Prion 6:3, 282-301; July/August 2012; © 2012 Landes Bioscience

Gene expression alterations in rocky mountain elk infected with chronic wasting disease

Urmila Basu,[1] Luciane M. Almeida,[1,†] Sandor Dudas,[2] Catherine E. Graham,[2] Stefanie Czub,[2] Stephen S. Moore[1,‡] and Le Luo Guan[1,*]

[1]Department of Agricultural, Food & Nutritional Science; University of Alberta; Edmonton, AB Canada; [2]Canadian Food Inspection Agency; National BSE Reference Laboratory; Lethbridge, AB Canada

Current affiliations: [†]Universidade Estadual de Goiás; Ipameri, Brazil; [‡]The University of Queensland; Centre for Animal Science; Queensland Alliance for Agriculture and Food Innovation; St. Lucia, Queensland, Australia.

Keywords: CWD, differentially expressed, microarray, neurological, signaling

Abbreviations: CWD, chronic wasting disease; DE, differentially expressed; PrP, prion protein; TSE, transmissible spongiform encephalopathy

Chronic wasting disease (CWD) is an invariably fatal neurologic disease that naturally infects mule deer, white tailed deer and elk. The understanding of CWD neurodegeneration at a molecular level is very limited. In this study, microarray analysis was performed to determine changes in the gene expression profiles in six different tissues including brain, midbrain, thalamus, spleen, RPLN and tonsil of CWD-infected elk in comparison to non-infected healthy elk, using 24,000 bovine specific oligo probes. In total, 329 genes were found to be differentially expressed (> 2.0-fold) between CWD negative and positive brain tissues, with 132 genes upregulated and 197 genes downregulated. There were 249 DE genes in the spleen (168 up and 81 downregulated), 30 DE genes in the retropharyngeal lymph node (RPLN) (18 up and 12 downregulated), and 55 DE genes in the tonsil (21 up and 34 downregulated). Using Gene Ontology (GO), the DE genes were assigned to functional groups associated with cellular process, biological regulation, metabolic process, and regulation of biological process. For all brain tissues, the highest ranking networks for DE genes identified by Ingenuity Pathway Analysis (IPA) were associated with neurological disease, cell morphology, cellular assembly and organization. Quantitative real-time PCR (qRT-PCR) validated the expression of DE genes primarily involved in different regulatory pathways, including neuronal signaling and synapse function, calcium signaling, apoptosis and cell death and immune cell trafficking and inflammatory response. This is the first study to evaluate altered gene expression in multiple organs including brain from orally infected elk and the results will improve our understanding of CWD neurodegeneration at the molecular level.

Introduction

Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy (TSE) affecting all members of the Cervidae family. Like other prion diseases, CWD is characterized by progressive dementia and/or ataxia.[1,2] CWD has a long incubation period usually lasting from two to four years followed by the appearance of clinical symptoms including weight loss and behavioral changes lasting a few weeks to months.[3] Despite some similarities, CWD differs from ovine scrapie and bovine spongiform encephalopathy (BSE) in that it occurs in two different non-domestic deer and elk, and epidemiologic observations suggest its horizontal transmission within species.[4,5] Although no definitive evidence has been found for higher levels of human TSE disease occurrence in CWD endemic areas where hunting and venison consumption are common,[6] research to define the potential of CWD to infect humans is ongoing.[7] A recent study has shown that cynomolgus macaques, which are evolutionarily close to humans, when exposed either orally or intracerebrally to CWD, did not develop any symptoms after 6 years of observation, although the direct relevance to humans is not definitive.[8] As the prevalence and distribution of CWD increases, there is also the risk that the disease may be transmitted to other species such as cattle, and this may result in increased risks to human health. Given these uncertainties and increasing CWD prevalence in North America, it is crucial to improve our understanding of the pathogenesis mechanisms of CWD disease.

Pathogenesis studies have revealed that disease associated prion proteins (PrP^{cwd} or PrP^{d}) are present in saliva, blood, urine and feces.[9-11] These prions are especially widespread in the tonsil and gut-associated lymphoid tissues, such as the spleen and lymph nodes, well before neuroinvasion, and they can occur

*Correspondence to: Le Luo Guan; Email: lguan@ualberta.ca
Submitted: 01/06/12; Revised: 02/22/12; Accepted: 03/05/12
http://dx.doi.org/10.4161/pri.19915

in the absence of spongiform lesions or clinical signs, spreading to the central nervous system and spinal cord with concurrent distribution in peripheral lymphoid tissues from the early incubation phase until death.[12-14] While physiological changes associated with TSE disease in the brain are well documented, the underlying molecular events involved in neurodegeneration are poorly defined. A number of studies using microarrays have identified global gene expression changes in TSE diseases such as scrapie in mouse and hamster models with identical genetic backgrounds[15-20] and BSE in natural hosts, such as cattle.[21-23] These studies have been useful for providing information about the involvement of multiple genes and identifying novel genes related to neurodegenerative pathway and pathogenesis mechanism including altered synapse function, regulation of apoptosis, stress response and calcium ion homeostasis.[14] However, no report has analyzed gene expression changes associated with CWD, and more gene expression profiling studies are needed in TSE-infected animals with different genetic backgrounds.

Based upon established homology between domestic cow (*Bos taurus*) and white-tailed deer chromosomes,[24] a recent study by massively parallel pyrosequencing of a reduced representation library and a random shotgun library from white-tailed deer and using comparative contig overlay method showed a substantial number of high quality contigs (> 92%) were unambiguously aligned to the bovine genome assembly.[25] In this study, we used microarray analysis to profile gene expression changes occurring in CWD-infected tissues from orally inoculated elk. Because cervine microarray platform is not available due to insufficient cervid sequence data and genomic information, we used cross-species microarray analysis (i.e., elk samples hybridized to a bovine DNA microarray) to identify differentially expressed (DE) genes in different brain (brain, midbrain and thalamus), spleen, tonsil and RPLN tissues of CWD-diseased elk. The DE genes identified in response to CWD infection are important to understand the molecular mechanisms involved in neuropathology and prion replication.

Results

Differentially expressed genes in brain, spleen, RPLN and tonsil tissues from CWD-infected elk. Global gene expression variations were determined in tissues from the brain, midbrain, thalamus, spleen, RPLN and tonsil from control and CWD-infected elk using a bovine microarray representing 16,846 unique gene loci and 5,943 ESTs from the bovine genome. Microarray analysis identified 329 genes (132 upregulated and 197 downregulated) that were DE at least 2.0-fold in the different brain tissues comparing the CWD-negative and the CWD-positive animals (**Table 1**). Among these, 152, 65 and 112 genes were DE in the brainstem, midbrain and thalamus, respectively. **Table 2** shows there were 249 DE genes (168 upregulated and 81 downregulated) in the spleen, 30 DE genes (18 upregulated and 12 downregulated) in the RPLN, and 55 DE genes (21 upregulated and 34 downregulated) in the tonsil.

Functional classification of DE genes. Overall, 122 DE genes from the brain tissues (**Table 1**) and 110 DE genes from the spleen, RPLN and tonsil tissues (**Table 2**) were associated with a function based on Gene Ontology (GO) analysis. The results showing categorization of DE genes from the GO analyses (modified Fisher's exact p value < 0.05) include cellular process, biological regulation, metabolic process, regulation of biological process, multicellular organismal process and development process (**Fig. 1 and Table S1**). According to the GO database, many DE genes fall into more than one category; however, for simplistic presentation, these genes have been presented under a single functional heading.

Table 3 provides a comprehensive list of DE genes for the different brain tissues and the spleen, RPLN and tonsil tissues in CWD-infected elk. While some of these genes have been identified in previous studies of other TSE models (**Table 3**), a number of these genes are being reported for the first time (**Table S4**). Nonetheless, 86 DE genes identified in this study in response to CWD in elk have been identified in previously published studies of various TSE diseases (**Table 3**).

We also used the Ingenuity Pathways Analysis (IPA) to annotate genes according to their functional relationships and to determine potential regulatory networks and pathways. **Table 4** provides the number of DE genes in the different brain tissues and in the spleen, RPLN and tonsil as identified by Genesifter analysis, and the Mapped IDs, network eligible genes and function eligible genes identified by IPA. The representative categories of biological functions/diseases of all of the DE genes in the different tissues using IPA are provided in **Table 5**. While neurological disease, genetic disorder, psychological disorder, skeletal disorders and cancer were the top five categories in both the brain and thalamus under "Diseases and disorders" in brain tissues, endocrine system disorder, metabolic disease, inflammatory response, genetic disease and inflammatory disease were the top functions in midbrain (**Table 5**). Among the DE genes in the brain tissues, cell morphology, cellular assembly and organization, cellular function and maintenance, free radical scavenging, cell signaling and molecular transport were the top molecular and cellular functions; the top physiological system development and function classifications were nervous system, skeletal and muscular system, connective tissue, behavior, tissue development and tissue morphology (**Table 5**).

The top functional categories for the DE genes were as follows: cancer, skeletal and muscular disorders, hematological disease, genetic disorder and reproductive system disease for the spleen tissues; genetic disorder, neurological disease, skeletal and muscular disorders, inflammatory response and renal and urological disease functional categories for RPLN tissues; and inflammatory response, reproductive system disease, infection mechanism, hypersensitivity response and connective tissue disorders for tonsil tissues under diseases and disorders (**Table 5**). Cellular movement, cellular compromise, cellular assembly and organization, cellular function and maintenance, cell cycle and cell death were the top molecular and cellular functions among DE genes for spleen, RPLN and tonsil tissues; the top physiological system development and function categories were immune cell trafficking and endocrine and hematological system development and function (**Table 5**).

Table 1. Gene ontology (GO) analysis of differentially expressed genes with ≥ 2.0-fold change, associated with CWD disease in brain tissues of elk

GO function (biological and molecular processes)	Gene ID	Gene name	BR	MB	TH
Apoptosis	Bt.49637	Superoxide dismutase 1	-3.96	-2.28	-2.01
	Bt.17392	Tax1 binding protein 1	-2.26		-2.21
	Bt.49336	Ras homolog gene family	-3.44		-2.70
	Bt.49674	Voltage-dependent anion channel 1	2.46		
	Bt.5427	Secretogranin II			
	Bt.87330	Scinderin			
Calcium binding and regulation	Bt.61440	Parvalbumin	-2.07		-3.77
	Bt.61911	Multiple coagulation factor deficiency 2	3.24		-2.79
	Bt.65801	Neurocalcin delta	-2.31		
	Bt.49702	Visinin-like			
Nervous system function and synaptic transmission	Bt.12770	Synuclein, α	-2.32	-2.49	-3.30
	Bt.12930	Tachykinin, precursor 1	-2.27	-3.36	-3.05
	Bt.20015	Cocaine and amphetamine responsive transcript	-2.40		-2.01
	Bt.22135	Neurensin 1	-3.74		-3.69
	Bt.30532	Cholecystokinin	-2.16		-2.22
	Bt.35938	Calcyon neuron-specific vesicular protein			-2.01
	Bt.40062	Amyloid β (A4) precursor protein			-2.88
	Bt.5457	Growth associated protein 43			-2.78
	Bt.67878	Neuron specific gene family member 1			-2.50
	Bt.89014	Neurofilament, medium polypeptide			-4.17
	Bt.42529	Synaptosomal-associated protein, 25 kDa			
Cytoskeleton organization	Bt.49700	Allograft inflammatory factor 1	2.03	2.16	-2.36
	Bt.13391	Capping protein muscle Z-line	-2.19		
	Bt.49547	Lymphocyte cytosolic protein 1	2.29		
	Bt.53255	Thy-1 cell surface antigen			
	Bt.6630	Calponin 1, basic, smooth muscle			

BR, brainstem; MB, midbrain; TH, thalamus.

Identification of biologically relevant networks. To investigate the global expression response to CWD and to define the interaction of individual regulated genes in specific pathways, potential networks of interacting DE genes were identified using IPA. All of the potential networks with a score > 9 (a score of 3 or greater is considered significant with $p < 0.001$) from different tissues have been provided in **Table S2**, with information on the network genes, score, the focus genes and top functions associated with the focus genes in each network. For all of the brain tissues, the highest ranked network (**Fig. 2**) identified by IPA was associated with neurological disease, cell morphology, cellular assembly and organization. This network contained genes (**Table S2**) that are primarily involved in apoptosis and cell death (26S proteasome, CDKN1C, MAPK3, MT1F, POLR2A, PRPH, NR4A2, RDX, SCIN, GNCA, SOD1, TAC1, TAX1BP1 and tubulin), signaling (actin, calmodulin, GNAS, SNAP25, SOD1, TAC1 and TAX1BP1), and cell movement, disassembly and differentiation (CAPZA2, ERMN, GDI, NEFH and GPNMB). The highest ranking networks were cancer, cell death and immune cell trafficking for the spleen and infection mechanism for both the RPLN and tonsil tissues (**Table S2**). The top five canonical pathways for all of the brain tissues and the spleen, RPLN and tonsil tissues have been provided in **Table S3**. Most of these pathways were related to signaling ncluding endothelin-signaling, androgen signaling, synaptic long-term depression and CXCR4 signaling for the brain tissues; B cell development, antigen presentation pathway, disease signaling and oxidative stress response for midbrain; and Parkinson signaling, OX40 signaling pathway, acute phase response signaling, IL-6 signaling for thalamus tissues. For the spleen, RPLN and tonsil tissues (**Table S3**), the most prominent pathways included the protein ubiquitination pathway, actin cytoskeleton signaling, aldosterone signaling and IGF-1 signaling.

Quantitative real-time PCR validation of DE genes. Quantitative RT-PCR analyses were performed to validate the differential expression of several genes from different pathways including the following: neuronal signaling and synapse function in neurological disease [α-Synuclein (SNCA), neuropeptide Y (NPY), cocaine and amphetamine responsive transcript (CART), synaptosomal associated protein 25 kDa (SNAP25) and tachykinin 1 (TAC1)]; calcium ion regulation [parvalbumin B (PVALB), visinin-like 1 (VSNL1), angiotensin II receptor

Table 1. Gene ontology (GO) analysis of differentially expressed genes with ≥ 2.0-fold change, associated with CWD disease in brain tissues of elk (continued)

Binding	Bt.13833	RING1 and YY1 binding protein	-2.37	-2.07	-2.00
	Bt.20597	Tetratricopeptide repeat domain 35	-2.40	3.97	-2.48
	Bt.22187	Regulator of chromosome condensation (RCC1) and BTB (POZ) domain	-2.12	2.23	-2.65
	Bt.24896		-2.22	-2.91	-2.63
	Bt.34711	Kinesin-associated protein 3	2.19	-2.26	4.89
	Bt.35816	Leucine rich repeat transmembrane neuronal 1	7.86	2.42	-2.27
	Bt.37226	Neurotrimin	2.01	-2.02	-2.81
	Bt.42457	Fibroblast growth factor 12	-2.17		-2.22
	Bt.42866	Hypothetical protein LOC506058	-2.88		2.93
	Bt.43398	Metallothionein 1E	-2.41		2.04
	Bt.49619	TRIM6-TRIM34	4.14		-2.44
	Bt.49713	Spastic paraplegia 3A	-4.36		-2.19
	Bt.5448	Creatine kinase, mitochondrial 1	-2.24		-2.28
	Bt.60794	Chromogranin B			-4.52
	Bt.62630	Alpha2-macroglobulin			
	Bt.64032	Mal, T-cell differentiation protein 2			
	Bt.6645	Lymphocyte antigen 75			
	Bt.6830	RNA-binding region (RNP1, RRM) containing 3			
	Bt.74143	Septin 7			
	Bt.76429	Ermin, ERM-like protein			
	Bt.76948	Ceruloplasmin (ferroxidase)			
	Bt.87082	FK506 binding protein 1B, 12.6 kDa			
	Bt.91195	GTP-binding protein 8 (putative)			
	Bt.207	Succinate-CoA ligase, ADP-forming,			
	Bt.34807	Transthyretin (prealbumin, amyloidosis)			
		Neurofilament, heavy polypeptide 200 kDa			
Immune and inflammatory response	Bt.1035	Fc fragment of IgG, receptor	3.22	2.01	2.02
	Bt.5356	Similar to MHC class II antigen	2.31		2.96
	Bt.611	Chemokine (C-X-C motif) ligand 2	2.54		-2.03
	Bt.64791	Acyloxyacyl hydrolase (neutrophil)			2.15
	Bt.73288	Complement component 4A			2.56
	Bt.8552	Major histocompatibility II, DR α			
	Bt.88538	Major histocompatibility II, DRB3			
Post-translational protein modification and protein folding	Bt.26656	Mitogen-activated protein kinase 10	-2.57	-2.69	-2.32
	Bt.36328	P21 (CDKN1A)-activated kinase 3		-2.52	-2.04
	Bt.39123	Protein tyrosine phosphatase			-2.43
	Bt.73670	Carboxypeptidase E			-2.12
	Bt.22615	Protein-L-isoaspartate O-methyltransferase			
	Bt.53009	Chaperonin containing TCP1			
Proteolysis	Bt.12553	Haptoglobin	2.14		2.33
	Bt.52393	Cathepsin H			-3.05
	Bt.58848	Ubiquitin carboxyl-terminal esterase L1			

BR, brainstem; MB, midbrain; TH, thalamus.

(AGTRL1)]; apoptosis and cell death [Tax 1 binding protein 1 (Tax1BP1) and superoxide dismutase (SOD1)]; and immune cell trafficking and inflammatory response [(chemokine 20 (CCL20), Serum amyloid A-like (SAA)].

All five genes, i.e., SNCA, NPY, CART, SNAP25 and TAC1, from the neuronal signaling and synapse function in neurological disease category showed reduced expression in both of the positive animals as compared with the control animals (**Fig. 3A**), validating our microarray data. The extent of differential expression varied in the brain, midbrain and thalamus tissues from the two animals; however, the trend remained similar in both animals. Both of the genes associated with apoptosis and cell

Table 1. Gene ontology (GO) analysis of differentially expressed genes with ≥ 2.0-fold change, associated with CWD disease in brain tissues of elk (continued)

Regulation of biological process	Bt.49566	SH3 domain binding glutamic acid-rich protein like 3	2.12		-2.42
	Bt.35977	Radixin	-2.00		-2.17
	Bt.4804	Cyclin-dependent kinase inhibitor 1C	-2.52		2.10
	Bt.67202	Protein tyrosine phosphatase-like A	-2.31		
	Bt.63542	Similar to Chain D, Crystal Structure Of The Adenylyl Cyclase Domain Of Anthrax Edema Factor In Complex With Calmodulin	-2.50		
	Bt.25016		-2.15		
	Bt.25332	Similar to EBF1 protein	-2.31		
	Bt.64131	Nuclear receptor subfamily 4	2.22		
	Bt.52808	RAB2A, member RAS oncogene family	-2.08		
	Bt.64620	TAF9 RNA polymerase II	-2.03		
	Bt.4832	Polymerase (RNA) II polypeptide A	2.53		
	Bt.49563	Ribosomal protein S15			
	Bt.4185	RNA binding motif protein 3			
	Bt.48705	NHP2 non-histone chromosome protein 2-like 1			
	Bt.48894	GINS complex subunit 4			
		Protein S (α)			
Signal transduction	Bt.3704	Stathmin-like 2	-2.50	-2.46	-2.77
	Bt.53744	Membrane-spanning 4-domains	-2.13	-2.09	-2.44
	Bt.53955	Chimerin (chimaerin) 1	-2.14		
	Bt.5546	Guanine nucleotide binding protein	-4.00		
	Bt.62010	Syndecan binding protein			
	Bt.64619	GNAS complex locus			
Transport	Bt.2235	GDP dissociation inhibitor 1	2.60	-2.16	-2.05
	Bt.26950	Solute carrier family 39	2.01	3.40	5.16
	Bt.4482	Macrophage scavenger receptor 1	-2.35	2.26	-2.40
	Bt.46431	K_v channel interacting protein 4			-2.30
	Bt.46903	Potassium inwardly-rectifying channel			-2.79
	Bt.48572	Guanine nucleotide binding protein			-2.09
	Bt.49570	ATPase, Na^+/K^+ transporting			-2.03
	Bt.52307	ATPase, H^+ transporting, lysosomal 42 kDa			-2.61
	Bt.61189	ATP synthase, H^+ transporting, mitochondrial F0 complex			-3.24
	Bt.62049	Sodium channel, voltage-gated			
	Bt.87586	Similar to Kinesin-like protein KIF3A			
	Bt.88575	Tubulin, β 2B			
	Bt.14573	ELMO/CED-12 domain containing 1			
Metabolic process	Bt.97115	Stearoyl-CoA desaturase	-2.69	-2.11	-2.15
	Bt.20383	Microsomal glutathione S-transferase 1	-2.41		
	Bt.60921	Citrate synthase			
	Bt.87389	Glyceraldehyde-3-phosphate dehydrogenase			
Glycolysis	Bt.15319	Phosphoglycerate mutase 1 (brain)		-2.36	-2.15
	Bt.5345	Malate dehydrogenase 1, NAD			-2.48
Cell adhesion	Bt.9656	Collagen, type XI, α1	-2.36		
	Bt.9807	Glycoprotein (transmembrane) nmb	2.71		
Hydrolase activity	Bt.49237	Transmembrane protein 55[a]			-2.04

BR, brainstem; MB, midbrain; TH, thalamus.

death, Tax1BP1 and SOD1, and three of the genes involved in calcium ion regulation, PVALB, VSNL1 and AGTRL1, showed decreased expression in all brain tissues from both animals (**Fig. 3A**). A higher CCL20 expression was observed in the elk-infected spleen, RPLN and tonsil tissues (**Fig. 3B**). We observed some inconsistency in SAA expression, which was increased in

Table 2. Gene ontology (GO) analysis of differentially expressed genes with ≥ 2.0-fold change, associated with CWD disease in spleen (SP), retropharyngeal lymph nodes (RPLN) and tonsil (TO) tissues of elk

GO function (biological and molecular processes)	Gene ID	Gene name	SP	RPLN	TO
Apoptosis and cell cycle	Bt.64777	Baculoviral IAP repeat-containing 3	2.03		-2.02
	Bt.64827	Cadherin 1, E-cadherin (epithelial)	2.72		
	Bt.2408	Chemokine (C-C motif) ligand 2	2.10		
	Bt.13573	CDC28 protein kinase regulatory subunit 2	2.38		
	Bt.15980	Cyclin B1			
	Bt.4539	Angiotensin II receptor, type 1	-2.34		-4.86
	Bt.34390	Cysteine and glycine-rich protein	2.46		-2.12
	Bt.4922	Myosin, light chain 1, alkali	2.11		-5.63
	Bt.6324	Myosin, light chain 9, regulatory	-3.79		
	Bt.357	S100 calcium binding protein A12 (calgranulin C)	2.69		
	Bt.76113	S100 calcium binding protein A14			
	Bt.87249	S100 calcium binding protein A9			
Calcium binding and regulation	Bt.66812	Sarcoglycan, β	-2.28		
	Bt.59155	Cytokeratin 19	2.21		
	Bt.15705	Destrin (actin depolymerizing factor)	-3.47		
	Bt.97	Fatty acid binding protein 4, adipocyte	-5.11		
	Bt.4057	Myosin, heavy chain 10, non-muscle	-2.15		
	Bt.120	Rho-associated, coiled-coil containing protein kinase 2	-2.67		
Cytoskeleton organization and cytokinesis	Bt.59726	Caldesmon 1	-5.00		-5.83
	Bt.16055	Transgelin	-3.00		-2.57
	Bt.9714	Actin, gamma2, smooth muscle, enteric	-3.81		-2.76
	Bt.575	Common salivary protein BSP30 form a	-3.91		
	Bt.6141	Desmin	2.02		
	Bt.45294	Elongation factor RNA polymerase II	-2.17		
	Bt.89332	Fibroblast growth factor-binding protein	2.77		
	Bt.45158	Filamin A, α	2.50		
	Bt.62630	Mal, T-cell differentiation protein 2	-2.25		
	Bt.4703	Myocilin	-3.45		
	Bt.33253	Myotilin	2.53		
	Bt.64757	Neurotrophic tyrosine kinase, receptor	-2.84		
	Bt.8880	SPARC-like 1 (mast9, hevin)	-3.37		
	Bt.49324	Stress-induced-phosphoprotein 1 (Hsp70/Hsp90-organizing protein)	2.02		
	Bt.74664	Tensin 3	2.75		
	Bt.53077	Tropomyosin 2 (β)			
	Bt.97350	Troponin C type 2 (fast)			
	Bt.8897	Von Ebner minor salivary gland protein			
	Bt.41406	Zinc finger protein 618			

spleen, RPLN and tonsil tissues from one of the infected elk and only in RPLN in tissue from the other animal. A reduced expression was observed for AGTRL1 in the spleen, RPLN and tonsil tissues (**Fig. 3B**).

Discussion

Due to the increasing prevalence of CWD and the potential risk of its transmission to domestic livestock or humans, it is important to gain an understanding of CWD neurodegeneration at the molecular level. This study is the first comprehensive analysis of DE genes and pathways associated with CWD in elk including qRT-PCR validation of the differential expression of a set of key genes from different pathways from multiple organs of CWD infected elk. Eighty-six DE genes identified in this study in response to CWD in elk have also been identified in studies of other TSE diseases, suggesting the involvement of similar mechanisms, while 92 genes identified only in elk suggesting a host species specific mechanism may also be part of the pathogenesis mechanism.

Table 2. Gene ontology (GO) analysis of differentially expressed genes with ≥ 2.0-fold change, associated with CWD disease in spleen (SP), retropharyngeal lymph nodes (RPLN) and tonsil (TO) tissues of elk (continued)

Immune and inflammatory response	Bt.91780	Cathelicidin 1	-6.61	-2.09	2.70
	Bt.4259	Cathelicidin 2	-9.89		-3.03
	Bt.88495	Cathelicidin 6	-2.98		
	Bt.9560	Chemokine (C-C motif) ligand 20	3.29		
	Bt.26536	Chemokine (C-C motif) ligand 21	2.51		
	Bt.13542	Complement factor B	-2.78		
	Bt.68530	Complement factor H	3.82		
	Bt.49740	Interleukin 8	2.05		
	Bt.89770	Lysozyme (renal amyloidosis)	4.51		
	Bt.29871	Major histocompatibility complex, class II, DY α			
	Bt.62645	MHC class I heavy chain			
	Bt.88760	Serum amyloid A-like			
Protein folding	Bt.88837	DnaJ (Hsp40) homolog, subfamily A, member 1	2.53	2.14	
	Bt.21932	F-box protein 7	2.24		
Proteolysis	Bt.77495	ADAM-like, decysin 1	3.07	3.32	
	Bt.12553	Haptoglobin	4.78		
	Bt.13092	Matrix metallopeptidase 7			
Regulation of transcription	Bt.22888	Nuclear receptor subfamily 3, group C	-3.05		
	Bt.87332	Similar to HMGB2 protein	2.38		
	Bt.45673	Spi-C transcription factor	-7.14		
Signal transduction	Bt.44951	Intersectin 1 (SH3 domain protein)	-2.19	-2.01	2.48
	Bt.53744	IQ motif containing GTPase activating protein 1	-2.38		2.01
	Bt.3081	Membrane-spanning 4-domains	-2.57		
	Bt.21143	Rho GTPase activating protein 29	-5.46		
	Bt.20059	Ribosomal protein S6 kinase, 70 kDa	-2.36		
	Bt.3704	Stathmin-like 2	-2.28		
	Bt.6915	CD163 molecule			
	Bt.9632	Deleted in malignant brain tumors 1			
	Bt.61075	Similar to macrophage receptor MARCO			
Transport	Bt.49731	Carbonic anhydrase II	-2.22	2.11	-2.69
	Bt.79147	Growth hormone receptor	-2.14		
	Bt.10591	Hemoglobin α chain	-7.54		
	Bt.45049	Hemoglobin, β	-19.6		
	Bt.4482	Macrophage scavenger receptor 1	-3.81		
	Bt.43781	NECAP endocytosis associated 2	-3.51		
	Bt.14388	Potassium channel tetramerisation domain containing 15	2.05		
	Bt.47454	Potassium voltage-gated channel	-2.00		
	Bt.52764	Proliferating cell nuclear antigen			
	Bt.22651	Solute carrier family 39 (zinc transporter)			

Neuronal signaling and synapse function in neurological disease in elk brain tissues. Synapse loss has been identified as an early and critical pathophysiological event in neurodegenerative diseases.[26] The altered levels of a number of other compounds and neuropeptides that are important for normal neuronal function have also been noted for prion diseases. All DE genes related to the nervous system function and synaptic transmission identified in this study (11 DE genes listed in **Table 6**) showed a reduced expression in infected animals as compared with control animals. Previous studies have shown that decreased CART and SNAP25 expression[20,27] and increased neuropeptide Y (NPY) and α-synuclein gene expression, are associated with prion infection in mice.[28,29] The presynaptic protein α-synuclein has been reported to be involved in Creutzfield-Jacob disease (CJD) and in scrapie-infected hamsters through its role in prion protein accumulation and neurodegeneration in prion diseases.[29] Abnormalities in synaptic plasticity are believed to overlap with the deposition of PrP^{Sc}, which is one of the earliest features of prion disease.[30] Additionally, prion responsive genes in mice have been found to be associated with a number of signaling pathways

Table 2. Gene ontology (GO) analysis of differentially expressed genes with ≥ 2.0-fold change, associated with CWD disease in spleen (SP), retropharyngeal lymph nodes (RPLN) and tonsil (TO) tissues of elk (continued)

Cellular process	Bt.48705	GINS complex subunit 4	2.02	2.28	
	Bt.37052	Hemicentin 1	-2.90	2.49	
	Bt.28966	Hypothetical protein LOC100125947	4.22		
	Bt.67194	Lysozyme 1	2.68		
	Bt.30108	Meiotic nuclear divisions 1 homolog	2.18		
	Bt.37111	Microtubule-associated protein 1B	-2.55		
	Bt.57571	PDZ and LIM domain 3	-2.15		
	Bt.4844	Pleiotrophin	-3.45		
	Bt.42764	Prosaposin	-2.14		
	Bt.61915	Similar to 90-kDa heat shock protein α	2.12		
	Bt.20277	Topoisomerase (DNA) II α 170 kDa	2.22		
	Bt.58848	Ubiquitin carboxyl-terminal esterase L1	-2.15		
	Bt.49598	Cysteine-rich, angiogenic inducer, 61			
Cell adhesion	Bt.63411	Cadherin 18, type 2	-2.03		
	Bt.44183	Fibulin 5	-2.69		
	Bt.9807	Glycoprotein (transmembrane) nmb	-2.92		
	Bt.20373	Neuropilin 1	-2.08		
	Bt.121	Frizzled-related protein	-2.21		
Structure Organization	Bt.6630	Calponin 1, basic, smooth muscle	-4.60		-2.03
	Bt.13391	Capping protein (actin filament) muscle Z-line, α 2			
Response to stimulus	Bt.4001	Heme oxygenase (decycling) 1	-3.81	3.14	
	Bt.51574	Caveolin 1, caveolae protein, 22 kDa	-2.02		
	Bt.8738	Heat shock 105 kDa/110 kDa protein 1	2.10		
	Bt.12309	Heat shock 70 kDa protein 8	2.44		
	Bt.96882	Similar to RIKEN cDNA 1110064P04 gene			
Angiogenese and axonogenesis	Bt.91353	Angiopoietin 1	-2.52	2.10	
	Bt.5240	Connective tissue growth factor	-2.12		
	Bt.40062	Amyloid β (A4) precursor protein (peptidase nexin-II, Alzheimer disease)	2.62		
	Bt.3137	Stathmin 1/oncoprotein 18			
Phosphatase activity	Bt.20846	CTD (C-terminal domain, RNA polymerase II, polypeptide A)	-2.00		

particularly related to synaptic long-term potentiation and calcium signaling.[20] NPY is a neurotransmitter that is known to be associated with a number of physiological processes in the brain and has been shown to be overexpressed in a number of TSE experimental models, including mouse, and in Alzheimer disease.[28,31,32] Another neurotransmitter, CART, which is a peptide that has been implicated in a variety of brain functions including the protection offered by estradiol against ischemic brain injury in stroke and other neurodegenerative diseases,[33] showed differential expression between infected and control brain samples, and its reduced expression was validated by qRT-PCR. Sorensen et al.[20] detected a lower expression of CART in mice with prion infection in microarray studies, which is consistent with our studies.

SNAP25 is a protein that is involved in neurotransmitter vesicle trafficking,[34] and a reduction in SNAP25 and a number of other crucial synaptic proteins have been observed in sporadic CJD,[26] and a lower expression of SNAP25 was found in all elk tissues. The reduced expression of TAC1, another neurotransmitter, was also observed in all brain tissues of CWD-infected animals. However, TAC1 differential expression has not been reported in previous mouse studies.[16,19,20,35] All of the DE genes associated with neuronal signaling may play a role in the loss of function of neurons, and disruption of this system may be important for CWD-induced neurodegeneration in elk, leading to the irreversible pathogenesis.

Apoptosis and cell death in elk brain tissues. There has been strong evidence, based on several studies, that the apoptotic death of neurons is an underlying cause of the spongiform degeneration of the central nervous system in prion diseases.[26] However, whether this programmed cell death is occurring as an effect of PrP^{Sc} accumulation, a loss of functional PrP^{C} or due to effects of the immune response remains unclear. Identification of six DE genes associated with apoptosis in CWD-infected elk, and further validation of a reduced expression of Tax1BP1 and SOD1 indicates the role of these genes in CWD disease in elk. The Tax1BP1 protein has the ability to deregulate the expression of a vast array of cellular genes,[36] and its DE was not reported in previous TSE studies. Among the pathological mechanisms of prion disease, neuroprotective and anti-apoptotic functions

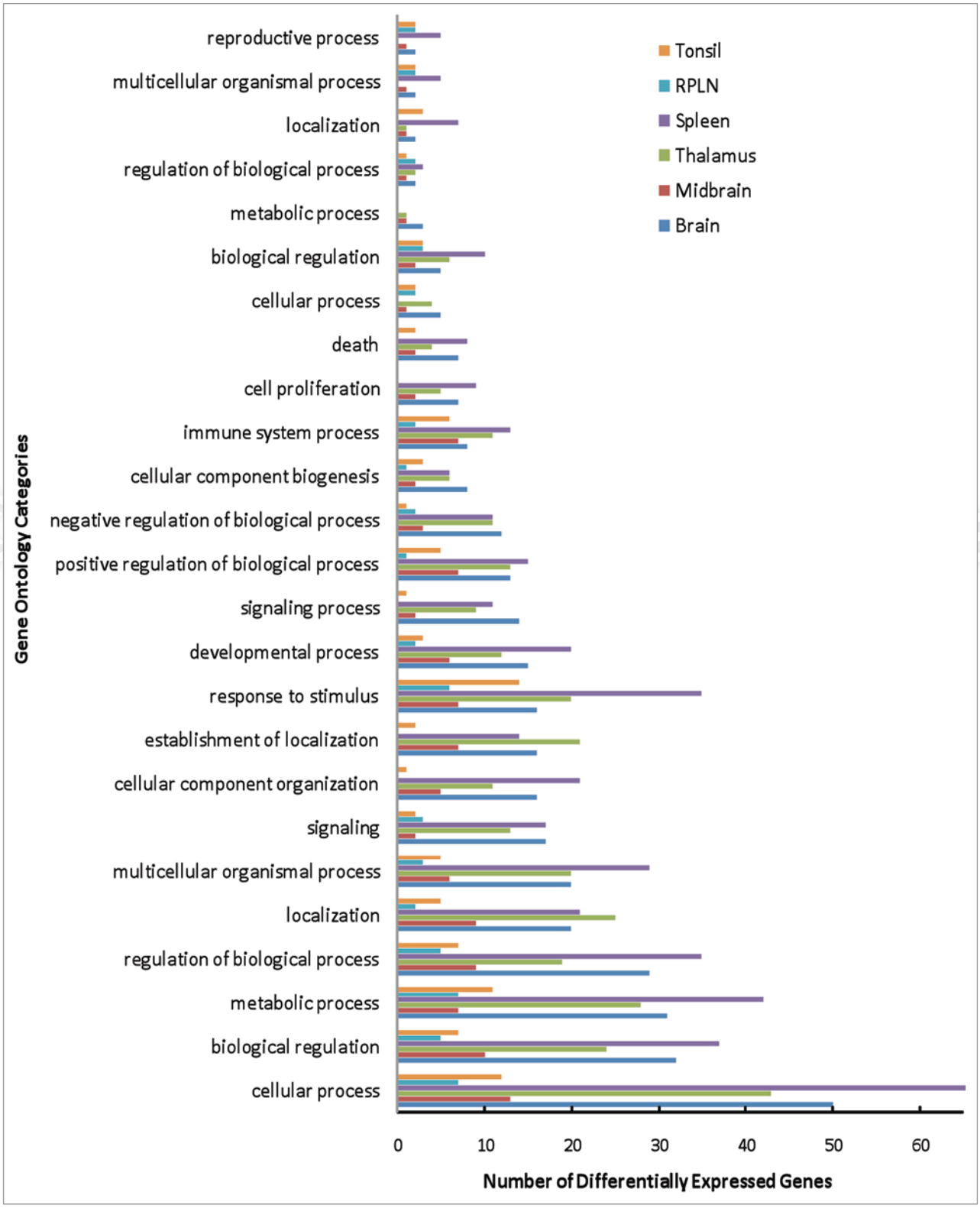

Figure 1. Functional classification of the differentially expressed genes in elk brain (brain, midbrain and thalamus), spleen, RPLN and tonsil tissues based on the Gene ontology (GO) analysis.

of PrP^C are the most significant due to the anti-oxidant activity of PrP^C.[37] Several experiments have shown the synergistic activity of PrP^C and the SOD1 enzyme, and both are dependent on copper uptake and are involved in the protection against oxidative stress.[38] Other studies have reported a significant upregulation of the SOD1 gene in the brain stem of H-type BSE.[39]

Table 3. Differentially expressed genes identified by previous studies of prion infected animals

Gene name	Previous studies	Organism	Tissue	Prion
Actin, gamma2, smooth muscle, enteric	Booth et al. 2004; Skinner et al. 2006; Sorensen et al. 2008	Mice	Brain	Scrapie
	Sawiris et al. 2007	Mice	Brain	BSE
ADAM-like, decysin 1	Sorensen et al. 2008	Mice	Brain	Scrapie
Amyloid β (A4) precursor protein	Sorensen et al. 2008	Mice	Brain	Scrapie
	Sawiris et al. 2007	Mice	Brain	BSE
ATP synthase, H^+ transporting, mitochondrial	Sawiris et al. 2007	Mice	Brain	BSE
ATPase, Na^+/K^+ transporting	Booth et al. 2004; Skinner et al. 2006; Sorensen et al. 2008	Mice	Brain	Scrapie
	Sawiris et al. 2007	Mice	Brain	BSE
Calponin 1	Almeida et al. 2011	Cow	Medulla	BSE
Carbonic anhydrase II	Skinner et al. 2006	Mice	Brain	Scrapie
Cadherin E	Khanyia et al. 2009	Cow	Peyer's Patch	BSE
	Almeida et al. 2011	Cow	Medulla	BSE
Cathepsin H	Xiang et al. 2004; Riemer et al. 2004; Sorensen et al. 2008	Mice	Brain	Scrapie
Chemokine (C-C motif) ligand 2, 20, 21,	Xiang et al. 2004; Sawiris et al. 2007	Mice	Brain	Scrapie
Citrate synthase	Sawiris et al. 2007	Mice	Brain	BSE
Cocaine and amphetamine responsive transcript	Sorensen et al. 2008; Almeida et al. 2011	Mice	Brain	Scrapie
		Cow	Medulla	BSE
Creatine kinase, mitochondrial 1	Greenwood et al. 2005	Mice	Neuroblastoma cells	Scrapie
Desmin	Almeida et al. 2011	Cow	Medulla	BSE
DnaJ (Hsp40) homolog, subfamily A, member 1	Sorensen et al. 2008	Mice	Brain	Scrapie
ELMO/CED-12 domain containing 1	Almeida et al. 2011	Cow	Medulla	BSE
Filamin A, α	Sorensen et al. 2008	Mice	Brain	Scrapie
	Almeida et al. 2011	Cow	Medulla	BSE
Fibulin 5	Sawiris et al. 2007	Mice	Brain	BSE
FK506 binding protein 1B, 12.6 kDa	Sorensen et al. 2008	Mice	Brain	Scrapie
Growth associated protein 43	Greenwood et al. 2005	Mice	Neuroblastoma cells	Scrapie
Guanine nucleotide binding protein	Sorensen et al. 2008	Mice	Brain	Scrapie
Heat shock 70 kDa protein	Sorensen et al. 2008	Mice	Brain	Scrapie
Interleukin 8	Booth et al. 2004; Skinner et al. 2006;	Mice	Brain	Scrapie
	Sorensen et al. 2008	Mice	Brain	BSE
Kinesin-associated protein 3	Skinner et al. 2006; Sawiris et al. 2007	Mice	Brain	Scrapie
		Mice	Brain	BSE
Leucine rich repeat transmembrane neuronal 1	Sorensen et al. 2008	Mice	Brain	Scrapie
Lymphocyte antigen 75	Skinner et al. 2006; Sorensen et al. 2008	Mice	Brain	Scrapie
Lymphocyte cytosolic protein 1	Xiang et al. 2004	Mice	Brain	Scrapie
Lysozyme 1	Riemer et al. 2004	Mice	Brain	Scrapie
Macrophage scavenger receptor 1	Xiang et al. 2004	Mice	Brain	Scrapie
Major histocompatibility complex, class II	Riemer et al. 2004	Mice	Brain	Scrapie
	Basu et al. 2011	Cow	Medulla	BSE
Matrix metallopeptidase 7	Sorensen et al. 2008	Mice	Brain	Scrapie
MHC class I heavy chain	Riemer et al. 2004	Mice	Brain	Scrapie
Malate dehydrogenase 1, NAD	Sawiris et al. 2007	Mice	Brain	BSE
	Skinner et al. 2006	Mice	Brain	Scrapie

Genes identified in elk brain, midbrain and thalamus (bold text) and in elk spleen, RPLN and tonsil tissues.

Table 3. Differentially expressed genes identified by previous studies of prion infected animals (continued)

Membrane-spanning 4-domains	Sawiris et al. 2007	Mice	Brain	BSE
Metallothionein 1E	Greenwood et al. 2005	Mice	Neuroblastoma cells	Scrapie
Mitogen-activated protein kinase 10	Riemer et al. 2004	Mice	Brain	Scrapie
Multiple coagulation factor deficiency 2	Martínez and Pascual, 2007	Human	Neuroblastoma cells	Peptide 106–126
Myosin, heavy chain 10, non-muscle	Martínez and Pascual, 2007	Human	Neuroblastoma cells	Peptide 106–126
Myosin, light chain 1; Myosin, light chain 9	Sorensen et al. 2008	Mice	Brain	Scrapie
Neurofilament, medium polypeptide	Skinner et al. 2006; Sorensen et al. 2008	Mice	Brain	Scrapie
Neurotrophic tyrosine kinase, receptor	Skinner et al. 2006	Mice	Brain	Scrapie
Neuropeptide Y	Diez et al. 2007	Mice	Brain	RML or ME7
PDZ and LIM domain 3	Sawiris et al. 2007	Mice	Brain	BSE
P21 (CDKN1A)-activated kinase 3	Sawiris et al. 2007	Mice	Brain	BSE
Parvalbumin	Voigtländer et al. 2008	Mice	Brain	Scrapie
	Almeida et al. 2011	Cow	Medulla	BSE
Phosphoglycerate mutase 1 (brain)	Sawiris et al. 2007	Mice	Brain	BSE
Pleiotrophin	Sorensen et al. 2008	Mice	Brain	Scrapie
Potassium voltage-gated channel	Sawiris et al. 2007	Mice	Brain	BSE
Potassium channel tetramerisation domain containing 15	Sorensen et al. 2008	Mice	Brain	Scrapie
	Sawiris et al. 2007	Mice	Brain	BSE
Potassium inwardly-rectifying channel	Skinner et al. 2006	Mice	Brain	Scrapie
Proliferating cell nuclear antigen	Sorensen et al. 2008	Mice	Brain	Scrapie
Protein S (α)	Xiang et al. 2004	Mice	Brain	Scrapie
Protein tyrosine phosphatase	Xiang et al. 2004; Sorensen et al. 2008	Mice	Brain	Scrapie
	Martínez and Pascual, 2007	Human	Neuroblastoma cells	Peptide 106–126
Ras homolog gene family	Sorensen et al. 2008	Mice	Brain	Scrapie
Regulator of chromosome condensation (RCC1)	Sorensen et al. 2008	Mice	Brain	Scrapie
Ribosomal protein S6 kinase, 70 kDa	Martínez and Pascual, 2007	Human	Neuroblastoma cells	Peptide 106–126
Ribosomal protein S15	Sorensen et al. 2008	Mice	Brain	Scrapie
	Sawiris et al. 2007	Mice	Brain	BSE
	Greenwood et al. 2005	Mice	Neuroblastoma cells	Scrapie
S100 calcium binding protein A9	Xiang et al. 2004	Mice	Brain	Scrapie
Sarcoglycan, β	Sorensen et al. 2008	Mice	Brain	Scrapie
Secretogranin II	Skinner et al. 2006	Mice	Brain	Scrapie
	Greenwood et al. 2005	Mice	Neuroblastoma cells	Scrapie
Septin 7	Booth et al. 2004; Skinner et al. 2004; Sorensen et al. 2008	Mice	Brain	Scrapie
Solute carrier family 39	Martínez and Pascual, 2007	Human	Neuroblastoma cells	Peptide 106–126
SPARC-like 1	Skinner et al. 2006	Mice	Brain	Scapie
Spastic paraplegia 3A	Sorensen et al. 2008	Mice	Brain	Scrapie
Stathmin-like 2	Greenwood et al. 2005	Mice	Neuroblastoma cells	Scrapie
Stearoyl-CoA desaturase	Booth et al. 2004; Sorensen et al. 2008	Mice	Brain	Scrapie
Succinate-CoA ligase, ADP-forming	Martínez and Pascual, 2007	Human	Neuroblastoma cells	Peptide 106–126

Genes identified in elk brain, midbrain and thalamus (bold text) and in elk spleen, RPLN and tonsil tissues.

Table 3. Differentially expressed genes identified by previous studies of prion infected animals (continued)

Superoxide dismutase 1	Greenwood et al. 2005 Larska et al. 2010	Mice Cattle	Neuroblastoma cells Brain	Scrapie BSE
Synaptosomal-associated protein, 25 kDa	Booth et al. 2004; Skinner et al. 2004; Sorensen et al. 2008 Basu et al. 2011; Almeida et al. 2011	Mice Cow	Brain Medulla	Scrapie BSE
Syndecan	Sorensen et al. 2008	Mice	Brain	Scrapie
Thy-1 cell surface antigen	Sorensen et al. 2008	Mice	Brain	Scrapie
Tachykinin, precursor 1	Almeida et al. 2011	Cow	Medulla	BSE
Transgelin	Sawiris et al. 2007	Mice	Brain	BSE
Transthyretin (prealbumin, amyloidosis)	Sorensen et al. 2008	Mice	Brain	Scrapie
Tropomyosin 2 (β)	Martínez and Pascual, 2007; Almeida et al. 2011	Human Cow	Neuroblastoma cells Medulla	Peptide 106–126 BSE
Tubulin, β 2B	Sorensen et al. 2008	Mice	Brain	Scrapie

Genes identified in elk brain, midbrain and thalamus (bold text) and in elk spleen, RPLN and tonsil tissues.

Table 4. Summary of the number of DE genes which were found to be network and function eligible using ingenuity pathway analysis (IPA) in comparison of control vs. infected of different tissues

Comparison (control vs. infected)	Number of DE genes*	Ingenuity pathway analysis		
		Mapped IDs	Network eligible	Functions eligible
Brain	152	82	77	77
Midbrain	66	27	22	24
Thalamus	11	73	69	66
Spleen	249	145	124	123
RPLN	30	15	14	14
Tonsil	55	29	23	22

*DE genes were determined using Genesifter analysis.

Calcium signaling in examined elk tissues. The altered regulation of calcium ion levels is proposed to be important in TSE-induced cell death.[40] The altered expression of calcium binding, transport and homeostasis genes has been observed in previous prion disease studies[17,20,41,42] and other neurodegenerative disorders, such as Down syndrome and Parkinson disease.[43,44] Recent research has also demonstrated that neurodegeneration induced by a PrP^{Sc}-like prion protein peptide is the result of ER stress and calcium ion release, which results in cytosolic Ca^{2+} elevation, cytochrome *c* release and apoptosis.[40] In this study, four DE genes were identified and two genes, Parvalbumin (PVALB) and Visinin-like 1 (VSNL1), which are related to modulation of the calcium ions levels, were further validated. PVALB is a calcium binding albumin protein present mainly in GABAergic interneurons in the nervous system that have been shown to influence the survival or death of neurons under pathological conditions.[45] VSNL1 is a neuronal Ca^{2+} sensor protein that modulates Ca^{2+}-dependent cell signaling; however, its function in other tissues remains largely unknown.[46] The reduced expression of VSNL1 in CWD-infected elk provides support to a previous study that showed VSNL1 downregulation in brain tissues of scrapie-infected mice.[19] Research has shown the involvement of calcium binding proteins in neurologic disorders[35] through a possible calcium trafficking role of functional PrP^{C};[40] thus, calcium deregulation may be important in the events leading to the characteristic pathology observed in TSE diseases.

Immune and inflammatory response in elk spleen and RPLN tissues. A distinct immune/inflammatory response has been identified in previous TSE disease microarray studies.[15,16,18] It has been suggested that these results are due to microglial and astrocyte activation in response to neurodegeneration; however, some researchers believe that this activation is a key element in neurodegeneration.[26] Activation of these resident immune cells can trigger the release of proinflammatory cytokines, reactive oxygen species, proteases and complement proteins, all of which may be cytotoxic and lead to neurodegeneration.[44] Many studies have mentioned the involvement of the lymphoreticular cells in PrP^{Sc} accumulation, replication and transport from the peripheral lymphoid organs to the central nervous system,[48] and the detection of anti-PrP antibody at the terminal stage of mouse scrapie.[49] Investigating local immunity may be important to understand prion disease progression in the body. In addition, the contribution of inflammation to the progression of prion disease is poorly understood.

Table 5. Summary of the highest represented functional categories (top 5) for the DE genes based on IPA analysis in brain, midbrain, thalamus, spleen, RPLN and tonsil tissues

Sample	Top biological functions	#genes	p value
Brain	Neurological Disease[a]	38	2.1E-08-1.0E-02
	Genetic Disorder[a]	57	1.2E-06-1.0E-02
	Psychological Disorders[a]	19	2.9E-06-1.0E-02
	Skeletal and Muscular Disorders[a]	31	4.5E-05-1.0E-02
	Cancer[a]	34	4.8E-05-1.0E-02
	Cell Morphology[b]	21	5.1E-07-1.0E-02
	Cellular Assembly and Organization[b]	31	5.1E-07-1.0E-02
	Cellular Function and Maintenance[b]	19	6.3E-06-1.0E-02
	Cell Signaling[b]	23	4.9E-05-8.0E-03
	Molecular Transport[b]	23	4.9E-05-9.7E-03
	Nervous System Development and Function[c]	22	5.1E-07-1.0E-02
	Skeletal and Muscular System Development and Function[c]	19	2.9E-05-1.0E-02
	Connective Tissue Development and Function[c]	57	5.6E-05-1.0E-02
	Tissue Development[c]	22	5.6E-05-1.0E-02
	Tissue Morphology[c]	20	8.3E-05-1.0E-02
Midbrain	Endocrine System Disorders[a]	9	3.6E-04-3.6E-02
	Metabolic Disease[a]	10	3.6E-04-3.6E-02
	Inflammatory Response[a]	6	5.0E-04-2.8E-02
	Genetic Disorder[a]	20	7.1E-04-4.5E-02
	Inflammatory Disease[a]	13	7.1E-04-4.5E-02
	Drug Metabolism[b]	3	7.1E-06-3.3E-02
	Free Radical Scavenging[b]	3	1.4E-05-4.6E-02
	Cellular Function and Maintenance[b]	3	2.9E-05-3.1E-02
	Small Molecule Biochemistry[b]	7	2.9E-05-5.0E-02
	Molecular Transport[b]	9	3.6E-05-5.0E-02
	Organ Morphology[c]	5	7.7E-04-2.2E-02
	Cardiovascular System Development and Function[c]	3	1.6E-03-4.2E-02
	Digestive System Development and Function[c]	3	1.6E-03-4.7E-03
	Embryonic Development[c]	4	1.6E-03-2.3E-02
	Nervous System Development and Function[c]	6	1.6E-03-4.69E-02
Thalamus	Neurological Disease[a]	49	4.0E-14-1.6E-02
	Genetic Disorder[a]	57	3.2E-10-1.2E-02
	Psychological Disorders[a]	27	3.8E-10-8.4E-03
	Skeletal and Muscular Disorders[a]	34	5.1E-09-1.4E-02
	Cancer[a]	29	2.1E-06-1.4E-02
	Cell Signaling[b]	20	2.1E-06-1.3E-02
	Molecular Transport[b]	28	2.1E-06-1.6E-02
	Vitamin and Mineral Metabolism[b]	15	2.1E-06-1.3E-02
	Cellular Assembly and Organization[b]	19	6.6E-06-1.6E-02
	Drug Metabolism[b]	9	1.6E-05-1.6E-02
	Skeletal and Muscular System Development and Function[c]	8	1.2E-05-1.6E-02
	Tissue Morphology[c]	8	1.2E-05-1.6E-02
	Behavior[c]	10	1.4E-05-1.2E-02
	Nervous System Development and Function[c]	19	3.3E-05-1.6E-02
	Renal and Urological System Development and Function[c]	3	1.5E-04-1.2E-02

[a]Diseases and disorders, [b]molecular and cellular functions, [c]physiological system development and function.

Table 5. Summary of the highest represented functional categories (top 5) for the DE genes based on IPA analysis in brain, midbrain, thalamus, spleen, RPLN and tonsil tissues (continued)

Spleen	Cancer[a]	70	4.2E-15-3.6E-03
	Skeletal and Muscular Disorders[a]	66	4.2E-13-3.1E-03
	Hematological Disease[a]	32	1.4E-09-3.6E-03
	Genetic Disorder[a]	93	7.5E-09-3.4E-03
	Reproductive System Disease[a]	45	7.5E-09-6.1E-04
	Cellular Movement[b]	41	1.6E-10-4.1E-03
	Antigen Presentation[b]	14	1.9E-08-4.1E-03
	Cellular Growth and Proliferation[b]	49	3.3E-07-3.4E-03
	Cell Cycle[b]	31	3.7E-07-3.2E-03
	Cell Death[b]	53	5.3E-07-4.4E-03
	Hematological System Development and Function[c]	31	1.9E-08-3.4E-03
	Immune Cell Trafficking[c]	20	1.9E-08-4.1E-03
	Hair and Skin Development and Function[c]	6	1.6E-07-4.7E-07
	Cardiovascular System Development and Function[c]	15	2.9E-07-4.5E-03
	Embryonic Development[c]	9	4.7E-07-1.0E-03
RPLN	Genetic Disorder[a]	12	3.9E-04-4.5E-02
	Neurological Disease[a]	9	3.9E-04-4.3E-02
	Skeletal and Muscular Disorders[a]	7	3.9E-04-2.7E-02
	Inflammatory Response[a]	6	4.1E-04-4.5E-02
	Renal and Urological Disease[a]	5	7.8E-04-2.4E-02
	Cellular Assembly and Organization[b]	6	8.0E-06-2.4E-02
	Cellular Compromise[b]	9	8.3E-06-1.6E-02
	Cellular Function and Maintenance[b]	7	8.3E-06-2.4E-02
	Drug Metabolism[b]	2	2.9E-05-2.9E-05
	Lipid Metabolism[b]	4	2.9E-05-4.2E-02
	Endocrine System Development and Function[c]	2	2.9E-05-2.9E-05
	Connective Tissue Development and Function[c]	4	1.3E-04-4.3E-02
	Hepatic System Development and Function[c]	2	1.3E-04-2.3E-02
	Tissue Development[c]	4	3.1E-04-4.4E-02
	Hematological System Development and Function[c]	5	4.1E-04-4.4E-02
Tonsil	Inflammatory Response[a]	7	6.1E-06-4.5E-02
	Reproductive System Disease[a]	6	2.4E-05-2.4E-05
	Infection Mechanism[a]	4	1.7E-04-3.1E-02
	Hypersensitivity Response[a]	3	2.6E-04-4.5E-02
	Connective Tissue Disorders[a]	3	3.0E-04-1.8E-02
	Cellular Movement[b]	8	5.9E-07-4.8E-02
	Cellular Compromise[b]	7	3.4E-05-2.4E-02
	Cellular Function and Maintenance[b]	7	3.4E-05-3.8E-02
	Drug Metabolism[b]	3	7.3E-05-2.3E-02
	Lipid Metabolism[b]	7	7.3E-05-4.4E-02
	Hematological System Development and Function[c]	6	5.9E-07-4.5E-02
	Immune Cell Trafficking[c]	5	5.9E-07-4.5E-02
	Hematopoiesis[c]	6	4.2E-05-4.5E-02
	Endocrine System Development and Function[c]	3	7.3E-05-3.4E-02
	Cardiovascular System Development and Function[c]	4	1.3E-04-4.0E-02

[a]Diseases and disorders, [b]molecular and cellular functions, [c]physiological system development and function.

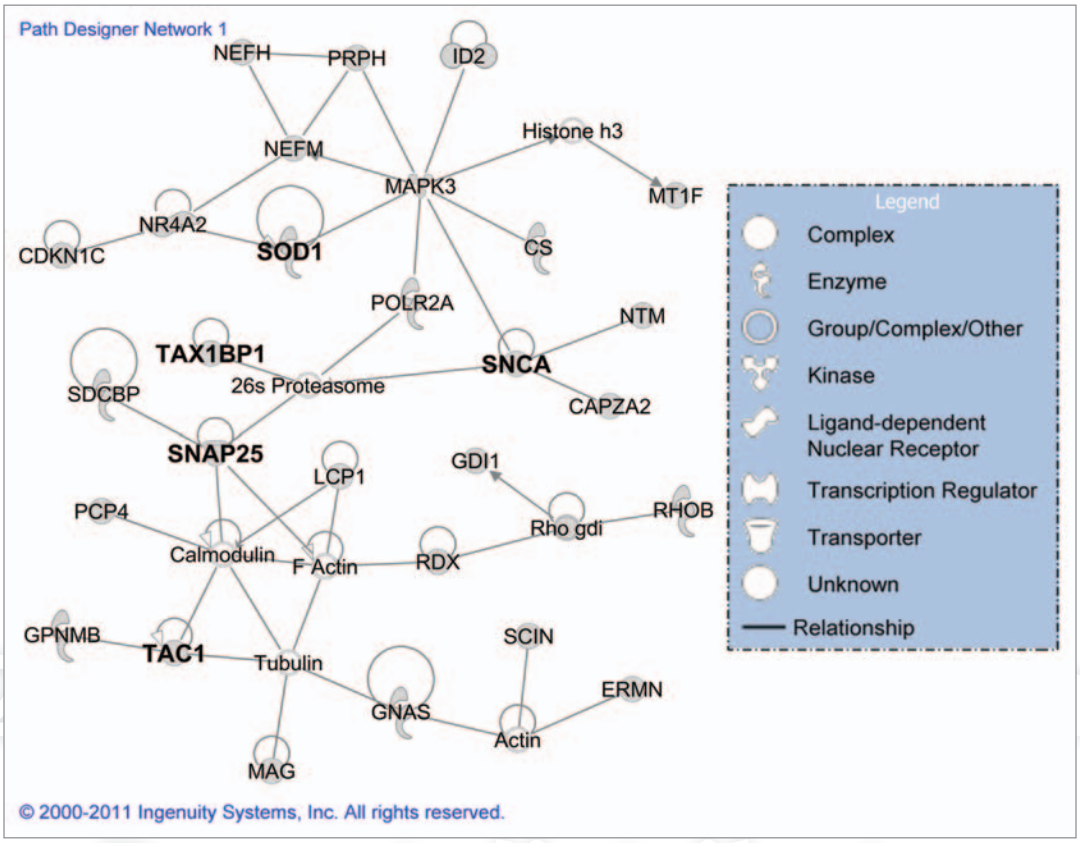

Figure 2. The highest scoring gene network based on classification of DE genes from elk brain tissues (brain, midbrain and thalamus) using the Ingenuity Pathway Analysis. The shape of the node indicates the major function of the protein and a line denotes binding of the products of the two genes. The genes in bold have been validated by qRT-PCR.

Serum amyloid (SAA) proteins are a family of apolipoproteins with biological functions that are not fully defined, although recent reports indicate that SAA induces proinflammatory cytokine expression.[50] SAA is mainly secreted by hepatocytes, and its concentration increases in the blood up to 1,000 times during an inflammatory response.[51] SAA expression was increased in all tissues from one infected elk and in the RPLN in the other animal. SAA has been associated with the top network of cancer and cell death in spleen tissues and with increased CCL20 expression, indicating its involvement in immune cell trafficking in spleen tissues (**Table S1**). A higher CCL20 expression was observed in elk-infected spleen tissues. The chemotactic actions of chemokines are known to be critical for the recruitment and activation of leukocytes, and they are important for the innate immune responders, such as neutrophils, dendritic cells and natural killer (NK) cells, as well as those cells involved in the adaptive immune response.[52] NK cells are capable of inducing enzyme-mediated apoptosis and may contribute to immune system-mediated apoptosis of CWD-infected neurons.

The angiotensin-converting enzyme (ACE) cleaves angiotensin I to angiotensin II.[53] Variations in the ACE gene have been associated with Alzheimer disease,[54,55] suggesting that ACE and ACE receptors may play a role in, or be affected by, neurodegenerative disease. Angiotensin II was downregulated in infected spleen, tonsil and RPLN tissues. Some of these genes could be considered presumptive biomarkers to distinguish CWD-infected elk from healthy animals; however, this needs to be assessed in further studies with more individuals.

Methods

Animals and sampling. Rocky mountain elk (*Cervus elaphus nelsoni*) were selected from two farms in central Alberta, Canada. All animal screening, selection, inoculation and experiments were approved by the Canadian Food Inspection Agency (CFIA) and the Lethbridge Laboratory Animal Care Committee and followed the guidelines of the Canadian Council for Animal Care (CCAC). Two animals were selected for the negative inoculation control group, and two animals were selected for the CWD-infected group. All animals in the experiment were females, and they were inoculated at approximately 4 months of age. The animals were kept in a biosafety level 3 facility for the remaining duration of the experiment. After a week of adjustment to the new environment, the animals were orally inoculated with 10 mL of a 10% brain homogenate (1 g tissue equivalent). The two control animals were fed normal elk brain tissue homogenate, while the CWD-infected group was fed brain tissue homogenate from confirmed CWD-positive elk.

Tissue collection, confirmation of PrP^{CWD} infection and RNA extraction. Once clinical signs had reached a predetermined

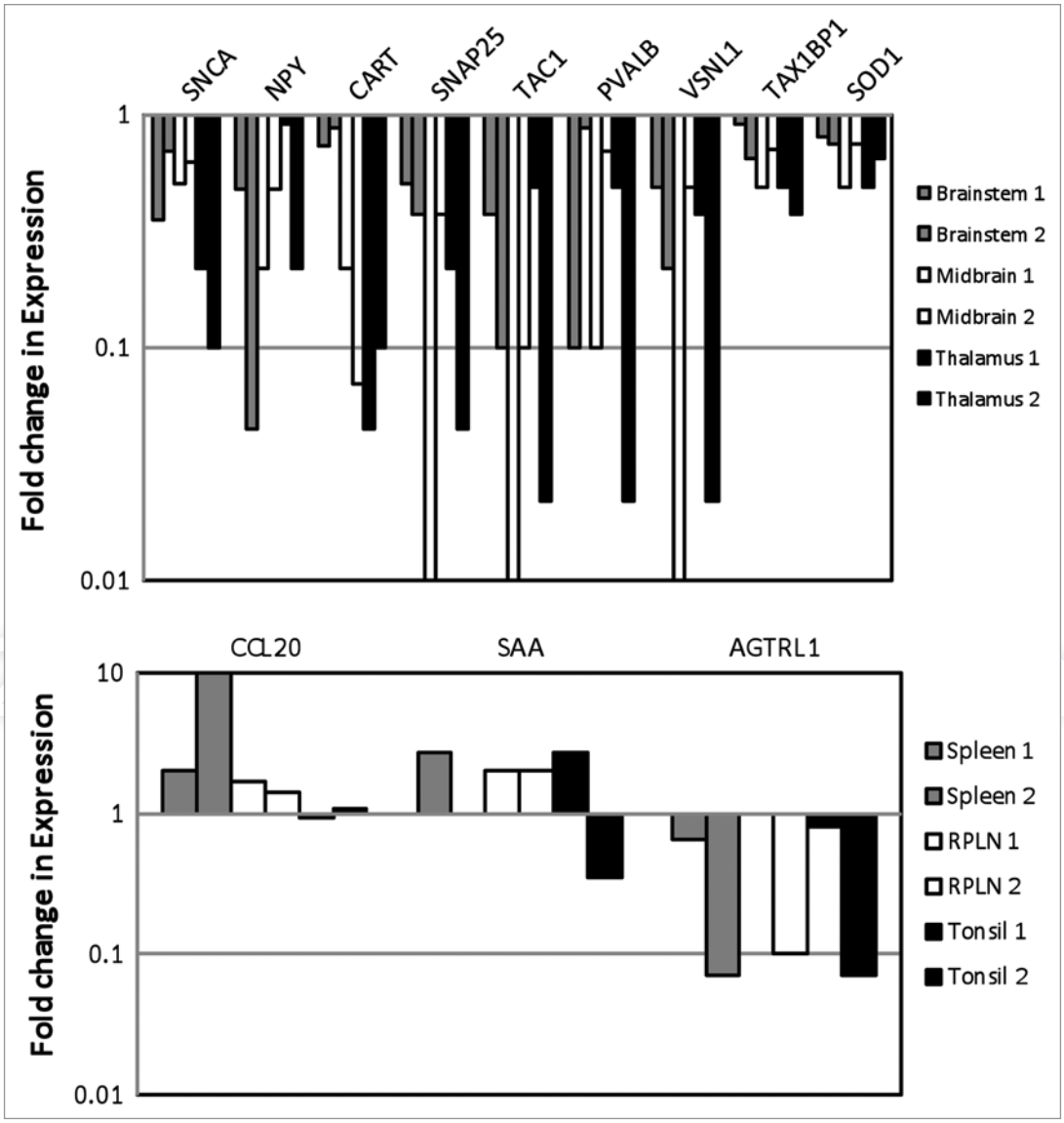

Figure 3. Quantitative real-time PCR analysis of DE genes in elk brain tissues (A) and elk spleen, RPLN and tonsil tissues (B) in two different animals. DE genes associated with neuronal signaling include: α-Synuclein A (SNCA); neuropeptide Y (NPY), cocaine amphetamine responsive transcript (CART); Synaptosomal-associated protein 25 KD (SNAP25), tachykinin (TAC1); calcium ion regulation: parvalbumin (PVALB), visinin-like (VNSL1) and angotensin II receptor (AGTRL); apoptosis: human T-cell leukemia virus type I (Tax1BP1) and superoxide dismutase (SOD1); immune and inflammatory response: chemokine 20 (CCL20) and serum amyloid A-like (SAA). Gene expression was compared between control and PRP^{CWD} infected elk.

level, the animals were euthanized (overdose of pentobarbital injected intravenously in the jugular vein), and an extensive post mortem examination was performed. Animals no. 8 and 28 were euthanized 738 d post-inoculation. Control animals no. 6 and 31 were euthanized 752 d post-inoculation. Weights of the animals at euthanasia were as follows: no. 8, 450 lbs; no. 28, 485 lbs; no. 6, 610 lbs; and no. 31, 530 lbs.

All of the tissues (i.e., the brain stem, midbrain, thalamus, RPLN, tonsil and spleen) were collected as quickly as possible and placed in RNAlater (Ambion) to preserve the integrity of the RNA samples. After 24 h at 4°C, the RNAlater was removed from the samples, and the preserved tissues were stored at -80°C until further processing. Tissues from each of the animals were tested during routine surveillance by Prionics Check Priostrip and Prionics Check western (Prionics AG), and the CWD status was confirmed by histology and immunohistochemistry.

Total RNA extraction from the infected and control tissues were performed using the Trizol/Chloroform protocol found in the Qiagen RNeasy extraction kit (Qiagen). The RNA quality and quantity were measured using a Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific), and the samples were stored at -80°C.

Microarray hybridization and data analysis. Global gene expression variations were determined by microarray analysis of two groups of control and CWD-infected animals (control animal no. 6 vs. infected animal no. 8 and control animal no. 31 vs. infected animal no. 28). Duplicates of 24,000 bovine oligonucleotide probes (www.Bovineoligo.org) representing 16,846

Table 6. Oligonucleotides and probes used for qRT-PCR analysis of the DE genes

Gene	Nucleotide sequence (5'-3')	Probe	Accession number
Synuclein A	F: GGA GCA GGG AGC ATT GCA R: TGC CCA TAT GAT CCT TTT TGC	CTG CCA CTG GCT TT	NM_001034041
Neuropeptide Y	F: CGG AGG ACT TGG CCA GAT AC R: TGC CTG GTG ATG AGA TTG ATG	ACT CAG CGC TGC GAC	NM_001014845
Cocaine and amphetamine responsive transcript (CART)	F: CGC GAG CCC TGG ACA TC R: GCT TCA ATC AGC TCC TTC TCA TG	TCC GCC GTG GAG GA	NM_001007820
Synaptosomal-associated protein (SNAP25)	F: CCG TCA TAT GGC CCT GGA T R: TGT CGA TCT GGC GGT TCT G	TGG GCA ATG AGA TTG ATA	NM_001076246
Tachykinin 1 (TAC1)	F: CCG TGG CAG TGA TTT TTT TCA R: CGT TGG CTC CGA TTT CTT CT	CTC CAC TCA ACT GTC TG	NM_174193
Parvalbumin (PVALB)	F: CCG GAA GAC GTG AAG AAG GTA T R: CTC GAT GAA GCC GCT CTT G	CCA CAT CCT GGA TAA AG	NM_001076114
Visinin-like 1 (VSNL1)	F: ATC ACC CGA GTG GAG ATG CT R: TCA CTG TGC CCA CCA TTT TG	AGA TCA TCG AGG CTA TC	NM_174490
Tax-1 Binding protein1 (Tax-1BP1)	F: TCA GGC AAC ACG GCA AGA A R: TTA TCC CGC ACA TTT ACA GCA T	CTG TCT TTC TGG CTA AAG	NM_001046409
Superoxide dismutase (SOD1)	F: TGG AGA CCT GGG CAA TGT G R: ACA ATA TCC ACG ATG GCA ACA C	CAG CTG ACA AAA AC	NM_174615
Angiotensin II receptor (AGTRL1)	F: CTC ATG AAC GTC TTC CCC TAC TG R: GGG TTG AGG CAG CTG TTG A	ACG TGC GTC AGC TAC	NM_001102524
Chemokine 20 (CCL20)	F: CCC AGT ATT CTT GTG GGC TTC A R: GCA TTG ATG TCA CAG GCT TCA	ACA GCA GCT GGC C	NM_004591
Serum amyloid A-like (SAA)	F: ACT CTG ACA TGA GAG AAG CCA ACT A R: CGC GGG CGT GGA AGT	AAG GGT GCA GAC AAA	NM_001075260
Bos taurus ribosomal protein L12 (RPL12)	F: AGG GTC TGA GGA TTA CAG TGA AAC R: GAT CAG GGC AGA AGC AGA AGG	ACC ATT CAG AAC AGA C	NM_205797

Primers were designed using the Primers Express 3.0 software of Applied Biosystems. F, forward primer; R, reverse primer.

different genes, with 5,943 ESTs from the bovine genome, were spotted onto ultragap slides (Corning) using Q-array2 (Genetix). One microgram of total RNA was reversed-transcribed, amino-allyl coupled [Applied Biosystemsand labeled with Cy3 or Cy5 fluorescent dyes (GE Healthcare)] according to the protocol of the manufacturer. For each tissue, four slides were used including dye swaps and two technical replicates. Thus, each gene was represented eight times in the statistical analysis. Hybridizations were performed in a hybridization chamber (Genetix) at 42°C overnight. Hybridized slides were washed with low stringency buffer [2x standard sodium citrate (SSC), 0.5% sodium dodecyl sulfate (SDS)], high stringency buffer (0.5x SSC, 0.2% SDS) and 0.05x SSC. The hybridized slides were scanned at 5 micron resolution and their signal intensities were detected by Q-Scan (Genetix).

Data analysis was performed using GeneSifter (VizX Labs). The background-corrected signal intensity for each spot was normalized by the locally weighted scatter plot smoothing (LOWESS) method and log base 2 transformed. Differences in the gene expression levels between control and CWD-infected tissues were analyzed using the t-test statistic method. The criteria for significant DE genes were a 2-fold or greater change in expression level with a $p < 0.01$, which was adjusted by the Bonferroni correction method.[57] The 95% quality filter was used to eliminate data from probes in any group that had an intensity variation between the probes greater than 5%.

Functional analysis of the DE genes. The characterization of the DE genes was performed by GeneSifter to annotate the GO of each gene into their respective biological and cellular processes and molecular function categories. The DE genes were also analyzed for their functions, pathways and networks using IPA (Ingenuity Systems, www.ingenuity.com/products/IPA/Free-Trial-Software.html). Each gene identifier was mapped to its corresponding gene, called Focus genes, in the Ingenuity Pathways database, which was overlaid into a global molecular network. Networks of these Focus genes were then algorithmically generated to reveal their connectivity, and functional analysis of these networks identified their association with biological function and/or disease.

Quantitative real-time PCR analysis of DE genes. For technical validation of the microarray analysis, 12 DE genes were further tested using qRT-PCR. **Table 6** provides information on the targeted genes and the primer and probe sequences for each targeted gene. We tested four genes for endogenous controls,

GPADH, 18S, Cyc and RPL. According to the Bestkeeper software,[58] RPL showed most stable expression and was selected as the endogenous control for subsequent analysis. RNA abundance was measured using the TaqMan Universal PCR Master Mix with gene specific MGB probes labeled with FAM and VIC fluorescent dyes (Applied Biosystems). Due to the limited amount of RNA, the samples were amplified using the reverse-transcribed synthesis amino-allyl kit (Applied Biosystems). The expression levels detected between total RNA and aRNA samples have been shown to be consistent.[59] Each reaction was performed in triplicate with 500 ng of aRNA using the StepOnePlus Real time PCR System (Applied Biosystems). The positive control (bovine cDNA) and the negative control (no template) control were always included on each plate. The thermal cycling conditions were as follows: 95°C for 20 sec followed by 40 cycles of 95°C for 1 sec and 60°C for 20 sec. The delta CT value for each gene was calculated by subtracting the reference gene CT from the targeted gene CT. Gene expression was compared between two control and two PrP^{CWD} infected elk in three brain (brain, midbrain and thalamus) and spleen, RPLN and tonsil tissues. The changes in gene expression between the control and infected animals were quantitatively measured relative to the RNA from the control sample. Relative quantification values were determined using $2^{-\Delta\Delta CT}$ method and were expressed as fold change in the infected vs. control animals.

Conclusions

CWD infects elk, white-tailed deer and mule deer, and with no treatment or early diagnostic tests available, the disease is invariably fatal. Gene profiling studies data in naturally oral infected cervid TSE are also very limited. Thus, gaining a better understanding about the molecular mechanisms involved in CWD infection will potentially shed light on the many unknowns that are hampering the effective control and management of this disease. This is the first study to perform a high-throughput gene expression analysis to identify changes in the brain, spleen, RPLN and tonsil tissues of CWD disease-infected elk, which may prove useful in testing the accessible lymphatic tissues, such as the tonsils and RPLN, in preclinical animals.

Some of the DE genes identified have been previously reported in mice, cattle and humans suggesting that some common mechanisms associated with prion pathogenesis may exist among different host species. The differential expression of the Tax1BP1 gene, which is known to regulate the expression of a vast array of cellular genes, was not reported in previous TSE studies and needs to be confirmed in further studies with a larger number of animals. DE genes associated with major regulatory and signaling networks, including neuronal signaling, synapse function in neurological disease, calcium signaling, apoptosis and cell death, and immune cell trafficking and inflammatory response, from both microarray and qRT-PCR analyses provide further support for the utility of a bovine DNA microarray for gene expression profiling of elk samples. We are aware of the limitations of the study due to the lack of several biological replicates. However, high dispersion in the results is expected even when the biological replicates are individuals affected by evolution and natural selection depending on the gender, breed or environmental factors.[56] These results may also provide direction for other research focused on diagnosis, treatment and prevention of TSE diseases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

U.B. performed IPA analysis and wrote the manuscript; L.M.A. worked on microarray and qRT-PCR experiments and contributed to manuscript writing; S.D. was involved in RNA extraction, preliminary microarray experiments and contributed to manuscript writing. C.E.G. and S.C. performed animal study and pathology study; S.S.M. contributed to the discussion and manuscript writing; L.L.G. was involved in experimental design, data analysis and manuscript writing. This research was supported by PrionNet Canada, Alberta Prion Institute and Alberta Bovine Genomic Program.

Supplemental Material

Supplemental material may be found here:
www.landesbioscience.com/journals/prion/article/19915

References

1. Prusiner SB. Prions. Proc Natl Acad Sci USA 1998; 95:13363-83; PMID:9811807; http://dx.doi.org/10.1073/pnas.95.23.13363.
2. Prusiner SB. Prion Biology and Diseases. Cold Spring Harbor Laboratory Press 2004; 2; ISBN: 0879696931.
3. Salman MD. Chronic wasting disease in deer and elk: scientific facts and findings. J Vet Med Sci 2003; 65:761-8; PMID:12939501; http://dx.doi.org/10.1292/jvms.65.761.
4. Williams ES. The transmissible spongiform encephalopathies: disease risks for North America. Vet Clin North Am Food Anim Pract 2002; 18:461-73; PMID:12442578; http://dx.doi.org/10.1016/S0749-0720(02)00032-4.
5. Williams ES. Chronic wasting disease. Vet Pathol 2005; 42:530-49; PMID:16145200; http://dx.doi.org/10.1354/vp.42-5-530.
6. Anderson CA, Bosque P, Filley CM, Arciniegas DB, Kleinschmidt-Demasters BK, Pape WJ, et al. Colorado surveillance program for chronic wasting disease transmission to humans: lessons from 2 highly suspicious but negative cases. Arch Neurol 2007; 64:439-41; PMID:17353391; http://dx.doi.org/10.1001/archneur.64.3.439.
7. Belay ED, Maddox RA, Williams ES, Miller MW, Gambetti P, Schonberger LB. Chronic wasting disease and potential transmission to humans. Emerg Infect Dis 2004; 10:977-84; PMID:15207045; http://dx.doi.org/10.3201/eid1006.031082.
8. Race B, Meade-White KD, Miller MW, Barbian KD, Rubenstein R, LaFauci G, et al. Susceptibilities of nonhuman primates to chronic wasting disease. Emerg Infect Dis 2009; 15:1366-76; PMID:19788803; http://dx.doi.org/10.3201/eid1509.090253.
9. Haley NJ, Seelig DM, Zabel MD, Telling GC, Hoover EA. Detection of CWD prions in urine and saliva of deer by transgenic mouse bioassay. PLoS One 2009; 4:4848; PMID:19293928; http://dx.doi.org/10.1371/journal.pone.0004848.
10. Tamgüney G, Giles K, Bouzamondo-Bernstein E, Bosque PJ, Miller MW, Safar J, et al. Transmission of elk and deer prions to transgenic mice. J Virol 2006; 80:9104-14; PMID:16940522; http://dx.doi.org/10.1128/JVI.00098-06.
11. Tamgüney G, Miller MW, Wolfe LL, Sirochman TM, Glidden DV, Palmer C, et al. Asymptomatic deer excrete infectious prions in faeces. Nature 2009; 461:529-32; PMID:19741608; http://dx.doi.org/10.1038/nature08289.
12. Gilch S, Chitoor N, Taguchi Y, Stuart M, Jewell JE, Schätzl HM. Chronic wasting disease. Top Curr Chem 2011; 305:51-77; PMID:21598099; http://dx.doi.org/10.1007/128_2011_159.
13. Spraker TR, O'Rourke KI, Balachandran A, Zink RR, Cummings BA, Miller MW, et al. Validation of monoclonal antibody F99/97.6.1 for immunohistochemical staining of brain and tonsil in mule deer (*Odocoileus hemionus*) with chronic wasting disease. J Vet Diagn Invest 2002; 14:3-7; PMID:12680636; http://dx.doi.org/10.1177/104063870201400102.

14. Huzarewich RLCH, Siemens CG, Booth SA. Application of "omics" to prion biomarker discovery. J Biomed Biotechnol 2010; 2010:613504; PMID:20224650; http://dx.doi.org/10.1155/2010/613504.
15. Booth S, Bowman C, Baumgartner R, Sorensen G, Robertson C, Coulthart M, et al. Identification of central nervous system genes involved in the host response to the scrapie agent during preclinical and clinical infection. J Gen Virol 2004; 85:3459-71; PMID:15483264; http://dx.doi.org/10.1099/vir.0.80110-0.
16. Xiang W, Windl O, Wünsch G, Dugas M, Kohlmann A, Dierkes N, et al. Identification of differentially expressed genes in scrapie-infected mouse brains by using global gene expression technology. J Virol 2004; 78:11051-60; PMID:15452225; http://dx.doi.org/10.1128/JVI.78.20.11051-60.2004.
17. Greenwood AD, Horsch M, Stengel A, Vorberg I, Lutzny G, Maas E, et al. Cell line dependent RNA expression profiles of prion-infected mouse neuronal cells. J Mol Biol 2005; 349:487-500; PMID:15896347; http://dx.doi.org/10.1016/j.jmb.2005.03.076.
18. Riemer C, Neidhold S, Burwinkel M, Schwarz A, Schultz J, Krätzschmar J, et al. Gene expression profiling of scrapie-infected brain tissue. Biochem Biophys Res Commun 2004; 323:556-64; PMID:15369787; http://dx.doi.org/10.1016/j.bbrc.2004.08.124.
19. Skinner PJ, Abbassi H, Chesebro B, Race RE, Reilly C, Haase AT. Gene expression alterations in brains of mice infected with three strains of scrapie. BMC Genomics 2006; 7:114-26; PMID:16700923; http://dx.doi.org/10.1186/1471-2164-7-114.
20. Sorensen G, Medina S, Parchaliuk D, Phillipson C, Robertson C, Booth SA. Comprehensive transcriptional profiling of prion infection in mouse models reveals networks of responsive genes. BMC Genomics 2008; 9:114-28; PMID:18315872; http://dx.doi.org/10.1186/1471-2164-9-114.
21. Khaniya B, Almeida L, Basu U, Taniguchi M, Williams JL, Barreda DR, et al. Microarray analysis of differentially expressed genes from Peyer's patches of cattle orally challenged with bovine spongiform encephalopathy. J Toxicol Environ Health A 2009; 72:1008-13; PMID:19697233; http://dx.doi.org/10.1080/15287390903084199.
22. Almeida LM, Basu U, Khaniya B, Taniguchi M, Williams JL, Moore SS, et al. Gene expression in the medulla following oral infection of cattle with bovine spongiform encephalopathy. J Toxicol Environ Health A 2011; 74:110-26; PMID:21218340; http://dx.doi.org/10.1080/15287394.2011.529061.
23. Basu U, Almeida LM, Olson NE, Meng Y, Williams JL, Moore SS, et al. Transcriptome analysis of the medulla tissue from cattle in response to bovine spongiform encephalopathy using digital gene expression tag profiling. J Toxicol Environ Health A 2011; 74:127-37; PMID:21218341; http://dx.doi.org/10.1080/15287394.2011.529062.
24. Gallagher DS Jr, Derr JN, Womack JE. Chromosome conservation among the advanced pecorans and determination of the primitive bovid karyotype. J Hered 1994; 85:204-10; PMID:8014460.
25. Seabury CM, Bhattarai EK, Taylor JF, Viswanathan GG, Cooper SM, Davis DS, et al. Genome-wide polymorphism and comparative analyses in the white-tailed eeer (*Odocoileus virginianus*): A model for conservation genomics. PLoS ONE 2011; 6:15811; PMID:21283515; http://dx.doi.org/10.1371/journal.pone.0015811.
26. Crozet C, Beranger F, Lehmann S. Cellular pathogenesis in prion diseases. Vet Res 2008; 39:44; PMID:18413130; http://dx.doi.org/10.1051/vetres:2008021.
27. Sisó S, Puig B, Varea R, Vidal E, Acín C, Prinz M, et al. Abnormal synaptic protein expression and cell death in murine scrapie. Acta Neuropathol 2002; 103:615-26; PMID:12012094; http://dx.doi.org/10.1007/s00401-001-0512-6.
28. Diez M, Groth D, DeArmond SJ, Prusiner SB, Hökfelt T. Changes in neuropeptide expression in mice infected with prions. Neurobiol Aging 2007; 28:748-65; PMID:16621165; http://dx.doi.org/10.1016/j.neurobiolaging.2006.02.017.
29. Haïk S, Privat N, Adjou KT, Sazdovitch V, Dormont D, Duyckaerts C, et al. Alpha-synuclein-immunoreactive deposits in human and animal prion diseases. Acta Neuropathol 2002; 103:516-20; PMID:11935269; http://dx.doi.org/10.1007/s00401-001-0499-z.
30. Johnston AR, Fraser JR, Jeffrey M, MacLeod N. Synaptic plasticity in the CA1 area of the hippocampus of scrapie-infected mice. Neurobiol Dis 1998; 5:188-95; PMID:9848090; http://dx.doi.org/10.1006/nbdi.1998.0194.
31. Diez M, DeArmond SJ, Groth D, Prusiner SB, Hökfelt T. Decreased MK-801 binding in discrete hippocampal regions of prion-infected mice. Neurobiol Dis 2001; 8:692-9; PMID:11493033; http://dx.doi.org/10.1006/nbdi.2001.0404.
32. Diez M, Danner S, Frey P, Sommer B, Staufenbiel M, Wiederhold KH, et al. Neuropeptide alterations in the hippocampal formation and cortex of transgenic mice overexpressing beta-amyloid precursor protein (APP) with the Swedish double mutation (APP23). Neurobiol Dis 2003; 14:579-94; PMID:14678773; http://dx.doi.org/10.1016/j.nbd.2003.08.003.
33. Xu Y, Zhang W, Klaus J, Young J, Koerner I, Sheldahl LC, et al. Role of cocaine- and amphetamine-regulated transcript in estradiol-mediated neuroprotection. Proc Natl Acad Sci USA 2006; 103:14489-94; PMID:16971488; http://dx.doi.org/10.1073/pnas.0602932103.
34. Söllner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempst P, et al. SNAP receptors implicated in vesicle targeting and fusion. Nature 1993; 362:318-24; PMID:8455717; http://dx.doi.org/10.1038/362318a0.
35. Sawiris GP, Becker KG, Elliott EJ, Moulden R, Rohwer RG. Molecular analysis of bovine spongiform encephalopathy infection by cDNA arrays. J Gen Virol 2007; 88:1356-62; PMID:17374782; http://dx.doi.org/10.1099/vir.0.82387-0.
36. Vajente N, Trevisan R, Saggioro D. HTLV-1 Tax protein cooperates with Ras in protecting cells from apoptosis. Apoptosis 2009; 14:153-63; PMID:19089619; http://dx.doi.org/10.1007/s10495-008-0289-3.
37. Vana K, Zuber CH, Nikles D, Weiss S. Novel aspects of prions, their receptor molecules and innovative approaches for TSE therapy. Cell Mol Neurobiol 2007; 27:107-28; PMID:17151946; http://dx.doi.org/10.1007/s10571-006-9121-1.
38. Brown DR, Besinger A. Prion protein expression and superoxide dismutase activity. Biochem J 1998; 334:423-9; PMID:9716501.
39. Larska M, Polak MP, Zmudzinski JF, Torres JM. Comparison of mRNA expression levels of selected genes in the brain stem of cattle naturally infected with classical and atypical BSE. Brain Res 2010; 1351:13-22; PMID:20654596; http://dx.doi.org/10.1016/j.brainres.2010.07.035.
40. Ferreiro E, Oliveira CR, Pereira CM. The release of calcium from the endoplasmic reticulum induced by amyloid-beta and prion peptides activates the mitochondrial apoptotic pathway. Neurobiol Dis 2008; 30:331-42; PMID:18420416; http://dx.doi.org/10.1016/j.nbd.2008.02.003.
41. Colling SB, Collinge J, Jefferys JG. Hippocampal slices from prion protein null mice: disrupted Ca(2+)-activated K^+ currents. Neurosci Lett 1996; 209:49-52; PMID:8734907; http://dx.doi.org/10.1016/0304-3940(96)12596-9.
42. Fuhrmann M, Bittner T, Mitteregger G, Haider N, Moosmang S, Kretzschmar H, et al. Loss of the cellular prion protein affects the Ca^{2+} homeostasis in hippocampal CA1 neurons. J Neurochem 2006; 98:1876-85; PMID:16945105; http://dx.doi.org/10.1111/j.1471-4159.2006.04011.x.
43. Griffin WS, Sheng JG, McKenzie JE, Royston MC, Gentleman SM, Brumback RA, et al. Life-long overexpression of S100beta in Down's syndrome: implications for Alzheimer pathogenesis. Neurobiol Aging 1998; 19:401-5; PMID:9880042; http://dx.doi.org/10.1016/S0197-4580(98)00074-8.
44. Muramatsu Y, Kurosaki R, Watanabe H, Michimata M, Matsubara M, Imai Y, et al. Cerebral alterations in a MPTP-mouse model of Parkinson's disease—an immunocytochemical study. J Neural Transm 2003; 110:1129-44; PMID:14523625; http://dx.doi.org/10.1007/s00702-003-0021-y.
45. Voigtländer T, Unterberger U, Guentchev M, Schwaller B, Celio MR, Meyer M, et al. The role of parvalbumin and calbindin D28k in experimental scrapie. Neuropathol Appl Neurobiol 2008; 34:435-45; PMID:18005331; http://dx.doi.org/10.1111/j.1365-2990.2007.00902.x.
46. Dai FF, Zhang Y, Kang Y, Wang Q, Gaisano HY, Braunewell KH, et al. The neuronal Ca^{+2} sensor protein-1 is expressed in pancreatic islet and regulates insulin secretion. J Biol Chem 2006; 281:21942-53; PMID:16731532; http://dx.doi.org/10.1074/jbc.M512924200.
47. Chiarini A, Dal Pra I, Whitfield JF, Armato U. The killing of neurons by beta-amyloid peptides, prions and pro-inflammatory cytokines. Ital J Anat Embryol 2006; 111:221-46; PMID:17385278.
48. Montrasio F, Frigg R, Glatzel M, Klein MA, Mackay F, Aguzzi A, et al. Impaired prion replication in spleens of mice lacking functional follicular dendritic cells. Science 2000; 288:1257-9; PMID:10818004; http://dx.doi.org/10.1126/science.288.5469.1257.
49. Sassa Y, Kataoka N, Inoshima Y, Ishiguro N. Anti-PrP antibodies detected at terminal stage of prion-affected mouse. Cell Immunol 2010; 263:212-8; PMID:20417929; http://dx.doi.org/10.1016/j.cellimm.2010.03.018.
50. Cheng N, He R, Tian J, Ye PP, Ye RD. Cutting edge: TLR2 is a functional receptor for acute-phase serum amyloid A. J Immunol 2008; 181:22-6; PMID:18566366.
51. Badolato R, Wang JM, Murphy WJ, Lloyd AR, Michiel DF, Bausserman LL, et al. Serum amyloid A is a chemoattractant: induction of migration, adhesion and tissue infiltration of monocytes and polymorphonuclear leukocytes. J Exp Med 1994; 180:203-9; PMID:7516407; http://dx.doi.org/10.1084/jem.180.1.203.
52. Esche C, Stellato C, Beck LA. Chemokines: key players in innate and adaptive immunity. J Invest Dermatol 2005; 125:615-28; PMID:16185259; http://dx.doi.org/10.1111/j.0022-202X.2005.23841.x.
53. Coates D. The angiotensin converting enzyme (ACE). Int J Biochem Cell Biol 2003; 35:769-73; PMID:12676162; http://dx.doi.org/10.1016/S1357-2725(02)00309-6.
54. Katzov H, Chalmers K, Palmgren J, Andreasen N, Johansson B, Cairns NJ, et al. Genetic variants of ABCA1 modify Alzheimer disease risk and quantitative traits related to beta-amyloid metabolism. Hum Mutat 2004; 23:358-67; PMID:15024730; http://dx.doi.org/10.1002/humu.20012.
55. Lehmann DJ, Cortina-Borja M, Warden DR, Smith AD, Sleegers K, Prince JA, et al. Large meta-analysis establishes the ACE insertion-deletion polymorphism as a marker of Alzheimer's disease. Am J Epidemiol 2005; 162:305-17; PMID:16033878; http://dx.doi.org/10.1093/aje/kwi202.
56. Whitehead A, Crawford DL. Neutral and adaptive variation in gene expression. Proc Natl Acad Sci USA 2006; 103:5425-30; PMID:16567645; http://dx.doi.org/10.1073/pnas.0507648103.
57. Bland JM, Altman DG. Multiple significance tests: the Bonferroni method. BMJ 1995; 310:170; PMID:7833759; http://dx.doi.org/10.1136/bmj.310.6973.170.

58. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper-Excel-based tool using pair-wise correlations. Biotechnol Lett 2004; 26:509-15; PMID:15127793; http://dx.doi.org/10.1023/B:BILE.0000019559.84305.47.

59. Taniguchi M, Guan LL, Zhang B, Dodson MV, Okine E, Moore SS. Adipogenesis of bovine perimuscular preadipocytes. Biochem Biophys Res Commun 2008; 366:54-9; PMID:18060854; http://dx.doi.org/10.1016/j.bbrc.2007.11.110.