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

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Article Type: Research Paper

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

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The Editor
Veterinary Microbiology

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,
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

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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$^{\text{Sc}}$ deposition and vacuolation. Peripheral infection of sheep with SSBP/1 showed consistent progression of neuropathology as assessed by the temporal course of PrP$^{\text{Sc}}$ 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 (PrPC) to disease-associated, insoluble PrPSc. 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 PrPSc 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 PrPSc, 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 PrPSc 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 RNAlater (Qiagen, Crawley, UK) prior to storage at -80°C, 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).
2.2. Gene expression analysis

Frozen brain tissue was converted to powder using a Mikro-Dismembrator U (Sartorius, Aubagne Cedex, Fr) and total RNA prepared using RNeasy Lipid Tissue Mini Kit (Qiagen) including DNase I digestion; RNA quality was assessed using a RNA Nano 6000 kit on the Agilent 2100 Bioanalyser and quantified using a NanoDrop ND-1000 Spectrophotometer. RT reactions were performed with 1 µg of total RNA from each sample with an anchored oligo(dT)$_{20}$ primer (Invitrogen, Paisley, UK) and M-MLV reverse transcriptase (Promega, Southampton, UK). A sample without RT was included as control.

Gene-specific primers (Table 1) were designed using Primer3 (Rozen and Skaltsky, 2000) and Net Primer (http://www.premierbiosoft.com/netprimer/index.html). BLAST searches were performed for all primer sequences to confirm gene specificity prior to synthesis (Sigma-Aldrich, Poole, UK.). Quantitative real-time RT-PCR (RT-qPCR) was performed in a Rotor-Gene™ 3000 (Qiagen) using FastStart Taq DNA Polymerase (Roche Diagnostics Ltd., Lewes, UK) with SYBR green detection in a final volume of 20 µl. Amplification conditions used were 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 at 72ºC. All reactions were performed in triplicate and ‘no template’ controls included for each gene. The cycle threshold value (Cq) was determined using the Rotorgene Software 6.0.34. Agarose gel electrophoresis and melt curve analysis confirmed single products, sequence analysis confirmed specificity. The linearity and efficiency of RT-qPCR amplification was determined for each primer pair using a standard curve generated by a dilution series of a pool of sample cDNAs for each tissue. Several genes were evaluated for expression stability and suitability as endogenous reference genes for each of the different tissues using GeNorm v3.4 (Vandesompele et al., 2002) and NormFinder v 0.953 (Andersen et al., 2004). Gene expression levels were 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 PrPSc 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, PrPSc accumulation was detected in the susceptible animals at preclinical stages. In VRQ/VRQ sheep, PrPSc 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 PrPSc was detected in all four brain areas of all infected, susceptible animals (Fig. 1). Vacuolation and PrPSc deposition were not observed in mock-infected sheep or in sheep of the ARR/ARR genotype.
3.2. Gene expression analysis in four brain regions

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

Analysis of expression levels of transcripts for the six target genes revealed that most significant changes occurred after PrP\(^{Sc}\) deposition (Tables 2 and 4). *C1QB* showed no consistent alterations in transcript expression between brain regions or within any one region over time; the only significant changes were a 7-fold increase in the thalamus and an 8-fold reduction in the cerebellum at the clinical disease time point. *CCL5* transcript levels were generally raised (1.27 - 2.94 fold) in medulla, thalamus and cerebellum at the earliest three time points after infection (P ≤ 0.05 only at 125 dpi in the thalamus and 75 dpi in the cerebellum), but reduced at the clinical time point in all four brain areas (P ≤ 0.05 only in medulla and frontal cortex). *CCR5* expression levels were variable at different time points and between different brain regions; showing a small, but significant increase (1.6-fold, P ≤ 0.03) at 75 dpi in the medulla and 1.9-fold (P ≤ 0.01) at 125 dpi in the thalamus. Similarly variable and inconsistent over time within each region and 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 \textit{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; \textit{EGR1} was also strongly repressed at the clinical time point (except cerebellum) but $P \geq 0.2$ in each case. The pro-apoptotic \textit{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 \textit{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\textsuperscript{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\textsuperscript{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; PrPSc 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 PrPSc deposition (Foster et al., 1996; Begara-McGorum et al., 2002).

Cholesterol has been shown to be important in the conversion of PrPC to PrPSc. 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.

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


Figure legends

Fig. 1. Immunohistological staining for PrP\textsuperscript{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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer 5’ → 3’</th>
<th>Reverse primer 5’ → 3’</th>
<th>Primer (nM)</th>
<th>Mg²⁺ (mM)</th>
<th>Size (bp)</th>
<th>*qPCR efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>GGTGATGCTGGTGCTGAGTA</td>
<td>TCATAAGTCCCTCCACGATG</td>
<td>300</td>
<td>3.5</td>
<td>265</td>
<td>95%</td>
</tr>
<tr>
<td>SDHA</td>
<td>ACCTGATGCTTTGTGCTCTGC</td>
<td>CCTGGATGGGGCTTGGAGTAA</td>
<td>300</td>
<td>2</td>
<td>126</td>
<td>98%</td>
</tr>
<tr>
<td>YWHAZ</td>
<td>TGTAGGAGCCGTAAGGTTCATC</td>
<td>TCTCTCTGTATTCTCGAGCCATC</td>
<td>600</td>
<td>3</td>
<td>101</td>
<td>94%</td>
</tr>
<tr>
<td>CCL5</td>
<td>CGCCAACCCAGAGAAGAAGT</td>
<td>CGCCACAAAGTTCAGGTTCAA</td>
<td>300</td>
<td>2.5</td>
<td>91</td>
<td>96%</td>
</tr>
<tr>
<td>CCR5</td>
<td>ATACGTGCAGCCCCACATRTC</td>
<td>GATCCCCGAGTAGCACAGACGA</td>
<td>600</td>
<td>2.5</td>
<td>98</td>
<td>99%</td>
</tr>
<tr>
<td>C1QB</td>
<td>AACGAGAATGGCGAGAAGG</td>
<td>CAGGTTGTGGTGTGATGGTG</td>
<td>300</td>
<td>3</td>
<td>191</td>
<td>95%</td>
</tr>
<tr>
<td>FDFT1</td>
<td>ACTGTCACTATGTTGCTGTC</td>
<td>CTTTCTCGTGGTCTTCC</td>
<td>600</td>
<td>3</td>
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</tr>
<tr>
<td>EGR1</td>
<td>CCACCTCCTACTCTCTCTCTG</td>
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<td>300</td>
<td>3</td>
<td>282</td>
<td>99%</td>
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<tr>
<td>NCKAP1</td>
<td>CAAGAGCAAGAGCTGGGACATC</td>
<td>AACTCGCCACCAGGACTTAGAG</td>
<td>600</td>
<td>3</td>
<td>108</td>
<td>98%</td>
</tr>
</tbody>
</table>

* Reaction efficiency was calculated using the equation $E=10^{(-1/\text{slope})}−1$. 
**Table 2.** Immunohistology for PrPSc of *PRNP* genotype-sheep challenged with SSBP/1 scrapie

<table>
<thead>
<tr>
<th>dpi</th>
<th>PRNP genotype</th>
<th>Brain Region</th>
<th>Medulla</th>
<th>Thalamus</th>
<th>Cerebellum</th>
<th>Frontal cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>VRQ/VRQ</td>
<td></td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td></td>
<td>VRQ/ARR</td>
<td></td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>125</td>
<td>VRQ/VRQ</td>
<td></td>
<td>3/3</td>
<td>1/3</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>VRQ/ARR</td>
<td></td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
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<tr>
<td>150</td>
<td>VRQ/ARR</td>
<td></td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>230</td>
<td>VRQ/ARR</td>
<td></td>
<td>1/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>Clinical 193</td>
<td>VRQ/VRQ</td>
<td></td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>VRQ/ARR</td>
<td></td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
</tbody>
</table>

Shaded rows are time points with PrPSc accumulation.
**Table 3.** Reference gene expression stability in four brain regions.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Medulla</th>
<th>Thalamus</th>
<th>Cerebellum</th>
<th>Frontal cortex</th>
</tr>
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<tr>
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<td>0.045</td>
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<td>0.111</td>
</tr>
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<td>0.073</td>
<td>0.037</td>
<td>0.050</td>
<td>0.050</td>
</tr>
<tr>
<td>YWHAZ</td>
<td>0.065</td>
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* 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

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* 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 ≤ 0.05. Shaded columns are time points with PrPSc accumulation.