Prion disease induced alterations in gene expression in spleen and brain prior to clinical symptoms

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Abstract: Prion diseases are fatal neurodegenerative disorders that affect animals and humans. There is a need to gain understanding of prion disease pathogenesis and to develop diagnostic assays to detect prion diseases prior to the onset of clinical symptoms. The goal of this study was to identify genes that show altered expression early in the disease process in the spleen and brain of prion disease-infected mice. Using Affymetrix microarrays, we identified 67 genes that showed increased expression in the brains of prion disease-infected mice prior to the onset of clinical symptoms. These genes function in many cellular processes including immunity, the endosome/lysosome system, hormone activity, and the cytoskeleton. We confirmed a subset of these gene expression alterations using other methods and determined the time course in which these changes occur. We also identified 14 genes showing altered expression prior to the onset of clinical symptoms in spleens of prion disease infected mice. Interestingly, four genes, Atp1b1, Gh, Anp32a, and Grn, were altered at the very early time of 46 days post-infection. These gene expression alterations provide insights into the molecular mechanisms underlying prion disease pathogenesis and may serve as surrogate markers for the early detection and diagnosis of prion disease.

Keywords: prion disease, microarrays, gene expression

Background

Transmissible spongiform encephalopathies (TSEs), also termed prion diseases, are fatal neurodegenerative disorders that occur in humans and various animal species. Examples include Creutzfeldt-Jakob disease (CJD) in humans; bovine spongiform encephalopathy (BSE) in cattle; chronic wasting disease (CWD) in deer and elk; and scrapie in sheep and experimental mice (Chesebro 2003). There is no test yet available for preclinical diagnosis of transmitted prion disease, nor is there an effective treatment available. In experimental models, certain drug treatments can prolong survival, but early intervention is essential to achieve this effect. We are faced with an urgent need to develop methods both to diagnose and to treat these diseases prior to the onset of clinical symptoms.

TSEs are characterized by vacuolation of the neuropil, neuron loss, activation of astrocytes and microglia, and in some situations the deposition of amyloid fibrils (Wells 1993; Budka et al 1995). These pathological changes are thought to depend on a misfolded partially protease-resistant isoform of endogenous prion protein, known as PrP^{sc} or PrP-res (Prusiner 1982; McKinley et al 1983). The molecular mechanisms underlying the pathology are far from clear (Aguzzi and Polymenidou 2004; Weissmann, 2004).

Oral infection is the major route of transmission for many TSEs, including BSE, CWD (Belay et al 2004), transmissible mink encephalopathy (Bartz et al 2003),

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scrapie (Andreoletti et al 2000; van Keulen et al 2000), and variant CJD (Bruce et al 1997). After oral transmission, infectivity passes through the gut to secondary immune tissues and then to the brain (Aguzzi and Heikenwalder 2005). Similar to oral infection, experimental intracerebral scrapie inoculation also leads to the secondary lymphoid organs rapidly accumulating infectivity and misfolded prion proteins (Fraser and Dickinson 1970; Hunter et al 1972).

We hypothesize that genes involved in protein folding and genes that function in the endosome/lysosome system are important cofactors in the misfolding and accumulation of misfolded prion proteins and the propagation of scrapie infectivity. The rationale for this hypothesis include that: 1) prion diseases involve prion protein misfolding (Prusiner 1982; Aguzzi 2006), 2) the misfolding of prion proteins is thought to occur on the cell surface or in the endosome/lysosome pathway (Caughey and Raymond 1991; Caughey et al 1991; Caughey 1993), 3) misfolded prion proteins accumulate in the endosome/lysosome system (McKinley et al 1991), 4) lysosomotropic agents and cysteine protease inhibitors inhibit the scrapie-associated accumulation of prion proteins (Doh-Ura et al 2000), and 5) we have identified a number of genes that function in protein folding and genes that function in the endosome/lysosome system that show increased expression during prion disease (Skinner et al 2006).

In a previous study using cDNA microarrays, we identified 22 genes that showed increased expression in the brains of scrapie-infected mice prior to the onset of clinical symptoms (Skinner et al 2006). One goal of the present study was to expand our gene expression studies using Affymetrix 430A microarrays (Santa Clara, CA), in order to identify additional alterations in expression that occur prior to the onset of clinical symptoms. The Affymetrix 430A microarrays contain over 14,000 unique genes, more than 7000 of which were not represented in the cDNA microarrays in our previous study (Skinner et al 2006). Thus, the Affymetrix microarrays allowed us to identify additional undescribed scrapie-associated gene expression alterations.

Several published TSE gene expression studies have sought to identify alterations in gene expression that occur in the brain. However, given the importance of the lymphoid organs in prion disease transmission and pathogenesis, there is a need to expand gene expression studies to the lymphoid organs. Another goal of the study presented here was to determine in scrapie-infected mice whether genes that show expression alterations in the brain also show expression alterations in spleen tissues.

In this study we used Affymetrix microarrays and identified 67 genes that show alterations in expression in the brains of scrapie-infected mice prior to the onset of clinical symptoms. We confirmed a number of these gene expression alterations using quantitative reverse transcription polymerase chain reaction (qRT-PCR). With qRT-PCR we also determined the time-course of several of these changes. We also determined the time course of astrocytosis and PrP^{sc} accumulation in mice used in these studies, and evaluated protein accumulation of two genes identified GFAP and Gh within the pituitary gland. In addition, we identified 15 genes that showed alterations in gene expression in both spleen and brain tissues from scrapie-infected mice. We further evaluated protein accumulation and localization of one of these genes, anp32a, within the spleen. Our results extend our understanding of prion disease pathogenesis and indicate certain gene products that may serve as surrogate markers for early disease diagnosis.

Methods

Scrapie-infected mice

Three strains of mouse scrapie were examined: ME7, 22L, and RML-Chandler (Chandler 1963; Zlotnik and Rennie 1963; Dickinson 1976). For Affymetrix microarray analyses, we used RNA isolated by the Chesebro lab and shipped to the Skinner lab, from five male C57BL/10 mice that were infected with 50 µl of a 1% scrapie brain homogenate derived from mice at the end stage of scrapie infection. The titer of each inoculum was at least 2×10^8 infectious units/gram brain and exceeded the amount needed to induce disease in 100% of the animals (Vorberg et al 2004). RNA was from the same mice used in our previous study (Skinner et al 2006). This included RNA from two mock-infected mice and one ME7-infected, one 22L-infected, and one RML-Chandler-infected mouse, sacrificed at 104 days post-inoculation (dpi).

For the real-time PCR studies a separate set of female C57BL/6 mice was used. For each strain, six C57BL/6 female mice were inoculated intracerebrally with 50 µl of a 1% scrapie brain homogenate derived from mice at the end stage of scrapie infection. The titer of each inoculum was at least 2×10^8 infectious units/gram brain and exceeded the amount needed to induce disease in 100% of the animals (Vorberg et al 2004). Six control mice were mock-inoculated with 1% homogenate of brain tissues from uninfected mice. Mock-infected mice served as a control for nonspecific effects induced by the intracerebral injection of brain inoculum. At 46 and 104 dpi (before the onset of clinical symptoms), and at the onset of clinical symptoms at 144 dpi, two mice

from each group were sacrificed. Mock- and scrapie-infected mice were sacrificed at the same time of day. Clinical symptoms included kyphosis, dull eyes, flattened stature, weight loss, and ataxia. Total RNA was extracted from whole brain and spleen homogenates using Trizol following the manufacturer's instructions (Invitrogen), resuspended in 100 µl of RNase free molecular biology grade water (Eppendorf), analyzed by spectrometry and gel electrophoresis for quantity and quality, diluted to 1 µg/µl, and stored at −80 °C.

For the western blot analysis nine female C57BL/6 mice were infected with RML-Chandler strain of scrapie and nine mice were mock-infected as described above and three from each group sacrificed at 45, 100, and 140 dpi.

For the immunohistochemistry staining of GFAP and Gh, one ME7, 22L, RML-Chandler, and one mock-infected C57BL/6 female mouse was infected and sacrificed at 45, 100, and 140 dpi. For anp32a immunohistochemistry three mice were infected with RML-Chandler, and three mock-infected as described above and sacrificed at 45 dpi.

Affymetrix microarrays

Total whole brain RNA from scrapie- and mock-infected mice sacrificed at 104 dpi was reverse transcribed and labeled following protocols provided by Affymetrix. Five Affymetrix Murine 430A chips were hybridized with RNA from two different mock-infected mice and RNA from one ME7-, one 22L-, and one RML-Chandler-infected mouse, following the manufacturer's instructions. Scan intensity data was extracted and normalized from the Affymetrix output files using the RMAExpress program (http://www.stat.berkeley.edu/~bolstad/RMAExpress/RMAExpress.html). MatchMiner (http://discover.nci.nih.gov/matchminer/index.jsp) was used to convert Affymetrix IDs to corresponding GenBank accession numbers.

Statistical analyses and data mining

We used significance of analysis of microarrays (SAM) with false discovery rates (FDR) and q-values to identify genes that showed differences in expression in scrapie-infected brains compared to mock-infected brains (Tusher et al 2001; Storey 2002). We performed a two-class test between results obtained from mock-infected mice and scrapie-infected mice, with data input parameters kept at the default values. We considered for further study those resultant genes that showed a 1.4-fold or greater change in scrapie-infected compared to mock-infected mice and had a q-value of $<10\%$. For simplicity of presentation here, in instances where a gene was represented by more that one Affymetrix probe set, we removed duplicates showing synonymous results.

To determine the function of the proteins encoded by genes showing scrapie-associated alterations in expression, we used published literature, the National Center for Biotechnology Information (NCBI), and the Source (Diehn et al 2003).

Quantitative real-time RT-PCR

For real-time qRT-PCR, we used total RNA from whole brains and spleens. We compared RNA from two mock-infected mice to RNA from six scrapie-infected mice at 46, 104, and 144 dpi. The six scrapie-infected mice included two ME7-, two 22L-, and two RML/Chandler-infected mice. RNA was treated with deoxyribonuclease I (Fermentas, Glen Burnie, MD) to remove genomic DNA contamination and reverse transcribed to cDNA using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). An M × 3000P qPCR machine (Stratagene, La Jolla, CA) and Platinum SYBR Green qPCR SuperMix-UDG kits (Invitrogen) were used following manufacturers' instructions. PCR primers were designed using MacVector software (Accelrys Inc., San Diego, CA), and spanned introns. Samples that were not treated with reverse transcriptase were included as a negative control. We previously made a stock of normal mouse brain RNA, treated it with DNAse, reverse transcribed it to cDNA, and purified and used it in each qPCR reaction as a standard to normalize results from experimental samples. The relative cDNA levels were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Actin was used as the reference gene using previously described primers (Lengacher et al 2004). PCR products were run on an agarose gel to verify the size of product. For each gene studied, a t-test was performed to verify the significance of the results. The primers and annealing temperatures used, as well as product sizes, are indicated in Table 2. Fold change values of less than 1 were converted using the equation −1/fold change, for ease of interpretation.

Western blot analysis

To detect levels of PrP^{sc} protein accumulation in the spleen and brain, 20% solutions of spleen and brain tissue were prepared in 0.01M TrisHCL pH 7.4, 0.005 M MgCl, treated with DNAse, and sonicated. Tissue lysates were mixed with an equal volume (1 ml) of 20% sarcosyl in 0.01 M Tris HCl pH 7.4 and spun in an ultracentrifuge for thirty minutes in a Beckman ultracentrifuge rotor Type 70.1 (Beckman Coulter, Fullerton, CA) at 5000 RPM. Supernatant were transferred

to a new tube and centrifuged at 55000 RPM for two hours. The resultant pellet was resuspended in 1 ml water, sonicated, and then treated with 100 μg/ml proteinase K for 1 hour at 37 °C. Reactions were terminated with 3 mM PMSF. Samples were again be spun at 55000 rpm for one hour and pellets resuspended in 2 × western blot sample buffer, sonicated, and boiled prior to loading on gels. For detection of Prp^c and anp32a, brain and spleen tissues were lysed in RIPA buffer (50 mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, and 0.5% sodium deoxycholate, 0.1% SDS) with protease inhibitors (Sigma P8340; Sigma, St. Louis, MO) and centrifuged at 12000 rpm for 20 minutes. BCA protein assay kit (Pierce Biotechnology, Rockford, IL) was used to determine protein concentrations of resultant supernatant. 6.25 mg/well of protein was loaded for the PrP blot and 12.5 mg/well was used for the anp32a blot. Samples were run using pre-cast polyacrylamide gels in Tris-glycine (4%–12% IM-6000 Invitrogen) and transferred to PVDF membranes (Millipore, Billerica, MA). Blots were probed with antibodies against PrP (SAF-83 from Cayman Labs, or anp32a from AbCam) and then were stripped and re-probed with antibodies directed against Gapdh (Chemicon) and densitometry determined levels of protein accumulation between samples from scrapie-infected and mock-infected mice. Levels of Gapdh were used to normalize each sample.

Immunohistochemistry

Mice were perfused and fixed overnight at 4 °C with freshly made paraformaldehyde. For Gh and GFAP staining tissues were dehydrated in alcohol and paraffin embedded. Sections were treated with antigen retrieval reagent (Diva Decloaker; Biocare, Concord, CA) and then blocked in blocking sniper solution (Biocare) for one hour, and then stained with either GFAP antibodies and counter stained with hematoxylin and eosin, or anti-human Gh antibodies (Biomeda, Foster City, CA) (1:1000) and counter stained with hematoxylin using the Vectastain ABC Kit (Vector Labs, Burlingame, CA). For anp32a staining, after fixation tissues were transferred to phosphate buffer solution (PBS) and stored at 4 °C. Tissues were cut into 50 μm thick sections using a vibratome and blocked with 2% normal goat serum in PBS containing 0.3% Triton-X100, and then stained free floating in 1 ml solution of rabbit anti-anp32a (AbCam) diluted 1:400 and rat anti-mouse-B220 antibodies (BD Pharmingen, San Diego, CA) diluted 1:200 in block solution on a rocking platform over night at 4 °C. Sections were then washed three times for at least 20 minutes with PBS and then incubated with goat anti-rabbit Alexa 488 (Invitrogen) diluted 1:1000 and goat anti-rat Cy3 diluted 1:5000 (Jackson ImmunoResearch, West Grove, PA) and incubated overnight at 4 °C on a rocking platform. Finally sections were again washed then mounted with glycerol-gelatin (Sigma) containing 4 mg/ml n-proply gallate as a fluorophore preservative, and analyzed using a confocal microscope.

Results

Affymetrix microarray analysis of brain gene expression in scrapie-infected mice

We used Affymetrix microarrays and identified 67 genes that showed increased expression in the brains of scrapie-infected mice prior to the onset of clinical symptoms at 104 dpi (Table 1). In this study, gene expression was determined in two mock and three scrapie-infected male C57BL/10 mice. The set of scrapie-infected mice included one mouse infected with scrapie strain ME7, one with scrapie strain RML-Chandler, and one with scrapie strain 22L in order to allow us to find scrapie-associated gene expression alterations that occur regardless of strain. A number of the genes we identified have previously been described in related studies of prion disease, primarily at the end stages of disease, and these are cited in Table 1 (Dormont et al 1981; Manuelidis et al 1987; Duguid et al 1988; Duguid and Dinauer 1990; Diedrich et al 1993; Duguid and Trzepacz 1993; Williams et al 1994; Dandoy-Dron et al 1998; Baker et al 1999, 2002, 2004; Doh-ura et al 2000; Gayrard et al 2000; Kopacek et al 2000; Riemer et al 2000, 2004; Baker and Manuelidis 2003; Rangon et al 2003; Zhang et al 2003; Booth et al 2004a, 2004b; Brown et al 2004; Lu et al 2004; Schultz et al 2004; Xiang et al 2004; Viguie et al 2004; Greenwood et al 2005; Rodriguez et al 2006; Skinner et al 2006). Eighteen of the genes we identified have not been previously reported in prion disease studies (Table 1). The genes identified function in multiple cellular processes including: immunity, the endosome lysosome system, the cytoskeleton, and hormone activity. Many of the genes function in multiple cellular processes, but are listed in a single functional category for ease of presentation.

Onset of pathology

In order to establish when pathological PrP^{sc} accumulation developed in the scrapie-infected mice, we performed western blot analysis of brain and spleen extracts from three mice infected with RML-Chandler strain of scrapie and three mock-infected mice at 45, 100, and 140 dpi. In the brain, PrP^c was readily detectible at all time points examined. No proteinase K resistant PrP^{sc} was detectible at 45 dpi, but was readily detectible at 100 and 140 dpi (Figure 1). This correlated with detection of increased GFAP immunoreactivity in

Table 1 Scrapie-associated expression alterations in brain at 104 dpi using Affymetrix microarrays

Function	Gene symbol	Fold change	Previous references
Immunity	B2m	2.4	Diedrich et al 1993; Dandoy-Dron et al 1994; Riemer et al 2000; Booth et al 2004a; Booth et al 2004b; Xiang et al 2004; Skinner et al 2006
	Ccl12	1.6	Xiang et al 2004
	Ccl9	1.4	Xiang et al 2004
	Cxcl10	1.5	Baker et al 2004; Lu et al 2004; Riemer et al 2004; Schulz et al 2004; Xiang et al 2004;
	C1qa	2.7	Klein et al 2001; Baker and Manuelidis 2003; Brown et al 2004; Riemer et al 2004; Xiang et al 2004; Skinner et al 2006
	C1qb	3.7	Baker and Manuelidis 2003; Baker et al 2004; Brown et al 2004; Riemer et al 2004; Xiang et al 2004
	C1qg	2.4	Riemer et al 2000; Riemer et al 2004; Xiang et al 2004
	C4	2.6	Xiang et al 2004
	Fcer1g	1.7	Baker and Manuelidis 2003; Booth et al 2004b; Xiang et al 2004
	Fcgr2b	2.0	Baker and Manuelidis 2003; Xiang et al 2004
	Fcgr3	1.5	Baker and Manuelidis 2003; Xiang et al 2004
	Gbp4	1.4	
	Grn	1.6	Baker and Manuelidis 2003; Booth et al 2004b
	H2-D1	2.2	Duguid and Trzepacz 1993; Riemer et al 2000; Booth et al 2004a; Riemer et al 2004; Xiang et al 2004
	H2-K1	1.7	Duguid and Trzepacz 1993; Riemer et al 2000; Booth et al 2004a; Riemer et al 2004; Xiang et al 2004
	H2-T23	1.6	
	Ifitm3	1.9	
	Ifit1	1.9	
	Ifit3	2.8	Booth et al 2004b
	Ifi27	1.8	
	Ly86	2.5	Baker et al 2004; Booth et al 2004b; Lu et al 2004; Riemer et al 2004; Xiang et al 2004
	Lyzs	1.9	Kopacek et al 2000; Xiang et al 2004
	Rtp4	1.9	
Lysosome	Hexb	2.0	Kopacek et al 2000; Xiang et al 2004; Skinner et al 2006
	Ctsc	1.6	Riemer et al 2004; Xiang et al 2004
	Ctsh	1.5	Baker and Manuelidis 2003; Riemer et al 2004; Xiang et al 2004
	Ctsl	1.4	Baker and Manuelidis 2003; Zhang et al 2003; Brown et al 2004; Lu et al 2004
	Ctss	2.2	Baker et al 1999; Baker et al 2002; Baker and Manuelidis 2003; Baker et al 2004; Booth et al 2004b; Brown et al 2004; Riemer et al 2004; Xiang et al 2004; Lu et al 2004
	Ctsz	2.0	Riemer et al 2004; Xiang et al 2004
	Cd68	1.8	Williams et al 1994; Baker et al 2002; Baker and Manuelidis 2003; Lu et al 2004; Riemer et al 2004; Xiang et al 2004
	Laptm5	1.6	Riemer et al 2000; Booth et al 2004b; Riemer et al 2004; Xiang et al 2004
	Prdx6	1.4	Kopacek et al 2000
Hormone activity	Cga	1.5	
	Gh	21.5	Viguie et al 2004
	Prl	15.0	
	Pomc1	2.4	Gayard et al 2000
Cytoskeleton	Aif1	1.6	Xiang et al 2004
	Cnn3	1.5	Booth et al 2004b; Xiang et al 2004

(*continued*)

Table 1 (*continued*)

Function	Gene symbol	Fold change	Previous references
	Gfap	3.9	Dormont et al 1981; Wietgrefe et al 1985; Diedrich et al 1987; Manuelidis et al 1987; Duguid et al 1988; Campbell et al 1994; Baker et al 1999; Doh-ura et al 2000; Riemer et al 2000; Klein et al 2001; Baker et al 2002; Baker et al 2004; Brown et al 2004; Xiang et al 2004; Skinner et al 2006
	Vim	2.0	Booth et al 2004a; Booth et al 2004b; Brown et al 2004; Riemer et al 2000; Xiang et al 2004
Protease Inhibitor	Cst7	2.2	Baker and Manuelidis 2003; Lu et al 2004; Riemer et al 2004; Xiang et al 2004
	Serpina3n	2.5	Riemer et al 2004; Xiang et al 2004
Calcium	Anxa3	1.4	Riemer et al 2004
	S100a6	1.5	Riemer et al 2004; Xiang et al 2004; Greenwood et al 2005
Scavenger receptor activity	Lgals3bp	2.5	Riemer et al 2004; Xiang et al 2004; Skinner et al 2006
	Msr2	1.5	Xiang et al 2004
Ubiquitin/Proteosome	Trim30	1.5	Xiang et al 2004
	Usp18	1.6	
Zinc Binding	Mt2	1.6	Duguid et al 1988; Dandoy-Dron et al 1994; Riemer et al 2000; Xiang et al 2004
	Pdlim4	1.4	
Other	Anp32a	1.6	
	Aqp4	1.8	Riemer et al 2000; Riemer et al 2004; Rodriguez et al 2006
	Cd52	1.5	Riemer et al 2004; Xiang et al 2004
	Cd53	1.6	Riemer et al 2004; Xiang et al 2004
	Cd9	1.7	Doh-ura et al 2000; Brown et al 2004; Riemer et al 2004; Xiang et al 2004
	Clecsf12	1.8	Xiang et al 2004
	Cyba	1.6	Baker and Manuelidis 2003; Riemer et al 2004; Xiang et al 2004
	Cyp7b1	1.5	
	Dbp	1.4	
	Hba-a1	1.4	Booth et al 2004b
	Olfml3	1.6	
	Ppp1r3c	1.4	
	Prg1	1.4	
	Pycard	1.6	Xiang et al 2004
	Stat1	1.8	
	Trem2	1.6	
	Tyrobp	2.3	Riemer et al 2004; Xiang et al 2004

astrocytes throughout the brain in mice infected with Me7, 22L, and RML-Chandler strains of scrapie at 104 and 146 dpi (Appendix 2). In addition, our western blot analysis of PrP^{c} and PrP^{sc} in spleen tissues from the same scrapie-infected mice revealed that the proteinase K digestion was incomplete, but nonetheless showed abundant PrP^{c} and PrP^{sc} at 45, 100, and 140 dpi (data not shown).

qRT-PCR confirmation of microarray results

We used quantitative RT-PCR (qRT-PCR) to confirm a subset of the scrapie-associated alterations in expression of the genes identified in our Affymetrix study that have not previously been described in association with TSEs, and to determine the time course over which these alterations occur. Twelve genes were selected that were representative of each functional category of genes identified in the Affymetrix microarray study: immunity, lysosome, ubiquitin/proteosome, and other. The genes evaluated were Usp18, Olfml3, Ifit1, Ifit3, Ifitm3, Ifit27, Rtp4, Stat1, Trem2, Grn, H2-T2, and Anp32a. Hormone genes were also evaluated and discussed in the next section. We evaluated gene expression in at three time points post-infection: prior to the onset of clinical symptoms (at 44 and 104 dpi) and at the time when symptoms became detectable (at 144 dpi).

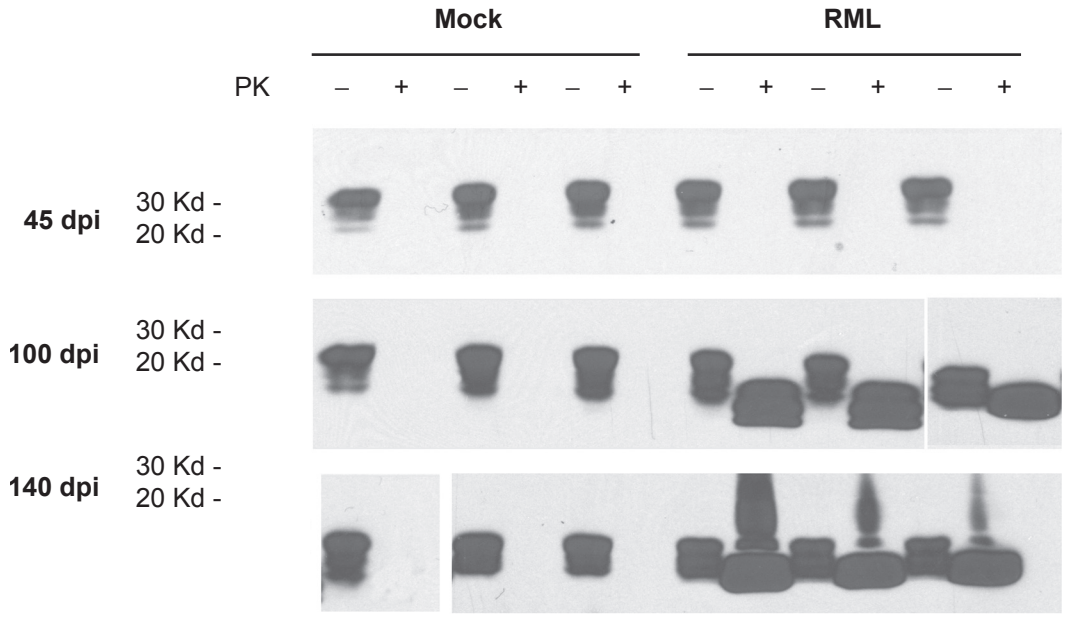


Figure 1 Prion protein and proteinase K resistant prion protein accumulation in brains from mock and RML-Chandler scrapie-infected mice at 45, 100, and 140 days post-infection. Western blot analysis showing prion protein antibody staining (SAF83) of protein extracts that were untreated or treated with proteinase K.

These time points were selected because they are similar to as those used in our previous study (Skinner et al 2006), and thus allow the two studies to be correlated. Gene expression levels were determined at each time point in two mock-infected and six scrapie-infected female C57BL/6 mice. The set of scrapie-infected mice included two infected with strain ME7, two with strain 22L, and two with strain RML-Chandler. As controls in this study, we used two genes: Atp1b1, because it showed decreased expression and GFAP, because it showed increased expression in the brains of scrapie-infected mice in our previous studies (Skinner et al 2006). Primers, annealing temperatures, and product sizes are presented in Table 2. We show the average expression from all three strains of scrapie-infected mice relative to mock-infected mice in Table 3, and we present in Appendix 1 our more detailed results for each scrapie strain: ME7, 22L, and RML-Chandler. In this study, none of the genes showed significantly altered expression at 46 dpi. Five of the 12 genes showed significantly increased expression at 104 dpi. These genes were Usp18, Olfml3, Ifit1, Ifit3, and Ifi27. At the onset of clinical symptoms (144 dpi), 11 of the 12 genes (all but Anp23a) showed significant increased expression in the brain of scrapie-infected mice relative to mock-infected mice. Overall, although not statistically significant in every case, all 12 experimental genes showed higher expression in scrapie-infected mice compared to mock-infected mice at some point in time. Although none of the experimental genes showed significant alteration in the brain at the very early time point 46 dpi, interestingly, the control gene Atp1b1 showed significantly decreased expression at 46 dpi, demonstrating that alterations in Atp1b1 occur very early in the course of scrapie infection.

Alterations in hormone expression in scrapie-infected mice

Four of the genes that showed increased expression in the brain of scrapie-infected mice prior to the onset of clinical symptoms encode hormones expressed primarily in the pituitary gland. These genes were Cga, Gh, Prl, and Pomc1. To confirm these increases and to determine the time course over which these increases in hormone expression take place, we performed qRT-PCR and evaluated gene expression in scrapie-infected and mock-infected mice at 46, 104, and 144 dpi. Results shown in Table 4 depict the average of all three scrapie strains. Results from individual strains are presented in Appendix 1. Gh showed significant increased expression at the very early time point 46 dpi. Gh, Prl, and Pomc1 showed increased expression at 104 and 144 dpi. In this study, Cga did not show significant increased expression at any time point using qRT-PCR.

We then used qRT-PCR to determine whether the upstream regulators of these hormones might themselves be upregulated in scrapie-infected mice. We evaluated the expression of Crh, Ghrh, Trh, and Gnrh1, which induce the expression and secretion of hormones in the pituitary gland. None of these genes showed significant expression alterations in presymptomatic mice. However, Crh showed increased expression at the onset of clinical symptoms (144 dpi)

Table 2 Primers and annealing temperatures used for qRT-PCR studies

Name	Unigene No.	Sequence	T C	Size
Prolactin-F	Mm.1270	5'- AACCTGCTGTTCTGCCAAAATG -3'	60	369bp
Prolactin-R		5'- TCTTGATAGGATGTATTCGGGGG -3'	60	
Pomc1-F	Mm.277996	5'- GCCACTGAACATCTTTGTCCCC -3'	60	296bp
Pomc1-R		5'- ATCTCCGTTGCCAGGAAACAC -3'	60	
Ifit3-F	Mm.271850	5'- TTCCCAGCAGCACAGAAAC -3'	60	96bp
Ifit3-R		5'- CCAGGTGAAATGGCACTTC-3'	60	
Granulin-F	Mm.1568	5'- CCTGCTTCCAGATGTCAGATAACC -3'	62	235bp
Granulin-R		5'- TTCTTTAGTAGGGTGTGGGTGCC -3'	62	
Anp32a-F	Mm.269088	5'- GAGCCGCTGAAGAAGTTAGAGAATC -3'	60	138bp
Anp32a-R		5'- GTTGTCCCTGTCATAGCCATCG -3'	60	
Gh-F	Mm.343934	5'- TGGCAATGGCTACAGACTCTCG -3'	60	118bp
Gh-R		5'- TTAGAAAACAGACTGGACAAGGGC -3'	60	
H2-T23-F	Mm.35016	5'- CAGAGTAACGACGAATCTCACACG -3'	60	263bp
H2-T23-R		5'- AGCCGTAGGTATCTATGGAGCCAC -3'	60	
Rtp4-F	Mm.390891	5'- GGTTCCAGTGTTCCAGATGCTG -3'	60	90bp
Rtp4-R		5'- CCTGCGATTTCAAAGTGTCCG -3'	60	
Ifi27-F	Mm.271275	5'- ACTCCAATCAGCAGGGGTCC -3'	60	193bp
Ifi27-R		5'- TTCTTTGACATCAGTGAGGGTTCTG -3'	60	
Stat1-F	Mm.277406	5'- GAGTGAGTGAGAGCCAGTCGTTTC -3'	62	171bp
Stat1-R		5'- CCAAATGCTTCCGTTCCCAC -3'	62	
Trem2-F	Mm.261623	5'- TGGTGGAGGTGCTGGAGGAC -3'	62	133bp
Trem2-R		5'- GGTGGGAAGGAGGTCTCTTGATTC -3'	62	
Olfml 3-F	Mm.211535	5'- TGCCTTAGAGGAACGGCTGG -3'	60	174bp
Olfml 3-R		5'- TCCCTTTCAAGACGGTCCACTC -3'	60	
Usp18-F	Mm.326911	5'- CCTCGGTGATACCAAGGAACAG -3'	60	144bp
Usp18-R		5'- TGTGAGTCATTGAAGCAGAACCAC -3'	60	
Ifit1-F	Mm.6718	5'- GAGGAGTTCTGCTCTGCTGAAAAC -3'	60	349bp
Ifit1-R		5'- ACAGTTGCCCCAGGTCGC -3'	60	
Atp1b1-F	Mm.4550	5'- CTCGGAGAAGAAGGAGTTTTTGG -3'	60	194bp
Atp1b1-R		5'- TCTGGGGAATCTGTGTCAATCC -3'	60	
Gfap-F	Mm.1239	5'- AGAAAACCGCATCACCATTCC -3'	60	128bp
Gfap-R		5'- GCATCTCCACAGTCTTTACCACG -3'	60	
Cga-F	Mm.1361	5'- ACACATCCCTCAAAAAGTCCAGAG -3'	60	223bp
Cga-R		5'- GAGAAGCAACAGCCCATACACTG -3'	60	
Hexb-F	Mm.27816	5'- GGGAGCGTTACGAGGTTTAGAGAC -3'	60	108bp
Hexb-R		5'- TGAGGGAATCTTGGAGAATCAGC -3'	60	
Ctsz-F	Mm.156919	5'- CCAAGGACCAAGACTGTGACAAG -3'	60	183bp
Ctsz-R		5'- CTGTTGCCATTATCCCGCAG -3'	60	
Hspa4-R	Mm.239865	5'- GCTTGAGGTGGTTGGTCCG -3'	60	115bp
Hspa4-F		5'- ATACTGAAGAGCAGCAGCAGCC -3'	62	
Hspa8-F	Mm.290774	5'- CGGGCATTCGTGTGGTCTC -3'	62	173bp
Hspa8-R		5'- CTTGGTGTGGTGCGGTTACC -3'	60	
Hspa12-R	Mm.39739	5'- TGAACTGCTTGGCTGGTTGC -3'	62	109bp
Hspa12-F		5'- CAGGCTCTGAAGGAACTGAGTGAC -3'	62	
Cd68-F	Mm.15819	5'- ACAGGCAGCACAGTGGACATTC -3'	62	134bp
Cd68-R		5'- GAGAGAGCAGGTCAAGGTGAACAG -3'	62	
Actb-F	Mm.391967	5-GCTTCTTTGCAGCTCCTTCGT-3	60	63bp
Actb -R		5-ATATCGTCATCCATGGCGAAC-3	60	
Ifitm3 - F	Mm.141021	5'- AGCCTATGCCTACTCCGTGAAGTC -3'	60	113 bp
Ifitm3 - R		5'- TGAGGACCAAGGTGCTGATGTTC -3'	60	
Lhb-F	Mm.57061	5'- GAGAGGCTCCAGGGGCTG -3'	60	214bp
Lhb-R		5'- GCACAGGAGGCAAAGCAG -3'	60	

(*continued*)

Table 2 (*Continued*)

Name	Unigene No	Sequence	T C	Size
Tshb-F	Mm.110730	5'- TCACTCATGCAAAGTAAGATCCTGC -3'	60	218bp
Tshb-R		5'- GGCACACTCTCTCCTATCCACG -3'	60	
Fshb-F	Mm.249525	5'- GCTGACTGCACAGGACGTAG -3'	60	209bp
Fshb-R		5'- CACCAGATCCCTAGTGTAGCAG -3'	60	
Gnrh1-F	Mm.358309	5'- TCACTCATGCAAAGTAAGATCCTGC -3'	60	230bp
Gnrh1-R		5'- GGCACACTCTCTCCTATCCACG -3'	60	
Trh-F	Mm.1363	5'- CCTGTGTATCCTATCCCAGTTCCC -3'	60	126bp
Trh-R		5'- CTGATTGGCTCTTTGAAGTTCCTG -3'	60	
Crh-F	Mm.290689	5'- AAGGGAGGAGAAGAGAGCG -3'	60	92bp
Crh-R		5'- CAAGGCAGGCAGGACGAC -3'	60	
Ghrh-F	Mm.144157	5'- AACTGTTCTCACCATCTAATCGGG -3'	60	128bp
Ghrh-R		5'- CCTTCACTCCTGGGTGGGACTC -3'	60	
Cts S-F	Mm.3619	5'-TGAGCACCACACTTCAGGATGACC-3'	60	210bp
Cts S-R		5'-TCTTTTCCCAGATGAGACGCCG-3'	60	

Notes: F and R refer to forward and reverse primers; T °C is the annealing temperature used for qPCR.

(Table 4). We also determined whether other hormone genes that are primarily expressed in the pituitary gland were altered in pre-symptomatic scrapie-infected mice by evaluating expression levels of Lhb, Tshb, and Fshb in scrapie- and mock-infected mice. These gene products bind to Cga (glycoprotein hormones alpha subunit) to form luteinizing hormone, thyroid-stimulating hormone, and follicle-stimulating hormone. None of these genes were significantly altered in scrapie-infected mice (Table 4). These results confirm the findings from our Affymetrix microarray study, which showed increased expression of Gh, Prl, and Pomc1 in the brains of scrapie-infected mice. The results also show that not all hormone-encoding genes that are primarily expressed in the pituitary gland are altered in scrapie-infected mice. The increased expression of hormone genes identified in our study is not likely due to increased expression of upstream regulators of hormones but rather by some other mechanism.

Increases GFAP and Gh in anterior pituitary gland of scrapie-infected mice

Two of the genes that were found to be upregulated in scrapie-infected mice were GFAP and Gh. We stained pituitary

Table 3 Confirmation of scrapie-associated gene expression alterations using qRT-PCR

Symbol	Unigene No	46 dpi		104 dpi		144 dpi	
		AVE. FC	p-value	AVE. FC	p-value	AVE. FC	p-value
Atp1b1	Mm.4550	−1.4	0.027	−1.3	0.013	−1.7	0.037
Usp18	Mm.326911	1.2	0.246	3.1	0.017	4.4	0.04
Gfap	Mm.1239	1.2	0.138	2.4	0.019	10.1	0.006
Olfml3	Mm.211535	1	0.459	2.8	0.027	3.1	0.018
Ifit1	Mm.6718	1.2	0.386	2.2	0.028	4.9	0.019
Ifi27	Mm.271275	1.1	0.356	2.7	0.036	2.9	0.021
Ifit3	Mm.271850	1.1	0.353	3.3	0.05	5.1	0.005
Rtp4	Mm.390891	1.3	0.573	1.2	0.416	5.4	0.007
Stat1	Mm.277406	1.3	0.531	1.2	0.275	2.4	0.01
Trem2	Mm.261623	1.6	0.131	1.3	0.227	4.3	0.012
Ifitm3	Mm.141021	1.2	0.284	1.2	0.343	3	0.014
Grn	Mm.1568	1.1	0.395	1	0.398	3.1	0.019
H2-T23	Mm.35016	1.2	0.67	1.2	0.285	2.4	0.022
Anp32a	Mm.269088	1.5	0.28	1.2	0.292	1.3	0.26

Notes: Statistically significant expression alterations are highlighted in bold dpi is days post-infection; AVE. FC is the average fold change in expression of scrapie samples relative to r.

Table 4 Hormone and hormone regulator gene expression in scrapie-infected vs mock-infected mice using qRT-PCR

		46 dpi		104 dpi		144 dpi	
Gene Symbol	**Unigene No**	**AVE. FC**	**p-value**	**AVE. FC**	**p-value**	**AVE. FC**	**p-value**
Gh	Mm.343934	1.5	0.012	14	0.015	13.9	0.014
Prl	Mm.1270	1.4	0.508	13.5	0.007	14.8	0.014
Pomc1	Mm.277996	1.1	0.379	5.1	0.03	5.3	0.015
Crh	Mm.290689	1.2	0.582	−1.4	0.213	2.8	0.026
Cga	Mm.1361	1.3	0.786	1	0.377	1.5	0.169
Tshb	Mm.110730	1.3	0.813	1.6	0.138	1	0.459
Lhb	Mm.57061	1.3	0.431	1.4	0.232	1.5	0.183
Ghrh	Mn.144157	1.5	0.394	1.3	0.27	1.1	0.106
Trh	Mm.1363	1	0.541	−1.3	0.274	2.7	0.157
Fshb	Mm.249525	1	0.486	1	0.302	1.1	0.453
Gnrh1	Mm.358309	1.4	0.512	1	0.358	1.4	0.236

Notes: Statistically significant expression alterations are highlighted in bold dpi is days post-infection; AVE. FC is the average fold change in expression detected in scrapie samples relative to mock samples.

glands from ME7, 22L, and RML Chandler-infected mice and mock-infected mice at 45, 100, and 145 dpi with GFAP and Gh antibodies. Interestingly, both GFAP and Gh antibodies showed increased immunoreactivity in glandular hormone secreting cells of the anterior pituitary gland (Figures 2 and 3).

Alterations in gene expression in the spleen of scrapie-infected mice

Experimental intracerebral scrapie inoculation leads to the rapid accumulation of infectivity and PrP^{sc} in secondary lymphoid organs including the spleen (Fraser and Dickinson 1970; Hunter et al 1972). To analyze gene expression in the spleen of scrapie-infected mice, we used qRT-PCR to evaluate relative expression levels of 22 genes that in our current or previous microarray studies (Skinner et al 2006) showed scrapie-associated expression alterations in the brain. These genes were selected 1) because they encode proteins that function either in protein folding or in the endosome/lysosome system (where misfolded prion proteins accumulate), 2) because they function in

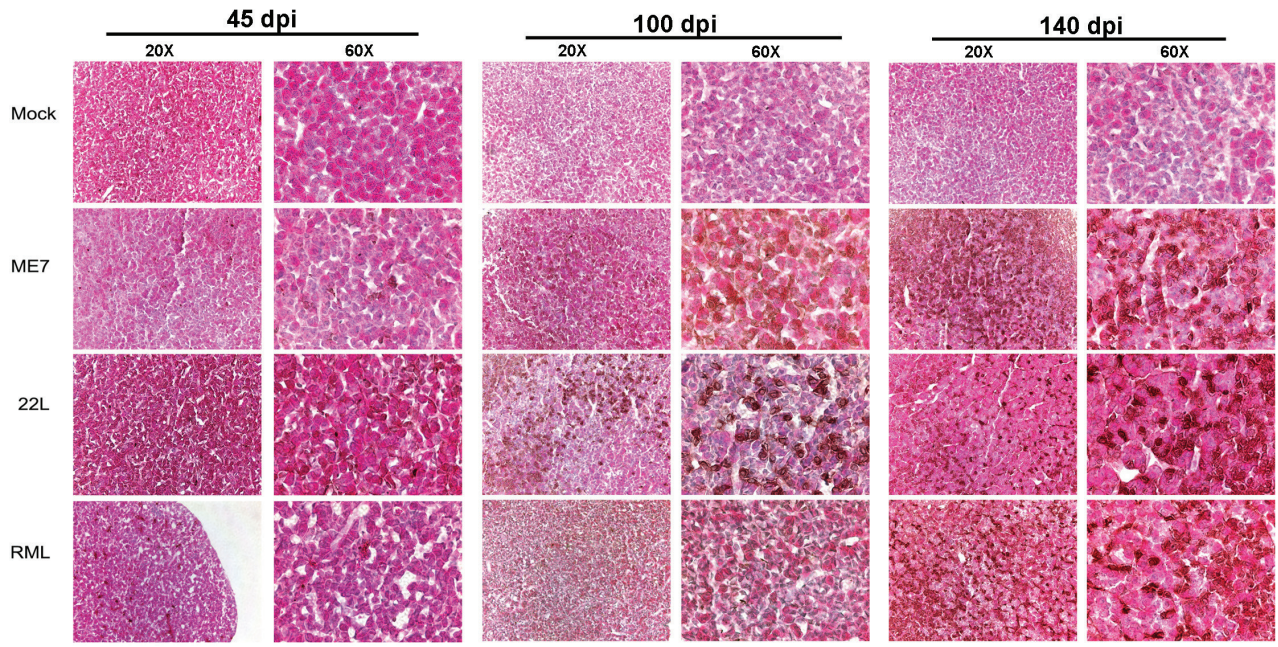


Figure 2 GFAP antibody staining in the anterior pituitary gland (adenohypophysis) from scrapie- and mock-infected mice at 45, 100, and 140 days post-infection. Sections were stained with GFAP antibodies (brown) and counterstained with hematoxylin (blue) and eosin (pink). Images were collected with 20 × and 60 × objectives as indicated. Note increased GFAP immunoreactivity in glandular cells of anterior pituitary in sections from scrapie-infected mice relative to mock-infected mice.

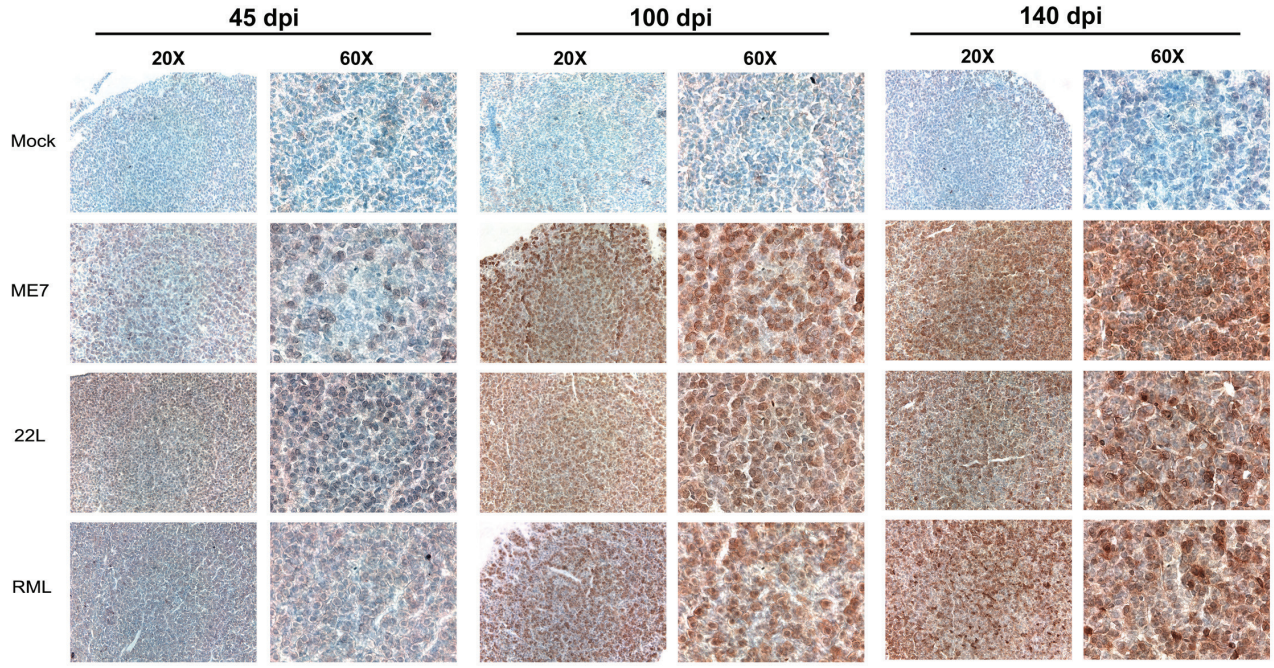


Figure 3 Growth hormone (Gh) antibody staining in the anterior pituitary gland (adenohypophysis) from scrapie- and mock-infected mice at 45, 100, and 140 days post-infection. Images show sections stained with Gh antibodies (brown) and counterstained with haematoxylin (blue). Images were collected with 20 × and 60 × objectives as indicated. Note increased Gh immunoreactivity in glandular cells of anterior pituitary in sections from scrapie-infected mice relative to mock-infected mice.

immunity (a major functional group of genes that show altered expression in the brain during prion disease), or 3) because they were novel genes that have never been described in association with prion diseases. These genes were Hspa8, Hspa12a, Hspa4, Ctsz, Ctss, HexB, H2-T23, Ifit1, Ifit3, Ifitm3, Ifi27, Rtp4, Cd68, Gh, Grn, Stat1, Usp18, Trem2, Olfml3, and Anp32a. We also included GFAP and Atp1b1 because in our previous studies they showed increased and decreased expression, respectively, in the brain early in the course of scrapie infection (Skinner et al 2006). We evaluated gene expression in the spleen at the same time points as in our brain studies: at two time points prior to the onset of clinical symptoms (at 46 and 104 dpi) and at the time when clinical symptoms appeared in the mice (at 144 dpi).

The results showed that all 22 genes evaluated were expressed in the spleen. At 46 dpi, two of the 22 genes – Anp32a and Grn – showed significant increases in expression in the spleens of scrapie-infected mice compared to mock-infected mice (Table 5). These two genes also showed significantly increased expression at the later time points, 104 and 144 dpi. At 104 dpi, in addition to Anp32a and Grn, 12 other genes showed significantly increased expression relative to mock-infected mice. These genes were Ctsz, HexB, Ctss, Hspa4, Hspa8, Hspa12a, Ifit1, Ifit3, Ifitm3, Cd68, Ifi27, and Olfml3 (Table 5). At the onset of clinical symptoms, at 144 dpi, these 14 genes continued to show significant increased levels of expression, and were joined by H2-T23 (Table 5). The remaining genes, Gh, Stat1, Usp18, Rtp4, Gfap, Trem2, and Atp1b1, were expressed in the spleen, but none showed significantly altered expression at any time point (Table 5). Table 5 shows the results from averaging the expression of all three scrapie strains relative to mock-infected mice. Appendix 3 shows results obtained for each strain individually. These results demonstrate that prior to the onset of clinical symptoms, scrapie induces multiple alterations in gene expression that occur in both the spleen and brain.

In order to determine if anp32a which showed very early increased expression in spleens of scrapie-infected mice also showed increased protein accumulation in the spleen or brain of scrapie-infected mice, we performed western blot analysis using brains and spleens from three mice infected with RML/Chandler strain of scrapie and three mock-infected mice sacrificed at 100 dpi. As shown in ure 4, we found anp32a protein accumulated 3.7 times more in spleens from scrapie-infected mice compared to mock-infected mice ($p = 0.03$). In the brain, anp32a showed 1.5 times more anp32a protein in scrapie-infected compared to mock-infected mice, but this trend was not significant ($p = 0.1$).

In order to localize anp32a protein expression within the spleen of scrapie-infected mice, we stained spleen sections

Table 5 Spleen gene expression in scrapie-infected vs mock-infected mice at 46, 104, and 144 dpi

		46dpi		104dpi		146dpi	
Symbol	**Unigene No**	**AVE. FC**	**p-value**	**AVE. FC**	**p-value**	**AVE. FC**	**p-value**
Grn	Mm.1568	2.2	0.03	2.5	0.009	1.7	0.027
Anp32a	Mm.269088	2.2	0.011	1.7	0.037	2.4	0.03
Hspa8	Mm.290774	1.9	0.382	2.6	0.028	1.7	0.029
Olfml3	Mm.211535	1.6	0.396	2.5	0.011	2.3	0.039
Ifit1	Mm.6718	1.9	0.37	2.1	0.045	1.9	0.019
Ifit3	Mm.271850	2.7	0.196	2.1	0.004	1.8	0.039
Hspa12a	Mm.39739	1.5	0.343	2	0.042	2	0.016
Ifitm3	Mm.141021	1.6	0.18	2	0.015	2	0.01
Cd68	Mm.15819	1.6	0.158	1.9	0.021	2	0.023
Ifi27	Mm.271275	1.6	0.419	1.7	0.029	1.7	0.029
Hspa4	Mm.239865	2.3	0.109	1.6	0.024	1.8	0.021
Ctsz	Mm.156919	2.5	0.091	1.6	0.027	1.9	0.024
HexB	Mm.27816	1.6	0.405	1.6	0.016	1.8	0.018
Ctss	Mm.3619	2.1	0.175	1.6	0.039	1.7	0.024
H2-T23	Mm.35016	1.8	0.036	1.2	0.303	1.8	0.036
Gh	Mm.343934	1.4	0.262	1.3	0.206	2	0.061
Stat1	Mm.277406	1.4	0.126	1.5	0.28	1.4	0.262
Usp18	Mm.326911	1.2	0.348	1.9	0.159	1.4	0.126
Rtp4	Mm.390891	1.1	0.832	1	0.442	1.2	0.348
Gfap	Mm.1239	1.1	0.362	1.5	0.28	1.1	0.832
Trem2	Mm.261623	−1.3	0.211	1.4	0.293	1.1	0.362
Atp1b1	Mm.009721	1.1	0.702	−1.1	0.293	−1.3	0.211

Notes: Statistically significant expression alterations are highlighted in bold. All genes presented here showed expression in the spleen in this study dpi is days post infection. AVE. FC is the average expression of scrapie samples/mock samples.

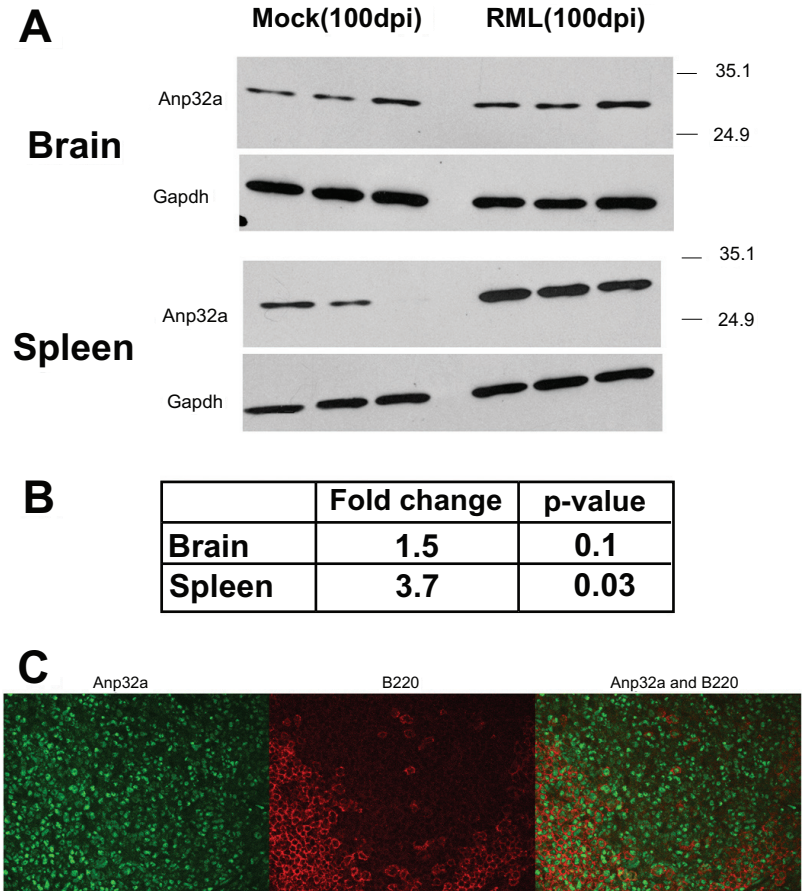
from RML/Chandler scrapie-infected mice with antibodies directed against anp32a and the B cell marker B220. The results showed that anp32a was expressed in B cells as well as other cell types (Figure 4C)

Discussion

These studies shed light on the complex molecular events that occur during prion disease and identify genes whose further study may yield new insights into TSE pathogenesis and transmission. We identified 67 genes that show altered expression in the brain and 15 genes that show altered expression in both the spleen and brain of scrapie-infected mice. All of the scrapie-associated alterations in gene expression in the brain, and 14 out of 15 in the spleen, occurred prior to the development of clinical symptoms and therefore may lead to the development of ante-mortem tests for TSEs.

Fourteen genes showed increased expression in the spleens of scrapie-infected mice prior to the onset of clinical symptoms. These same genes showed increased expression in the brains of scrapie infected mice as presented here or in our previous studies (Skinner et al 2006). Because these increases in gene expression occur in both the brain and the spleen prior to the onset of disease, and because prion infectivity and PrP^{sc} accumulates in both the spleen and brain of scrapie infected mice, these genes may be important contributors to the mechanism by which prion proteins misfold and accumulate and to the dissemination of TSE infectivity.

Importantly, all seven of the genes evaluated in the spleen that function in protein folding or in the endosome/lysosome system showed increased expression in scrapie-infected mice relative to mock-infected mice. These genes were Hspa8, Hspa4, Hspa12a, Cd68, Ctsz, Ctss, and HexB. These results support our hypothesis that genes that function in protein folding or in the endosome/lysosome system contribute to prion disease pathogenesis and are important cofactors in the misfolding and accumulation if prion proteins. Most of the genes involved in immunity also showed alterations in expression in the spleens of scrapie-infected mice. These genes were Grn, Ifit1, Ifit3, fitm3, Ifi27, and H2-T23. In addition, Anp32a, which functions in signal transduction, and



B

	Fold change	p-value
Brain	1.5	0.1
Spleen	3.7	0.03

Figure 4 Anp32a protein accumulation in brain and spleen of scrapie-and mock-infected mice. **A**) Western blot results from three mock and three scrapie-infected mice at 100 days post-infection stained with anp32a antibodies, and stripped and reprobed with Gapdh antibodies. **B**) Densitometry analysis of western blot results showing relative amounts of anp32a accumulation in scrapie verses mock-infected mice. **C**) Anp32a (green) and B220 (red) antibody staining in spleen tissue from scrapie-infected mouse. **Note:** B220 is a B cell marker.

Olfml3, a gene of unknown function, were also found to show increased expression in spleens of scrapie-infected compared to mock-infected mice. Gene alterations in the spleen may prove useful as markers for the preclinical diagnosis of prion diseases. It will be particularly important to identify which cell types alterations occur in and whether these alterations also occur in cells in the blood.

Some genes differ with regard to the time post-infection at which altered expression was first observed in the spleen compared to brain. Grn and Anp32a showed increased expression in the spleen at 46 dpi, but did not show altered expression in the brain until 104 dpi. Gh showed increased expression, and Atb1b1 showed decreased expression in the brains of scrapie-infected mice at 46, 104, and 144 dpi, but neither showed significant alterations in expression at any of these time points in the spleen. Olfml3 and Ifit1 showed altered expression at 104 dpi in both brain and spleen tissues. These similarities and differences in the time course of alterations between the spleen and brain tissues might be attributed to similar and different pathological changes that are occurring in each of these tissue compartments during the course of disease.

It is unclear why there was a delay in detection upregulated genes in our qRT-PCR study relative to our microarray study, but may be due to differences in the sex and stains of mice used in the studies. A set of male C57BL/10 mice was used for the microarray studies, and a different set of female C57BL/6 mice was used for the qRT-PCR studies. Differences may also be due to small sample numbers. Importantly, because our qRT-PCR results did show that 11 of 12 genes examined showed significant increased expression at 146 dpi, these results confirm that these 11 genes show scrapie-associated expression alterations and validate the microarray results.

Very little is known about the molecular changes that take place very early after TSE infection. Four genes in this study showed altered expression at the very early time point, 46 dpi. In the brain, we observed significantly decreased expression

of ATPase Na+/K+ transporting beta 1 polypeptide (Atp1b1) and increased expression in Gh. In the spleen acidic leucine-rich nuclear phosphoprotein 32 family member A (Anp32a) and granulin (Grn) showed significant increased expression. Atb1b1 is an intrinsic membrane protein that functions in the active transport of Na+ and K+ ions in most animal cells, is important for transmission of action potentials in neurons, and also is associated with caveolae and intracellular signal transduction events (Kaplan 2002; Liu and Askari 2006). Gh, in addition to stimulating growth, also functions in an autocrine and paracrine fashion in reproduction processes, immunity, tooth development, retinal development, and placental function (Harvey et al 2003; Harvey and Hull 2003). Anp32a is thought to function in signal transduction and interacts with the cytoskeleton (Opal et al 2003; Matilla and Radrizzani 2005), and has been implicated in other neurodegenerative diseases. For example, its expression is decreased in spinocerebellar ataxia type 3 (Evert et al 2003), and it binds to and colocalizes with mutant ataxin-1, the protein that causes spinocerebellar ataxia type 1 (Matilla et al 1997). Full length Grn gets processed into a variety of 6 kDa peptides and full length protein and peptides are important for, regulation of cell growth, normal development, wound healing, and tumorigenesis. Interestingly, mutations in Grn have recently been discovered to cause frontotemporal dementia linked to chromosome 17 (FTD-17) (Baker et al 2006; Cruts et al 2006; Mukherjee et al 2006). Additional studies are needed to expand our understanding of the contributions to TSE disease pathogenesis and progression of Gh, Atp1b1, Anp32a and Grn, as well as the other scrapie-induced alterations in gene expression identified in this study that occur later in the progression of the disease. In addition, we evaluated only 22 genes at the very early time-point 46 days post-infection. Additional large scale microarray studies of tissues from scrapie-infected compared to mock-infected mice at very early time-points post infection are warranted.

Our studies also found that the pituitary hormones prolactin (Prl), Gh, and pro-opiomelanocortin-alpha (Pomc1) showed increased expression in the brains of scrapie-infected mice prior to the development of clinical symptoms. Pomc1 encodes a 235 amino acid protein that gets processed to produce at least eight peptides including: adrenocorticotropic hormone (ACTH), lipotropins, endorphins, and melanocyte-stimulating hormones (MSHs). The scrapie-associated upregulation of these genes was not associated with a concurrent increase in the expression in upstream regulators of these hormones, Crh, Gnrh1, Trh, and Ghrh, indicating that some other mechanism is responsible for the upregulation pituitary hormones identified in our study. Previous studies in hamsters showed that scrapie strain 139H, but not strain 263K, highly impacts the pituitary gland, inducing neuropathology including vacuolization, atrophy, cytoplasmic vesicles, and hypertrophy (Ye and Carp 1996). This neuropathology in the pituitary gland is associated with hyperplasia and hypertrophy of islets of Langerhan cells, thyroid glands, adrenal glands, liver, and kidneys (Ye and Carp 1996). In addition, increased plasma levels of Gh, ACTH, and cortisol are evident during natural sheep scrapie (Gayrard et al 2000; Viguie et al 2004). Taken together, these studies indicate that alterations in the expression of pituitary hormones may be responsible for some of the clinical symptoms observed during scrapie infection in mice, hamsters, and sheep.

This study identified alterations in gene expression that occur in three strains of scrapie in mice. Mice infected intracerebrally with the scrapie strains ME7, 22L and RML-Chandler differ in incubation period, in neuropathology, and in disease associated alterations in gene expression (Dickinson 1976; Bruce and Fraser 1991; Kascsak et al 1991; Booth et al 2004a; Skinner et al 2006). Alterations that occur regardless of strain and in multiple TSEs are more likely to play a critical role in TSE pathogenesis and disease progression, whereas alterations in gene expression that do not occur in all TSEs or in only some strains are more likely to be associated with strain-specific neuropathological changes.

These studies show that as few as five mice, two mock-infected and three scrapie-infected (with scrapie strain ME7, 22L, and RML-Chandler, respectively), are sufficient to identify alterations in gene expression that occur during the course of scrapie infection in C57BL mice. Our analysis identified 67 gene expression alterations in scrapie-infected mice relative to mock-infected mice. These alterations were verified by the fact that 1) most of the genes identified in our study have been described by us or others in related prion disease studies, 2) the novel gene alterations described here were confirmed in the brain a separate set of scrapie-infected mice using qRT-PCR, and 3) several of these genes were also found to show significant increased expression in spleen tissues from scrapie-infected mice relative to mock-infected mice. Thus, Affymetrix microarray studies using only five mice can prove useful for the identification of gene expression alterations in scrapie-infected mice. At the same time, the small sample size with the microarray study likely limited the total amount of gene alterations identified. Additional studies with increased numbers of mice may yield more

genes that show significant scrapie-associated changes in gene expression.

Although many of the genes identified in this study showed relatively large (greater than two-fold) changes in expression relative to mock-infected mice, many other genes showed relatively low (less than two-fold) changes. Relatively low-fold changes in gene expression may lead to substantial effects in the brain that are important to prion disease pathogenesis. Also, because our studies used RNA from total brain tissue including multiple cell types from multiple regions of the brain, we anticipate that gene alterations that occur in a single cell type or only in a particular region of the brain will show a low fold change in this analysis due to dilution by mRNA from surrounding cells or brain regions in which the particular gene alteration is not occurring. Thus, for gene expression analyses involving complex tissues such as brain, low fold changes in gene expression may indicate large fold changes in a subset of cells or cells in a particular region of the tissue and should not be overlooked.

Future studies are needed to determine whether the scrapie-associated alterations identified in mice also occur in other animals and humans with TSEs, and whether the alterations are specific to prion disease. We also need to determine what the range is of normal variation in expression levels among individuals. To our knowledge, the decrease in erythroid differentiation-related factor (EDRF) expression is the only other nonneuronal alteration in gene expression that is associated with prion diseases described to date (Miele et al 2001). Decreased EDRF expression levels were found in spleen, bone marrow, and blood of scrapie-infected rodents, and were also found in tissues from BSE-infected cows and scrapie-infected sheep. As researchers attempted to translate these findings to a diagnostic assay for human TSEs, they discovered that EDRF expression levels in blood are highly variable amongst individual humans, making it difficult to use EDRF expression levels as a marker for disease (Glock et al 2003). We are hopeful that some of the genes identified in this study that show scrapie-associated changes, will show similar disease-associated changes in humans and show low variability in expression between humans and thus be good surrogate markers for the early diagnosis of prion disease in humans and animals.

Conclusions

We identified a set of genes that show altered expression prior to the onset of clinical symptoms in spleen and brain tissues from scrapie-infected mice. These studies take us several steps forward in our understanding of the molecular events underlying scrapie pathogenesis. Some of the genes identified in this study may serve as surrogate markers for prion disease diagnosis and lead to the identification of targets for drug therapies.

Disclosure

HK performed scrapie infections of female mice, sacrificed and dissected mice, processed tissues, performed qRT-PCR analyses and GFAP and Gh immunohistochemistry; GS performed Affymetrix microarray analyses and helped with scrapie infections; TB performed Anp32a immunohistochemistry; BC provided RNA from male scrapie-infected mice, and helped with manuscript preparation; RR did the scrapie infections and extracted the RNA from male mice used for the microarray studies; and PS was the project leader, oversaw experimentation, and drafted this manuscript.

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Appendix 1 Brain gene expression in mice infected with ME7, 22L, and RML-Chandler strains of scrapie vs. mock-infected mice using qRT-PCR

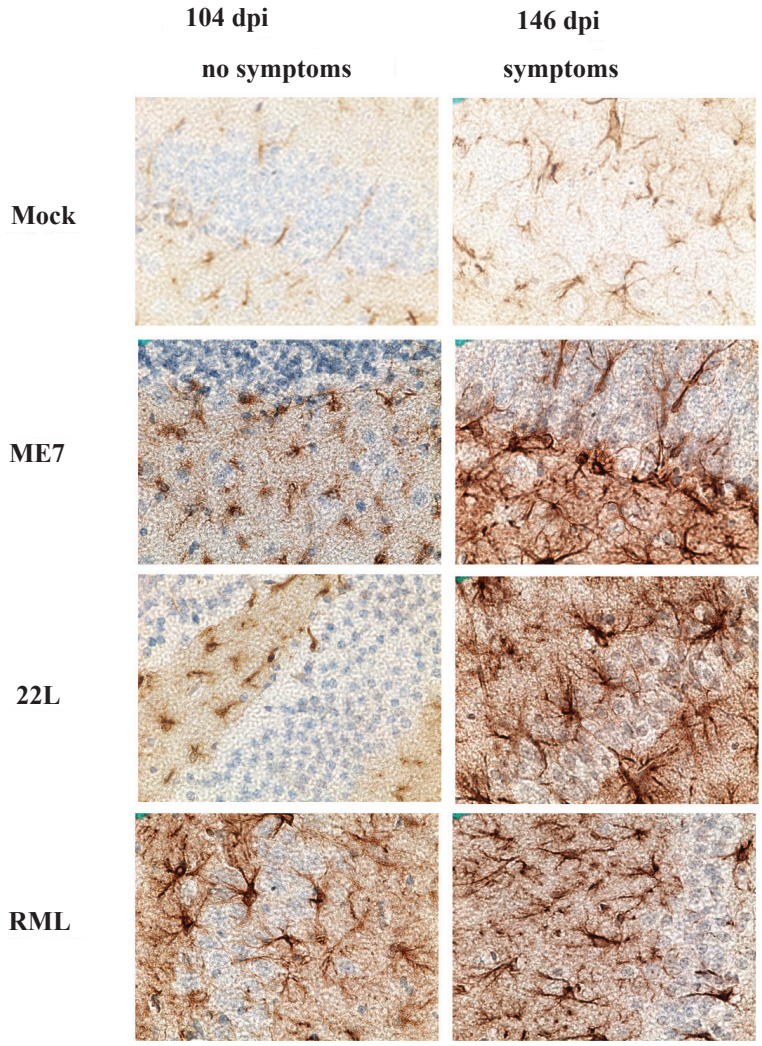
D.P.I.	Gene symbol	Unigene	RT-PCR fold change, p-value, and standard deviation											
			ME7	p-value	SD	RML	p-value	SD	22L	p-value	SD	AVE.	p-value	SD
46	Gh	Mm.343934	1.5	0.01	0.264	1.3	0.017	0.324	1.6	0.008	0.271	1.5	0.012	0.286
	Atp1b1	Mm.4550	0.7	0.045	0.115	0.7	0.003	0.246	0.7	0.033	0.121	0.7	0.027	0.164
	Gfap	Mm.1239	1.2	0.142	0.115	1.2	0.153	0.074	1.2	0.119	0.042	1.2	0.138	0.077
	Usp18	Mm.326911	1.3	0.047	0.343	1.2	0.289	0.455	1.1	0.402	0.336	1.2	0.246	0.378
	Ifit3	Mm.271850	1.1	0.366	0.321	1.2	0.198	0.433	1.1	0.494	0.344	1.1	0.353	0.366
	Ifi27	Mm.271275	1.1	0.374	0.281	1.1	0.411	0.334	1.2	0.284	0.321	1.1	0.356	0.312
	Pomc1	Mm.277996	1.2	0.24	0.164	1.1	0.46	0.243	1	0.436	0.343	1.1	0.379	0.250
	Ifit1	Mm.6718	1.5	0.262	0.417	1	0.452	0.544	1.1	0.444	0.470	1.2	0.386	0.477
	Olfml3	Mm.211535	1	0.496	0.457	1	0.436	0.392	1	0.444	0.363	1	0.459	0.404
	Prl	Mm.1270	1.4	0.657	0.464	1.4	0.296	0.430	1.3	0.572	0.544	1.4	0.508	0.479
	Rtp4	Mm.390891	1.2	0.52	0.436	1.6	0.641	0.473	1.1	0.557	0.445	1.3	0.573	0.451
	Trem2	Mm.261623	1.4	0.066	0.281	2.2	0.106	0.458	1.3	0.222	0.234	1.6	0.131	0.324
	Grn	Mm.1568	1.3	0.226	0.244	1	0.499	0.278	1	0.461	0.303	1.1	0.395	0.275
	Ifitm3	Mm.141021	1.2	0.188	0.306	1.3	0.429	0.425	1.2	0.236	0.314	1.2	0.284	0.348
	H2-T23	Mm.35016	1.1	0.667	0.316	1.3	0.569	0.347	1.1	0.773	0.450	1.2	0.67	0.371
	Stat1	Mm.277406	1.4	0.305	0.249	1.1	0.69	0.201	1.3	0.599	0.252	1.3	0.531	0.234
	Cga	Mm.1361	1.3	0.786	0.343	1.4	0.737	0.219	1.4	0.715	0.255	1.4	0.746	0.272
	Anp32a	Mm.269088	1.6	0.476	0.268	1.4	0.28	0.342	1.4	0.085	0.320	1.5	0.28	0.310
	Crh	Mm.290689	0.9	0.786	0.173	1.3	0.373	0.238	1.3	0.588	0.211	1.2	0.582	0.207
	Trh	Mm.1363	0.9	0.585	0.324	1.1	0.464	0.207	1.2	0.572	0.308	1	0.541	0.280
	Lhb	Mm.57061	1.2	0.366	0.157	1.5	0.572	0.423	1.2	0.355	0.158	1.3	0.431	0.246
	Gnrh1	Mm.358309	1.6	0.514	0.788	1.3	0.436	0.478	1.5	0.586	0.765	1.4	0.512	0.677
	Fshb	Mm.249525	1	0.488	0.198	1	0.492	0.352	1	0.48	0.489	1	0.486	0.347
	Ghrh	Mn.144157	1.4	0.443	0.393	1.6	0.27	0.440	1.6	0.468	0.285	1.5	0.394	0.373
	Tshb	Mm.110730	1.5	0.792	0.292	1.2	0.84	0.465	1.3	0.801	0.423	1.3	0.813	0.393
	Actb	Mm.391967	1.2	0.658	0.256	1.2	0.714	0.252	1.1	0.776	0.233	1.2	0.716	0.247
104	Gh	Mm.343934	14.7	0	0.320	14.4	0.007	0.304	13	0.037	0.095	14	0.015	0.031
	Atp1b1	Mm.4550	0.9	0.013	0.000	0.8	0.011	0.262	0.6	0.013	0.147	0.8	0.013	0.136
	Gfap	Mm.1239	2.4	0.002	0.318	2.3	0.02	0.318	2.6	0.035	0.286	2.4	0.019	0.281
	Usp18	Mm.326911	2.8	0.005	0.075	2.9	0.037	0.156	3.5	0.007	0.257	3.1	0.017	0.027
	Ifit3	Mm.271850	5.5	0.05	0.275	1.8	0.05	0.217	2.7	0.048	0.303	3.3	0.05	0.158
	Ifi27	Mm.271275	2.6	0.012	0.217	1.6	0.479	0.184	3.1	0.051	0.163	2.7	0.036	0.188
	Pomc1	Mm.277996	4.7	0.031	0.360	5.2	0.025	0.283	5.4	0.035	0.266	5.1	0.03	0.303
	Ifit1	Mm.6718	2.4	0.012	0.399	1.9	0.048	0.344	1.6	0.025	0.354	2.2	0.028	0.365
	Olfml3	Mm.211535	1.8	0.03	0.137	2.9	0.038	0.177	3.5	0.012	0.219	2.8	0.027	0.178
	Prl	Mm.1270	13	0.013	0.264	14.5	0.004	0.248	13.1	0.004	0.293	13.5	0.007	0.268
	Rtp4	Mm.390891	1.1	0.493	0.243	0.9	0.479	0.344	1.6	0.276	0.190	1.2	0.416	0.366
	Trem2	Mm.261623	1.2	0.333	0.194	1.3	0.299	0.177	1.3	0.05	0.421	1.3	0.227	0.264
	Grn	Mm.1568	0.9	0.484	0.440	1.1	0.338	0.243	1.1	0.372	0.243	1	0.398	0.308
	Ifitm3	Mm.141021	1.2	0.34	0.186	1	0.391	0.093	1.2	0.3	0.093	1.2	0.343	0.124
	H2-T23	Mm.35016	1.1	0.274	0.238	1.1	0.376	0.118	1.5	0.205	0.289	1.2	0.285	0.215
	Stat1	Mm.277406	1	0.452	0.246	1.2	0.247	0.381	1.4	0.125	0.377	1.2	0.275	0.280
	Cga	Mm.1361	1	0.438	0.115	1	0.258	0.140	1.1	0.434	0.201	1	0.377	0.152
	Anp32a	Mm.269088	1.3	0.015	0.185	1	0.434	0.114	1.3	0.296	0.382	1.2	0.292	0.227
	Crh	Mm.290689	0.8	0.465	0.343	0.7	0.072	0.197	0.8	0.103	0.160	0.7	0.213	0.233

(*Continued*)

Appendix 1 (*Continued*)

D.P.I.	Gene symbol	Unigene	RT-PCR fold change, p-value, and standard deviation											
			ME7	p-value	SD	RML	p-value	SD	22L	p-value	SD	AVE.	p-value	SD
	Trh	Mm.1363	0.8	0.4	0.168	0.8	0.292	0.276	0.7	0.129	0.247	0.8	0.274	0.230
	Lhb	Mm.57061	1.1	0.239	0.228	1.5	0.222	0.179	1.7	0.233	0.421	1.4	0.232	0.276
	Gnrh1	Mm.358309	1	0.473	0.543	1	0.388	0.287	1	0.212	0.192	1	0.358	0.312
	Fshb	Mm.249525	1	0.338	0.374	1	0.284	0.223	1	0.284	0.312	1	0.302	0.303
	Ghrh	Mn.144157	1.4	0.141	0.200	1.2	0.242	0.125	1.4	0.425	0.386	1.3	0.27	0.237
	Tshb	Mm.110730	1.9	0.117	0.276	1	0.103	0.353	1.9	0.196	0.430	1.6	0.138	0.357
	Actb	Mm.391967	1	0.494	0.281	1.1	0.477	0.197	1.1	0.451	0.112	1.1	0.474	0.197
146	Gh	Mm.343934	14.8	0.009	0.173	13.7	0.029	0.251	13.3	0.005	0.131	13.9	0.014	0.185
	Atp1b1	Mm.4550	0.7	0.05	0.141	0.5	0.027	0.100	0.7	0.033	0.327	0.6	0.037	0.189
	Gfap	Mm.1239	9.3	0	0.140	6.4	0.018	0.169	14.5	0.001	0.162	10.1	0.006	0.157
	Usp18	Mm.326911	4.8	0.03	0.268	3.2	0.04	0.253	5.2	0.05	0.122	4.4	0.04	0.215
	Ifit3	Mm.271850	5.9	0	0.380	3.6	0.014	0.178	5.8	0.002	0.277	5.1	0.005	0.278
	Ifi27	Mm.271275	2.8	0.024	0.142	2.8	0.034	0.149	3.3	0.004	0.195	2.9	0.021	0.162
	Pomc1	Mm.277996	6.8	0	0.145	3.7	0.009	0.353	5.4	0.036	0.188	5.3	0.015	0.229
	Ifit1	Mm.6718	5.2	0	0.319	3.7	0.034	0.192	5.8	0.024	0.142	4.9	0.019	0.218
	Olfml3	Mm.211535	2.7	0.012	0.256	2.3	0.042	0.171	4.4	0.001	0.387	3.1	0.018	0.271
	Prl	Mm.1270	17.1	0	0.424	11.6	0.014	0.129	15.8	0.027	0.234	14.8	0.014	0.262
	Rtp4	Mm.390891	5.2	0.002	0.123	5.2	0.005	0.155	6	0.014	0.256	5.4	0.007	0.178
	Trem2	Mm.261623	4.8	0	0.174	3.4	0.027	0.258	4.6	0.01	0.270	4.3	0.012	0.234
	Grn	Mm.1568	2.9	0.007	0.250	2.5	0.047	0.298	3.8	0.002	0.180	3.1	0.019	0.243
	Ifitm3	Mm.141021	3.3	0.005	0.195	2.1	0.019	0.166	3.7	0.019	0.155	3	0.014	0.172
	H2-T23	Mm.35016	2.1	0.018	0.148	1.9	0.042	0.175	3.2	0.006	0.112	2.4	0.022	0.145
	Stat1	Mm.277406	2	0	0.033	1.8	0.001	0.044	3.3	0.028	0.049	2.4	0.01	0.042
	Cga	Mm.1361	2.8	0.069	0.596	1.6	0.281	0.421	1.6	0.157	0.308	1.5	0.169	0.442
	Anp32a	Mm.269088	1.2	0.259	0.110	1	0.483	0.157	1.8	0.039	0.147	1.3	0.26	0.138
	Crh	Mm.290689	3.4	0.023	0.202	2	0.037	0.141	3	0.017	0.133	2.8	0.026	0.159
	Trh	Mm.1363	2.8	0.081	0.264	1.4	0.335	0.160	3.9	0.055	0.293	2.7	0.157	0.239
	Lhb	Mm.57061	1.7	0.005	0.468	1	0.437	0.367	1.7	0.107	0.598	1.5	0.183	0.478
	Gnrh1	Mm.358309	1.6	0.113	0.027	1	0.46	0.159	1.5	0.134	0.313	1.4	0.236	0.166
	Fshb	Mm.249525	1.1	0.45	0.489	1	0.482	0.160	1.1	0.427	0.126	1.1	0.453	0.258
	Ghrh	Mn.144157	1.1	0.01	0.291	1	0.133	0.345	1.1	0.281	0.160	1.1	0.106	0.266
	Tshb	Mm.110730	1.1	0.4	0.334	1	0.497	0.668	1	0.48	0.492	1	0.459	0.498
	Actb	Mm.391967	1.2	0.406	0.240	1.1	0.844	0.149	1	0.808	0.241	1.1	0.686	0.210

Notes: Statistically significant expression alterations are highlighted in bold. DPI is the day post-infection. Fold change is average fold change of scrapie vs. mock-infected. Ave. is the average expression of scrapie samples/mock samples. SD is the standard deviation.



Appendix 2 GFAP immunoreactivity in scrapie-infected and mock-infected mice at 104 and 146 dpi. Hippocampus region of the brain from sections from ME7, 22L, and RML-Chandler-infected mice stained with GFAP antibodies (brown) and counterstained with hematoxylin (blue). Note the increase in GFAP immunoreactivity in the scrapie compared to mock-infected mice.

Appendix 3 Spleen gene expression in mice infected with ME7, 22L, and RML-Chandler strains of scrapie vs. mock-infected mice using qRT-PCR

D.P.I.	Gene symbol	Unigene	qRT-PCR fold change, p-value, and standard deviation											
			ME7	p-value	SD	RML	p-value	SD	22L	p-value	SD	AVE.	p-value	SD
46	Anp32a	Mm.269088	2.1	0.015	0.263	2.4	0.001	0.327	2.0	0.018	0.279	2.2	0.011	0.290
	Grn	Mm.1568	2.2	0.045	0.056	2.3	0.004	0.133	2.1	0.043	0.252	2.2	0.030	0.147
	Ctsz	Mm.156919	2.4	0.050	0.280	2.3	0.085	0.357	2.7	0.138	0.257	2.5	0.091	0.298
	Hspa4	Mm.239865	2.5	0.066	0.433	2.4	0.202	0.268	1.8	0.059	0.216	2.3	0.109	0.306
	Cd68	Mm.15819	1.6	0.103	0.242	1.7	0.135	0.283	1.5	0.237	0.282	1.6	0.158	0.269
	Ctss	Mm.3619	2.1	0.157	0.107	2.2	0.242	0.148	1.9	0.127	0.170	2.1	0.175	0.142
	Ifitm3	Mm.141021	1.6	0.172	0.435	1.9	0.182	0.365	1.5	0.187	0.357	1.6	0.180	0.329
	Ifit3	Mm.271850	3.0	0.021	0.341	2.9	0.288	0.139	2.3	0.278	0.176	2.7	0.196	0.219
	Hspa12a	Mm.39739	1.6	0.273	0.271	1.6	0.267	0.230	1.4	0.490	0.223	1.5	0.343	0.242
	Ifit1	Mm.6718	2.4	0.172	0.112	2.1	0.344	0.117	1.2	0.595	0.427	1.9	0.370	0.219
	Hspa8	Mm.290774	2.2	0.171	0.143	1.7	0.529	0.115	1.9	0.447	0.116	1.9	0.382	0.125

(*Continued*)

Appendix 3 (*Continued*)

D.P.I.	Gene symbol	Unigene	qRT-PCR fold change, p-value, and standard deviation											
			ME7	p-value	SD	RML	p-value	SD	22L	p-value	SD	AVE.	p-value	SD
	Olfml3	Mm.211535	1.9	0.183	0.193	1.7	0.438	0.120	1.4	0.567	0.193	1.6	0.396	0.169
	HexB	Mm.27816	1.6	0.184	0.233	1.6	0.557	0.177	1.7	0.473	0.170	1.6	0.405	0.193
	Ifi27	Mm.271275	1.6	0.317	0.209	1.8	0.267	0.270	1.5	0.673	0.320	1.6	0.419	0.266
	Gh	Mm.343934	1.5	0.428	0.095	1.9	0.340	0.158	1.6	0.492	0.161	1.7	0.420	0.138
	H2-T23	Mm.35016	1.6	0.330	0.279	1.5	0.254	0.208	1.6	0.493	0.412	1.5	0.359	0.300
	Stat1	Mm.277406	1.5	0.569	0.107	1.8	0.459	0.154	1.4	0.567	0.087	1.6	0.532	0.116
	Usp18	Mm.326911	1.5	0.341	0.250	1.4	0.515	0.178	1.3	0.567	0.244	1.4	0.474	0.224
	Rtp4	Mm.390891	1.2	0.493	0.130	1.5	0.410	0.382	1.3	0.551	0.341	1.3	0.485	0.284
	Gfap	Mm.1239	1.4	0.474	0.251	1.3	0.352	0.268	1.3	0.543	0.154	1.3	0.456	0.224
	Trem2	Mm.261623	1.9	0.155	0.256	2.0	0.156	0.358	1.4	0.412	0.389	1.8	0.241	0.335
	Atp1b1	Mm.4550	0.9	0.472	0.374	0.7	0.186	0.159	0.7	0.254	0.431	0.8	0.304	0.321
	Actb	Mm.391967	1.2	0.658	0.096	1.2	0.714	0.133	1.1	0.776	0.093	1.2	0.716	0.108
104	Anp32a	Mm.269088	1.8	0.031	0.271	1.6	0.044	0.283	1.6	0.037	0.178	1.7	0.037	0.244
	Grn	Mm.1568	2.6	0.000	0.203	2.0	0.024	0.241	3.0	0.004	0.248	2.5	0.009	0.230
	Ctsz	Mm.156919	1.9	0.037	0.143	1.4	0.037	0.084	1.6	0.007	0.121	1.6	0.027	0.116
	Hspa4	Mm.239865	1.5	0.029	0.146	1.5	0.042	0.151	1.9	0.001	0.281	1.6	0.024	0.192
	Cd68	Mm.15819	1.9	0.038	0.151	1.9	0.011	0.296	2.0	0.016	0.119	1.9	0.021	0.188
	Ctss	Mm.3619	1.5	0.023	0.139	1.7	0.046	0.103	1.6	0.049	0.186	1.6	0.039	0.142
	Ifitm3	Mm.141021	1.7	0.044	0.016	2.0	0.000	0.027	2.2	0.000	0.168	2.0	0.015	0.070
	Ifit3	Mm.271850	1.8	0.005	0.372	2.0	0.002	0.264	2.4	0.004	0.174	2.1	0.004	0.270
	Hspa12a	Mm.39739	2.1	0.043	0.295	1.9	0.041	0.179	2.0	0.041	0.155	2.0	0.042	0.210
	Ifit1	Mm.6718	2.1	0.044	0.223	2.2	0.044	0.253	2.1	0.046	0.260	2.1	0.045	0.245
	Hspa8	Mm.290774	2.4	0.026	0.250	2.2	0.041	0.153	3.0	0.016	0.184	2.6	0.028	0.196
	Olfml3	Mm.211535	2.1	0.003	0.161	2.3	0.029	0.193	3.2	0.001	0.178	2.5	0.011	0.177
	HexB	Mm.27816	1.4	0.025	0.181	1.5	0.012	0.226	1.8	0.012	0.141	1.6	0.016	0.183
	Ifi27	Mm.271275	1.7	0.038	0.253	1.4	0.047	0.195	2.1	0.001	0.219	1.7	0.029	0.222
	Gh	Mm.343934	1.1	0.518	0.394	1.7	0.059	0.362	1.1	0.040	0.454	1.3	0.206	0.403
	H2-T23	Mm.35016	1.2	0.280	0.139	1.1	0.424	0.157	1.4	0.204	0.106	1.2	0.303	0.134
	Stat1	Mm.277406	1.0	0.448	0.101	1.5	0.207	0.123	2.0	0.185	0.431	1.5	0.280	0.218
	Usp18	Mm.326911	2.0	0.165	0.376	1.6	0.265	0.441	2.2	0.046	0.461	1.9	0.159	0.426
	Rtp4	Mm.390891	1.0	0.496	0.033	1.0	0.477	0.011	1.1	0.355	0.129	1.0	0.442	0.057
	Gfap	Mm.1239	1.1	0.437	0.121	1.9	0.178	0.199	1.6	0.226	0.115	1.5	0.280	0.145
	Trem2	Mm.261623	1.5	0.227	0.191	1.2	0.358	0.262	1.6	0.292	0.325	1.4	0.293	0.259
	Atp1b1	Mm.4550	0.9	0.365	0.156	0.7	0.148	0.222	1.1	0.367	0.133	0.9	0.293	0.170
	Actb	Mm.391967	1.1	0.727	0.097	1.0	0.938	0.070	1.1	0.836	0.227	1.1	0.833	0.131
146	Anp32a	Mm.269088	2.2	0.037	0.093	2.3	0.029	0.188	2.7	0.022	0.110	2.4	0.030	0.130
	Grn	Mm.1568	1.3	0.044	0.275	1.8	0.024	0.196	2.0	0.014	0.106	1.7	0.027	0.236
	Ctsz	Mm.156919	1.6	0.041	0.214	2.0	0.011	0.152	2.1	0.021	0.234	1.9	0.024	0.200
	Hspa4	Mm.239865	1.8	0.022	0.147	1.7	0.034	0.152	1.9	0.007	0.179	1.8	0.021	0.159
	Cd68	Mm.15819	2.0	0.014	0.172	1.9	0.038	0.105	2.1	0.018	0.101	2.0	0.023	0.126
	Ctss	Mm.3619	1.5	0.032	0.359	1.7	0.012	0.278	1.7	0.027	0.143	1.7	0.024	0.260
	Ifitm3	Mm.141021	1.9	0.007	0.223	2.2	0.002	0.252	1.9	0.022	0.137	2.0	0.010	0.204
	Ifit3	Mm.271850	1.6	0.037	0.234	1.6	0.032	0.175	2.0	0.048	0.284	1.8	0.039	0.231
	Hspa12a	Mm.39739	2.0	0.010	0.047	2.0	0.022	0.063	2.1	0.017	0.066	2.0	0.016	0.059
	Ifit1	Mm.6718	2.0	0.038	0.140	1.8	0.016	0.065	1.8	0.002	0.065	1.9	0.019	0.082
	Hspa8	Mm.290774	1.6	0.038	0.050	1.6	0.034	0.149	1.8	0.017	0.153	1.7	0.029	0.117

(*Continued*)

Appendix 3 (*Continued*)

D.P.I.	Gene symbol	Unigene	qRT-PCR fold change, p-value, and standard deviation										
			ME7	**p-value**	**SD**	**RML**	**p-value**	**SD**	**22L**	**p-value**	**SD**	**AVE.**	**p-value**	**SD**
	Olfml3	Mm.211535	2.3	0.046	0.260	2.5	0.028	0.145	2.2	0.043	0.000	2.3	0.039	0.135
	HexB	Mm.27816	1.7	0.020	0.183	1.8	0.014	0.244	1.9	0.019	0.190	1.8	0.018	0.206
	Ifi27	Mm.271275	1.5	0.032	0.056	1.7	0.014	0.247	1.8	0.042	0.145	1.7	0.029	0.149
	Gh	Mm.343934	1.9	0.016	0.036	2.2	0.004	0.344	1.9	0.162	0.170	2.0	0.061	0.184
	H2-T23	Mm.35016	1.7	0.041	0.299	1.5	0.028	0.389	2.3	0.039	0.184	1.8	0.036	0.291
	Stat1	Mm.277406	1.5	0.265	0.192	1.2	0.334	0.379	1.5	0.188	0.114	1.4	0.262	0.228
	Usp18	Mm.326911	1.3	0.101	0.247	1.4	0.110	0.102	1.6	0.168	0.239	1.4	0.126	0.196
	Rtp4	Mm.390891	1.1	0.422	0.194	1.1	0.404	0.089	1.5	0.217	0.259	1.2	0.348	0.181
	Gfap	Mm.1239	1.4	0.512	0.193	1.0	0.994	0.123	1.0	0.986	0.200	1.1	0.832	0.172
	Trem2	Mm.261623	1.2	0.246	0.312	1.1	0.350	0.217	1.0	0.491	0.252	1.1	0.362	0.260
	Atp1b1	Mm.4550	0.8	0.242	0.096	0.7	0.059	0.196	0.8	0.331	0.156	0.8	0.211	0.149
	Actb	Mm.391967	1.1	0.785	0.149	1.2	0.557	0.147	1.2	0.764	0.196	1.1	0.702	0.164

Note: Statistically significant expression alterations are highlighted in bold.

Abbreviations: D.P.I., day post-infection; Ave, average expression of scrapie samples relative to mock samples; SD, standard deviation.