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Microarray analysis in caudal medulla of cattle orally challenged with bovine spongiform encephalopathy

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ABSTRACT. Bovine spongiform encephalopathy (BSE) is a fatal disorder in cattle characterized by progressive neurodegeneration of the central nervous system. We investigated the molecular mechanisms involved in neurodegeneration during prion infection through the identification of genes that are differentially expressed (DE) between experimentally infected and non-challenged cattle. Gene expression of caudal medulla from control and orally infected animals was compared by microarray analysis using 24,000 bovine oligonucleotides representing 16,846 different genes to identify DE genes associated with BSE disease. In total, 182 DE genes were identified between normal and BSE-infected tissues (>2.0-fold change, $P < 0.01$); 81 DE genes had gene ontology functions, which included synapse function, calcium ion regulation, immune and inflammatory response, apoptosis, and cytoskeleton organization; 13 of these genes were found to be involved in 26 different Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. The expression of five DE genes associated with synapse function (tachykinin, synuclein, neuropeptide Y, cocaine,

amphetamine-responsive transcript, and synaptosomal-associated protein 25 kDa) and three DE genes associated with calcium ion regulation (parvalbumin, visinin-like, and cadherin) was further validated in the medulla tissue of cattle at different infection times (6, 12, 42, and 45 months post-infection) by qRT-PCR. These data will contribute to a better understanding of the molecular mechanisms of neuropathology in bovine species.

Key words: Microarray; Transmissible spongiform encephalopathy; $Prion^{BSE}$; Orally challenged

INTRODUCTION

Bovine Spongiform Encephalopathy (BSE), an infectious neurodegenerative disease in cattle, is one of the fatal transmissible sponge encephalopathies (TSEs) that have been identified in many mammalian species including humans. These diseases are characterized by neurodegeneration and aggregation of aberrantly folded prion protein (Aguzzi et al., 2008). While the physiological changes associated with TSE disease in the brain are well documented, the underlying molecular events involved in neurodegeneration are poorly defined. Some studies have shown that the probability of infection and the outcome of the disease are genetically controlled (Prusiner and Scott, 1997). In sheep, mice and human, a large part of the natural susceptibility to TSE depends on alleles of the PRPN gene (Moreno et al., 2003). However, in cattle the association between *Prpn* variations and disease incidence is less well defined (Juling et al., 2006), which suggests that genes other than PRPN may be involved in susceptibility of cattle to BSE. Quantitative trait loci (QTL) studies have identified genomic regions associated with disease susceptibility, on *Bos tauros* chromosomes (BTA) 5, 10, and 20 (Hernandez-Sanches et al., 2002); BTA1, -6, -13, -17, -19 and -X (Zhang et al., 2004); and BTA2, -14, -16, -20, -21 and 28 (Murdoch et al., 2010).

In addition to QTL analysis, other approaches have been used to investigate the molecular mechanisms involved in neurodegeneration associated with prion diseases by identification of genes differentially expressed (DE) between normal and infected tissues These studies have detected differential gene expression by the analysis of cDNA libraries (Diedrich et al., 1991), mRNA differential display (Dandoy-Dron et al., 1998), suppression subtractive hybridization (Kopacek et al., 2000) and more recently using microarrays (Riemer et al., 2004; Greenwood et al., 2005; Sorensen et al., 2008). These studies have revealed multiple genes and signaling pathways that may be involved in TSE pathogenesis.

Recently, we reported 101 DE genes between normal and BSE-infected Peyer's patch tissues (Khaniya et al., 2009) and 966 DE genes in medulla (Almeida et al., 2011) in cattle orally infected with BSE agent 12 and 45 months post-infection. In the present study, a pooling approach was used in order to analyze a large number of animals and to decrease the individual variation. In total 182 DE genes were identified, from which 45% had previously been detected in a comparison between controls and both 12- and 45-month post-infection animals. Differences in gene expression associated with synapse function and calcium ion regulation were validated by qRT-PCR. Global gene expression analyses through identification of genes DE in response to BSE will help to understand the disease process and may result

in the discovery of biomarkers for disease progression, therapeutic targets and elucidate the mechanisms of neuropathology and prion replication.

MATERIAL AND METHODS

BSE challenge of cattle

Two hundred steers were selected randomly from farms with no history of BSE. These steers were randomly allocated to 2 groups of about 90 individuals in each group. Both groups were housed at the same location, but separately, in the same barn, and managed under the same conditions. The first group remained as unexposed controls; the second group was exposed orally at approximately 6 months of age in August and September 1998, with 100 grams of BSE brain homogenate with a titre of $10^{3.1}$ mouse (i.c./i.p.) units LD_{50}/g. Infected and control steers slaughtered at similar time points post-infection (PI) and tissues for transcriptome analysis were snap frozen in liquid nitrogen rapidly following slaughter. RNA was extracted from caudal medulla tissue of animals 12, 18, 40, 45 and 61 months PI in the control group, and in animals at 6, 9, 12, 42 and 45 months PI in the infected group. The challenged animals were tested for signs of disease by immunohistochemistry test (IHC) analysis of obex and medulla tissues at slaughter. Control animals did not display clinical signs of BSE and IHC test of medulla tissues were negative for PrP^{BSE}, while infected animals at 42 and 45 PI showed possible pathological signs of BSE (nervousness, hunched posture, hindlimb paresis) and IHC tests were positive for medulla and obex, confirming the BSE status of these animals.

RNA extraction

Total RNA was extracted using the RNA-Easy Column method (Qiagen, UK) according to the manufacturer instruction. The quality and quantity of RNA was measured using a Bioanalyzer Nano 6000 assay (Agilent, USA).

Microarray hybridization and data analysis

Samples from controls (N = 5) from different time points post-infection were pooled as were samples from infected (N = 5) animals. The pooling approach was used and as only a few samples were available (Kendziorski et al., 2005) in the present study only one animal was slaughtered at each time point. One microgram of the pooled RNA samples was used for microarray experiments.

Duplicates of 24,000 bovine oligonucleotide probes (www.Bovineoligo.org) representing 16,846 different genes from the bovine genome were spotted onto ultragap slides (Corning, USA) using a Q-array2 spotter (Genetix, UK). One microgram of total RNA was reverse-transcribed using Amino Allyl MessageAmp™ antisense RNA (aRNA) amplification Kit (Applied Biosystems, USA) and labeled with Cy3 or Cy5 fluorescent dyes (GE Healthcare, USA) according to manufacturer instructions. In total, 4 slides were used to compare control and infected animals, including dye swaps, and two technical replicates (two sets of 24,000 oligonucletide probes). Therefore, each gene was represented 8 times in the statistical analysis. Hybridizations were carried out in a hybridization chamber (Genetix, UK) at 42°C

overnight. Hybridized slides were washed with low stringency buffer (2X SSC and 0.5% SDS) then high stringency buffer (0.5X SSC and 0.2% SDS) and 0.05X SSC. Hybridized slides were scanned at 5 micron resolution and their signal intensities were detected by Q-Scan (Genetix, UK).

Data analysis was performed using GeneSifter™ (VizX Labs, USA). Differences in gene expression levels at different times post-infection were analyzed using the t-test statistical method. Criteria for identifying DE genes were a 2-fold or greater change in expression level, with $P < 0.01$, which was adjusted for multiple testing using Bonferroni correction (Bland and Altman, 1995). The quality filter of 95% was used to eliminate data from probes in any group that had an intensity variation larger than 5%. Gene ontology was used to determine the function of DE genes (http://www.geneontology.org).

Quantitative real-time PCR analysis

The DE genes identified were validated by qRT-PCR using individual samples from both infected and control groups (N = 10). The qRT-PCR analyses of selected genes were performed using amplified RNA due to the very limited amounts of total RNA available. Previous studies have shown that similar values for comparative expression levels are obtained when using either total RNA or aRNA (Taniguchi et al., 2008). The RNA abundance was measured using the TaqMan® Universal PCR Master Mix with gene specific minor grove binder (MGB) probes labeled with FAM and VIC fluorescent dyes (Applied Biosystems, USA). The templates used were 500 ng aRNA obtained by reverse-transcribed synthesis using an aminoallyl kit (Applied Biosystems, USA). The targeted genes, primer and probe sequences for each targeted gene are described in Table 1. Each reaction was carried out in triplicate using StepOnePlus-Real time PCR System (Applied Biosystems, USA). The thermal cycling conditions were as follows: 95°C for 20 s followed by 40 cycles of 95°C for 1 s and 60°C for 20 s. Four genes were tested as the endogenous control: GPADH, 18S rRNA, Cyc and RPL12. According to Bestkeeper software (Pfaffl et al., 2004), RPL12 showed the most stable expression in medulla tissues, and therefore was selected as an endogenous control. Cycle threshold (C_T) values for each gene were obtained for each sample. Delta Ct values for each gene were calculated by subtracting the Ct value of reference gene Ct from that of the targeted gene. Gene expressions for infected samples were quantitatively measured relative to RNA levels from control animals at a similar time post-infection (6, 12, 42, and 45 months PI). Relative quantification values were determined using the $2^{-\Delta\Delta CT}$ method and expressed as fold change in infected versus control animals.

RESULTS AND DISCUSSION

Microarray analysis of DE genes between control and BSE-infected cattle

The microarray analysis identified 182 DE genes (133 up-regulated and 49 down-regulated) between caudal medulla tissues of BSE-infected animals and control animals. Among these DE genes 81 genes (67 up-regulated and 14 down-regulated) could be assigned a function according to Gene Ontology (GO) analysis (Table 2). The main categories of GO terms assigned to the DE genes were: cellular process (35 genes), metabolic process (29 genes),

Table 1. Oligonucleotides and probes used in qRT-PCR studies.

Gene	Nucleotide sequence (5'-3')	Probe	Accession number	Gene position/exon
Synuclein	F: GGAGCAGGGAGCATTGCA	CTGCCACTGGCTTT	NM_001034041	307-361 nt/ 1
	R: TGCCCATATGATCCTTTTTGC			
Neuropeptide Y	F: CGGAGGACTTGGCCAGATAC	ACTCAGCGCTGCGAC	NM_001014845	429-490 nt/ 1
	R: TGCCTGGTGATGAGATTGATG			
Cocaine and amphetamine responsive transcript (CART)	F: CGCGAGCCCTGGACATC	TCCGCCGTGGAGGA	NM_001007820	126-188 nt/ 1
	R: GCTTCAATCAGCTCCTTCTCATG			
Synaptosomal-associated protein (SNAP25)	F: CCGTCATATGGCCCTGGAT	TGGGCAATGAGATTGATA	NM_001076246	673-731 nt /1
	R: TGTCGATCTGGCGGTTCTG			
Tachykinin 1 (TAC1)	F: CCGTGGCAGTGATTTTTTTCA	CTCCACTCAACTGTCTG	NM_174193	160-200 nt /1
	R: CGTTGGCTCCGATTTCTTCT			
Parvalbumin (PVALB)	F: CCGGAAGACGTGAAGAAGGTAT	CCACATCCTGGATAAAG	NM_001076114	357-375 nt /1
	R: CTCGATGAAGCCGCTCTTG			
Visinin-like 1 (VSNL1)	F: ATCACCCGAGTGGAGATGCT	AGATCATCGAGGCTATC	NM_174490	600-650 nt /1
	R: TCACTGTGCCCACCATTTTG			
Cadherin	F: GCCGTGTCTTCAAATGGACAA	CCATTGAAGAGCCTATG	NM_001002763	700-759 nt/1
	R: CGGTCACGGTGATCACAATC			
Bos taurus ribosomal protein L12 (RPL12)	F: AGGGTCTGAGGATTACAGTGAAA	ACCATTCAGAACAGAC	NM_205797	217-279 nt/1
	R: GATCAGGGCAGAAGCAGAAGG			

Table 2. Differentially expressed genes (DE) associated with BSE disease in caudal medulla tissues detected by microarray.

Biological process	Gene ID	Gene Name	Fold change	Previously detected
Nervous system function and synaptic transmission	Bt.12770	Synuclein, alpha*	2.0	Sisó et al., 2002
	Bt.12930	Tachykinin 1*	3.3	
	Bt.20015	Cocaine and amphetamine responsive transcript*	2.1	Sorensen et al., 2008
	Bt.33726	Neuropeptide Y*	-2.4	Diez et al., 2007
	Bt.19972	Solute carrier family 15	2.4	
	Bt.57772	Actinin, alpha 1	4.0	Greenwood et al., 2005
	Bt.42529	Synaptosomal-associated protein, 25kDa*	2.7	Skinner et al., 2006
Molecular binding				
Calcium ion binding	Bt.61440	Parvalbumin*	2.6	Voigtländer et al., 2008
	Bt.49702	Visinin-like*	10.4	Skinner et al., 2006
	Bt.64827	Cadherin 1*	-2.3	Khaniya et al., 2009
	Bt.6324	Myosin	4.8	Sorensen et al., 2008
	Bt.357	S100 calcium binding protein A12	2.4	Sorensen et al., 2008
	Bt.49037	Calmodulin-like 4	2.2	Sorensen et al., 2008
	Bt.96910	Phospholipid scramblase 1	2.2	
Zinc ion binding	Bt.85458	Similar to testis derived transcript	2.5	
	Bt.32813	SET and MYND domain containing 3	-2.2	
Actin binding	Bt.53077	Tropomyosin 2 (beta)	6.5	
	Bt.45158	Filamin A, alpha	2.9	Greenwood et al., 2005
GTP binding	Bt.60959	GTPase, IMAP family member 4	5.8	
	Bt.41393	GTPase, IMAP family member 1	2.2	
	Bt.91120	GTPase, IMAP family member 7	2.2	
Protein binding	Bt.45257'	PDZ domain containing 1	2.9	
	Bt.32810	PDZ and LIM domain 1	2.3	
	Bt.9636	Serine peptidase inhibitor, Kazal type 1	11.3	
Sugar binding	Bt.9082	Lectin, galactoside-binding, soluble, 4	3.3	Greenwood et al., 2005
Immune response				
Immune response	Bt.24326	Chemokine ligand 5	3.7	Xiang et al., 2004
	Bt.4046	Histocompatibility complex, class II, DQ alpha	-9.5	
	Bt.89024	Similar to retinoic acid early transcript	2.1	
	Bt.64557	Beta-2-microglobulin	2.7	Xiang et al., 2004
	Bt.91164	Non-classical MHC class I	3.0	
Metabolic process				
Cellular metabolic process	Bt.17717	Ubiquitin specific peptidase like 1	2.3	Sawiris et al., 2007
	Bt.1286	Retinoic acid receptor responder	2.5	
Arginine biosynthetic process	Bt.1370	Argininosuccinate synthetase 1	2.2	Greenwood et al., 2005
Metabolic process	Bt.46035	Aldolase B, fructose-bisphosphate	6.2	
	Bt.58935	Arylacetamide deacetylase	4.7	
	Bt.28307	Hypothetical LOC525823	2.9	
	Bt.43514	Double C2-like domains	2.4	
Phosphate metabolic process	Bt.20923	Eukaryotic translation initiation factor 2 kinase 2	-2.4	Sawiris et al., 2007

Continued on next page

Table 2. Continued.

Biological process	Gene ID	Gene Name	Fold change	Previously detected
	Bt.67184	Similar to AKR1C1 protein	4.1	
	Bt.66508	Similar to prostaglandin F synthase-like1	15.3	
	Bt.64820	Similar to prostaglandin F synthetase II	8.3	
	Bt.91779	Myosin IA	4.2	
Nucleoside metabolic process	Bt.34369	Phosphoribosyl pyrophosphate synthetase 2	-11.0	
Retinoid metabolic process	Bt.24055	Complement component 5	2.6	
Multicellular organismal process	Bt.16055	Transgelin	3.0	Sawiris et al., 2007
Establishment of localization				
Establishment of localization	Bt.9838	Fatty acid binding protein 6	3.9	
	Bt.26050	Solute carrier family 11	-17.1	Xiang et al., 2004
	Bt.97287	Fatty acid binding protein 2	16.4	
	Bt.74237	Organic solute transporter β	2.0	
Biological regulation				
Regulation of ARF GTPase activity	Bt.9714	Actin, gamma 2	7.7	
	Bt.3726	Development and differentiation enhancing factor 2	-7.7	
Regulation of transcription	Bt.23286	Telomeric repeat binding factor 1	-2.5	
Regulation of ATPase activity	Bt.28966	Hypothetical protein LOC100125947	4.0	
Proteolysis				
Proteolysis	Bt.27994	Alanyl (membrane) aminopeptidase	10.2	Greenwood et al., 2005
	Bt.3417	Matrix metallopeptidase 1	29.1	Sorensen et al., 2008
	Bt.11790	Glutamyl aminopeptidase	11.1	
	Bt.29080	Tubulointerstitial nephritis antigen	8.3	
	Bt.48813	Dipeptidase 1 (renal)	4.6	
Cell activity				
Enzyme activator activity	Bt.61300	Guanylate cyclase activator 2B	2.3	
Hormone activity	Bt.89771	Prolactin-related protein 2	-11.1	Tang et al., 2009
Hydrolase activity	Bt.2203	Abhydrolase domain containing	2.4	Sawiris et al., 2007
Structural molecule activity	Bt.6141	Desmin	5.5	
	Bt.28231	Claudin 7	2.6	
Transcription activity	Bt.227	Glutathione S-transferase A	2.9	Sorensen et al., 2008
Oxidoreductase activity	Bt.15632	Similar to Aldo-keto reductase family 1	20.4	Tang et al., 2009
Transport				
Sodium ion transport	Bt.62645	MHC class I heavy chain	2.9	
	Bt.48865	Nuclear receptor subfamily 1, group H, member 4	2.8	
	Bt.62049	Sodium channel, voltage-gated, type III, beta	4.4	
Neuron migration	Bt.53763	Nulear receptor subfamily 2, group F, member 2	-18.4	
Response to external stimulus				
Response to external stimulus	Bt.59155	Cytokeratin 19	6.6	
Oxidation				
Protein amino acid phosphorylation	Bt.33433	Similar to KIAA0999 protein	-19.4	
	Bt.11993	Hydroxysteroid (17-beta) dehydrogenase 11	2.4	

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Table 2. Continued.

Biological process	Gene ID	Gene Name	Fold change	Previously detected
Oxidation reduction	Bt.49451	Hydroxysteroid (11-beta) dehydrogenase 2	2.1	
Apoptosis				
Apoptosis	Bt.56652	Keratin	2.5	Booth et al., 2004
Cell cycle	Bt.91789	Structural maintenance of chromosomes 1A	3.3	
Cytoskeleton organization				
Cytoskeleton organization and biogenesis	Bt.24710	Villin 1	3.2	
	Bt.2798	Thymosin beta 10	-6.6	
Organelle organization and biogenesis	Bt.6630	Calponin 1	8.8	Xiang et al., 2004
Signaling				
Signal transduction	Bt.28525	Solute carrier family 13	2.7	
Others				
Phagocytosis	Bt.14573	ELMO/CED-12 domain containing 1	-2.0	
Urea cycle	Bt.63426	UDP glucuronosyltransferase 2	2.2	Xiang et al., 2004

*Genes selected for further qRT-PCR analysis. Gene ID = gene identification.

biological regulation (17 genes), regulation of biological process (16 genes), localization (17 genes), establishment of localization (13 genes), response to stimulus (13 genes), developmental process (10 genes) and immune system process (9 genes). Many DE genes belong to more than one GO category and for simplicity these genes are presented under biological process functional headings in Table 2. Some DE genes found in this study have been previously reported from the studies of gene expression of TSE progression using mice models, which include: Cocaine and amphetamine responsive transcript, S100 calcium binding, Myosin, Calmodulin, Matrix metalopeptidase, Glutathione S-transferase (Sorensen et al., 2008); Synuclein (Sisó et al., 2002); Parvalbumin (Voigtländer et al., 2008) Neuropeptide Y (Diez et al., 2007); Abhydrolase domain containing, Transgelin, Ubiquitin specific protease (Sawiris et al., 2007); Visinin-like (Skinner et al., 2006); Lectin galactoside-binding, Actinin, Tropomyosin, Arginine succinate synthetase, Alanyl aminopeptidase (Greenwood et al., 2005); β 2 microglobulin, Solute carrier family 11, UDP-glucuronosyltransferase, Chemokine (Xiang et al., 2004), Synaptosomal associated protein 25 kDa, and Keratin (Booth et al., 2004). Many other genes have been found to be differentially expressed in those mice studies and were not detected to be differentially expressed in our analysis in cattle. Besides being different species, the difference in infection method also could be influencing the result. The common method for BSE agent infection in mice is intraperitoneally, while in this study cattle were orally infected. Although gene expression profiling studies in mouse models of BSE infection can provide valuable insights into the possible pathological mechanisms of the disease process, studies in the natural target species (cattle) infected by natural route (oral infection) are crucial for understanding the disease processes.

Recently, Tang et al. (2009) identified 114 DE genes in brains of orally infected cattle *vs* uninfected controls and 10 DE genes identified by the authors were in common with our analysis. These genes may be an important key in pathogenic processes and need to be better investigated in animals with different genotypes, ages, doses of BSE infection and environment. The common genes detected between our analysis and Tang et al. (2009) were: S100 calcium binding and Calmodulin (up-regulated in both studies); Prolactin-related protein (down-regulated in both studies); GTPase, IMAP family member, Histocompatibility complex, class II, Metallopeptidase (variable gene expression levels in the first study and up-regulated in our analyses) and Myosin, Glutathione S transferase A, Aldo-Keto reductase family and Nuclear receptor subfamily group H (down-regulated in the first study and up-regulated in our analysis). Notwithstanding these 10 genes that are in common between the studies, many genes identified were specific to one or the other study, which most likely is because different brain regions were used in the two studies: medulla was used in the present study while brain stem was examined by Tang et al. (2009). Other factors that may have influenced the difference in results include the variation in the genetic composition of the animals; different microarray platforms and experimental conditions. In the last decade many studies have identified a large number of genes that are DE between control and TSE infected animals; however, only a few have been shown to contribute directly to prion pathogenesis (Tamgüney et al., 2008).

The DE genes identified in the present study were mapped to biochemical pathways by the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. In total, 13 DE genes were found to be associated with 26 different networks including: Neuroactive ligand-receptor interaction; MAPK signaling pathway; cell adhesion molecules (CAMs); cell communication; cell cycle; focal adhesion; PPAR signaling pathway; SNARE interactions in vesicular

transport; and regulation of actin cytoskeleton, among others (Table 3). Further work will be necessary to link these changes in expression to the biological functions involved in the development of BSE disease.

Table 3. KEGG pathway analysis of DE genes detected by microarray.

KEGG pathway analysis	Gene identified using microarray
Adherens junction	Actin, gamma 2, smooth muscle, enteric
	Cadherin 1, type 1, E-cadherin (epithelial)
Androgen and estrogen metabolism	Hydroxysteroid (11-beta) dehydrogenase 2
Focal adhesion	Actin, gamma 2, smooth muscle, enteric
Filamin A, alpha (actin binding protein 280)	
Metabolism of xenobiotics by cytochrome P450	Cytochrome P450, subfamily IIIA, polypeptide 4
UDP glucuronosyltransferase 2 family, polypeptide B10	
PPAR signaling pathway	Fatty acid binding protein 6, ileal (gastrotropin)
	Matrix metallopeptidase 1 (interstitial collagenase)
Neuroactive ligand-receptor interaction	Tachykinin 1
SNARE interactions in vesicular transport	Synaptosomal-associated protein, 25kD
MAPK signaling pathway	Filamin A, alpha (actin binding protein 280)
C21-Steroid hormone	Hydroxysteroid (11-beta) dehydrogenase 2
Cell adhesion molecules (CAMs)	Cadherin 1, type 1, E-cadherin (epithelial)
Cell cycle	gamma-Hexachlorocyclohexane degradation
Cytochrome P450, subfamily IIIA, polypeptide 4	
Glutathione metabolism	Alanyl (membrane) aminopeptidase (aminopeptidase N, aminopeptidase M, microsomal
Hematopoietic cell lineage	Alanyl (membrane) aminopeptidase (aminopeptidase N, aminopeptidase M, microsomal
Leukocyte transendothelial migration	Actin, gamma 2, smooth muscle, enteric
Linoleic acid metabolism	Cytochrome P450, subfamily IIIA, polypeptide
Melanoma	Cadherin 1, type 1, E-cadherin (epithelial)
Pentose and glucuronate interconversions	UDP glucuronosyltransferase 2 family, polypeptide B10
Porphyrin and chlorophyll metabolism	UDP glucuronosyltransferase 2 family, polypeptide B10
Regulation of actin cytoskeleton	Actin, gamma 2, smooth muscle, enteric
Renin-angiotensin system	Alanyl (membrane) aminopeptidase (aminopeptidase N, aminopeptidase M, microsomal
Glutamyl aminopeptidase (aminopeptidase A)	
Starch and sucrose metabolism	UDP glucuronosyltransferase 2 family, polypeptide B10
Thyroid cancer	Cadherin 1, type 1, E-cadherin (epithelial)
Tight junction	Actin, gamma 2, smooth muscle, enteric

Functional classification of DE genes

Synapse loss has been identified as an early and critical pathophysiological event in neurodegenerative diseases. Electron microscopic studies have shown that degeneration of synapses precedes neuronal degeneration in scrapie-infected murine hippocampus (Jeffrey et al., 2000). In addition to synapse loss, the calcium homeostasis network has also been reported to be associated with prion diseases by various researchers (Greenwood et al., 2005; Sorensen et al., 2008). Therefore, in the present study the DE genes associated with these two networks were validated by qRT-PCR (Figure 1).

Degeneration of synapses associated DE genes in BSE-infected medulla tissues

For a better understanding of the molecular events involved in synapses degeneration during disease progression in BSE-infected cattle, five genes were selected for qRT-PCR analysis at different times post-infection. Three genes (Tachykinin, Synuclein, Cocaine and

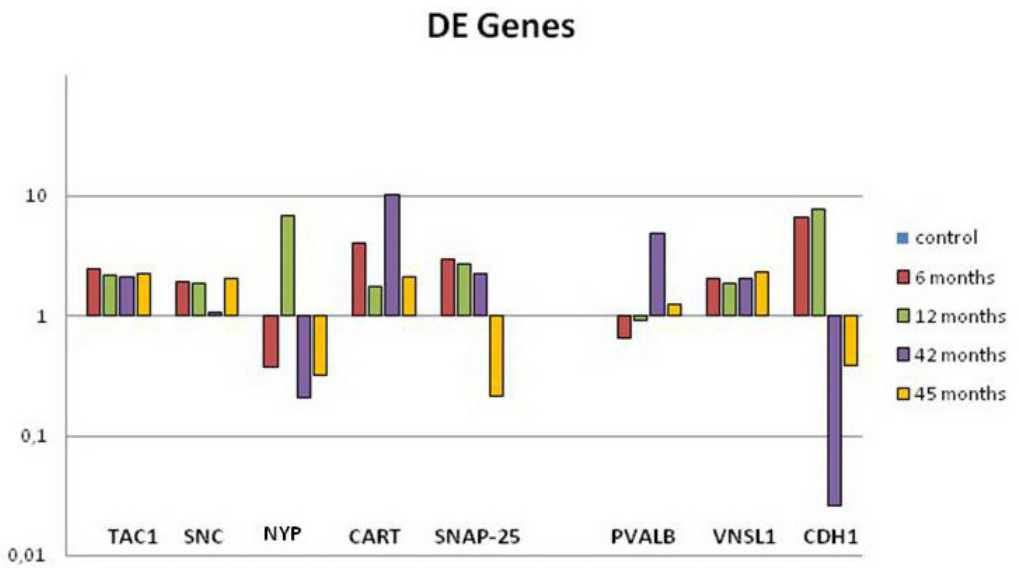

Figure 1. Quantitative real-time PCR analysis of DE genes: tachykinin (TACH1); synuclein (SNC); neuropeptide Y (NPY), cocaine amphetamine-responsive transcript (CART) and synaptosomal-associated protein 25 kDa (SNAP-25), parvalbumin (PVALB), visinin-like (VNSL1) and cadherin 1 (CDH1). Gene expression was compared between control animals and 100g infected animals at 6, 12, 42 and 45 months post-infection. Gene expressions of infected animals were quantitatively measured relative to RNA levels from control animals.

amphetamine responsive transcript) were found to be up-regulated in all time points from 6 to 45 months post-infection; the synaptosomal associated protein 25 kDa was up-regulated in almost all times post-infection except 45 months PI; while Neuropeptide Y was down-regulated in almost all times post-infection except at 12 months PI.

The biological functions of some of the DE genes identified in this study and also in previous studies were investigated further. CART codes for 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 (Xu et al., 2006). CART was found to be 2-fold overexpressed in pooled samples from infected animals vs. the control pool. This upregulation associated with infection was validated by qRT-PCR at 6, 12, 42 and 45 months PI with a 3.9-, 1.7-, 10.0- and 2.0-fold change respectively. A recent publication by Murdoch et al. (2010) using sib-TDT analysis identified two significant SNPs on BTA 20 associated with BSE incidence within the CART gene. These data suggest that there may be a genetic effect controlling susceptibility to BSE which may be mediated through the expression level of CART, which may also be a signature of the BSE infection. In addition, CART has previously been found to be DE expressed in the brain of mice infected with scrapie (Sorensen et al., 2008) and interestingly in Peyer's patches of BSE-infected cattle (Khaniya et al., 2009). These observations further suggest a role of this gene in the disease process.

Tachykinin (TAC1) is a neurotransmitter in the central and peripheral nervous system (Graham et al., 2004), smooth muscle (Savi et al., 1992), as well as being involved in immunologic and inflammatory processes (Brain, 1997). In the present study, TAC1 was found to be overexpressed in the medulla of infected animals (3.3 fold in microarray study and 2.4,

2.1, 2.0 and 2.2 at 6, 12, 42 and 45 months respectively PI by qRT-PCR). The variation in expression of TAC1 in response to prion disease was not previously detected. Further studies will therefore be necessary to investigate if changes in TAC1 expression are associated with various TSE infection models.

Presence of amyloid fibrils is considered to be a signature of the neurodegeneration in some TSEs although the mechanism behind the cellular degeneration associated with fibril formation remains unknown (Bhak et al., 2009). α-Synuclein (SNC) is an amyloidogenic protein with presynaptic and chaperone-like activity (Haik et al., 2002). This protein is associated with Parkinson's disease and contributes to the formation of intracellular Lewy body and its radiating filamentous structures (Bhak et al., 2009). SNC has also been associated with prion protein accumulation and neurodegeneration in a hamster scrapie model (Haik et al., 2002). In this study, SNC showed increased expression in samples from all infected animals, both in the pooled infected and control samples analyzed by microarray (2.0 fold) and by qRT-PCR, showing a 1.9-, 1.8-, 1.0- and 2.0-fold change at 6, 12, 42 and 45 months PI.

Neuropeptide Y (NPY) is a neurotransmitter known to be associated with a number of physiologic processes in the brain. NPY has been shown to be overexpressed in a number of TSE experimental mice models (Diez et al., 2007). According to Diez et al. (2007), the overexpression of this gene could reflect a protective and/or regenerative response counteracting neuronal degeneration resulting from cerebral nerve injury caused by PrP^{TSE} infection. However, in contrast to previous studies expression of NYP was found to decrease in infected animals (-2.4-fold change in the pooled samples) which was validated by the qRT-PCR which showed a 0.3-, 0.2- and 0.3-fold change at 6, 42 and 45 months PI, respectively.

SNAP-25 is involved in the regulation of neurotransmitter vesicle trafficking (Sollner et al., 1993), and its expression is widely distributed in the brain. Reduced SNAP-25 expression has been reported in the brain of scrapie infected mice (Sisó et al., 2002). In the present analyses, the expression of SNAP-25 was up-regulated at 6, 12 and 42 months PI (2.9-, 2.6- and 2.2-fold change respectively). However, in late stages of the disease (45 months PI), the expression of SNAP-25 was reduced (to 0.7 fold of the control value). Down-regulation of the SNAP-25 gene and dysfunction of neurotransmitter vesicle trafficking may be hypothesized in late stages of the disease with associated loss of synapse function in the medulla.

The differences of expression patterns of NPY, CART, TAC1 and SNAP-25 associated with BSE infection of cattle reported here compared with results from studies in mice suggest that responses to prion infection may differ between mice and cattle. These differences in gene expression patterns between experimental models could also be due to individual biological variation, differences in experimental design and route of infection.

Calcium homeostasis associated DE genes in BSE-infected medulla tissues

Changes in calcium homeostasis have been associated with prion disease (Greenwood et al., 2005; Sorensen et al., 2008) and other neurodegenerative disorders, such as Down's syndrome and Parkinson's disease (Muramatsu et al., 2003). The microarray of pooled infected samples compared with control samples identified a down-regulation of seven genes associated with calcium homeostasis (Table 2). Among these genes encoding Parvalbumin (PVALB), Visinin-like 1(VSNL1) and Cadherin 1 were selected for qRT-PCR analysis.

PVALB is a calcium binding albumin protein present mainly in GABAergic inter-neu-

rons of the nervous system that have been shown to influence the survival or death of neurons (Voigtländer et al., 2008). PVALB was found to be over-expressed in the caudal medulla of BSE-infected animals compared with controls (2.6 fold comparing pooled infected samples with control samples and 4.7 and 1.2 fold, respectively by qRT-PCR at 42 and 45 months PI) and down-expressed in early stage of disease (6 and 12 months PI). VSNL1 is a member of neuronal Ca^{2+} sensor protein which modulates Ca^{2+} dependent cell signaling; however, its function in other tissues remains largely unknown (Dai et al., 2006). VSNL1 was also found be down-regulated in brain tissues of scrapie infected mice (Skinner et al., 2006); however, in the present study, VSNL-1 was found to be up-regulated in both pooled samples (10.4 fold in infected *vs* controls) and qRT-PCR analyses (2.0; 1.8, 2.0 and 2.3 fold at 6, 12, 42 and 45 months PI respectively). CDH1 is a calcium dependent cell-cell adhesion membrane glycoprotein that plays an important role in adherens junctions between epithelial cells and down-regulating cell growth (Nelson, 2008). CDH1 is an important cell surface receptor of Schwann cells, and plays a pivotal role in the maintenance and regeneration of axons in the peripheral nervous system (Bhatheja and Field, 2006). This protein was found to be up-regulated at 6 and 12 months PI (6.6 and 7.7 fold, respectively) and down-regulated at 42 and 45 months PI (0.02 and 0.3 fold, respectively). Altered expression of CDH1 has not been reported previously from other TSE infected brain tissues. However, Khaniya et al. (2009) detected over-expression of CDH1 in Peyer's patch tissue of BSE-infected cattle, suggesting that it may be involved in BSE progression.

CONCLUSIONS

A better understanding of the molecular mechanism involved in BSE infection will hopefully shed some light on the mechanisms of TSE disease progression. This study identified genes that are differentially expressed in caudal medulla from BSE-infected *vs* control animals using microarray analysis. Many of the DE genes identified here have also been reported in mice models, revealing some common mechanisms associated with prion pathogenesis among different species. DE genes associated with two different biological functions (synapse function and calcium homeostasis) were validated by qRT-PCR, showing that expression patterns vary during the time course of the disease. However the study reported here used a small number of samples and additional studies are required using a larger number of animals at each time point to confirm the general validity of these results It would also be appropriate to examine the expression of these in different environments. The identification of variations in gene expression in response to BSE infection may help to understand the disease process and also provide indications of target genes and proteins that could be used in diagnosing infection or in treatment and prevention of TSE diseases.

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