652.

326 Ehehalt, R., Keller, P., Haass, C., Thiele, C., Simons, K., 2003. Amyloidogenic processing of the
327 Alzheimer beta-amyloid precursor protein depends on lipid rafts. J Cell Biol 160, 113-
328 123.

329 Foster, J., Goldmann, W., Parnham, D., Chong, A., Hunter, N., 2001. Partial dissociation of
330 PrP(Sc) deposition and vacuolation in the brains of scrapie and BSE experimentally
331 affected goats. J Gen Virol 82, 267-273.

332 Foster, J.D., Wilson, M., Hunter, N., 1996. Immunolocalisation of the prion protein (PrP) in the
333 brains of sheep with scrapie. Vet Rec 139, 512-515.

334 Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., Vandesompele, J., 2007. qBase relative
335 quantification framework and software for management and automated analysis of real-
336 time quantitative PCR data. Genome Biol 8, R19.

337 Houston, E.F., Halliday, S.I., Jeffrey, M., Goldmann, W., Hunter, N., 2002. New Zealand sheep
338 with scrapie-susceptible PrP genotypes succumb to experimental challenge with a sheep-
339 passaged scrapie isolate (SSBP/1). J Gen Virol 83, 1247-1250.

340 Hunter, N., 2007. Scrapie: uncertainties, biology and molecular approaches. Biochim Biophys
341 Acta 1772, 619-628.

342 Hwang, D., Lee, I.Y., Yoo, H., Gehlenborg, N., Cho, J.H., Petritis.B, Baxter, D., Pitstick, R.,
343 Young, R., Spicer, D., Price, N.D., Hohmann, J.G., Carlson, G.A., Hood, L.E., 2009. A
344 systems approach to prion disease. Molecular Systems Biology 5, 1-23.

345 Jeffrey, M., González, L., 2007. Classical sheep transmissible spongiform encephalopathies:
346 pathogenesis, pathological phenotypes and clinical disease. Neuropathol Appl Neurobiol
347 33, 373-394.

348 Jeffrey, M., Martin, S., Gonzalez, L., Ryder, S.J., Bellworthy, S.J., Jackman, R., 2001.
349 Differential diagnosis of infections with the bovine spongiform encephalopathy (BSE)
350 and scrapie agents in sheep. J Comp Pathol 125, 271-284.

351 Kimberlin, R.H., Walker, C.A., Fraser, H., 1989. The genomic identity of different strains of
352 mouse scrapie is expressed in hamsters and preserved on reisolation in mice. J Gen Virol
353 70, 2017-2025.

354 Lee, H.P., Jun, Y.C., Choi, J.K., Kim, J.I., Carp, I., Kim, Y.S., 2005. The expression of RANTES
355 and chemokine receptors in the brains of scrapie-infected mice. J. Neuroimmunol. 158,
356 26-33.

357 Mabbott, N.A., Bruce, M.E., Botto, M., Walport, M.J., Pepys, M.B., 2001. Temporary depletion
358 of complement component C3 or genetic deficiency of C1q significantly delays onset of
359 scrapie. Nat Med 7, 485-487.

MacGibbon, G.A., Lawlor, P.A., Walton, M., Sirimanne, E., Faull, R.L., Synek, B., Mee, E., Connor, B., Dragunow, M., 1997. Expression of Fos, Jun, and Krox family proteins in Alzheimer's disease. Exp Neurol 147, 316-332.

Manson, J.C., Clarke, A.R., McBride, P.A., McConnell, I., Hope, J., 1994. PrP gene dosage determines the timing but not the final intensity or distribution of lesions in scrapie pathology. Neurodegeneration 3, 331-340.

Marella, M., Gaggioli, C., Batoz, M., Deckert, M., Tartare-Deckert, S., Chabry, J., 2005. Pathological prion protein exposure switches on neuronal mitogen-activated protein kinase pathway resulting in microglia recruitment. J Biol Chem 280, 1529-1534.

Outram, G.W., Fraser, H., Wilson, D.T., 1973. Scrapie in mice. Some effects on the brain lesion profile of ME7 agent due to genotype of donor, route of injection and genotype of recipient. J Comp Pathol 83, 19-28.

Patterson, C.E., Daley, J.K., Echols, L.A., Lane, T.E., Rall, G.F., 2003. Measles Virus Infection Induces Chemokine Synthesis by Neurons. J Immunol 171, 3102-3109.

Riemer, C., Neidhold, S., Burwinkel, M., Schwarz, A., Schultz, J., Kratzschmar, J., Monning, U., Baier, M., 2004. Gene expression profiling of scrapie-infected brain tissue. Biochem Biophys Res Commun 323, 556-564.

Riemer, C., Queck, I., Simon, D., Kurth, R., Baier, M., 2000. Identification of upregulated genes in scrapie-infected brain tissue. J Virol 74, 10245-10248.

379 Rozen, S., Skaletsky, H., 2000. Primer3 on the WWW for general users and for biologist
380 programmers. Methods Mol Biol 132, 365-386.

381 Skinner, P., Abbassi, H., Chesebro, B., Race, R., Reilly, C., Haase, A., 2006. Gene expression
382 alterations in brains of mice infected with three strains of scrapie. BMC Genomics 7, 114.

383 Suzuki, T., Nishiyama, K., Yamamoto, A., Inazawa, J., Iwaki, T., Yamada, T., Kanazawa, I.,
384 Sakaki, Y., 2000. Molecular cloning of a novel apoptosis-related gene, human Nap1
385 (NCKAP1), and its possible relation to Alzheimer disease. Genomics 63, 246-254.

386 Tamgüney, G., Giles, K., Glidden, D.V., Lessard, P., Wille, H., Tremblay, P., Groth, D.F.,
387 Yehiely, F., Korth, C., Moore, R.C., Tatzelt, J., Rubinstein, E., Boucheix, C., Yang, X.,
388 Stanley, P., Lisanti, M.P., Dwek, R.A., Rudd, P.M., Moskovitz, J., Epstein, C.J., Cruz,
389 T.D., Kuziel, W.A., Maeda, N., Sap, J., Ashe, K.H., Carlson, G.A., Tesseur, I., Wyss-
390 Coray, T., Mucke, L., Weisgraber, K.H., Mahley, R.W., Cohen, F.E., Prusiner, S.B.,
391 2008. Genes contributing to prion pathogenesis. J Gen Virol 89, 1777-1788.

392 Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F.,
393 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric
394 averaging of multiple internal control genes. Genome Biology 3, research0034.1-
395 research0034.11.

396 Virolle, T., Krones-Herzig, A., Baron, V., De Gregorio, G., Adamson, E.D., Mercola, D., 2003.
397 Egr1 promotes growth and survival of prostate cancer cells. Identification of novel Egr1
398 target genes. J Biol Chem 278, 11802-11810.

399 Wilson, D.R., Anderson, R.D., Smith, W., 1950. Studies in scrapie. J Comp Pathol 60, 267-282.

400 Xiang, W., Hummel, M., Mitteregger, G., Pace, C., Windl, O., Mansmann, U., Kretzschmar,
401 H.A., 2007. Transcriptome analysis reveals altered cholesterol metabolism during the
402 neurodegeneration in mouse scrapie model. J Neurochem 102, 834-847.

403 Xiang, W., Windl, O., Westner, I.M., Neumann, M., Zerr, I., Lederer, R.M., Kretzschmar, H.A.,
404 2005. Cerebral gene expression profiles in sporadic Creutzfeldt-Jakob disease. Ann
405 Neurol 58, 242-257.

406 Xiang, W., Windl, O., Wunsch, G., Dugas, M., Kohlmann, A., Dierkes, N., Westner, I.M.,
407 Kretzschmar, H.A., 2004. Identification of differentially expressed genes in scrapie-
408 infected mouse brains by using global gene expression technology. J Virol 78, 11051-
409 11060.

410 Yamamoto, A., Suzuki, T., Sakaki, Y., 2001. Isolation of hNap1BP which interacts with human
411 Nap1 (NCKAP1) whose expression is down-regulated in Alzheimer's disease. Gene 271,
412 159-169.
413
414
415

Figure legends

Fig. 1. Immunohistological staining for PrP^{Sc} with the BG4 antibody of VRQ/VRQ sheep brain at clinical time point with SSBP/1 scrapie. (A) medulla (obex); (B) thalamus; (C) cerebellum and (D) frontal cortex. Magnification x10.

Table 1. Primers for real-time qRT-PCR

Gene	*Forward primer 5' → 3'*	*Reverse primer 5' → 3'*	*Primer (nM)*	*Mg^{2+} (mM)*	*Size (bp)*	**qPCR efficiency*
GAPDH	GGTGATGCTGGTGCTGAGTA	TCATAAGTCCCTCCACGATG	300	3.5	265	95%
SDHA	ACCTGATGCTTTGTGCTCTGC	CCTGGATGGGCTTGGAGTAA	300	2	126	98%
YWHAZ	TGTAGGAGCCCGTAGGTCATC	TCTCTCTGTATTCTCGAGCCATC	600	3	101	94%
CCL5	CGCCAACCCAGAGAAGAAGT	CGCCACAAAGTTCAGGTTCAA	300	2.5	91	96%
CCR5	ATACGTGCAGCCCACATTTC	GATTCCCGAGTAGCAGACGA	600	2.5	98	99%
C1QB	AACGAGAATGGCGAGAAGG	CAGGTGGTGGTTGATGGTG	300	3	191	95%
FDFT1	ACTGTCACTATGTTGCTGGTC	CCTTCTCGCTGGTCTTCC	600	3	169	100%
EGR1	CCACCTCCTACTCCTCTCCTG	CCATCTCCTCCTCCTGTCCT	300	3	282	99%
NCKAP1	CAAGAGCAAGAGCTGGACATC	AACTCGCCACCAGGACTTAGAG	600	3	108	98%

* Reaction efficiency was calculated using the equation $E=10^{(-1/slope)}-1$.

Table 2. Immunohistology for PrP^{Sc} of *PRNP* genotype-sheep challenged with SSBP/1 scrapie

		Brain Region			
dpi	*PRNP genotype*	*Medulla*	*Thalamus*	*Cerebellum*	*Frontal cortex*
100	VRQ/VRQ	0/0	0/0	0/0	0/0
	VRQ/ARR	0/0	0/0	0/0	0/0
125	VRQ/VRQ	3/3	1/3	0/3	0/3
	VRQ/ARR	0/3	0/3	0/3	0/3
150	VRQ/ARR	0/3	0/3	0/3	0/3
230	VRQ/ARR	1/3	0/3	0/3	0/3
Clinical 193	VRQ/VRQ	3/3	3/3	3/3	3/3
328	VRQ/ARR	3/3	3/3	3/3	3/3

Shaded rows are time points with PrP^{Sc} accumulation.

Table 3. Reference gene expression stability in four brain regions.

Gene	*Medulla*	*Thalamus*	*Cerebellum*	*Frontal cortex*
GAPDH	0.083*	**0.045**	0.111	0.111
SDHA	**0.073**	**0.037**	**0.050**	**0.050**
YWHAZ	**0.065**	0.136	**0.069**	**0.098**

* Gene expression normalization factor calculated by GeNorm and NormFinder; lowest value is most stable (least variable). Bold are the best combination of two genes for particular brain region.

Table 4. Transcript expression levels in brain of scrapie VRQ/VRQ infected sheep

	Medulla (obex)				*Thalamus*				*Cerebellum*				*Frontal Cortex*			
dpi	*25*	*75*	*125*	*clin**	*25*	*75*	*125*	*clin*	*25*	*75*	*125*	*clin*	*25*	*75*	*125*	*clin*
C1QB‡																
Fold	2.55	1.01	-1.31	1.85	†	†	†	**7.01**	1.05	1.21	-1.10	**-8.06**	-1.49	-1.26	-1.2	-1.35
P value	0.08	0.98	0.73	0.21	†	†	†	**<0.01**	0.96	0.59	0.87	**<0.01**	0.06	0.75	0.79	0.61
CCL5																
Fold	2.16	1.27	1.54	**-2.06**	2.94	1.90	**2.30**	-3.47	1.55	**2.21**	1.30	-17.38	1.09	-1.12	1.12	**-3.66**
P value	0.27	0.72	0.07	**<0.03**	0.06	0.06	**<0.05**	0.24	0.07	**<0.01**	0.56	0.27	0.86	0.8	0.84	**<0.01**
CCR5																
Fold	1.31	**1.60**	-1.23	-1.34	1.92	1.15	**1.90**	1.49	-1.89	-1.06	1.12	-8.84	1.86	1.37	-1.29	**-2.16**
P value	0.61	**<0.03**	0.18	0.18	0.22	0.76	**<0.01**	0.31	0.34	0.88	0.77	0.13	0.19	0.56	0.07	**0.03**
EGR1																
Fold	1.26	-1.91	1.10	-2.87	-2.20	1.06	1.32	-25.82	1.01	1.10	1.01	1.22	**-2.28**	-1.51	-1.03	-4.6
P value	0.77	0.10	0.92	0.22	0.10	0.87	0.46	0.33	0.99	0.79	0.98	0.48	**<0.05**	0.68	0.91	0.34
NCKAP1																
Fold	-1.11	-1.02	1.48	**2.89**	1.24	1.07	**1.44**	-1.62	-1.25	-1.22	1.26	**6.32**	-1.14	-1.75	1.16	2.27
P value	0.62	0.69	0.41	**<0.01**	0.41	0.83	**<0.02**	0.32	0.41	0.16	0.41	**<0.01**	0.58	0.45	0.70	0.12
FDFT1																
Fold	1.45	1.05	-1.08	**-2.63**	1.10	1.07	1.35	-1.16	**2.57**	1.42	†	-5.14	-1.39	1.07	-1.34	1.29
P value	0.20	0.72	0.74	**<0.02**	0.52	0.85	0.38	0.41	**<0.01**	0.44	†	0.17	0.17	0.64	0.13	0.53

* clinical disease time point 193 ± 12 dpi . ‡ Data are expressed as fold change, scrapie infected vs mock infected. † Below level of detection limit.

P values shown in bold are significant $P \leq 0.05$. Shaded columns are time points with PrP^{Sc} accumulation.

Figure 1

Click here to download high resolution image