University of Veterinary Medicine Hannover

Early Pathogenesis of Classical Bovine Spongiform Encephalopathy in Young Calves

Inaugural-Dissertation
in partial fulfillment of the requirements for the degree
Doctor of Veterinary Medicine
- Doctor medicinae veterinariae -
(Dr. med. vet.)

submitted by
Ivett Ackermann
Leinefelde

Hannover 2018

Academic supervision:

Prof. Dr. Martin Groschup
Institute of Novel and Emerging Infectious Diseases
Friedrich-Loeffler-Institute
Federal Research Institute for Animal Health
Greifswald - Insel Riems

PD Dr. Anne Balkema-Buschmann
Institute of Novel and Emerging Infectious Diseases
Friedrich-Loeffler-Institute
Federal Research Institute for Animal Health
Greifswald - Insel Riems

1. Referees:

Prof. Dr. Martin Groschup
Institute of Novel and Emerging Infectious Diseases
Friedrich-Loeffler-Institute
Federal Research Institute for Animal Health
Greifswald - Insel Riems

PD Dr. Anne Balkema-Buschmann
Institute of Novel and Emerging Infectious Diseases
Friedrich-Loeffler-Institute
Federal Research Institute for Animal Health
Greifswald - Insel Riems

2. Referee:

Univ.-Prof. Dr. med. vet. Wolfgang Baumgärtner
Institute of Pathology
University of Veterinary Medicine Hannover
Hannover

Day of the oral examination: 05.11.2018

Sponsorship:
This work was partially supported by a grant by WALA Heilmittel GmbH.

To my family

"How often have I said to you that
when you have eliminated the impossible,
whatever remains, however improbable,
must be the truth."
Sherlock Holmes in The Sign of the Four (1890) by Arthur Conan Doyle

Manuscripts extracted from the doctorate project:

Detection of PrP^{BSE} and prion infectivity in the ileal Peyer's patch of young calves as early as 2 months after oral challenge with classical bovine spongiform encephalopathy
Ivett Ackermann, Anne Balkema-Buschmann, Reiner Ulrich, Kerstin Tauscher, James C. Shawulu, Markus Keller, Olanrewaju I. Fatola, Paul Brown, Martin H. Groschup

Veterinary Research 2017 Dec 19; Volume 48, Issue 1, Page 88;
DOI: 10.1186/s13567-017-0495-5

Exploring PMCA as a potential *in-vitro* alternative method to mouse bioassays for the highly sensitive detection of BSE prions
Evaluierung der PMCA als in-vitro-Ersatzmethode zum Maus-Bioassay zur hochsensitiven Detektion von BSE-Prionen
Ivett Ackermann, James C. Shawulu, Markus Keller, Olanrewaju I. Fatola, Martin H. Groschup, Anne Balkema-Buschmann

Berliner und Münchener Tierärztliche Wochenschrift 2018 June 26; Volume OA, Issue 18021;
DOI: 10.2376/0005-9366-18021

Prion infectivity and PrP^{BSE} in the peripheral and central nervous system of cattle 8 months post oral BSE challenge
Ivett Ackermann, Reiner Ulrich, Kerstin Tauscher, Olanrewaju I. Fatola, Markus Keller, James C. Shawulu, Stefanie Czub, Martin H. Groschup, Anne Balkema-Buschmann

(to be submitted)

Parts of the results have already been published in poster presentations:

Junior Scientist Zoonoses Meeting 2015, 8th – 10th of June 2015, Munich, Germany:
Early pathogenesis of bovine spongiform encephalopathy in young calves after oral infection
Ivett Ackermann, Anne Balkema-Buschmann, Markus Keller, Martin H. Groschup

Junior Scientist Zoonoses Meeting 2016, 1st – 3rd of June 2016, Göttingen, Germany:
Early pathogenesis of bovine spongiform encephalopathy in young calves after oral infection
Ivett Ackermann, Anne Balkema-Buschmann, Markus Keller, Martin H. Groschup

Prion 2017: Deciphering Neurodegenerative Disorders, 23th – 26th of May 2017, Edinburgh, United Kingdom:
Detection of classical BSE prions in the ileal Peyer's patch of unweaned calves from 2 months after oral challenge
Ivett Ackermann, Anne Balkema-Buschmann, Reiner Ulrich, Kerstin Tauscher, Christine Fast, Markus Keller, James C. Shawulu and Martin H. Groschup

Table of Contents

List of abbreviations

ANS	autonomic nervous system
BSE	bovine spongiform encephalopathy
C-BSE	classical bovine spongiform encephalopathy
CJD	Creutzfeldt-Jacob disease
CMGC	coeliac mesenteric ganglion complex
CNS	central nervous system
DMNV	dorsal motor nucleus of the vagus nerve
ENS	enteric nervous system
FAE	follicle associated epithelium
FDC	follicular dendritic cell
GALT	gut-associated lymphoid tissue
GPI	glycosylphosphatidylinositol
H-BSE and L-BSE	atypical forms of bovine spongiform encephalopathy
IHC	immunohistochemistry
IPP	ileal Peyer's patch
kDa	kilodalton
LD_{50}	median lethal dose / lethal dose 50%
LRS	lymphoreticular system
M cells	microfold cells
mAb	monoclonal antibody
MBM	meat and bone meal
mpi	months post infection
PK	proteinase K
PMCA	protein misfolding cyclic amplification
PNS	peripheral nervous system
PRNP	prion protein gene
PrP	prion protein
PrP^{BSE}	BSE associated pathological prion protein
PrP^{C}	cellular prion protein
PrP^{Sc}	scrapie associated pathological prion protein
PrP^{TSE}	TSE associated prion protein (pathological isoform of the prion protein)
rPrP	recombinant prion protein
SEM	standard error of the mean
SLO	secondary lymphoid organs
SRM	specified risk materials
TBM	tingible body macrophage
Tgbov XV	bovine PrP transgenic mouse line Tgbov XV
TSE	transmissible spongiform encephalopathy
vCJD	variant form of Creutzfeldt-Jacob disease
WHO	World Health Organization

List of figures

List of tables

Chapter 1 Introduction

Transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative disorders including bovine spongiform encephalopathy (BSE) and scrapie in animals as well as Creutzfeldt-Jacob disease (CJD) in humans, as shown by the brief overview given in table 1.1.

Table 1.1 Prion diseases of humans and animals and their mechanism of pathogenesis

host species	prion disease	mechanism of pathogenesis
Human	iatrogenic CJD (iCJD)	infection from medical exposure to prion-contaminated tissues or tissue products
	sporadic CJD (sCJD)	unknown, somatic mutation or spontaneous conversion of PrP^{C} to PrP^{CJD}
	familial CJD (fCJD)	hereditary; germ-line mutations in PRNP gene
	variant CJD (vCJD)	infection through consumption of BSE-contaminated beef products (or transfusion of blood products from CJD-infected donor)
	Gerstmann-Sträussler-Scheinker syndrome (GSS)	hereditary; germ-line mutations in PRNP gene
	fatal familial insomnia (FFI)	hereditary; germ-line mutations in PRNP gene
	fatal sporadic insomnia (FSI)	unknown, somatic mutation or spontaneous conversion of PrP^{C} to PrP^{FSI}
	Kuru	infection through ritualistic cannibalism
Cattle	classical bovine spongiform encephalopathy (C-BSE)	infection through contaminated meat and bone meal or milk replacer
	atypical bovine spongiform encephalopathy (L-BSE and H-BSE)	supposedly spontaneous conversion of PrP^{C} to PrP^{BSE}
Sheep, Goats	classical scrapie	infection via ingestion and horizontal transmission (vertical transmission unclear)
	atypical scrapie	supposedly spontaneous conversion of PrP^{C} to PrP^{Sc}
Cervids	chronic wasting disease (CWD)	infection via ingestion and horizontal transmission (vertical transmission unclear)
Mink	transmissible mink encephalopathy (TME)	supposedly ingestion of BSE- or scrapie-contaminated food
Felines	feline spongiform encephalopathy (FSE)	infection through ingestion of BSE-contaminated food
Kudu, Nyala and Oryx	exotic ungulate encephalopathy (EUE)	infection through ingestion of BSE-contaminated food

Legend: CJD: Creutzfeldt-Jakob disease; modified according to PRUSINER (1998) and DONALDSON & MABBOTT (2016)

Also referred to as prion diseases, these disorders are implicated with the formation and deposition of an abnormal misfolded isoform of the cellular (i.e. endogenous) prion protein (PrP) (PRUSINER 1982, 1998).

1.1 Agent hypothesis

PRUSINER (1982, 1997) postulated that the TSE agent is a "proteinaceous infectious particle that lacks nucleic acid", referred to as prion. This *protein only hypothesis* has been deduced from the observation that scrapie infectivity was reduced using procedures modifying proteins, while the scrapie agent was resistant to treatments altering nucleic acids (PRUSINER 1982).

Other theories proposed two alternative agent hypotheses: The *virus hypothesis* argued that TSEs are caused by a slow virus infection, in which the prion protein might function as a receptor for a virus (DIRINGER et al. 1994; MANUELIDIS 1994). According to the *virino hypothesis* the infective agent is assumed to be a viral nucleic acid with a protective protein shell (DICKINSON & OUTRAM 1988).

The nature of the TSE agent has not been fully deciphered yet, although several studies provided evidence supporting the prion hypothesis. The generation of PrP^{C}-knockout mice by ablation of the prion protein gene made these animals resistant to prion disease (BÜELER et al. 1993), supporting the importance of PrP^{C} for the TSE pathogenesis. Eventually, the *in vitro* conversion and aggregation of bacterially expressed recombinant prion protein (rPrP) followed by the infection of transgenic mice was successfully (LEGNAME et al. 2004). Moreover, CASTILLA et al. (2005a) reported the *in vitro* generation of prions, using the protein misfolding cyclic amplification (PMCA) method, and these prions induced infection of wild-type hamsters. Also, infectious prions have been generated *de novo* from rPrP, and have caused prion disease upon inoculation of wild-type mice and hamsters (WANG et al. 2010; MAKARAVA et al. 2010, 2011). These recent findings were interpreted as strong evidence, if not as breakthrough, in favour of the prion hypothesis. As summarized by COLBY & PRUSINER (2011), several studies indicated that the occurrence of different prion strains (showing different biochemical, neuropathological and transmission characteristics) might rather be due to conformational variability of the associated PrP^{TSE} than due to the genetic variability of the agent (COLBY & PRUSINER 2011).

1.2 The cellular prion protein (PrP^C)

The cellular prion protein (PrP^C) is a physiological cell surface protein, which is encoded by the host's prion protein gene (PRNP) (OESCH et al. 1985) and is highly conserved among mammals (WOPFNER et al. 1999). During posttranslational modification in the endoplasmatic reticulum and the Golgi apparatus, a glycosylphosphatidylinositol (GPI) anchor is added, which binds the protein to the cell membrane after transport to the cell surface (STAHL et al. 1987; CAUGHEY et al. 1989). PrP^C is characterised by a secondary structure consisting of 42 % α-helices and only few (3 %) β-sheets (PAN et al. 1993) and a molecular mass of 33 to 35 kilodalton (kDa) (OESCH et al. 1985). Typical biochemical properties of PrP^C include its protease sensitivity and solubility in non-denaturing detergents (OESCH et al. 1985; MEYER et al. 1986).

While highest expression levels are observed in the central and peripheral nervous system (CNS and PNS), PrP^C was also found in various other organs and tissues (BENDHEIM et al. 1992; FORD et al. 2002). Also, PrP^C is abundant in the enteric nervous system, a finding that had inspired the hypothesis that PrP^C is involved in ileal contractility regulation, which was proven by a study in PrP^C-knockout mice (MARTIN et al. 2012). Changes in sleep patterns and circadian rhythms were observed for PrP^C-knockout mice (TOBLER et al. 1996), which might be connected with reported alterations in melatonin levels due to the ablation of PrP^C expression (BROWN et al. 2002). When interpreting functional studies performed in PrP^C-knockout mice, it must however be kept in mind that other proteins may be able to take over physiological functions of PrP^C, if the prion protein is lacking already during the embryonal stage (CHADI et al. 2010). As concluded by CASTLE & GILL (2017), the physiological function of PrP^C still remains largely unresolved: For instance, increasing evidence suggest a relevant role of PrP^C in myelin maintenance in the PNS, in modulation of cell proliferation and differentiation in several cell types, as well as an involvement in functions of the immune system. On the other hand, earlier indications of PrP^C involvement in copper homeostasis, stress protection and neuronal excitability modulation have been questioned by recent research.

1.3 The pathological isoform of the prion protein (PrP^{TSE})

The pathological isoform, also referred to as scrapie associated prion protein (PrP^{Sc}), is infectious and differs from the physiological PrP^C in structure as well as in its biochemical properties. In contrast to PrP^C, PrP^{TSE} has an increased β-sheet content of 43 % and a reduced α-helices content of 30% (PAN et al. 1993) and is insoluble in mild detergents (MEYER et al. 1986). Its partial resistance to proteolysis by Proteinase K or other proteases, resulting in the formation of a protease-resistant protein fragment with a mass of 27 to 30 kDa (PrP 27-30) (BOLTON et

al. 1982; OESCH et al. 1985), is routinely used in the diagnostic protein biochemical differentiation from PrP^{C}. Due to the elevated β-sheet content, PrP^{TSE} is prone to aggregate into amyloid fibrils (Figure 1.1), referred to as prion rods or scrapie associated fibrils (PRUSINER et al. 1983; PAN et al. 1993).

1.4 Conversion of PrP^{C} to PrP^{TSE}

The prion disease pathogenesis is based on the conversion of PrP^{C} to PrP^{TSE} (Figure 1.1), which involves a conformational change whereby the β-sheet content increases due to partial refolding of the α-helical structure (PAN et al. 1993; PRUSINER 1998). Further in this conversion process, PrP^{TSE} is thought to provide a template for the refolding of PrP^{C} into nascent PrP^{TSE} (PRUSINER 1998). This might be assisted by a co-factor designated protein X (TELLING et al. 1995; KANEKO et al. 1997).

Two models have been discussed to causally explain this replication mechanism, which is yet not finally clarified. Comparative kinetic analysis conducted by EIGEN (1996) suggest the correctness of both models. EIGEN (1996) emphasised that both postulated mechanisms necessarily involve an aggregated state, which is finally favoured at equilibrium and presumably a necessary precondition for infection. The *nucleated polymerisation model* (JARRETT & LANSBURY 1993; ORGEL 1996) assumes the existence of a thermodynamic equilibrium between PrP^{C} and PrP^{TSE}, until the further addition of PrP^{TSE} monomers favours forming of a polymer (primary nucleation). This primary nucleus grows to an ordered PrP^{TSE} aggregate, that can detach buds or decay into fragments, which then again act as seeds (secondary nucleation), explaining the autocatalytic nature of the replication process (JARRETT & LANSBURY 1993; ORGEL 1996). COHEN et al. (1994) proposed the *heterodimer model*, describing an intermediate partially unfolded state of PrP^{C}, named PrP*. Such a PrP* monomer can enter the formation of a heterodimer upon addition of a PrP^{TSE} monomer. Fragmentation of heterodimers provide new monomers for further conversion and exogenous supply of PrP^{TSE} (infectious prion disease) would trigger this irreversible reaction. The *heterodimer model* explains the occurrence of sporadic prion disease as a rare event in which sufficient quantity of PrP* is cumulated for the development of PrP^{TSE} (COHEN et al. 1994).

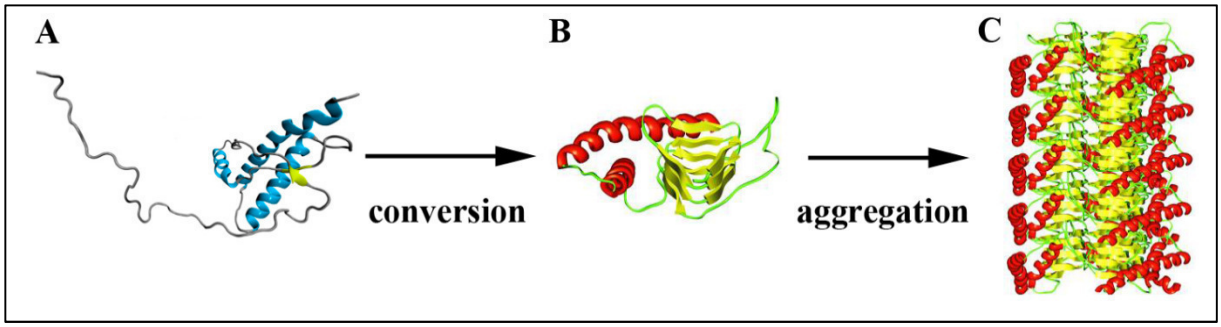

Figure 1.1 Different structures of the prion protein. A: cellular prion protein PrP^{C}, mainly consisting of α-helices (blue) and few β-sheet (yellow); **B:** after conversion to the abnormal isoform, the pathological prion protein PrP^{TSE} contains an increased β-sheet content (yellow) and less α-helices (red); **C:** following aggregation leads to the formation of scrapie associated fibrils; (illustration modified according to GOVAERTS et al. (2004) and GIACHIN et al. (2015))

1.4.1 Co-factors in prion infection and conversion

As explained above, PrP^{C}-knockout mice are resistant to prion infection, which has led to the conclusion, that it is necessary for successful prion propagation and accumulation, which is required for development of CNS disease (BÜELER et al. 1993; PRUSINER et al. 1993). However, the efficient conversion of PrP^{C} to PrP^{TSE} may still depend on co-factors, as the before mentioned protein X (TELLING et al. 1995; KANEKO et al. 1997). This is supported by *in vitro* studies, which have shown that purified hamster PrP^{C} was not converted in presence of purified PrP^{Sc}, while restitution of conversion was possible upon addition of PrP^{C}-free cell lysate or alternatively of polyanionic compounds or various other molecules (SABORÍO et al. 1999; DELEAULT et al. 2005; ABID et al. 2010). Moreover, results of an oral scrapie challenge study in sheep suggested that also in the *in vivo* situation, infectivity may not be linked to the PrP^{TSE} fraction that is detectable by immunological methods, but may resident in a different sub-fraction of PrP^{TSE} or even in molecules different from PrP (JEFFREY et al. 2006). In addition, studies performed in mice, sheep and cattle emphasise the distinction between infectivity indicated by animal bioassays and PrP^{TSE} detected by immunohistochemistry (IHC) and immunoblotting (LASMÉZAS et al. 1997; BUSCHMANN & GROSCHUP 2005; BARRON et al. 2007; BALKEMA-BUSCHMANN et al. 2011a; GONZÁLEZ et al. 2012). As summarised by SOTO (2011), cofactors participating in prion transmission can be sub-divided: Firstly, there are the above described factors contained in the infectious particle, which would therefore not exclusively consist of PrP^{TSE}. On the other hand, host factors or molecules taking part in conformational change could be necessary for prion replication, by contributing to conversion while being physiological constituents of the infected cell.

1.5 Pathogenesis of infectious prion diseases

Naturally acquired infectious TSEs are mostly due to the oral ingestion of prion infectivity, which is followed by prion uptake through the intestinal mucosa and agent replication in Peyer's patches of the small intestine. During the following neuroinvasion, prions enter intestinal nerves and spread towards the brain as the target organ.

1.5.1 Prion uptake via the intestinal mucosa after oral infection

After entering the body via the oral route, prions cross the intestinal barrier. Different mechanisms have been discussed for this yet not completely resolved process.

Microfold (M) cells, located in the follicle associated epithelium (FAE) of Peyer's patches, are specialised epithelial cells ensuring the transport of macromolecules and antigens, and initially have been shown to provide transcytosis of prions *in vitro* (HEPPNER et al. 2001; MABBOTT & MACPHERSON 2006). Recently, strong evidence of their role in prion transport *in vivo* has been provided (FOSTER & MACPHERSON 2010; TAKAKURA et al. 2011; DONALDSON et al. 2012, 2016). Mice were resistant to scrapie due to the experimentally induced depletion of M cells, which inhibited the accumulation of prions in Peyer's patches and neuroinvasion (DONALDSON et al. 2012). Vice versa, increasing the density of M cells in the intestine of mice, by treatment with RANKL (receptor activator of nuclear factor-κB ligand), improved prion uptake and led to about 10-fold higher susceptibility as well as shorter scrapie incubation times (DONALDSON et al. 2016). Moreover, prion susceptibility may vary due to variable densities of M cells or variable functionality (BORGHESI et al. 1999; TAHOUN et al. 2012; KOBAYASHI et al. 2013; BENNETT et al. 2016; DONALDSON et al. 2016): M cell densities may be increased by pathogens and inflammation or reduced with aging. M cell functionality is altered by aging for example.

Besides, different M cell independent uptake mechanism have been proposed by several earlier studies. JEFFREY et al. (2006) concluded that prion uptake can occur via the absorptive villous epithelium, as PrP^{Sc} has been detected in villi lacteals 15 min after inoculation of intestinal loops in lambs, while no PrP^{Sc} was immunohistochemically detectable in the FAE or the dome of Peyer's patch follicles underlying the FAE. This was supported by ÅKESSON et al. (2011), who evaluated the same sheep gut loop model using electron microscopy, and observed a prion transport through the villi, as PrP^{Sc} was detectable in the lacteal and submucosal lymphatics, while no PrP^{Sc} was traceable in active transcytotic FAE exosomes. Moreover, in this model, ovine rPrP was transported via macrophages through the villous epithelium, which argues that prion uptake might occur in a pattern similar to the uptake of macromolecules (ÅKESSON et

al. 2012). An M cell independent transcytosis of prions in endosomal compartments of specialised enterocytes in the FAE has been shown in the murine intestine (KUJALA et al. 2011). Furthermore, migratory classical dendritic cells can sample antigens from the intestinal lumen by inserting dendrites through the tight junctions assembling the epithelial cells, which provides another possible mechanism for the uptake of prions (RESCIGNO et al. 2001; HUANG et al. 2002; RAYMOND et al. 2007).

1.5.2 Early pathogenesis in the Peyer's patch of the ileum

Migratory classical dendritic cells as well as macrophage-like mononuclear phagocytic cells have been discussed to incorporate the transcytosed prions and transport those to lymphoid tissues and lymphoid sites of agent replication (HUANG et al. 2002; ÅKESSON et al. 2008; KUJALA et al. 2011). Further prion replication is dependent on PrP^C expression (BÜELER et al. 1993; PRUSINER et al. 1993) on the surface of follicular dendritic cells (FDCs) (BROWN et al. 1999) and in enteric neuronal cells, while prion uptake seems to occur independently of endogenous PrP^C, as it has also been observed in PrP^C-knockout mice (KUJALA et al. 2011; TAKAKURA et al. 2011).

The gut-associated lymphoid tissue (GALT) including the tonsils and Peyer's patches in the intestine as well as the mesenteric lymph nodes, represent important sites of initial prion replication and accumulation, which may be a prerequisite for neuroinvasion (BEEKES & MCBRIDE 2000; MABBOTT & MACPHERSON 2006). In orally acquired prion infection, intestinal Peyer's patches play a crucial role in the early pathogenesis, where especially the GALT in the small intestine represent sites of initial prion replication (PRINZ et al. 2003b; DONALDSON et al. 2015). In young cattle, the ileal Peyer's patch (IPP) is a packed lymphatic structure with a maximal extension of about 4 metres length at 12 – 18 months of age, which undergoes an involution that is completed at approximately 2 years of age remaining with a length of about 0.3 metres (CARLENS 1928). Peyer's patches lymphoid follicles are covered by the FAE and the underlying sub-epithelial dome (MABBOTT & MACPHERSON 2006).

As it has been shown that prions are transported by migratory classical dendritic cells or macrophage-like mononuclear phagocytic cells, prion infectivity seems to be delivered by these cells from the sub-epithelial dome to the FDCs in the germinal centre of the Peyer's patch follicles (HUANG et al. 2002; RAYMOND et al. 2007; KUJALA et al. 2011; BRADFORD et al. 2017). FDCs are long-living non-migratory, non-phagocytotic cells in the germinal centres of B cell follicles, which feature a large surface for the trapping and storing of antigens, thereby functioning as prerequisite locations of prion replication (reviewed in MABBOTT & MACPHERSON (2006) and MABBOTT (2017)). Furthermore, FDCs have been reported to

enable prion amplification above the level that is necessary for neuroinvasion, while upon their depletion, prion neuroinvasion was blocked (MABBOTT et al. 2003; GLAYSHER & MABBOTT 2007; MCCULLOCH et al. 2011). Meanwhile macrophages are able to acquire prions from FDC processes (HERRMANN et al. 2003) and seem to degrade prion infectivity without completely clearing the infection (CARP & CALLAHAN 1982; BÉRINGUE et al. 2000; MAIGNIEN et al. 2005; SASSA et al. 2010). Until the breakdown of these clearance efforts, an initial balance between prion degradation by macrophages and prion accumulation in FDCs persists (MABBOTT & BRUCE 2001). Thus, immunohistochemically detectable amounts of PrP^{TSE} in the Peyer's patch lymphoid follicles are initially observable in tingible body macrophages (TBMs) and subsequently traceable in FDCs after the clearance breakdown (VAN KEULEN et al. 2002; HERRMANN et al. 2003; HOFFMANN et al. 2011). JEFFREY & GONZÁLEZ (2007) described the temporal progression of PrP^{TSE} accumulation in lymphoid follicles in more detail: Single puncta are initially seen in TBMs, followed by weak curvilinear patterns associated with PrP^{TSE} accumulation on the surface of FDCs. Subsequently, PrP^{TSE} granules are observed in TBMs of the follicles dark zone. Later, FDC process networks are diffusively involved and multigranular accumulation in TBMs is prominent in light and dark zones of a follicle.

1.5.3 Limited immune response to prion diseases

The conflicting role of the host immune system in prion disease pathogenesis is characterised by facilitating prion replication and transport, while also trying to combat prion infection. This is especially the case for the non-specific response of the innate immune system (which is described in detail in paragraph 1.5.2): On the one hand, M cells for instance pass infectious prions through the intestinal barrier to antigen presenting cells, such as macrophages and dendritic cells, in the lumen. Subsequently, dendritic cells transport prions to FDCs, on which prion replication occurs (contrasting their normal function of trapping antibody or complement opsonized antigens). On the other hand, macrophages of GALT lymphoid follicles phagocytise prions from FDC processes (HERRMANN et al. 2003) and degrade prion infectivity but cannot completely clear the infection (CARP & CALLAHAN 1982; BÉRINGUE et al. 2000; MAIGNIEN et al. 2005; SASSA et al. 2010) as in those cases eventually developing a clinical TSE. Also, during CNS disease, microglia (serving as macrophages in the CNS) undertake a neuroprotective role by devouring prions and prion-affected cells (MABBOTT 2017).

As summarized by ZABEL & AVERY (2015), there is no prion-specific response by the adaptive immune system: Briefly, antigen representing cells cannot process and present this antigen to B cells, as these cells are blocked up and stressed by prions. Usually, large antigens are processed by the external endosomal pathway, but lysosomal proteases, like Cathepsin, cannot

efficiently digest prions. Furthermore, B cells can indeed recognise misfolded prion protein, while those are not further activated by T cells. T cells which may recognise prions would be eliminated because of their autoreaction against the endogenous protein PrP^{C}, as PrP^{TSE} and PrP^{C} share the same primary amino acid sequence. Finally, there is no humoral immune response, as antibody-producing plasma cells do not differentiate from B cells due to the failure of secondary B cell activation.

1.5.4 Involvement of the lymphoreticular system (LRS)

Generally, secondary lymphoid organs (SLO), such as lymph nodes, spleen and GALT are important sites of agent replication, which depends on prion accumulation on FDCs (as mentioned in paragraph 1.5.2).

Although individual studies reported prion infectivity in the tonsil of experimentally infected bovines (WELLS et al. 2005; ESPINOSA et al. 2007) which is most probably related to the oral uptake of infectivity, other reports showed the absence of infectivity in other LRS components, such as spleen and peripheral lymph nodes (BUSCHMANN & GROSCHUP 2005; WELLS et al. 2005; ESPINOSA et al. 2007). In summary, the LRS is, despite for the IPP, rarely involved in the BSE pathogenesis in cattle (TERRY et al. 2003; HOFFMANN et al. 2011; STACK et al. 2011; FAST et al. 2013). In contrast, initial PrP^{Sc} accumulation is commonly detectable in the tonsils of sheep (ANDRÉOLETTI et al. 2000; VAN KEULEN et al. 2002).

In contrast to the situation in cattle BSE, the substantial involvement of SLO is a specific feature of the pathogenesis of some other prion diseases, e.g. human vCJD as well as murine or ovine scrapie and cervid CWD (VAN KEULEN et al. 1996; HILL et al. 1997b; HILTON et al. 1998; MAIGNIEN et al. 1999; SIGURDSON et al. 1999; ANDRÉOLETTI et al. 2000; HEGGEBØ et al. 2000; VAN KEULEN et al. 2002; MABBOTT et al. 2003; PRINZ et al. 2003b; GLAYSHER & MABBOTT 2007; BROWN & MABBOTT 2014). After replication in the draining lymphoid tissue of those species, prions disseminate towards other SLO, like lymph nodes and spleen (MABBOTT et al. 2003; GLAYSHER & MABBOTT 2007; VAN KEULEN et al. 2008), which is thought to be provided by B cells capturing antigens from the FDCs and travelling via lymph and blood (TURNER et al. 1997; SUZUKI et al. 2009; MOK et al. 2012). As shown by studies performed in rodents, the spleen may play no or only a minor role for neuroinvasion upon oral infection (KIMBERLIN & WALKER 1989; BEEKES et al. 1996; BALDAUF et al. 1997), as splenectomy had no influence on the incubation period after intragastric infection of mice (KIMBERLIN & WALKER 1989).

1.5.5 Neuroinvasion

The term of neuroinvasion describes the prion infection of the enteric nervous system (ENS), the subsequent prion spread towards the CNS and the invasion of the brain. Whether agent replication in the GALT is a prerequisite for neuroinvasion is still uncertain.

On the one hand, agent replication upon FDCs in the GALT was found to be crucial for prion infection of the enteric nervous system (ENS), subsequent centripetal spread (towards the CNS) and invasion of the brain (BROWN et al. 1999; MABBOTT & MACPHERSON 2006; GLAYSHER & MABBOTT 2007). Also, the proximity of FDCs to sympathetic nerve endings has been shown to influence the efficiency of neuroinvasion in the spleen of mice (PRINZ et al. 2003a). But as summarized by MABBOTT (2017), the cellular mechanism by which prions are transported from the FDCs to peripheral nerves is yet unresolved, while classical dendritic cells, exosomes (small endosomal-derived vesicles) as well as tunnelling nanotubes (membrane-bound cylinders of cytoplasm) have been discussed to play a role in this transfer. The GPI anchor of the prion protein seems to play a role in this process, as impaired neuroinvasion has been observed in transgenic mice expressing PrP lacking the GPI anchor (KLINGEBORN et al. 2011).

On the other hand, as concluded by BEEKES & MCBRIDE (2000), there are three possibilities regarding the involvement of the GALT in neuroinvasion after oral infection: The GALT may either play an essential role, they may act as optional intermediaries, or they may not be involved in the neuronal infection after oral uptake. This is reflected by contradicting reports regarding the innervation of GALT lymphoid follicles. While lymphoid follicles have been reported to be poorly innervated by some groups (FELTEN et al. 1985; MARRUCHELLA et al. 2009), others observed nerve fibres in the capsule as well as within lymphoid nodules when examining ileal and jejunal Peyer's patches of 20 to 24 months old sheep using immunolabeling and electron microscopy (HEGGEBØ et al. 2003). The latter finding is supported by immunohistochemical detection of nerve fibres in the follicles sub-capsular region and the dome (CHIOCCHETTI et al. 2008).

Apart from that, the possibility of a direct neuroinvasion occurring immediately underneath the intestinal epithelium without GALT involvement has been indicated by studies showing the presence of nerve fibres in the sub-epithelial dome and by the detection of PrP^{Sc}-positive ENS plexuses with neighbouring PrP^{Sc} negative follicles (HEGGEBØ et al. 2003; JEFFREY et al. 2006; MARRUCHELLA et al. 2009; HOFFMANN et al. 2011). Moreover, nerve fibres are present in close vicinity to the lacteal epithelium and villous enterocytes (HEGGEBØ et al. 2003). Besides, BEEKES & MCBRIDE (2007) considered that this direct infection of the nervous system could be facilitated upon oral exposure to high agent doses or to highly neuroinvasive prion strains. However, the innervation of Peyer's patches seems to be rarely influenced

by age (BROWN et al. 2009; MARRUCHELLA et al. 2009), although MARRUCHELLA et al. (2009) observed fibres directly under the FAE only in adult sheep.

After infection of the ENS, prions utilize efferent fibres of the sympathetic (splanchnic nerve circuitry) and parasympathetic (vagal nerve circuitry) PNS for the retrograde ascent towards the CNS, particularly to the intermediolateral column of the grey matter in the thoracic spinal cord and the dorsal motor nucleus of the vagus (DMNV) in the brain (BEEKES et al. 1996; BALDAUF et al. 1997; BEEKES et al. 1998; GROSCHUP et al. 1999; MCBRIDE & BEEKES 1999; BEEKES & MCBRIDE 2000; MCBRIDE et al. 2001). In the spinal cord, the infection can spread both in the cranial as well as in the caudal direction (KIMBERLIN & WALKER 1982, 1989; BEEKES et al. 1996; BALDAUF et al. 1997; BEEKES et al. 1998). Moreover, the importance of the sympathetic nervous system for prion spread is indicated by an impaired neuroinvasion from the SLO of sympathectomised mice (GLATZEL et al. 2001).

Prions are thought to be in transit in peripheral nerves without active replication, which therein may impede the detection by IHC (MCBRIDE et al. 2001; HEGGEBØ et al. 2003; HOFFMANN et al. 2007). Different mechanisms of agent spread along the nerve fibres have been discussed. On the one hand, studies comparing incubation periods of rodents with the estimated axonal propagation speed, proposed the occurrence of axonal prion transport (KIMBERLIN et al. 1983, 1987; MCBRIDE et al. 2001). Recently, SHEARIN & BESSEN (2014) observed endosomal trafficking of PrP^{Sc} in axons of nerve bundles in the tongue of TME-challenged hamsters. Otherwise, non-axonal transport has been suggested, as adaxonal PrP^{TSE} accumulation between the axon and the myelin sheet has been observed (GROSCHUP et al. 1999), and experimental restriction of axonal transport did not reveal any influence on the incubation period (KUNZI et al. 2002; HAFEZPARAST et al. 2005; KRATZEL et al. 2007 a, b). As Schwann cells express PrP^{C} *in vivo*, their role in prion transport was considered after prion conversion in these glial cell of the PNS has been shown i*n vitro* (FOLLET et al. 2002). Also, conversion of PrP^{C} to PrP^{TSE} in a domino-like manner along the nerves has been considered as a possible transport mechanism (GLATZEL & AGUZZI 2000).

Different mechanisms of prion spread were considered to occur in the CNS: Ultrastructural studies of scrapie-infected mouse brains revealed that PrP^{Sc} can be released from dendrites to the extracellular space, where it accumulates prior to aggregating into fibrils (JEFFREY et al. 1994). PrP^{Sc} was also detected in endosome-like organelles, which were assumed to function as chambers for the conversion of PrP^{C} to PrP^{Sc} (ARNOLD et al. 1995). Tunnelling nanotubes, which connect cells to provide biomolecule transfer, may play a role for prion transfer within the CNS and also in the peripheral centripetal spread (CAUGHEY et al. 2009; GERDES 2009; GOUSSET et al. 2009). PrP^{Sc} was observed to be transferred between cultured neuronal cells as well as dendritic cells and neurons only *in vitro* (GOUSSET et al. 2009).

1.6 Neuropathology of prion diseases

In all TSE forms, the characteristic histopathology of the CNS involves vacuoles in neurons and in the neuropil of the grey matter (spongiform changes), neuronal degeneration and neuronal loss, astrocytic reaction as well as cerebral amyloidosis (WELLS et al. 1991; JEFFREY & GONZÁLEZ 2007).

No gross pathological changes have been reported in BSE-affected cattle, where the microscopic appearance includes remarkable spongiform changes due to neuropil vacuolation, but rarely includes neuronal vacuoles or reactive astrogliosis or neuronal degeneration (WELLS et al. 1987; WELLS & WILESMITH 1995; CORONA et al. 2017). In this process, the medulla oblongata (brainstem) is the most severely affected brain region, showing a bilateral symmetry of the histopathology in naturally BSE-affected cattle (WELLS & WILESMITH 1995; SIMMONS et al. 1996). The solitary tract nucleus (NST), the spinal tract nucleus of the trigeminal nerve (fifth [V.] cranial nerve) (NSTV) and also the dorsal motor nucleus of the vagus nerve (DMNV) in the brainstem at the level of the obex are the important target areas for the diagnostic of C-BSE (Figure 1.2).

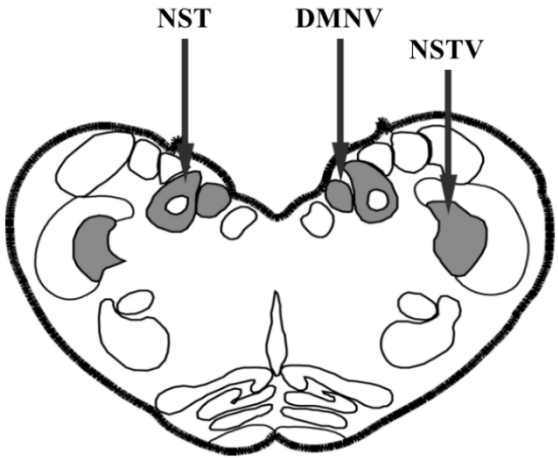

Figure 1.2 Histopathological and immunohistochemical target areas of C-BSE diagnostic shown in an illustrated cross section of the brainstem at the level of the obex. **NST:** solitary tract nucleus; **NSTV:** spinal tract nucleus of the trigeminal nerve (fifth [V.] cranial nerve); **DMNV:** dorsal motor nucleus of the vagus nerve; (illustration modified according to the OIE 'Manual of Diagnostic Tests and Vaccines for Terrestrial Animals' Chapter 2.4.5. BSE in the adopted version of May 2016)

As known from experimental BSE challenge studies, the accumulation of PrP^{BSE} occurs prior to the development of vacuolar changes and / or clinical signs (WELLS et al. 1998). Immunohistochemistry (IHC) can visualise the accumulated PrP^{BSE}, which is generally distributed in the same neuroanatomical localisations as the vacuolar changes (WELLS & WILESMITH 1995), which in C-BSE include the grey mater neuropil of the brainstem (NST, NSTV, also DMNV), midbrain and thalamus (WELLS & WILESMITH 1995; JEFFREY & GONZÁLEZ 2004; CASALONE et al. 2006; CORONA et al. 2017). The PrP^{BSE} accumulation patterns observed by IHC comprise intracellular (intraneuronal, intraglial) and extracellular (perineuronal, linear, fine punctate, coarse granular / particulate, coalescing; glial / stellate) PrP^{BSE} deposits, while vascular amyloid and classical plaques are extremely rarely seen in C-BSE-affected brains (WELLS & WILESMITH 1995; JEFFREY & GONZÁLEZ 2004; CASALONE et al. 2006; CORONA et al. 2017).

Ultrastructural studies provided evidence that most of the extracellular PrP^{TSE} co-localises with the extracellular space or is in close contact to the cell membrane of affected cells (irrespective of the accumulation morphology observed by light microscopy), while intracellular PrP^{TSE} co-localises with lysosomes (JEFFREY & GONZÁLEZ 2007; JEFFREY et al. 2011). As summarized by JEFFREY et al. (2011), the direct cause of pathological changes and clinical disease in animal TSE is still uncertain, but however may not demand PrP^{TSE}: On the one hand, as consistently observed in animal TSEs, PrP^{TSE} aggregates on membranes co-localise with toxic changes (mostly on dendritic membranes), which constitutes evidence for PrP^{TSE} toxicity. On the other hand, a loss of normal PrP^{C} function is considered to be the proximate disease cause, as some TSE-specific sub-cellular changes, such as gliosis or neuronal loss, are co-localised with PrP^{TSE} depositions, while for instance vacuoles, apoptosis or axonal terminal degeneration are not. Moreover, different studies did not reveal any direct correlation between PrP^{TSE} aggregates, morphological changes and clinical disease (JEFFREY & GONZÁLEZ 2007; JEFFREY et al. 2011).

1.7 Age-dependant susceptibility to prion infection

A negative correlation between host age and susceptibility to prion infections has been strongly indicated by studies in sheep and rodents. Susceptibility to peripheral prion infection was decreased in aged mice, as characterized by subclinical disease, which was proposedly due to an age-dependent reduction of FDCs, impairing the agent accumulation in lymphoid tissues and thus neuroinvasion (BROWN et al. 2009; BROWN & MABBOTT 2014). Similarly, ileal Peyer's patches of young lambs were shown to have better developed FDC networks than those of adult sheep, which might determine enhanced susceptibility (MARRUCHELLA et al. 2012). Indeed, susceptibility was decreased in weaned sheep (3 and 6 months old and adult) orally

challenged with BSE, as compared to lambs 2 to 3 week of age (HUNTER et al. 2012). HUNTER et al. (2012) suggested that the higher BSE susceptibility of unweaned lambs might also have been the result of a facilitated prion uptake. This is conceivable as the uptake of macromolecules across the intestinal mucosa is increased in young animals (UDALL et al. 1981; UDALL & WALKER 1982). Regarding the age-dependent susceptibility of cattle the data situation is less clear, as experimental BSE infections of young unweaned calves have not been performed thus far. Albeit, epidemiological data indicate that calves up to 6 months of age must have had a higher infection risk during the recent BSE epidemic (ARNOLD & WILESMITH 2004), while it was not determined whether this was due to a higher susceptibility or to a different feeding management in this young age group. Moreover, incorporation of proteins rich in β-sheet structure occurs more easily during the suckling period, as shown by an oral experimental infection of 2 weeks old and 6 months old calves with β-amyloid protein (ANO et al. 2008). A comparative study by ST. ROSE et al. (2006) suggested that the age-dependent development of the ileal Peyer's patch might be associated with the different risk of a prion infection in certain age groups. As mentioned above, aged animals show a reduced susceptibility to oral prion infection, which was postulated to be also partly due to a decreased density of functional mature M cells in the FAE resulting in inefficient prion uptake (KOBAYASHI et al. 2013).

The genetic susceptibility to prion disease plays an important role in sheep, but is almost negligible in cattle. While the prion protein gene (PRNP) is highly conserved among mammals (WOPFNER et al. 1999), showing a sequence homology of > 99 % in sheep and goats (GOLDMANN et al. 1996; BILLINIS et al. 2002), individual point mutations can cause polymorphisms. Amino acid exchanges in the open reading frame (ORF), the coding region, of PRNP of sheep, goats, deer and humans have been reported to modulate their susceptibility to prion infection, the disease progression, incubation period as well as the observed clinical symptoms (GOLDMANN et al. 1990, 1991a, 1996; PRUSINER 1998; BILLINIS et al. 2002; WILSON et al. 2009; SABA & BOOTH 2013). For example in sheep, polymorphisms at codons 136, 154 and 171 in the ORF are most important, resulting in 5 different alleles and various genotypes that are associated with different susceptibilities to classical scrapie and BSE (GOLDMANN et al. 1990, 1991a; LAPLANCHE et al. 1993; HUNTER et al. 1994; WESTAWAY et al. 1994; CLOUSCARD et al. 1995; HUNTER 1997; O'ROURKE et al. 1997). In contrast, coding polymorphisms seem to have a minor or negligible role in bovines, as only a polymorphism at codon E211K has been reported for one cow with atypical BSE in the USA (NICHOLSON et al. 2008; RICHT & HALL 2008). Even so, the prevalence of this E211K variant has been found to be very low in the US cattle population (HEATON et al. 2008), while the corresponding amino acid exchange of glutamate (E) to lysine (K) is a frequent mutation in human (E200K) hereditary CJD cases (KOVACS et al. 2005). Nevertheless, variations in regions outside the ORF of the bovine PRNP seem to be linked to an altered BSE

susceptibility of cattle (GOLDMANN et al. 1991b; SANDER et al. 2004, 2005; JULING et al. 2006; HAASE et al. 2007; CLAWSON et al. 2008; MURDOCH et al. 2010). Two alleles, a 23-base pair insertion/deletion in the promotor region of bovine PRNP and a 12-base insertion/deletion in intron 1, were shown to be associated with an elevated susceptibility to classical BSE (SANDER et al. 2004, 2005; JULING et al. 2006; HAASE et al. 2007).

Inflammatory conditions seem to facilitate the prion uptake via the intestinal mucosa (PAMMER et al. 2000; HUANG et al. 2002; SÖDERHOLM et al. 2004; SIGURDSON et al. 2009) and therefore enhance neuroinvasion. Challenge studies in mice and sheep with scrapie indicate that prion accumulation in chronically inflamed organs, such as pancreas, kidneys, liver or mammary gland (HEIKENWALDER et al. 2005; LIGIOS et al. 2005; SEEGER et al. 2005), which are usually free of detectable PrP^{TSE} in TSE-affected animals, may thus trigger prion excretion and transmission (SEEGER et al. 2005; LIGIOS et al. 2011; MAESTRALE et al. 2013). Also, accumulation of PrP^{Sc} has been detected in lymphoid nodules contained in inflammatory foci present in the mucosa of the abomasum, duodenum and large intestine of scrapie-challenged sheep, whereas only sparse PrP^{Sc} amounts were seen in the ENS of these locations (HEGGEBØ et al. 2002, 2003).

1.8 Classical BSE in cattle

In 1987, WELLS et al. described the first case of C-BSE that was diagnosed 1986 in the UK (WELLS et al. 1987). In Germany, the first case was reported in 1994, occurring in a Scottish Highland cow that had been imported from the UK before the implementation of the import ban (KAADEN et al. 1994). Besides gradual emaciation and a decline of milk yield, typical clinical symptoms of C-BSE include behavioural disorders (such as nervousness and anxiety or aggressiveness), as well as sensibility and movement disorders (progressively stiff gait to ataxia or lameness to recumbency) (WELLS et al. 1987; WILESMITH et al. 1988; BRAUN et al. 1998). According to BRAUN et al. (1997, 1998, 2002), sensibility disorders display as hypersensitivity to optical, acoustic and tactile stimuli, while affected animals do not necessarily show an equal level of overreaction to all three stimuli (BRAUN 2002).

According to TSE regulation (EC) No 999/2001, the competent authorities have to be informed of a clinical BSE suspicion in a herd, and the farm is put under control, which includes moving restrictions. Once the BSE suspicion has been confirmed by the National Reference Laboratory, culling and eradication measures have to be applied. Conclusive diagnostics is only possible by postmortal examination of the brainstem by laboratory methods as defined by the OIE (in the ‘Manual of Diagnostic Tests and Vaccines for Terrestrial Animals’ Chapter 2.4.5. BSE in the

adopted version of May 2016). The diagnostic methods are described in detail below (see paragraph 1.11.1).

In natural field cases, BSE is characterised by mean incubation times of about 4,5 to 5,5 years (WILESMITH et al. 1988; FERGUSON et al. 1997; ARNOLD & WILESMITH 2004), which may equate with an experimentally determined single dose of 0.1 g to 1 g of a brainstem homogenate pool of clinically diseased cattle (ARNOLD et al. 2007; WELLS et al. 2007). Albeit, for field infections of cattle it seems likely that animals have been repeatedly exposed to infectious material, which was shown to affect disease incidence and incubation periods in scrapie-challenged Syrian hamsters (DIRINGER et al. 1998; GRAVENOR et al. 2003). Generally, a reduction of the time period between repetitive challenges increased the probability of infection (DIRINGER et al. 1998; GRAVENOR et al. 2003), so that the risk of infection was highest upon a single high dose challenge as compared to repeated challenges using the same total dose (GRAVENOR et al. 2003). Similarly, PrP^{BSE} accumulation in the brainstem occurs earlier relative to the onset of clinical signs in cattle experimentally challenged with a high dose of 100g BSE-positive brain, as compared to animals challenged with lower doses (ARNOLD et al. 2007). Moreover, a 100 g dose was shown to result in a 100 % attack rate in cattle (WELLS et al. 2007) and was therefore frequently used in subsequent challenge experiments in cattle. However, such a high exposure dose seems highly unlikely to have occurred under field conditions (WELLS et al. 2007). However, the minimal infective threshold dose has not been determined for prion infections in cattle as well as in mice (MCLEAN & BOSTOCK 2000; WELLS et al. 2007).

1.8.1 C-BSE epidemic and BSE surveillance

Three decades ago, a huge BSE epidemic emerged in which infections of cattle were caused by the consumption of infected meat and bone meal (MBM) as well as milk replacer (WILESMITH et al. 1988, 1991). Ten years later the BSE epidemic was followed by the detection of vCJD cases in humans, being supposedly a consequence of the fact that about 500,000 preclinically infected cattle were slaughtered for human consumption in the UK (WILESMITH 1993; VALLERON et al. 2001).

The origin of the initial BSE cases, leading to the epidemic by entering the feed chain, is still an enigma. One theory proposes that scrapie prions have endured the treatment during MBM production and thereby may have been transmitted and adapted to cattle by this oral route (WILESMITH et al. 1988). However, there is no evidence that cattle develop BSE after oral scrapie transmission at first passage, as animals showed no signs of the disease 8 to 10 years after an oral scrapie challenge (CUTLIP et al. 2001; KONOLD et al. 2013) and intracerebrally

challenges in cattle resulted in disease phenotypes different from C-BSE (CUTLIP et al. 1994; CLARK et al. 1995; ROBINSON et al. 1995; KONOLD et al. 2006, 2015; BOLEA et al. 2017). Nevertheless, it cannot be excluded that under field conditions, cattle were exposed to a higher or accumulated dose and that the phenotype of the disease may have been altered and adapted to that of cattle BSE. Another hypothesis assumes that a sporadic case of BSE may have been responsible for the BSE epidemic onset (EDDY 1995). The conversion of the atypical H-BSE phenotype to C-BSE upon subpassage in bovine PrP transgenic and nontransgenic mice supports the theory that C-BSE may eventually have evolved from a sporadic BSE case after several interspecies passages (BARON et al. 2011; TORRES et al. 2011). In contrast, transmission of L-BSE to bovine PrP transgenic mice retained the H-BSE phenotype, while the C-BSE phenotype propagated in ovine PrP transgenic and wildtype mice (BUSCHMANN et al. 2006; BÉRINGUE et al. 2007; CAPOBIANCO et al. 2007). Recently, OKADA et al. (2017) reported a successful oral transmission of atypical L-BSE to one of 16 cattle by showing PrP^{BSE} detection in the brainstem at 88 months after infection, and immunoblot analyses revealed the PrP^{BSE} profile characteristics of L-BSE. Nevertheless, in another study of oral challenge with atypical BSE, one L-BSE- and one H-BSE-challenged cattle developed progressive clinical signs indicative of BSE after 19 and 17 mpi, respectively, while the remaining 4 animals of each group orally inoculated with these atypical BSE forms did not succumb to clinical disease thus far after 96 and 87 mpi, respectively (personal communication by S. Czub, July 2018). COLCHESTER & COLCHESTER (2005) postulated a third theory, assuming that MBM imported from India was contaminated by CJD-infected human cadavers.

As defined in Regulation (EC) No 999/2001 (EUROPEAN PARLIAMENT 2001), the feed ban for ruminant-derived MBM to ruminants (from 1988 in the UK and 2001 in the EU), the introduction of rapid testing of slaughtered cattle as well as the definition of specified risk materials (SRM) have been implemented as surveillance measures, in order to control the BSE epidemic in Europe, and have successfully brought it to a halt. Regulations have partly been lifted since the number of BSE cases declined and the BSE epidemic has mostly been overcome. Since 2013, European Member States that have implemented strict control measures have been allowed to discontinue the testing of healthy slaughtered cattle (while fallen stock surveillance is still obligatory for animals over 48 months of age) according to Decision 2009/719/EC, which was implemented in Germany in April 2015. As defined by the OIE, countries are grouped having a negligible, controlled or undetermined BSE risk status (according to the 'Terrestrial Animal Health Code' as of 2017, Chapter 11.4. Bovine spongiform encephalopathy, Article 11.4.2). Specified risk materials (SRM), which possibly contain BSE infectivity in incubating cattle, have been banned from manufacturing of food, health products and animal feedstuff, in order to minimize the risk of BSE exposure for humans and ruminants. Currently the SRM list of bovines born in EU member states with negligible BSE risk - as defined in Regulation (EC) No 999/2001 (consolidated version as of 05.2017) - includes the skull (excluding the mandible)

with brain and eyes as well as the spinal cord of animals over 12 months of age. Additionally, in case of cattle originating from countries with a controlled or undetermined BSE risk, the last 4 metres of the small intestine, tonsils, caecum, mesentery including mesenteric ganglion complex, nerves and fat from animals of all ages as well as parts of the vertebral column (including dorsal root ganglia) of animals over 30 months have to be removed and destroyed.

Consumer protection measures regarding medicinal products are regulated by the EU commission through the 'Note for guidance on minimising the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products' (EMA/410/01 rev.3) (EUROPEAN COMMISSION, 2011). This regulation defines that medicinal products shall not, or only in justified exceptions, be produced from high-infectivity tissues such as CNS and anatomically related tissues (category IA based on the WHO tables; WHO 2010). However, tissues are grouped into three major categories (IA, IB and IC with decreasing infectivity content), regardless of the stage of disease and thus the age of the animals (Note for guidance EMA/410/01 rev.3 (EUROPEAN COMMISSION 2011)). According to 'Products with risk of transmitting agents of animal spongiform encephalopathies' (a monograph enclosed in the European Pharmacopoeia), products and extracts from ruminant-derived materials may only be used in manufacturing of pharmaceuticals if the absence of BSE prion infectivity can be either shown by biological evidence or excluded due to geographical origin.

1.8.2 Intraspecies BSE transmissibility

As described above oral C-BSE infection is highly effective. But, although a slightly increased risk of BSE was estimated for the offspring of BSE-affected mothers, there is no evidence for a true vertical or horizontal BSE transmission in cattle, as the BSE agent is not shed by secretions or excretions or placental tissues (KIMBERLIN 1992; BRADBURY 1996; DONNELLY et al. 1997; WILESMITH & RYAN 1997; WILESMITH et al. 1997; VAN KEULEN et al. 2008). Recently, MURAYAMA et al. (2010) reported the detection of seeding activity by PMCA in the cerebrospinal fluid of clinically affected cattle and in the saliva of one asymptomatic cow and several clinically affected animals orally challenged with C-BSE. Furthermore, despite a single study that revealed BSE infectivity in bone marrow (WELLS et al. 1998, 1999; SOHN et al. 2009), no infectivity was detectable in any blood components or in the spleen of cattle (BRADLEY 1999; WELLS et al. 2005; BUSCHMANN & GROSCHUP 2005; ESPINOSA et al. 2007; BANNACH et al. 2013). However, MURAMAYA et al. (2010) reported scattered PrP^{BSE} amplification by PMCA in 3 out of 10 spleen samples of one clinically C-BSE-affected cow. Moreover, an intraspecies blood transfusion experiment in cattle proved the absence of infectivity in the blood of BSE-affected cattle, since the recipient cattle remained clinically healthy for 10 years, and all brainstem samples gave negative results by PMCA (oral

presentation by Balkema-Buschmann et al., 2018). Taken together, the absence of the BSE agent in the body fluids of BSE-challenged cattle seems to inhibit BSE transmission between cattle.

1.8.3 C-BSE pathogenesis

After an oral C-BSE infection, the ileal Peyer's patch functions as the primary site of entry for the agent, as PrP^{BSE} and BSE infectivity were detectable in this location from early after infection (TERRY et al. 2003; ARNOLD et al. 2009; HOFFMANN et al. 2011; STACK et al. 2011; FAST et al. 2013). Apart from that, the lymphoreticular system was shown to be rarely involved in disease pathogenesis (BUSCHMANN & GROSCHUP 2005; WELLS et al. 2005; ESPINOSA et al. 2007). The BSE agent enters the peripheral nervous system via infection of the ENS, while this neuroinvasion was shown to either occur subsequently to agent replication in the IPP (VAN KEULEN et al. 2000; HEGGEBØ et al. 2003), or directly via nerve fibres underneath the intestinal epithelium (HEGGEBØ et al. 2003; JEFFREY et al. 2006). HOFFMANN et al. (2011) observed indications for both routes in preclinical BSE-challenged cattle, but could show that the general involvement of the ENS in the PrP^{BSE} propagation seemed less pronounced in cattle than in scrapie-infected sheep (HOFFMANN et al. 2011). From 16 months post challenge, BSE infectivity was frequently present in the coeliac mesenteric ganglion complex (CMGC) (KAATZ et al. 2012). The CMGC functions as the regulator of the digestive tract and is a mixed ganglion, containing sympathetic and parasympathetic fibres. KAATZ et al. (2012) pondered, whether in their study the CMGC thereby operated as the further route of spread through these different components of the autonomic nervous system (ANS). Subsequently, BSE prions spread centripetally to the brain mainly via the ANS, which was reported to occur primarily by sympathetic structures like splanchnic nerves, the sympathetic ganglia chain and the cranial cervical ganglion (HOFFMANN et al. 2007; KAATZ et al. 2012). Parasympathetic fibres, like the vagal nerve and nodal ganglion, were involved to a lesser extent and considered as alternative routes (HOFFMANN et al. 2007; KAATZ et al. 2012). Besides, the spinal cord seemed to constitute an additional route (HOFFMANN et al. 2007; KAATZ et al. 2012). These routes resulted in PrP^{BSE} accumulation in the sympathetic cranial cervical ganglion, via which the agent then may have entered the brain at the level of the obex (KAATZ et al. 2012) as well as in parasympathetic regions of the obex (DMNV) (HOFFMANN et al. 2007). A detailed overview of the mentioned tissues involved in C-BSE pathogenesis of cattle with the time points of earliest prion detection are summarized in Table 1.2.

A centrifugal spread from the CNS via peripheral nerves to peripheral tissues in the final stage of disease is conceivable, as infectivity was detectable in various muscles, the adrenal gland,

the nasal mucosa and tongue of clinically BSE-affected cattle (BUSCHMANN & GROSCHUP 2005; MASUJIN et al. 2007; BALKEMA-BUSCHMANN et al. 2011a; FRANZ et al. 2012; OKADA et al. 2014).

Table 1.2 Lymphoreticular and nervous tissues involved in C-BSE pathogenesis with the earliest time points (in mpi) of detection of PrP^{BSE} and infectivity

				tissue	**PrP^{BSE}**		**infectivity**	
					mpi	detection method	mpi	bioassay mouse line
gut-associated lymphoid tissue (GALT)				IPP	4 (1)	IHC	4 (2)	Tgbov XV
				tonsil	46 (3)	IHC	20 (4)	BoPrP-Tg110
peripheral nervous system (PNS)	enteric nervous system (ENS)				16 (5)	IHC	n. a.	n. a.
	autonomic nervous system (ANS)	mixed sympathetic and parasympathetic ganglia		coeliac ganglion	24 (5)	IHC	16 (6)	Tgbov XV
				caudal mesenteric ganglion	24 (5)	IHC	32 (6)	Tgbov XV
		sympathetic nervous system		splanchnic nerve	n. d.	n. a.	16 (6)	Tgbov XV
				stellate ganglion	34 (7)	WB	24 (6)	Tgbov XV
				cranial cervical ganglion	36 (6)	IHC	16 (6)	Tgbov XV
		parasympathetic nervous system		vagal nerve (thoracic / cervical part)	44 (6) / 32 (6)	IHC	28 (6) / 20 (6)	Tgbov XV
				nodal ganglion	44 (6)	IHC	20 (6)	Tgbov XV
	somatic nervous system			trigeminal ganglion	32 (8)	IHC	36 / 38 (9)	C57bl / RIII
central nervous system (CNS)	thoracic spinal cord				24 (6)	IHC	16 (6)	Tgbov XV
	brainstem:			obex / caudal medulla	24 (5)	IHC	24 (6)	Tgbov XV

Legend: mpi: months post infection (experimental oral challenge); IPP: ileal Peyer's patch; n. d.: not detected; n. a.: not applicable; IHC: immunohistochemistry; WB: Western blot; PMCA: protein misfolding cyclic amplification;
(1) (HOFFMANN et al. 2011) / **(2)** (FAST et al. 2013) / **(3)** single finding by OKADA et al. (2011a) / **(4)** (ESPINOSA et al. 2007) / **(5)** (HOFFMANN et al. 2007) / **(6)** (KAATZ et al. 2012) / **(7)** (MURAYAMA et al. 2010) / **(8)** (ARNOLD et al. 2007; SIMMONS et al. 2011; KAATZ et al. 2012) / **(9)** (ARNOLD et al. 2009)

1.8.4 Zoonotic potential of BSE

The occurrence a new variant form of CJD (vCJD) 10 years after the notification of the first BSE cases and in the most affected countries raised concerns that BSE may have been transmitted to humans (WILL et al. 1996). BSE was then confirmed to be a zoonotic disease, when vCJD-infected mice showed uniform pattern of incubation times and brain pathology like that of BSE-infected mice and thereby brought strong evidence that the ingestion of BSE-contaminated food may cause vCJD (BRUCE et al. 1997; HILL et al. 1997a). Passaging BSE prions in human PrP transgenic mice propagated prions of the vCJD like phenotype but also of a sporadic CJD phenotype, which was considered as evidence that some sporadic CJD cases may have arisen due to BSE exposure (ASANTE et al. 2002). Moreover, the zoonotic potential of BSE was confirmed, when JONES et al. (2009) reproduced *in vitro* the conversion of BSE prions to human prions as shown by the PMCA amplification of BSE prions in a brain substrate of transgenic mice expressing the human PrP^{C}, while no amplification was observed when sheep scrapie prions were used as a seed.

1.9 Atypical BSE in cattle

In 2004, 2 atypical forms of BSE were first identified in France and Italy (BIACABE et al. 2004; CASALONE et al. 2004) and have, amongst other countries, also been detected in Germany (BUSCHMANN et al. 2006). These two distinct BSE strains are characterised by different PrP^{BSE} profiles in immunoblot analyses: The H-BSE strain shows, similar to classical scrapie, a higher (STACK et al. 2002; BIACABE et al. 2004) and L-BSE a lower (CASALONE et al. 2004) molecular mass of the nonglycosylated PrP^{BSE} moiety, as compared to that of C-BSE. Moreover, especially the L-BSE strain shows a decreased proportion of diglycosylated PrP^{BSE} and displays a predominance of the monoglycosylated moiety (BIACABE et al. 2004; CASALONE et al. 2004; BARON et al. 2007). Upon transmission of atypical BSE strains to bovine PrP transgenic mice, a H-BSE infection resulted in prolonged incubation times, while L-BSE challenge provoked shortened incubation periods (BUSCHMANN et al. 2006) as compared to the incubation times of C-BSE in this mouse line. Contrasting to the typical granular and linear IHC accumulation pattern in brainstems of C-BSE-infected cattle, a significant different nature of brain lesions was seen in L-BSE: Amyloid plaques were observed mainly in the thalamus and cortex, which led to the original designation bovine amyloidotic spongiform encephalopathy (BASE) (CASALONE et al. 2004).

Atypical BSE cases have occurred in cattle older than 8 years and have initially been detected during routine surveillance (BIACABE et al. 2004; CASALONE et al. 2004; BUSCHMANN et al. 2006; BALKEMA-BUSCHMANN et al. 2011b; STACK et al. 2013). In accordance to

this, atypical BSE-challenged animals have developed clinical signs after slightly prolonged incubation period with a shortened and more progressive disease course, as compared to intracerebrally C-BSE-challenged cattle (DAWSON et al. 1990; LOMBARDI et al. 2008; BALKEMA-BUSCHMANN et al. 2011c; KONOLD et al. 2012). In cattle challenged experimentally with atypical BSE, the clinical picture included weight loss and depression, while the observed ataxia and hyperaesthesia has impaired the distinction from classical BSE symptoms (LOMBARDI et al. 2008; FUKUDA et al. 2009; BALKEMA-BUSCHMANN et al. 2011c; OKADA et al. 2011b; KONOLD et al. 2012). Moreover, the pattern of PrP^{BSE} accumulation in peripheral tissues was similar to that observed for C-BSE (BALKEMA-BUSCHMANN et al. 2011b). In cattle intracerebrally challenged with atypical BSE, PrP^{BSE} has been detected in peripheral nerves and ganglia (IWAMARU et al. 2010; OKADA et al. 2011b; KONOLD et al. 2012; OKADA et al. 2013) as well as muscles (KONOLD et al. 2012; SUARDI et al. 2012), while no PrP^{BSE} has been detectable in lymphoid tissues including tonsil and Peyer's patches or the ENS (IWAMARU et al. 2010; BALKEMA-BUSCHMANN et al. 2011b; OKADA et al. 2011b; KONOLD et al. 2012; OKADA et al. 2013). Absence of the detectable PrP^{BSE} in the ileal Peyer's patch is comprehensible, as intracerebral challenge is bypassing the gastrointestinal tract (KONOLD et al. 2012), which may mirror the situation in natural atypical BSE cases.

Furthermore, the lower level of involvement of the brainstem including the DMNV compared to other brain regions suggest that the route of prion spread is independent from the gastrointestinal tract (CASALONE et al. 2004). This finding together with the detection of atypical BSE that is independent on the C-BSE endemic situation as well as the occurrence of H- and L-BSE exclusively in older animals, argue for a sporadic origin of atypical BSE (CASALONE et al. 2004; JACOBS et al. 2007; BALKEMA-BUSCHMANN et al. 2011b). BOUJON et al. (2016) concluded, that control measures may never succeed in eradicating atypical BSE forms, if those are actually sporadic and therefore a low level of L- and H-BSE might be constantly existent in the cattle population. Considering that rapid testing of healthy slaughtered cattle is no longer designated by European regulation, it should be noted that transmission studies to macaques (COMOY et al. 2008) and human PrP transgenic mice (BÉRINGUE et al. 2007; KONG et al. 2008) provided indications of a higher zoonotic potential of L-BSE as compared to C-BSE (LOMBARDI et al. 2008; BALKEMA-BUSCHMANN et al. 2011b).

1.10 BSE in small ruminants

Since 2002, according to European regulation (EC) 999/2000, surveillance measures for TSE include also the examination of small ruminants. Experimental challenge proved that sheep and goats are susceptible to BSE infection (FOSTER et al. 1993). In contrast to cattle, sheep BSE was shown to be transmissible by an experimental blood transfusion (HOUSTON et al. 2000,

2008). BSE in sheep shows a progressive disease course including primarily pruritus, ataxia and trembling (FOSTER et al. 2001; HOUSTON & GRAVENOR 2003), while clinical signs in goats comprise lethargy, progressive weight loss and ataxia (FOSTER et al. 2001; KONOLD et al. 2010). Thereby, clinical BSE in small ruminants exhibits symptoms similar to classical scrapie. In sheep and goats, natural classical scrapie clinically manifests as nervousness and aggressiveness, pruritus, trembling, loss of body condition, hypermetric gait and hindlimb ataxia, proceeding to recumbency and finally death (PARRY 1983; WOOD et al. 1992; CAPUCCHIO et al. 2001; FOSTER et al. 2001; HEALY et al. 2003; KONOLD et al. 2007, 2010). A TSE monitoring programme was established in the European Union (as defined in Regulation (EC) No 214/2005) to determine the incidence of BSE infections in small ruminants in order to evaluate the exposure risk for consumers. Only 2 natural BSE cases in goats have been reported to date (ELOIT et al. 2005; SPIROPOULOS et al. 2011), while no natural BSE case in sheep has been detected. Nevertheless, precautionary TSE monitoring measures seem advisable, considering that 2 studies showed the generation of phenotypically vCJD like prions by passaging experimental sheep and goat BSE prions in human PrP transgenic mice (PADILLA et al. 2011; JOINER et al. 2018).

1.11 Diagnostic possibilities for the detection of BSE in cattle

This paragraph provides an overview of methods currently used in routine BSE diagnostics as well as for research purposes regarding BSE pathogenesis, with focus on those methods used during the early BSE pathogenesis study presented in this thesis. Detailed procedures and protocols can be found in manuscripts I to III (Chapters 2, 3 and 4).

1.11.1 Methods applied in routine diagnostics

BSE can only be diagnosed upon postmortal examination of the brainstem region and the therefor used routine diagnostic methods are defined by the OIE ‘Manual of Diagnostic Tests and Vaccines for Terrestrial Animals’ (Chapter 2.4.5. BSE in the adopted version of May 2016). For monitoring purposes (active surveillance measure for TSE of ruminants according to Regulation (EC) 999/2001) a rapid test can be performed on a sample taken from 1 cm rostral or caudal of the obex (cranial and caudal medulla, respectively). In case of investigating a suspect clinical case, besides the obex sample collected for initial diagnostics, the whole brain and other tissues should be reserved for additional examination.

Protein biochemical methods are based on the partial resistance of the pathological prion protein (PrP^{TSE}) to proteolysis by Proteinase K (PK), resulting in the formation of a protease-resistant

protein fragment with a mass of 27 to 30 kDa (PrP 27-30) (BOLTON et al. 1982; OESCH et al. 1985), while the physiological PrP^{C} is completely hydrolysed. After this digestion step, anti-PrP antibodies bind to the PK resistant PrP^{BSE}, which provides the basis for most of the approved rapid and confirmatory tests. In Germany, for example, the BioRad TeSeE™ SAP rapid test (BioRad, Marnes-la-Coquette, France) as well as the IDEXX HerdChek® BSE-Scrapie Antigen Test Kit (IDEXX Europe B.V., Hoofddorp, Netherlands) are most frequently used (according to European Regulation (EC) 999/2001 Annex C Chapter X). In case that the sample gives repeatedly reactive results in rapid test performed by the regional laboratory, this TSE suspect case has to be further investigated by confirmatory methods in the National Reference Laboratory for TSEs. In the German National Reference Laboratory, the sample is subjected to a preparation of the scrapie-associated fibrils (SAF) in order to concentrate PrP^{BSE}, followed by electrophoretic separation of PrP^{BSE} in an immunoblot (Western blot). Application of this method allows the interpretation of the glycosylation pattern of the 3 characteristic protein bands (displaying the di-, mono- and non-glycosylated moieties of the prion protein) for strain characterisation (KUCZIUS et al. 1998). Therefore, in case of a C-BSE-positive result, the presence of PrP^{BSE} is confirmed by 3 bands migrating at approximately 30, 25 and 19 kDa with the diglycosylated band representing the strongest signal (KUCZIUS et al. 1998). The different molecular mass profiles have been described above for the atypical BSE strains (in paragraph 1.9).

Moreover, histopathological analyses to a lesser extent, but especially immunochemistry (IHC) are further confirmatory methods, which are performed on the same formalin-fixed paraffin-embedded sample of the obex region. For histopathological evaluation, haematoxylin and eosin stained sections are analysed for peri- and intraneuronal vacuolations. IHC demonstrates the presence of PrP^{TSE} accumulation in tissue samples based on the binding of mono- or polyclonal anti-PrP antibodies to specific epitopes of PrP^{TSE} and enables the assessment of the PrP^{TSE} depositions on a cellular level (MILLER et al. 1993; HARDT et al. 2000). While autolysis of the brain would render a reliable histopathological diagnosis impossible, the IHC is able to detect PrP^{TSE} also in autolytic or prior frozen samples (MILLER et al. 1993). The histopathological findings and IHC accumulation patterns have been described in more detail above (in paragraph 1.6).

1.11.2 Detection of BSE infectivity using mouse bioassays

Animal bioassays provide the exclusive option to determine prion infectivity in a tissue sample. WATTS & PRUSINER (2014) summarised that in general, bioassays in mice are preferred to those in hamsters, due to the better availability of tools for mouse genome manipulation and

thus the availability of a variety of highly sensitive prion transgenic mouse models. Prion-challenged mice display all characteristics of prion disease neuropathology, such as spongiform changes, astrogliosis and PrP^{TSE} accumulation, while 100% of the animals succumb to the disease upon a challenge with a high-titre sample (WATTS & PRUSINER 2014). In contrast, PrP-knockout mice ($Prnp^{0/0}$ mice) are resistant to prion disease (BÜELER et al. 1993) and thereby provided evidence that PrP^{C} is crucial for prion propagation.

$Prnp^{0/0}$ mice carrying the PrP gene of another species were developed as TSE models which lack the species barrier, thereby representing a highly sensitive method for the detection of even small amounts of prion infectivity in tissue samples. Thus, a transgenic mouse bioassay is frequently used to trace BSE infectivity in samples from preclinical cattle experimentally challenged with BSE. Bovine PrP transgenic mouse lines featuring an 8-fold over-expression of bovine PrP^{C} include Tgbov XV (BUSCHMANN et al. 2000; BUSCHMANN & GROSCHUP 2005), Tg4092 (SCOTT et al. 1997; SAFAR et al. 2002) and boTg110 (CASTILLA et al. 2003). Transgenic Tgbov XV mice are highly susceptible to the BSE agent, showing shorter incubation periods and providing a 10,000-fold sensitivity as compared to conventional RIII mice (BUSCHMANN et al. 2000; BUSCHMANN & GROSCHUP 2005). By end-point titration in Tgbov XV mice (BUSCHMANN & GROSCHUP 2005) the infectivity load (LD_{50}-titer) of a brainstem pool used for the challenge of experimental cattle can be determined (which is described in more detail in the methods section of manuscript I). In BSE pathogenesis studies, the Tgbov XV mice bioassay is used to analyse the presence of BSE infectivity in tissues sampled from the experimental animals. A detailed protocol of the bioassay procedure and mouse brain analyses is given in manuscript I. The transgenic mouse bioassay is the gold standard method of prion detection as it displays a very high sensitivity, although it is costly and time-consuming (as mentioned in manuscript II).

1.11.3 *In vitro* amplification of PrP^{BSE} by PMCA

In vitro amplification techniques detecting the prion seeding activity associated with replication, like Protein Misfolding Cyclic Amplification (PMCA) (SABORÍO et al. 2001) and Real-Time Quaking-Induced Conversion (RT-QuIC) (ATARASHI et al. 2008; WILHAM et al. 2010) have enabled the detection of low quantities of prions. To date, these assays are only used for research purposes, as have not yet been adjusted as a routine procedure for disease diagnostics (SAÁ & CERVENAKOVA 2015). Nevertheless, these methods can be considered a successful step towards rapid and sensitive high-throughput techniques (CORONA et al. 2017). Moreover, PMCA and RT-QuIC allow the quantification of the amplified PrP^{TSE} products (CHEN et al. 2010; WILHAM et al. 2010; ATARASHI et al. 2011; HENDERSON et al. 2015).

The PMCA, initially described in 2001 by SABORÍO et al., is based on the cyclic amplification of the misfolded prion protein: A PrP^{TSE} aggregate (seed) provides a template that enlarges by a fast conversion of excess PrP^{C} (substrate), which is followed by cycles of sonication and incubation (SABORÍO et al. 2001). Sonication is used to break up the grown PrP^{TSE} seed, which then makes new seeds available for the conversion of PrP^{C} to PrP^{TSE} during the following incubation cycle (SABORÍO et al. 2001). The method was successively developed further by using programmable sonicators (automated PMCA), as well as by performing several rounds of PMCA by diluting an aliquot from the reaction mix after various cycles of amplification into fresh substrate for a new round of amplification / sonication cycles (serial PMCA) (BIESCHKE et al. 2004; CASTILLA et al. 2005a, b, 2006; SAÁ et al. 2006). Thereby, PMCA made prion detection in samples with even minor amounts of the agent possible, as it amplifies PrP^{TSE} to levels which can be detected by standard protein biochemical methods, such as immunoblot (SABORÍO et al. 2001; SAÁ & CERVENAKOVA 2015). Moreover, quantitative PMCA allows an estimated PrP^{TSE} quantification in a sample, which is premised on the relation between the number of PMCA rounds or cycles needed for the PrP^{TSE} detection and the PrP^{TSE} amount in a predefined sample (CHEN et al. 2010). It has to be noted that increasing numbers of amplification rounds can favour spontaneous *de novo* generation of PrP^{TSE} and therefore the numbers of rounds should be restricted considering PMCA protocol implementation (SAÁ et al. 2006; FRANZ et al. 2012; MORALES et al. 2012). Additionally, adequate negative controls are particularly important to monitor the risk of cross-contamination during the performance of such an ultra-sensitive method which has been reported to enable the detection of a single infectious PrP^{TSE} unit (SAÁ et al. 2006; SAÁ & CERVENAKOVA 2015).

The PMCA method was initially developed using the hamster-adapted scrapie strain 263K, for which meanwhile protocols have been reported that provide comparable or even higher sensitivities to scrapie bioassays in Syrian hamsters and bank voles (SAÁ et al. 2006; BOERNER et al. 2013; MORALES et al. 2013; CHIANINI et al. 2015). Such an ultra-sensitive detection of scrapie prions argues for the replacement of the hamster bioassay for the detection of 263K scrapie and moreover indicates that this *in-vitro* test may also be suitable as an alternative to animal bioassays for other species. Albeit, the PMCA amplification of bovine BSE prions was not that successful for a long time, but recently reported protocols let anticipate similar improvements focused on at least comparable sensitivities to bioassays in transgenic mice (MURAYAMA et al. 2010; BALKEMA-BUSCHMANN et al. 2011a; FRANZ et al. 2012). Nevertheless, the analytical sensitivity and the reproducibility of this method still should be optimised in order to improve the PMCA to a reliable alternative method for mouse bioassays in line with the principle of the 3Rs (Replace, Reduce, Refine) (first published as "*The Principles of Humane Experimental Technique*" by RUSSEL & BURCH in 1959). A replacement of

mouse bioassays by PMCA would reduce the numbers of experimental mice used in TSE research to a minimum, while in the still necessary bioassays would be refined as the inoculated animals would rarely succumb to clinical disease.

1.12 Aims of this thesis

Thus far, oral C-BSE pathogenesis studies in cattle were focused on relatively late time points after oral challenge, in order to decipher the principles of the BSE pathogenesis and the agent distribution in late stages of the disease (ARNOLD et al. 2007; HOFFMANN et al. 2007; ARNOLD et al. 2009; KAATZ et al. 2012). Thereby, the neuronal pathways of BSE prion spread in cattle were revealed. Despite identifying the ileal Peyer's patch as the entry port for the BSE agent in the intestine (TERRY et al. 2003; ARNOLD et al. 2009; HOFFMANN et al. 2011; STACK et al. 2011; FAST et al. 2013), data on the early phase of pathogenesis are rare. In these studies cattle were challenged at 4 to 6 months of age (WELLS et al. 1996; HOFFMANN et al. 2007). However, epidemiological data suggested that calves up to 6 months of age had a higher risk of infection during the BSE epidemic (ARNOLD & WILESMITH 2004), while it was not determined whether this was due to a higher susceptibility or to a different feeding regime in calves. An age-related evaluation appears to be crucial, as studies in sheep and rodents indicated a higher susceptibility of young animals to prion infection (BROWN et al. 2009; HUNTER et al. 2012; BROWN & MABBOTT 2014). However, an experimental BSE infection of young unweaned calves had never been performed thus far.

In the study described in this thesis, calves were inoculated before weaning at 4 to 6 weeks of age, so as to simulate the worst-case scenario of supposedly highest prion susceptibility. Twenty unweaned Simmental calves were orally challenged with C-BSE, while two additional animals served as negative controls. Then, eighteen infected calves were euthanised at predetermined time points of 1 week as well as 2, 4, 6 and 8 months after oral challenge. During necropsy, a wide range of tissue samples were taken under TSE-sterile conditions (Table 9.1 in Chapter 9).

The first aim of this study was to track the initial phase of C-BSE infection and to understand the time course of the centripetal spread towards the brain (manuscripts I and III). This also includes to determine whether the BSE infection progress is influence by the age of cattle by using unweaned calves (manuscript I and III).

This thesis put emphasis on examining the early BSE pathogenesis in the ileal Peyer's patch (manuscript I) and in nervous tissues in the vicinity to the intestine (manuscript III). As a working hypothesis based on the current state of knowledge (ARNOLD et al. 2007; HOFFMANN et al. 2007; ARNOLD et al. 2009; KAATZ et al. 2012), this study postulated that the peripheral and central nervous system are most probably free of PrP^{BSE} and BSE prion infectivity at such

early time points after oral infection. Therefore, only highly sensitive detection methods (IHC, PMCA and Tgbov XV mouse bioassay) (BUSCHMANN & GROSCHUP 2005; BALKEMA-BUSCHMANN et al. 2011a; HOFFMANN et al. 2011) were applied, as less sensitive methods such as Western blot and rapid test (ARNOLD et al. 2007; BARRON et al. 2007; BALKEMA-BUSCHMANN et al. 2011a; HOFFMANN et al. 2011; GONZÁLEZ et al. 2012) were considered not to be adequate to prove or dismiss the working hypothesis. This thesis is based on the results obtained in this comprehensive study so far, while several PMCA analyses and mouse bioassays are still ongoing.

The second aim of this study was to compare the diagnostic sensitivities of the methods used here. Thus, the analytical sensitivities of the current PMCA protocol and the Tgbov XV mouse bioassay were compared, in order to explore the potential of the PMCA as an *in-vitro* alternative method to mouse bioassays (manuscript II). Thereby, it was evaluated whether mouse bioassays, constituting often lethal animal experiments, could be reduced in future BSE pathogenesis studies.

Chapter 2 Manuscript I

Detection of PrP^{BSE} and prion infectivity in the ileal Peyer's patch of young calves as early as 2 months after oral challenge with classical bovine spongiform encephalopathy

Ivett Ackermann[1], Anne Balkema-Buschmann[1], Reiner Ulrich[2], Kerstin Tauscher[2], James C. Shawulu[1], Markus Keller[1], Olanrewaju I. Fatola[1], Paul Brown[3] and Martin H. Groschup[1]

[1] Institute of Novel and Emerging Infectious Diseases, Friedrich-Loeffler-Institute, Greifswald-Insel Riems, Germany

[2] Department of Experimental Animal Facilities and Biorisk Management, Friedrich-Loeffler-Institute, Greifswald-Insel Riems, Germany

[3] National Institute of Neurological Diseases and Stroke, National Institutes of Health, Bethesda, Maryland, USA

Abstract

In classical bovine spongiform encephalopathy (C-BSE), an orally acquired prion disease of cattle, the ileal Peyer's patch (IPP) represents the main entry port for the BSE agent. In earlier C-BSE pathogenesis studies, cattle at 4–6 months of age were orally challenged, while there are strong indications that the risk of infection is highest in young animals. In the present study, unweaned calves aged 4–6 weeks were orally challenged to determine the earliest time point at which newly formed PrP^{BSE} and BSE infectivity are detectable in the IPP. For this purpose, calves were culled 1 week as well as 2, 4, 6 and 8 months post-infection (mpi) and IPPs were examined for BSE infectivity using a bovine PrP transgenic mouse bioassay, and for PrP^{BSE} by immunohistochemistry (IHC) and protein misfolding cyclic amplification (PMCA) assays. For the first time, BSE prions were detected in the IPP as early as 2 mpi by transgenic mouse bioassay and PMCA and 4 mpi by IHC in the follicular dendritic cells (FDCs) of the IPP follicles. These data indicate that BSE prions propagate in the IPP of unweaned calves within 2 months of oral uptake of the agent.

Veterinary Research 2017 Dec 19, Volume 48, Issue 1, Page 88
https://veterinaryresearch.biomedcentral.com/articles/10.1186/s13567-017-0495-5
DOI: 10.1186/s13567-017-0495-5

Chapter 3 Manuscript II

Exploring PMCA as a potential *in-vitro* alternative method to mouse bioassays for the highly sensitive detection of BSE prions

Ivett Ackermann[1], James C. Shawulu[1], Markus Keller[1], Olanrewaju I. Fatola[1], Martin H. Groschup[1], Anne Balkema-Buschmann[1]

[1] Institute of Novel and Emerging Infectious Diseases, Friedrich-Loeffler-Institute, Greifswald-Insel Riems, Germany

Abstract

Classical bovine spongiform encephalopathy (C-BSE) belongs to the transmissible spongiform encephalopathies (TSE), which are also designated prion diseases since they are caused by the conversion of the host-encoded cellular prion protein PrP^{C} to its pathological isoform PrP^{TSE}. BSE carries a zoonotic potential as BSE prions cause variant Creutzfeldt-Jakob disease in humans. To date, C-BSE infectivity can only be detected by bioassay, e.g. highly sensitive bovine PrP transgenic mice (e.g. Tgbov XV mice). Recently, highly sensitive *in-vitro* prion seeding activity assays, such as the Protein Misfolding Cyclic Amplification (PMCA), have been developed, which work particularly well for the template-assisted prion conversion of scrapie prions, while a similarly efficient bovine C-BSE-prion amplification remained unavailable. In the here described study, we have therefore compared the analytical sensitivities of the transgenic Tgbov XV mouse bioassay and our C-BSE PMCA protocol by analysing serial dilutions of a BSE-positive bovine brainstem homogenate pool. As both methods were shown to possess comparable sensitivities, we propose the C-BSE PMCA as a potential in-vitro replacement method, allowing the reduction and refinement of mouse bioassays for the detection of cattle derived classical BSE prions by reducing them to only specific analytical applications.

Berliner und Münchener Tierärztliche Wochenschrift 2018 June 26, Volume OA, Issue 18021
https://vetline.de/exploring-pmca-as-a-potential-in-vitro-alternative-method-to-mouse-bioassays-for-the-highly-sensitive-detection-of-bse-prions/150/3216/108497/
DOI: 10.2376/0005-9366-18021

Chapter 4 Manuscript III

Prion infectivity and PrP^{BSE} in the peripheral and central nervous system of cattle 8 months post oral BSE challenge

Ivett Ackermann[1], Reiner Ulrich[2], Kerstin Tauscher[2], Olanrewaju I. Fatola[1], Markus Keller[1], James C. Shawulu[1], Stefanie Czub[3], Martin H. Groschup[1] and Anne Balkema-Buschmann[1]

[1] Institute of Novel and Emerging Infectious Diseases, Friedrich-Loeffler-Institute, Greifswald-Insel Riems, Germany

[2] Department of Experimental Animal Facilities and Biorisk Management, Friedrich-Loeffler-Institute, Greifswald-Insel Riems, Germany

[3] Canadian Food Inspection Agency, Lethbridge Laboratory, Lethbridge, Alberta, Canada

This manuscript was prepared for submission.

4.1 Abstract

After oral exposure of cattle with the agent of classical bovine spongiform encephalopathy (C-BSE), the infectious agent ascends from the gut to the central nervous system (CNS) primarily via the autonomic nervous system. However, the timeline of this progression has so far remained widely undetermined, firstly because previous studies were focused on later time points after oral exposure, and secondly because in those studies, animals were already 4 to 6 months old when challenged. In this present study, we have therefore challenged unweaned calves aged 4 to 6 weeks in order to determine whether BSE infectivity and PrP^{BSE} were detectable in peripheral nervous tissues early during the incubation phase (time points of 1 week as well as 2, 4, 6 and 8 months post infection (mpi)). Peripheral and central nervous tissues were examined for BSE infectivity using a bovine PrP transgenic mouse bioassay and for PrP^{BSE} depositions by immunohistochemistry (IHC) and by protein misfolding cyclic amplification (PMCA). We were able to show for the first time that as early as 8 mpi the thoracic spinal cord, as part of the CNS, as well as the parasympathetic nodal ganglion of cattle, that were challenged at a young age, may contain PrP^{BSE} and BSE infectivity. These results indicate that the centripetal prion spread starts early after challenge at least in this age group, which represents an essential piece of information for the risk assessments for food, feed and pharmaceutical products produced from young calves.

4.2 Introduction

This paragraph provides an abridged version of the manuscripts introduction, as the overview of the relevant literature was included in Chapter 1.

In previous C-BSE pathogenesis studies, cattle were challenged at 4 to 6 months of age (WELLS et al. 1996; HOFFMANN et al. 2007). In this study, we performed an experimental BSE infection of young unweaned calves, which we reported for the first time in a study on intestinal C-BSE-pathogenesis of those animals (ACKERMANN et al. 2017).

Here, we present data of this early pathogenesis study in unweaned calves orally challenged with C-BSE to understand the early centripetal spread towards the brain. We examined peripheral and central nervous tissues of animals sacrificed up to 8 months post challenge, in order to determine if and when prion protein could be detected in these tissues. By providing such detailed analyses of tissues from young calves for the first time, our study therefore fills the gap of data on early C-BSE pathogenesis especially in young cattle and provides new knowledge on early prion spread with relevance for risk assessment regarding SRMs and pharmaceuticals.

4.3 Material and Methods

4.3.1 Animals

Twenty unweaned Simmental calves aged 4-6 weeks were orally challenged with classical BSE using a brainstem homogenate of clinically diseased cattle, as described before (ACKERMANN et al. 2017). The inoculum had a LD_{50}-titer of $10^{-5.730}$ (95 % confidence interval, $10^{-6.569}$ - $10^{-4.891}$) (ACKERMANN et al. 2017) as experimentally determined by titration in Tgbov XV mice (BUSCHMANN & GROSCHUP 2005). Eighteen infected calves were euthanized and necropsied at predetermined time points of 1 week as well as 2, 4, 6 and 8 months post inoculation (mpi). During necropsy a wide range of tissue samples from the central and peripheral nervous system, the lymphoreticular system and the gastrointestinal tract were collected under TSE-sterile conditions. As positive controls, 2 infected animals were kept to monitor the development of clinical symptoms of BSE, and were examined by a monthly neurological check-up starting from 24 mpi (ACKERMANN et al. 2017). Additionally, 2 calves were inoculated with a BSE-negative brainstem homogenate and served as negative controls (Table 4.S1).

4.3.1.1 Ethical statement

The challenge experiments in cattle and mice described in this manuscript were approved by the competent authority of the Federal State of Mecklenburg-Western Pomerania, Germany on the basis of national and European legislation, namely the EU council directive 2010/63/EU for the protection of animals used for experiments (file number: 7221.3-1.1-037/13), on the basis of national (Tierschutzgesetz, Tierschutz-Versuchstierverordnung) and European (RL 2010/63/EU) legislation, which also includes the Ethic Committee of Mecklenburg-Western

Pomerania. In addition, animal studies are continuously monitored by the Animal Welfare Officer and were approved by the Institutional Animal Care and Use Committee (IACUC).

4.3.2 Tissue samples

During necropsy, tissue samples were cut in halves for formalin-fixed tissue used to perform the IHC analyses and frozen material for the protein misfolding cyclic amplification (PMCA) and bioassay studies. Samples of the central nervous system (CNS) and peripheral nervous system (PNS) were examined from the 20 BSE-challenged animals and the 2 negative controls (n = 22). The current state of knowledge led to the working hypothesis, that nervous tissues of the PNS and CNS are most probably free of PrP^{BSE} and BSE prion infectivity up to 8 mpi.
Therefore, for analyses of cerebellum, frontal cortex and peripheral nervous tissues located close to the brain (stellate, nodal, trigeminal and cranial cervical ganglia) the priority was given to the highly sensitive mouse bioassay and PMCA to be able to detect even trace amounts if present in these samples. Moreover, transgenic Tgbov XV mouse bioassays were confined on selected samples from the calves of the 4 and 8 mpi groups, thereby aiming for reduction of bioassay mice numbers to a minimum.
Peripheral tissue samples in vicinity to the intestine, in detail the coeliac and caudal mesenteric ganglia, splanchnic nerve, vagal nerve and sympathetic trunk as wells as the CNS samples of the brainstem and the thoracic spinal cord segment T 7 were examined by mouse bioassay and PMCA. These samples were also analysed by immunohistochemistry (IHC) to evaluate the involved cellular compartments of any PrP^{BSE} accumulation that may be traceable in those samples.

4.3.2.1 Comparative analysis of samples from cattle challenged 4-6 months of age

In order to be able to compare some unexpected results obtained for samples of the thoracic spinal cord and the nodal ganglion from calves at 4 and 8 mpi, the corresponding samples that had been collected at 4 and 8 mpi during a BSE pathogenesis study performed in cattle that were challenged at the age of 4 to 6 months (HOFFMANN et al. 2007; HOFFMANN et al. 2011) were also analysed by PMCA.

4.3.3 Immunohistochemistry (IHC) and histopathological examination

Tissue samples were processed and immunohistochemical staining was performed as described before (ACKERMANN et al. 2017). Samples were fixed in 4% neutral buffered formaldehyde for at least 2 weeks. Fixed tissues were cut into blocks, dehydrated and embedded in paraffin wax according to standard histopathological methods. Sections (3 µm) were prepared and mounted on SuperFrost Plus slides (Thermo Fisher Scientific Gerhard Menzel, Braunschweig, Germany). For samples of the 18 challenged calves between 1 week and 8 months post inoculation (n = 18) and negative controls (n = 2), a serial section procedure (HOFFMANN et al. 2011) was used to examine 5 different levels per block with a plane distance of about 30 µm

and to obtain a depth of approximately 195 µm per block. As opposed to that, for tissue samples from the 2 positive control animals (WAIT 01 and 04, n = 2) where positive results were expected, initially one level per block was analysed by IHC and only in the case of a negative result, additional levels were examined.
For the histopathological examination, a haematoxylin-eosin staining was performed according to standard histological methods.
With modifications depending on the type of tissues, IHC staining was performed as described before (ACKERMANN et al. 2017). Two PrP-specific monoclonal antibodies (mAbs) were used: Routinely IHC staining was performed using mAb 6C2 (Wageningen Bioveterinary Research, Lelystad, Netherlands), whereas additionally sections were stained with mAb F99 (F99/97.6.1, VMRD, Pullman, USA) for verification of inconclusive results. As a negative control antibody, on corresponding sections the anti-PrP mAb 3F4 (CHEMICON International, Temecula, USA) was applied, which does not bind to bovine PrP (HARDT et al. 2000).
After rehydration, sections were pretreated with 98% formic acid for 15 min and rinsed in tap water for 5 min. Endogenous peroxidase was blocked with 3% H_2O_2 in distilled water for 30 min. Sections underwent hydrated autoclaving in citrate buffer at 121°C for 20 min. As the central nervous system is rich in endogenous biotin, for samples of obex and spinal cord segment T 7, inhibition of endogenous biotin was accomplished using an avidin/biotin blocking kit (Vector Laboratories, Burlingame, USA) as described before (ACKERMANN et al. 2017). All slides were analysed for PrP^{BSE} depositions by light microscopy.

4.3.4 Tgbov XV mouse bioassay

Selected samples were examined by bioassay, using transgenic mice over-expressing bovine PrP (BUSCHMANN & GROSCHUP 2005). Therefore, depending on the probability that the tissue might carry BSE infectivity, 20 or 40 Tgbov XV mice per group were intracerebrally inoculated using 30 µl of a 10% tissue homogenate diluted in sterile 0.9% saline solution. In case the quantity of tissue samples was low and therefore not allowing the preparation of a 10% homogenate, a 2% tissue homogenate had to be used instead.
Tissues in vicinity to the intestine, precisely the coeliac ganglia, splanchnic and vagal nerves, from the infected calves sacrificed between 1 week and 8 months post challenge (n = 18) and negative controls (n = 2) were analysed by transgenic mouse bioassay, inoculating 20 Tgbov XV mice per group.
In addition, mouse bioassays using 40 mice per group were performed for selected samples from infected calves of 6 and 8 months post challenge (n = 10). These tissue samples included the caudal mesenteric ganglia, the central nervous system (thoracic spinal cord segment T 7, cranial medulla and cerebellum) and peripheral tissues nearby the brain (sympathetic trunk, nodal, trigeminal and cranial cervical ganglia).
All mice were monitored for the onset of clinical signs at least twice per week. Animals showing at least 2 clinical symptoms indicative of a BSE infection, such as hind limb paresis, abnormal tail tonus, behavioural changes and weight loss over several consecutive days (BUSCHMANN

& GROSCHUP 2005) were sacrificed and brain samples were collected. The brains were analysed for the presence of PrP^{BSE} by precipitation using phosphotungstic acid (PTA) followed by digestion with 50 µg/ml Proteinase K at 55°C for 1 h and Western blot using mAb L42 at a concentration of 0.4 µg/ml (r-biopharm, Darmstadt, Germany) as detection antibody (GRETZSCHEL et al. 2005). Results of mice incubating at least 100 dpi were taken into evaluation.

4.3.5 Protein misfolding cyclic amplification (PMCA)

The earlier described PMCA protocol (BALKEMA-BUSCHMANN et al. 2011a; FRANZ et al. 2012) was applied with some modifications as described before (ACKERMANN et al. 2017, 2018). Briefly, brain tissue from Tgbov XV transgenic mice (BUSCHMANN et al. 2000), that were collected after perfusion of the mice with PBS containing 5 mmol/l EDTA and immediately frozen in liquid nitrogen, was used as the PrP^{C} source for the PMCA reaction. Brain samples were homogenized to a concentration of 10% (w/v) in PMCA conversion buffer to prepare the substrate solution. Analyte tissue samples were homogenized at 10% (w/v) in 0.9% saline solution. 10 µl aliquots of the analyte homogenates were suspended in 90 µl Tgbov XV brain substrate and transferred into 0.5 ml reaction tubes. The template for the positive control PMCA reaction was a 10% (w/v) homogenate of bovine brain tissue in PBS of which serial dilutions (10^{-3}, 10^{-6}, 10^{-9}) were prepared in substrate solution. A brain sample of a confirmed BSE-negative cattle served as a negative control.
Samples were routinely subjected to 3 rounds of PMCA with each 48 cycles of sonication for 20 s a time at a potency of 210 – 250 W (level 8), followed by a 30 min incubation. For samples that were close to the PMCA detection limit, a fourth round was performed in addition, including the positive and negative control samples.
The experiment was considered valid if at least the 10^{-3} and 10^{-6} dilutions were clearly identified as positive, and the negative control gave a negative result.
The obtained results were interpreted as follows: a clear PrP^{BSE} signal in all three PMCA rounds was interpreted as +++ positive, a signal in the second and third PMCA round was interpreted as ++ positive, and a signal only in the third PMCA round was interpreted as + positive. For samples where a fourth round was performed, the results were reported as (+) if only the fourth round gave a positive result.

4.4 Results

In this study, we investigated the early BSE pathogenesis up to 8 months post infection (mpi) in peripheral and central nervous tissues of 18 unweaned calves orally challenged with classical BSE. To do that, we applied highly sensitive detection methods as described above. By the time of preparing this manuscript, the PMCA analyses as well as the Tgbov XV bioassays were still ongoing.

The tissue samples of peripheral nervous system (PNS) were grouped into tissues located in close vicinity to the intestine (as shown in Table 4.1) and tissue nearby the brain (Table 4.2). The results obtained for samples from the central nervous system are shown in Table 4.3.
Proof of successful BSE challenge of these 18 preclinical calves was shown by positive ileal Peyer's patch results, as described before (ACKERMANN et al. 2017).

4.4.1 Peripheral nervous tissues in vicinity to the intestine

The vagal and splanchnic nerves as well as the coeliac ganglia of the 18 infected preclinical calves up to 8 mpi did not contain any detectable PrP^{BSE}, as determined by IHC as well as by PMCA. In addition, no BSE infectivity was revealed by Tgbov XV bioassay of those tissues, as all mouse brains available for examination were negative by Western blot (Table 4.1). The caudal mesenteric ganglion as well as the sympathetic trunk including paravertebral ganglia of all 18 calves were negative by IHC. Those samples were also bioassayed from the calves of the 6 and 8 mpi groups and all available mouse brains remained negative.

4.4.2 Peripheral nervous tissues in vicinity to the brain

Surprisingly, so far 2 out of the 36 mice inoculated with nodal ganglion from WAIT 02 (8 mpi) were BSE-positive (Table 4.2). Moreover, PrP^{BSE} was detectable in this nodal ganglion sample (WAIT 02; 8 mpi), as shown by a positive result from the third PMCA round (Fig 4.1). All other tissues near the brain that were examined for WAIT 02 (8 mpi) were negative by bioassay (Table 4.2).
Bioassay was performed for the nodal, trigeminal and cranial cervical ganglia from the remaining 7 calves sacrificed at 6 and 8 mpi and so far, all Tgbov XV mouse brains were negative by Western blot (Table 4.2).

4.4.3 Central nervous system

Surprisingly, we detected BSE infectivity in the thoracic spinal cord segment T 7 from WAIT 02 (8 mpi) and also obtained a positive reaction in all 3 PMCA rounds for the same homogenate (Fig 4.2 A). Interestingly, testing a different location within the same tissue sample, revealed a + positive PMCA result (Fig 4.2 B). After the detection of PrP^{BSE} by PMCA in the thoracic spinal cord of WAIT 2 (8mpi), we decided to analyse the thoracic spinal cord samples obtained during an earlier pathogenesis study (HOFFMANN et al. 2007; HOFFMANN et al. 2011) of preclinical cattle incubating up to 4 and 8 months after an oral BSE challenge at 4 to 6 months of age (Table 4.S2). PMCA revealed seeding activity in the thoracic spinal cord of 2 out of 4 examined cattle at 8 mpi (Fig 4.S1).
The brainstem (Table 4.1) of all 18 infected calves up to 8 mpi was free of PrP^{BSE} by IHC and PMCA and all brainstem samples from the 6 and 8 mpi calves analysed by Tgbov XV mouse bioassay were negative. Similar bioassay results were obtained for the cerebellum from calves at 6 and 8 mpi (Table 4.3).

4.4.4 Positive controls

These positive control cattle developed clinical symptoms of BSE after relatively short incubation times of 32 mpi (WAIT 04) and 36 mpi (WAIT 01), respectively, and were confirmed as BSE-positive by IHC of the obex region as described before (ACKERMANN et al. 2017).
In both animals, the coeliac ganglion and caudal mesenteric ganglion were detected positive by IHC. In immunohistochemically stained sections, individual (WAIT 04; 35 mpi) to few (WAIT 01; 36 mpi) ganglia cells showed a fine to coarse granular intracytoplasmatic as well as perineuronal PrP^{BSE} accumulation (Fig 4.3 A, B, D, E). Surprisingly, the PMCA revealed seeding activity in the coeliac ganglia (Fig 4.4 A, D), while it revealed a negative result for the caudal mesenteric ganglia of both animals (Fig 4.4 B, E) (Table 4.1). In individual ganglia cells in the vagal nerve of WAIT 04 (35 mpi) an intracytoplasmatic fine to coarse granular staining reaction was observed (Fig 4.3 H), while for WAIT 01 (36 mpi) the IHC of this nerve remained inconclusive with both mAbs 6C2 and F99. Nevertheless, PMCA confirmed the presence of PrP^{BSE} in the vagal nerve of WAIT 01 as wells as of WAIT 04 (Fig 4.4 G, H) (Table 4.1). Similarly, the IHC of the splanchnic nerve of both positive control animals remained inconclusive, while PMCA enabled PrP^{BSE} amplification in these samples of WAIT 01 and 04 (Fig 4.4 J, K) (Table 4.1). PrP^{BSE} was immunohistochemically detectable in the thoracic spinal cord (T 7) of these animals (Table 4.3) but the sympathetic trunk was negative by IHC (Table 4.2).
Due to animal welfare reasons and considering the comparably low impact of mouse bioassay results of the tissue samples from the positive control animals, no mouse bioassays were performed on tissues of positive control cattle.

4.5 Discussion

In this study, we aimed at expanding the currently still incomplete data regarding the early C-BSE pathogenesis, especially in young cattle, by analysing peripheral nerves and ganglia as well as central nervous tissues sampled from calves at particularly short time points between 1 week and 8 months after oral challenge. As recent studies (ARNOLD et al. 2007; HOFFMANN et al. 2007; ARNOLD et al. 2009; KAATZ et al. 2012) suggested that in calves neither PrP^{BSE} nor BSE infectivity were expected to be present in nervous tissues, we used highly sensitive methods (IHC, PMCA and Tgbov XV mouse bioassay) (BUSCHMANN & GROSCHUP 2005; BALKEMA-BUSCHMANN et al. 2011a; HOFFMANN et al. 2011) to prove or dismiss this hypothesis, while less sensitive methods like Western Blot and rapid test (ARNOLD et al. 2007; BARRON et al. 2007; BALKEMA-BUSCHMANN et al. 2011a; HOFFMANN et al. 2011; GONZÁLEZ et al. 2012) were considered not to be adequate for this task. Moreover, we used a C-BSE-PMCA protocol providing an analytical sensitivity comparable to that of Tgbov XV mouse bioassay, as reported by our recent (ACKERMANN et al. 2018) and earlier studies (BALKEMA-BUSCHMANN et al. 2011a; FRANZ et al. 2012).
We have reported that young unweaned calves are susceptible to a BSE infection, as indicated by significant amounts of PrP^{BSE} and high infectivity loads in some of the analysed ileal Peyer's patch (IPP) samples (ACKERMANN et al. 2017). However, just one inexplicit indication for a

successful neuroinvasion was observed, since PrP^{BSE} was detectable in the enteric nervous system (ENS) of only one calf (ACKERMANN et al. 2017). Nevertheless, rare PrP^{BSE} accumulation in the ENS cannot be interpreted as proof for a restriction of agent replication to the intestine, as in the current study we were able to show that peripheral and central nervous tissues up to 8 mpi may contain PrP^{BSE} as well as BSE infectivity.

We have shown for the first time that as early as 8 mpi the thoracic spinal cord T 7 (WAIT 02), as a part of the central nervous system, may contain the BSE agent, as proven by detection of PrP^{BSE} using PMCA, as well as BSE infectivity by mouse bioassay. This finding was surprising as infectivity in the spinal cord had so far only been reported from 16 mpi onwards in our earlier study (KAATZ et al. 2012). Therefore, we additionally analysed thoracic spinal cord samples from cattle incubating for 4 and 8 months after C-BSE challenge at the age of 4 to 6 months. Two of these 8 mpi cattle indeed carried PrP^{BSE} (as detected by PMCA) at this location, which supports our results for the calf sacrificed at 8 mpi. By the time of writing, the brainstem seems to be free of the agent up to 8 months after infection, as indicated by negative IHC and PMCA results as well as all bioassay results that are available so far for all infected calves up to 8 mpi. These brainstem results are in line with our earlier studies showing prion accumulation by IHC and Tgbov XV mouse bioassay in the brainstem starting from 24 mpi (HOFFMANN et al. 2007; KAATZ et al. 2012). Moreover, the parasympathetic nodal ganglion, located close to the brain, was positive for PrP^{BSE} and infectivity in the same 8 mpi calf (WAIT 02) that carried the agent in the thoracic spinal cord. This is in contrast to earlier reports where agent detection was described from 20 mpi (mouse bioassay) for the parasympathetic nodal ganglion (KAATZ et al. 2012). Concluding, our novel findings, that were only made possible by the application of several highly sensitive detection methods, indicate that a time of up to 8 mpi is sufficient for further spread of the agent to peripheral as well as central nervous tissues.

However, we revealed negative results by IHC, PMCA and mouse bioassay for the coeliac and caudal mesenteric ganglion, the splanchnic and vagal nerves from calves that were sacrificed at these early time points after oral challenge. This seems surprising, as earlier studies showed that prion spread via the coeliac mesenteric ganglion complex (CMGC) and subsequently by the sympathetic splanchnic nerve and the parasympathetic vagal nerve preceded infection of the thoracic spinal cord and the nodal ganglion, respectively (HOFFMANN et al. 2007; KAATZ et al. 2012). Those studies reported the earliest detection of BSE infectivity at 16 mpi and PrP^{BSE} by IHC at 24 mpi in the CMGC, a mixed ganglion that functions in the centripetal spread via parasympathetic and sympathetic fibres (HOFFMANN et al. 2007; KAATZ et al. 2012). Infectivity was also present in the sympathetic splanchnic nerves from 16 mpi as well as in the parasympathetic vagal nerve from 28 mpi in the thoracical part and from 20 mpi in its cervical part, respectively (KAATZ et al. 2012). We were able to confirm these pathways for our positive control animals WAIT 01 and 04, which showed PrP^{BSE} accumulation in vagal and splanchnic nerves as determined by PMCA. It has been discussed that prions might be in transit without actively replication in nerves (MCBRIDE et al. 2001), which has been considered as a reason for negative results obtained for nerve samples before (HEGGEBØ et al. 2003; HOFFMANN et al. 2007).

Otherwise, a remaining risk of false negative results, in case of the CMGC of WAIT 02, cannot be excluded, as different locations of one sampled tissue may possibly contain different amounts of the BSE agent, as shown by differing PMCA results for 2 locations of the same spinal cord segment from WAIT 02. Moreover, this might explain the negative PMCA results for the caudal mesenteric ganglion of the positive control animals, while by IHC we were able to detect PrP^{BSE} accumulation in individual ganglia cells. Vice versa, PrP^{BSE} amplification by PMCA can correct negative or inconclusive IHC results in samples with low titres, as suggested by the results for splanchnic and vagal nerves from our positive controls. This is supported by our earlier study, where ileal Peyer's patch samples of two preclinically infected calves were negative or inconclusive by IHC, while a positive PMCA result was in agreement with infectivity detection by mouse bioassay (ACKERMANN et al. 2017).

Finally, our study demonstrates that tissues from infected animals may constitute a risk of infection for humans regardless of the bovines' age. However, it should be considered that for challenge of the examined cattle we used a 100 g dose in order to receive a complete attack rate (WELLS et al. 2007). Such a high exposure dose is highly unlikely under field conditions (ARNOLD et al. 2009). Moreover, the removal of the skull, including the brain and eyes, as well as the spinal cord at slaughter of bovines over 12 months of age, as defined in Regulation (EC) No 999/2001 (as of 05.2017), can be considered an adequate measure for consumer protection in countries with a negligible BSE risk. But our data argue that in countries with a controlled or undetermined BSE risk, the removal of the spinal cord from cattle of all age groups should be considered in terms of public health and consumer protection. Besides, EU legislation provides different regulations for medicinal products, which shall not be, or only in justified exceptions, produced from high-infectivity tissues (category IA based on WHO tables (WHO 2010), while the tissues in these 3 major infectivity categories are grouped regardless of the stage of disease and thus the age of the animals (Note for guidance EMA/410/01 rev.3 (EUROPEAN COMMISSION 2011)). These important regulations regarding consumer protection should be reviewed now in order to take into account the data of this study.

These novel data also provide indications regarding the age-dependent susceptibility of bovines to BSE infections. Our earlier study indicated that an increased prion uptake in combination with a decreased clearance favoured the agent accumulation in the ileal Peyer's patch of these young calves challenged at 4 to 6 weeks of age (before weaning) already after 2 months post oral challenge (ACKERMANN et al. 2017). The neuroinvasion progress of the C-BSE infection to the thoracic spinal cord, as shown by PMCA, seems to take place at about 8 mpi in both challenged age groups, 4 to 6 weeks as well as 4 to 6 months of age. Surprisingly, this is in contrast to a BSE challenge experiment in sheep, which proved the higher BSE susceptibility of young unweaned lambs compared to older weaned lambs and adults and also discussed an increased uptake as one reason for this observation (HUNTER et al. 2012). Indeed the clearing efforts of macrophages in the ileal Peyer's patch follicles of the young calves failed earlier during the infection, as indicated by PrP^{BSE} accumulation upon follicular dendritic cells (FDCs) as early as 4 mpi (ACKERMANN et al. 2017) when compared to PrP^{BSE}-positive FDCs from 12 mpi in cattle challenged as older calves with 4 to 6 months of age (HOFFMANN et al. 2011). Albeit, this apparently did not influence neuroinvasion or the speed of prion spread through the

nervous system, as illustrated by the presence of PrP^{BSE} at 8 mpi in the thoracic spinal cord of both age groups. The FDC status and the development of the FDC networks have been shown to influence age-dependent susceptibility in mice (BROWN et al. 2009; BROWN & MABBOTT 2014) and sheep (MARRUCHELLA et al. 2012), respectively. Nevertheless, the thereby reduced agent accumulation and neuroinvasion resulting in decreased disease susceptibility of aged (> 600 days old) mice compared to young (6 to 8 weeks old) mice (BROWN et al. 2009; BROWN & MABBOTT 2014) may not apply for the bovines examined in this study, as upon challenge even those bovines challenged at 4 to 6 months old were still in the age group of calves, just older and after weaning as compared to the calves infected at 4 to 6 weeks of age. Otherwise, direct neuroinvasion in the lamina propria mucosae under the intestinal epithelium (HEGGEBØ et al. 2003; JEFFREY et al. 2006) is independent from FDC status or development of FDC networks, and may therefore provide another explanation for our observations in these cattle. The accumulation of PrP^{BSE} in a submucosal plexus of the ENS from calf at 2 mpi in the absence of detectable PrP^{BSE} in the ileal Peyer's patch follicles may reflect this direct neuroinvasion (ACKERMANN et al. 2017). Finally, a third explanation should consider that replication upon FDCs in other lymphoreticular organs may play a role for the further progression of prion infection as occurring in sheep and mice (MABBOTT et al. 2003; GLAYSHER & MABBOTT 2007; MCCULLOCH et al. 2011). And thus, the thereby acceleration of disease progression may not occur in bovines as the lymphoreticular system – except for the IPP – is rarely involved in cattle BSE pathogenesis (BUSCHMANN & GROSCHUP 2005; WELLS et al. 2005; ESPINOSA et al. 2007).

In summary, we detected PrP^{BSE} and BSE infectivity as early as 8 mpi in the nodal ganglion as well as in the thoracic spinal cord from one calf challenged before weaning in this study and also at 8 mpi in the thoracic spinal cord sampled from cattle challenged at 4 to 6 months of age during an earlier pathogenesis study (HOFFMANN et al. 2007; HOFFMANN et al. 2011). This current study expands data on early C-BSE pathogenesis by demonstrating that tissues of the peripheral and central nervous system from cattle after short time periods up to 8 months after oral infection can contain PrP^{BSE} and BSE infectivity, which should be considered a relevant information for risk assessments for food and pharmaceutical products.

4.6 Tables

Table 4.1 Results for peripheral nervous tissues in vicinity to the intestine obtained by IHC, PMCA and Tgbov XV mouse bioassay

Time point post infection	Animal ID	Ganglion coeliacum			Ganglion mesenteriale caudale			Nervus splanchnicus major			Truncus sympathicus (incl. paravertebral ganglia)			Nervus vagus (thoracic part)		
		IHC	PMCA	BA	IHC	PMCA	BA	IHC	PMCA	BA	IHC	PMCA	BA	IHC	PMCA	BA
1 week	WAIT 17	neg.	neg.	0/20, > 730	neg.†	neg.	n. d.	neg.	neg.	0/19, > 730	neg.	o. a.	n. d.	neg.	neg.	0/20, > 730
	WAIT 18	neg.	neg.	0/20, > 730	neg.	neg.	n. d.	neg.	neg.	0/20, > 730	neg.	o. a.	n. d.	neg.	neg.	0/19, > 730
2 months	WAIT 15	neg.	neg.	0/9, > 730	neg.	neg.	n. d.	neg.	neg.	0/20, > 730	neg.	o. a.	n. d.	neg.	neg.	(~) 0/21, neg. (16), > 730
	WAIT 16	neg.	neg.	0/18, > 730	neg.	neg.	n. d.	neg.	neg.	0/20, > 730	neg.	o. a.	n. d.	neg.	neg.	0/20, > 730
4 months	WAIT 11	neg.	neg.	0/20, > 730	neg.†	o. a.	n. d.	neg.	neg.	0/20, > 730	neg.	o. a.	n. d.	neg.	neg.	0/20, > 730
	WAIT 12	neg.	neg.	(~) 0/20, neg. (17), > 730	neg.	o. a.	n. d.	neg.	neg.	(~) 0/20, neg. (12), > 677	neg.	o. a.	n. d.	neg.	neg.	(~) 0/20, neg. (15), > 687
	WAIT 13	neg.	neg.	(~) 0/20, neg. (15), > 632	neg.	o. a.	n. d.	neg.	neg.	(~) 0/20, neg. (17), > 637	neg.	o. a.	n. d.	neg.	neg.	(~) 0/20, neg. (17), > 601
	WAIT 14	neg.	neg.	0/20, > 693	neg.	o. a.	n. d.	neg.	neg.	0/20, > 730	neg.	o. a.	n. d.	neg.	neg.	0/20, > 730
	WAIT 19	neg.	neg.	(~) 0/20, neg. (13), > 642	neg.	neg.	n. d.	neg.	neg.	(~) 0/20, neg. (13), > 664	neg.	o. a.	n. d.	neg.	neg.	(~) 0/20, neg. (14), > 640
	WAIT 20	neg.	neg.	(~) 0/19, neg. (11), > 680	neg.	neg.	n. d.	neg.	neg.	(~) 0/20, neg. (13), > 661	neg.	o. a.	n. d.	neg.	neg.	(~) 0/20, neg. (10), > 594
6 months	WAIT 05	neg.	neg.	(~) 0/20, neg. (11), > 585	neg.†	neg.	(~) 0/40, neg. (9), > 486	neg.	neg.	(~) 0/20, neg. (10), > 610	neg.	o. a.	(~) 0/40, neg. (5), > 469	neg.	neg.	(~) 0/20, neg. (12), > 608
	WAIT 06	neg.	neg.	(~) 0/19, neg. (17), > 692	neg.	neg.#	(~) 0/40, neg. (4), > 350 #	neg.	neg.	(~) 0/20, neg. (8), > 730	neg.	o. a.	(~) 0/40, neg. (7), > 446	neg.	neg.	(~) 0/20, neg. (13), > 695
	WAIT 07	neg.	neg.	(~) 0/20, neg. (8), > 582	neg.	o. a.	(~) 0/40, neg. (8), > 496	neg.	neg.	(~) 0/20, neg. (15), > 610	neg.	o. a.	(~) 0/40, neg. (5), > 385	neg.	neg.	(~) 0/20, neg. (8), > 582
	WAIT 08	neg.	neg.	0/20, > 730	neg.	o. a.	(~) 0/40, neg. (38), > 730	neg.	neg.	(~) 0/18, neg. (16), > 730	neg.	o. a.	(~) 0/40, neg. (23), > 673	neg.	neg.	0/18, > 730
	WAIT 09	neg.	neg.	0/20, > 730	neg.	o. a.	(~) 0/38, neg. (7), > 493	neg.	neg.	0/20, > 730	neg.	o. a.	(~) 0/39, neg. (5), > 385	neg.	neg.	0/20, > 730
	WAIT 10	neg.	neg.	(~) 0/20, neg. (16), > 629	neg.	o. a. #	(~) 0/40, neg. (2), > 413 #	neg.	neg.	(~) 0/20, neg. (13), > 637	neg.	o. a.	(~) 0/40, neg. (3), > 398	neg.	neg.	(~) 0/20, neg. (13), > 625
8 months	WAIT 02	neg.	neg.	0/20, > 730	neg.	neg.	0/39, > 730	neg.	neg.	0/20, > 730	neg.	o. a.	0/40, > 730	neg.	neg.	0/20, > 730
	WAIT 03	neg.	neg.	0/20, > 730	neg.	neg.	(~) 0/40, neg. (4), > 438	neg.	neg.	0/19, > 730	neg.	o. a.	(~) 0/39, neg. (10), > 465	neg.	neg.	0/20, > 730
35 months	WAIT 04	pos.*	++	n. d.	pos.*	neg.	n. d.	inconcl.	+	n. d.	neg.	o. a.	n. d.	pos.	+	n. d.
36 months	WAIT 01	pos.*	+++	n. d.	pos.*	neg.	n. d.	inconcl.	+	n. d.	neg.	o. a.	n. d.	inconcl.	+	n. d.

Legend: IHC: immunohistochemistry, neg.: negative, pos.: positive, * in one level of the block; †no ganglia cells, but only nerve fibres were available for analyses; PMCA: protein misfolding cyclic amplification, +: positive in the third round, + +: positive from the second round, + + +: positive from the first round; neg.: negative, o. a.: ongoing analysis, n. d.: not done, # a 2% homogenate was analysed due to tissue size; BA: bioassay in Tgbov XV mice: positive / inoculated mice and mean incubation time in days ± standard error of the mean (SEM) or survival time of oldest negative mice, (~) bioassay ongoing: positive / inoculated mice, actual result (with number of tested mice), survival time of oldest negative mouse;

Table 4.2 Results for peripheral nervous tissues closer by the brain achieved by PMCA and Tgbov XV mouse bioassay

Time point post infection	Animal ID	Ganglion cervicale craniale		Ganglion stellatum		Ganglion nodosum		Ganglion trigeminale	
		PMCA	BA	PMCA	BA	PMCA	BA	PMCA	BA
1 week	WAIT 17	o. a.	n. d.	o. a.	n. d.	o. a.	n. d.	o. a.	n. d.
	WAIT 18	o. a.	n. d.	o. a.	n. d.	o. a.	n. d.	o. a.	n. d.
2 months	WAIT 15	o. a.	n. d.	o. a.	n. d.	o. a.	n. d.	o. a.	n. d.
	WAIT 16	o. a.	n. d.	o. a.	n. d.	o. a.	n. d.	o. a.	n. d.
4 months	WAIT 11	o. a.	n. d.	o. a.	n. d.	o. a.	n. d.	o. a.	n. d.
	WAIT 12	o. a.	n. d.	o. a.	n. d.	o. a.	n. d.	o. a.	n. d.
	WAIT 13	o. a.	n. d.	o. a.	n. d.	o. a.	n. d.	o. a.	n. d.
	WAIT 14	o. a.	n. d.	o. a.	n. d.	o. a.	n. d.	o. a.	n. d.
	WAIT 19	o. a.	n. d.	o. a.	n. d.	o. a.	n. d.	o. a.	n. d.
	WAIT 20	o. a.	n. d.	o. a.	n. d.	o. a.	n. d.	o. a.	n. d.
6 months	WAIT 05	o. a.	(~) 0/40, neg. (6), > 409	o. a.	n. d.	o. a.	(~) 0/40, neg. (5), > 415	o. a.	(~) 0/40, neg. (6), > 469
	WAIT 06	o. a.	(~) 0/38, neg. (4), > 415	o. a.	n. d.	o. a.	(~) 0/40, neg. (3), > 374	o. a.	(~) 0/39, neg. (8), > 463
	WAIT 07	o. a.	(~) 0/40, neg. (6), > 423	o. a.	n. d.	o. a.	(~) 0/40, neg. (6), > 389	o. a.	(~) 0/40, neg. (10), > 468
	WAIT 08	o. a.	(~) 0/40, neg. (37), > 730	o. a.	n. d.	o. a.	(~) 0/30, neg. (26), > 730	o. a.	0/39, 730
	WAIT 09	o. a.	(~) 0/39, neg. (8), > 410	o. a.	n. d.	o. a. #	(~) 0/40, neg. (1), > 166 #	o. a.	(~) 0/40, neg. (6), > 469
	WAIT 10	o. a. #	(~) 0/39, neg. (2), > 334 #	o. a.	n. d.	o. a.	(~) 0/40, neg. (4), > 406	o. a.	(~) 0/39, neg. (8), > 465
8 months	WAIT 02	o. a.	0/39, > 730	o. a.	n. d.	+	2/36, 490 ± 66	o. a.	0/40, > 730
	WAIT 03	o. a.	(~) 0/40, neg. (8), > 423	o. a.	n. d.	o. a.	(~) 0/40, neg. (3), > 129	o. a.	(~) 0/40, neg. (8), > 500
35 months	WAIT 04	o. a.	n. d.	o. a.	n. d.	o. a.	n. d.	o. a.	n. d.
36 months	WAIT 01	o. a.	n. d.	o. a.	n. d.	o. a.	n. d.	o. a.	n. d.

Legend: PMCA: protein misfolding cyclic amplification, +: positive in the third round; neg.: negative, o. a.: ongoing analysis, n. d.: not done; # a 2% homogenate was analysed due to tissue size; BA: bioassay in Tgbov XV mice: positive / inoculated mice and mean incubation time in days ± standard error of the mean (SEM) or survival time of oldest negative mice, (~) bioassay is ongoing: positive / inoculated mice, actual result (with number of tested mice), survival time of oldest negative mouse.

Table 4.3 Results obtained for samples of the central nervous system together with clinical status of all challenged calves

Time point post infection	Animal ID	Status	Obex	Cranial medulla		Frontal cortex		Cerebellum		Thoracic spinal cord T7		
			IHC	PMCA	BA	PMCA	BA	PMCA	BA	IHC	PMCA	BA
1 week	WAIT 17	Preclinical	neg.	neg.	n. d.	o. a.	n. d.	o. a.	n. d.	neg.	o. a.	n. d.
(n = 2)	WAIT 18	Preclinical	neg.	neg.	n. d.	o. a.	n. d.	o. a.	n. d.	neg.	o. a.	n. d.
2 months	WAIT 15	Preclinical	neg.	neg.	n. d.	o. a.	n. d.	o. a.	n. d.	neg.	o. a.	n. d.
(n = 2)	WAIT 16	Preclinical	neg.	neg.	n. d.	o. a.	n. d.	o. a.	n. d.	neg.	o. a.	n. d.
4 months	WAIT 11	Preclinical	neg.	neg.	n. d.	o. a.	n. d.	o. a.	n. d.	neg.	o. a.	n. d.
(n = 6)	WAIT 12	Preclinical	neg.	neg.	n. d.	o. a.	n. d.	o. a.	n. d.	neg.	o. a.	n. d.
	WAIT 13	Preclinical	neg.	neg.	n. d.	o. a.	n. d.	o. a.	n. d.	neg.	o. a.	n. d.
	WAIT 14	Preclinical	neg.	neg.	n. d.	o. a.	n. d.	o. a.	n. d.	neg.	o. a.	n. d.
	WAIT 19	Preclinical	neg.	neg.	n. d.	o. a.	n. d.	o. a.	n. d.	neg.	o. a.	n. d.
	WAIT 20	Preclinical	neg.	neg.	n. d.	o. a.	n. d.	o. a.	n. d.	neg.	o. a.	n. d.
6 months	WAIT 05	Preclinical	neg.	neg.	(~) 0/40, neg. (27), > 603	o. a.	n. d.	o. a.	(~) 0/39, neg. (7), > 582	neg.	neg.	(~) 0/40, neg. (19), > 587
(n = 6)	WAIT 06	Preclinical	neg.	neg.	(~) 0/40, neg. (21), > 560	o. a.	n. d.	o. a.	(~) 0/43, neg. (9), > 515	neg.	o. a.	(~) 0/37, neg. (16), > 589
	WAIT 07	Preclinical	neg.	neg.	(~) 0/40, neg. (18), > 593	o. a.	n. d.	o. a.	(~) 0/40, neg. (11), > 553	neg.	o. a.	(~) 0/39, neg. (14), > 522
	WAIT 08	Preclinical	neg.	neg.	(~) 0/40, neg. (38), > 730	o. a.	n. d.	o. a.	(~) 0/40, neg. (37), > 730	neg.	o. a.	0/38, > 730
	WAIT 09	Preclinical	neg.	neg.	(~) 0/40, neg. (20), > 601	o. a.	n. d.	o. a.	(~) 0/37, neg. (13), > 502	neg.	o. a.	(~) 0/40, neg. (16), > 587
	WAIT 10	Preclinical	neg.	neg.	(~) 0/40, neg. (17), > 581	o. a.	n. d.	o. a.	(~) 0/38, neg. (11), > 514	neg.	o. a.	(~) 0/39, neg. (15), > 580
8 months	WAIT 02	Preclinical	neg.	neg.	(~) 0/30, neg. (38), > 730	o. a.	n. d.	o. a.	0/37, > 730	neg.	(+)++	8/39, 498 ± 24
(n = 2)	WAIT 03	Preclinical	neg.	neg.	(~) 0/40, neg. (25), > 599	o. a.	n. d.	o. a.	(~) 0/43, neg. (11), > 526	neg.	neg.	(~) 0/40, neg. (14), > 574
35 months (n = 1)	WAIT 04	3 (Positive control)	pos.*	+++	n. d.	o. a.	n. d.	o. a.	n. d.	pos.*	o. a.	n. d.
36 months (n = 1)	WAIT 01	2 (Positive control)	pos.*	+++	n. d.	o. a.	n. d.	o. a.	n. d.	pos.*	o. a.	n. d.

Legend: IHC: immunohistochemistry, neg.: negative, pos.: positive, * in one level of the block; PMCA: protein misfolding cyclic amplification, + + +: positive from the first round; neg.: negative, o. a.: ongoing analysis: n. d.: not done; BA: bioassay in Tgbov XV mice: positive / inoculated mice and mean incubation time in days ± standard error of the mean (SEM) or survival time of oldest negative mice, (~) bioassay is ongoing: positive / inoculated mice, actual result (with number of tested mice), survival time of oldest negative mouse.

4.7 Figures

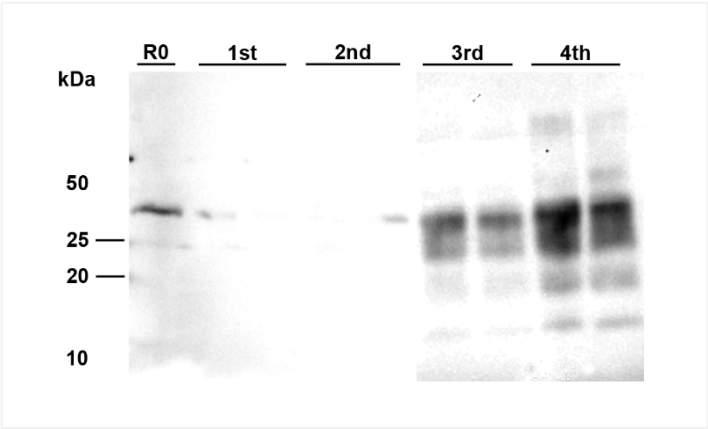

Figure 4.1 PrP^{BSE} amplification by PMCA in the nodal ganglion at 8 months after challenge. From the third round of PMCA, seeding activity was shown in the nodal ganglion from calf WAIT 02 (8 mpi); this sample was analysed in duplicate and subjected to 4 rounds (1st, 2nd, 3rd, 4th) of PMCA; M: marker, R0: analyte homogenate diluted 1:10 in Tgbov XV brain substrate without sonication.

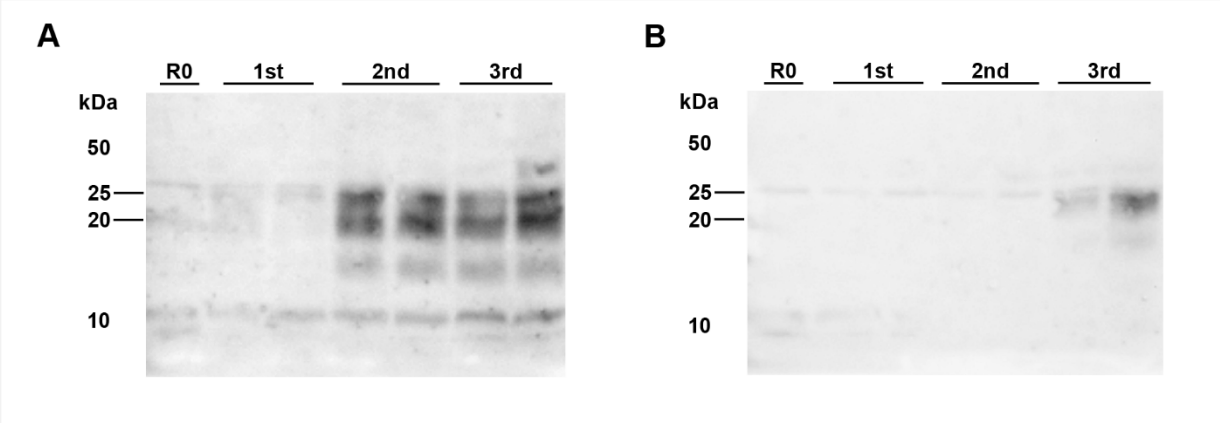

Figure 4.2 PrP^{BSE} amplification by PMCA in the thoracic spinal cord from a calf at 8 months after challenge. PMCA detected PrP^{BSE} in the thoracic spinal cord segment T7 of WAIT 02 (8 mpi); **A:** (+)++ positive PMCA reaction obtained when testing the homogenate used for inoculation of bioassay mice; **B:** analysing a different location of the same T7 sample revealed amplification only in the third round (+ positive reaction); each sample was analysed in duplicate and subjected to 3 rounds (1st, 2nd, 3rd) of PMCA, M: marker, R0: analyte homogenate diluted 1:10 in Tgbov XV brain substrate without sonication.

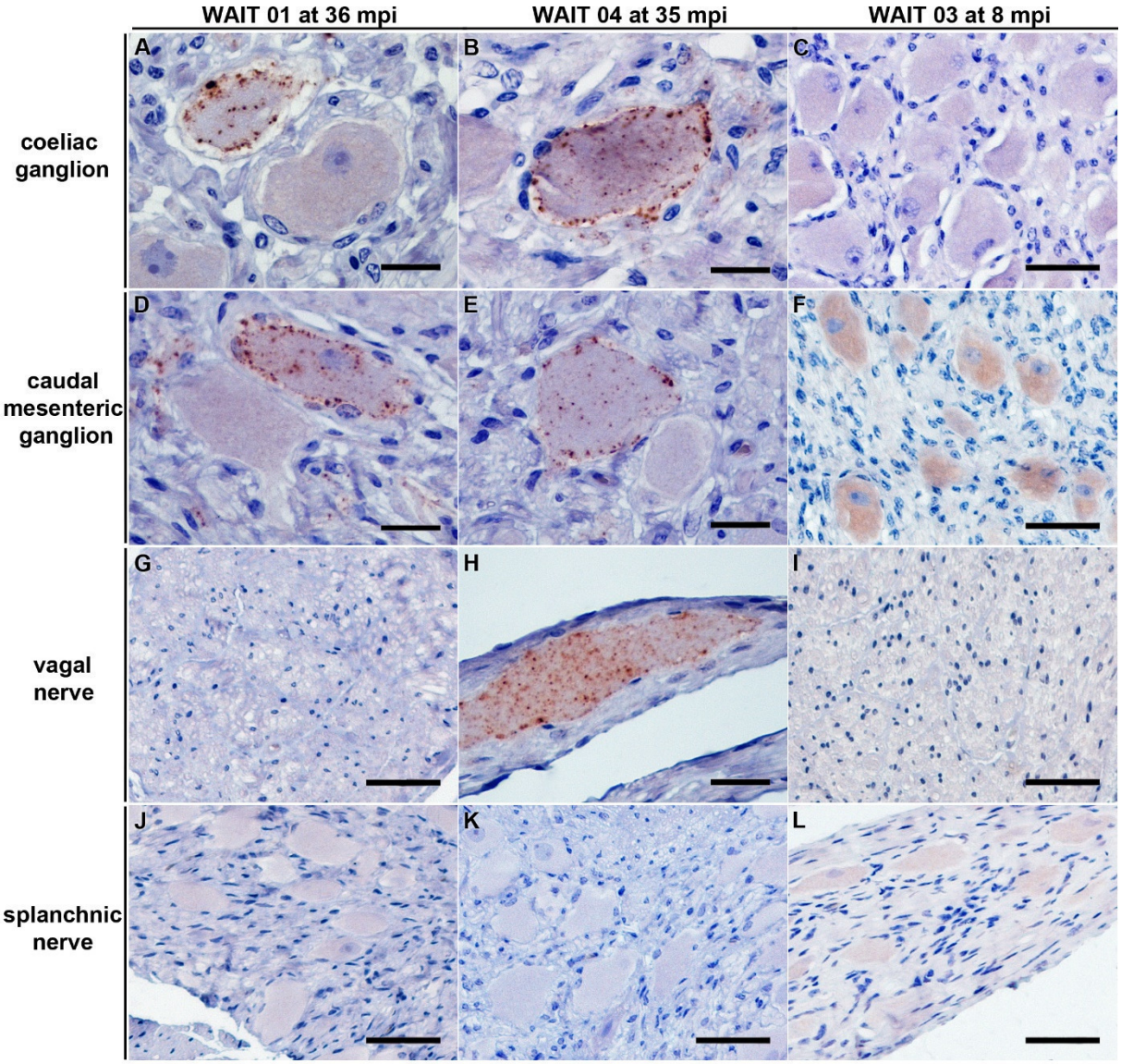

Figure 4.3 PrP^{BSE} accumulation in the coeliac and caudal mesenteric ganglion as well as the vagal nerve of positive control cattle. IHC results obtained for positive controls WAIT 01 (36 mpi; A, D, G and J) and WAIT 04 (35 mpi; B, E, H and K) as well as preclinical calf WAIT 03 (8 mpi; C, F, I and L); **A, B, D, E:** fine to coarse granular intracytoplasmatic as well as perineuronal PrP^{BSE} accumulation in a ganglia cell of the coeliac ganglion (A, B) and the caudal mesenteric ganglion (D, E) of WAIT 01 (36 mpi; A, D) and WAIT 04 (35 mpi; B, E); **H:** intracytoplasmatic fine to coarse granular accumulation of PrP^{BSE} in a ganglia cell contained in the vagal nerve of WAIT 04 (35 mpi); **C, F, G, I – L:** no staining reaction in samples from nervous tissues of calf WAIT 03 (8 mpi; C, F, I, L) as well as vagal nerve of WAIT 01 (G) and splanchnic nerves of WAIT 01 (J) and 04 (K), while the diffuse staining reaction in neural cells in the caudal mesenteric ganglion of WAIT 03 (F) represents an nonspecific background staining; A – L: Immunohistochemistry, PrP mAb 6C2; A, B, D, E, H: bar 20 µm; C, F, G, I – L: bar 50 µm.

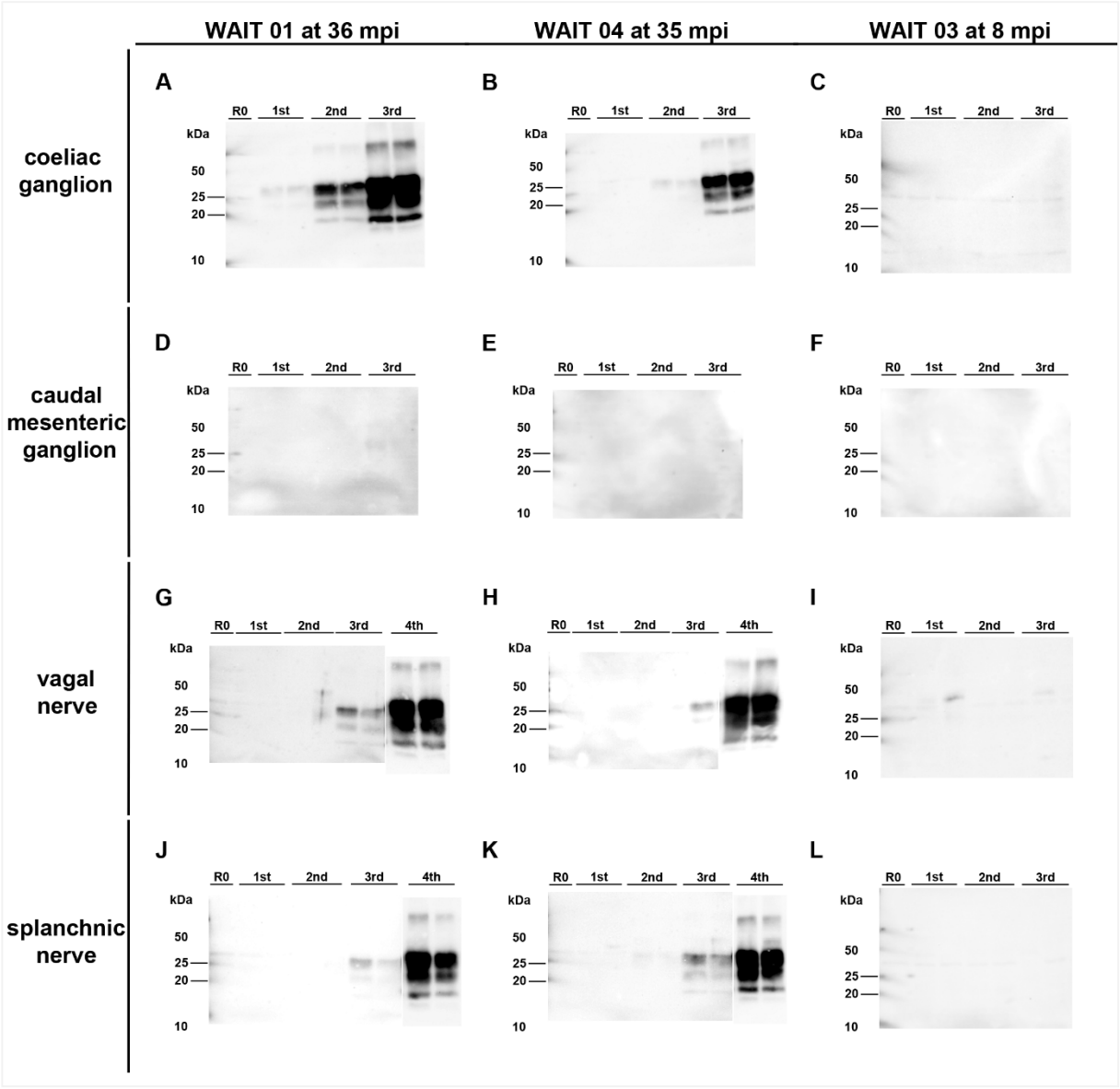

Figure 4.4 PrP^{BSE} amplification by PMCA in nervous tissue samples from positive control cattle. A, B, D, E: Seeding activity was present in the coeliac ganglion of the positive controls WAIT 01 (36 mpi; A) and 04 (35 mpi; B) as shown by +++ (A) and ++ (B) positive PMCA reactions, while – in contrast to IHC – no PrP^{BSE} was detectable by PMCA in the caudal mesenteric ganglion of WAIT 01 (D) and 04 (E); **G, H, J, K:** + positive PMCA reactions confirmed the presence of PrP^{BSE} in the parasympathetic vagal nerve (G, H) and sympathetic splanchnic nerve (J, K) of WAIT 01 (G, J) and 04 (H, K); **C, F, I, L:** the mentioned peripheral ganglia and nerves of WAIT 03 (8 mpi) were negative by PMCA; All analyte tissue samples were analysed in duplicate and subjected to 3 rounds (1st, 2nd, 3rd) of PMCA. An additional fourth (4th) round confirmed PrP^{BSE} amplification of weak positive results obtained with the initial PMCA run of some samples. M: marker, R0: analyte homogenate diluted 1:10 in Tgbov XV brain substrate without sonication.

4.8 Supporting information

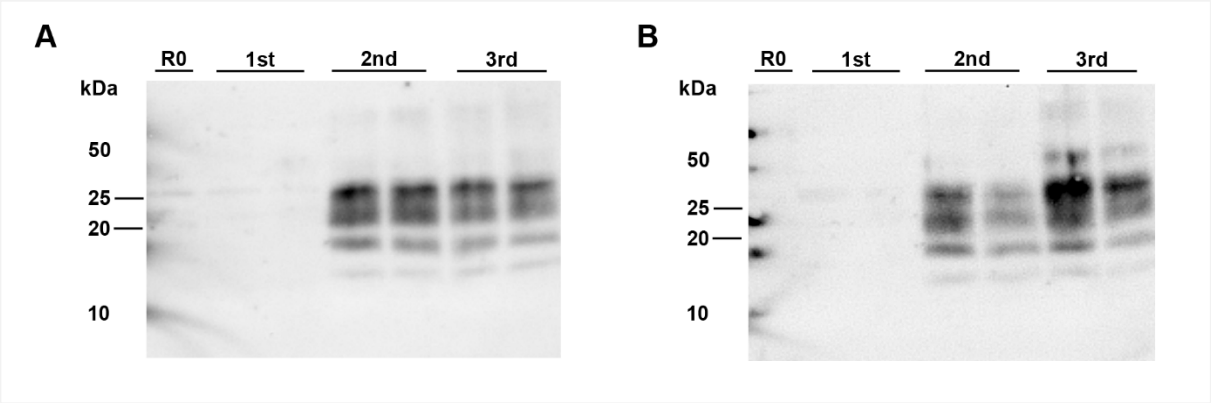

Figure 4.S1 PrP^{BSE} amplification by PMCA in the thoracic spinal cord of cattle incubating 8 months. PMCA detected PrP^{BSE} in the thoracic spinal cord of 2 cattle at 8 mpi (IT 39 and IT 20) which were challenged as older calves of 4 to 6 months of age (during the FLI pathogenesis study); **A:** analysing the thoracic spinal cord sample from IT 39 (8 mpi) revealed amplification from the second round (++ positive reaction); **B: a** ++ positive PMCA reaction was obtained for the sample from IT 20 (8 mpi); each sample was analysed in duplicate and subjected to three rounds (1st, 2nd, 3rd) of PMCA; M: marker, R0: analyte homogenate diluted 1:10 in Tgbov XV brain substrate without sonication. These tissue samples were obtained during an earlier BSE pathogenesis study (HOFFMANN et al. 2007; HOFFMANN et al. 2011).

Table 4.S1 Results for tissue samples of negative control calves

Tissue sample	WAKT 01			WAKT 02		
	IHC	PMCA	BA	IHC	PMCA	BA
Obex	neg.	n. d.	n. d.	neg.	n. d.	n. d.
Cranial medulla	n. d.	neg.	0/20, > 730	n. d.	neg.	0/20, > 730
Ganglion coeliacum	neg.	neg.	0/20, > 730	neg.	neg.	0/20, > 730
Ganglion mesenteriale caudale	neg.	neg.	n. d.	neg.	neg.	n. d.
Nervus splanchnicus major	neg.	neg.	0/20, > 730	neg.	neg.	0/20, > 730
Truncus sympathicus (incl. paravertebral ganglia)	neg.	o. a.	n. d.	neg.	o. a.	n. d.
Nervus vagus (thoracic part)	neg.	neg.	0/20, > 730	neg.	neg.	0/19, > 730
Thoracic spinal cord T7	neg.	neg.	0/20, > 730	neg.	o. a.	0/19, > 730
Frontal cortex	n. d.	o. a.	n. d.	n. d.	o. a.	n. d.
Cerebellum	n. d.	o. a.	0/20, > 730	n. d.	o. a.	0/20, > 730
Ganglion cervicale craniale	n. d.	o. a.	n. d.	n. d.	o. a.	n. d.
Ganglion stellatum	n. d.	o. a.	n. d.	n. d.	o. a.	n. d.
Ganglion nodosum	n. d.	o. a.	0/20, > 730	n. d.	o. a.	0/20, > 730
Ganglion trigeminale	n. d.	o. a.	0/20, > 730	n. d.	o. a.	0/19, > 730

Legend: IHC: immunohistochemistry; PMCA: protein misfolding cyclic amplification; neg.: negative; n. d.: not done, o. a. ongoing analysis; BA: bioassay in Tgbov XV mice, positive / inoculated mice and mean incubation time in days post infection

Table 4.S2 PMCA results of nervous tissue samples from cattle challenged at 4 to 6 months of age

Time point post infection	Animal ID	**Thoracic spinal cord** PMCA	**Ganglion nodosum** PMCA
4 months (n = 2)	IT 19	neg.	o. a.
	IT 45	neg.	o. a.
8 months (n = 2)	IT 14	neg.	o. a.
	IT 20	++	o. a.
	IT 39	++	o. a.
	IT 55	neg.	o. a.

Legend: PMCA: protein misfolding cyclic amplification, + +: positive from the second round, o.a.: ongoing analysis, neg.: negative. These tissue samples were obtained during an earlier BSE pathogenesis study (HOFFMANN et al. 2007; HOFFMANN et al. 2011).

4.9 Authors' contributions

This study was designed by ABB, IA, SC and MHG and the experimental work was carried out by IA, ABB, KT, RU, MK, OIF and JCS. Data were analysed by IA, ABB and MHG. The manuscript was written by IA, ABB, SC and MHG.

4.10 Acknowledgements

We thank the Scientific Advisory Group (SAG), namely Thierry Baron (ANSES Lyon), Michael Beekes (RKI Berlin), Jim Hope, Marion Simmons, John Spiropoulos (all APHA Weybridge) and Paul Brown for their advice and input at the design of this study. Julia Herger (protein biochemical analyses of mice), Daniel Balkema (PMCA) and Bärbel Hammerschmidt (mice inoculations) are acknowledged for their extensive skilful technical assistance. We are thankful to Lukas Steinke, Nicole Sinkwitz, Kerstin Kerstel and Doreen Fiedler for excellent care of the bioassay mice. We are grateful to Stefanie Marzahl, Ben Schiller and Volker Netz for the great care and handling of the experimental calves. This work was partially supported by a grant by WALA Heilmittel GmbH.

Chapter 5 General discussion

Classical bovine spongiform encephalopathy (C-BSE) of cattle is a neurodegenerative disease, in which the affected animals develop progressive behaviour, sensibility and movement disorders and which inevitably ends fatal (WELLS et al. 1987; WILESMITH et al. 1988; BRAUN et al. 1998; ARNOLD & WILESMITH 2004). Three decades ago a huge epidemic in cattle was caused by the feeding of contaminated meat and bone meal and milk replacer to cattle (WILESMITH et al. 1988, 1991). BSE is considered a zoonotic disease as the consumption of BSE-contaminated food may cause the variant form of Creutzfeldt-Jakob disease (BRUCE et al. 1997; HILL et al. 1997a). As BSE can only be diagnosed by postmortem examination, data on C-BSE pathogenesis in incubating cattle can only be obtained by experimental challenge studies.

In all oral C-BSE infection studies realised so far, cattle have been challenged at about 4 to 6 months of age (WELLS et al. 1996; HOFFMANN et al. 2007). By this approach, prions have been detected from 4 months post infection (mpi) to contain BSE infectivity in the ileal Peyer's patch (TERRY et al. 2003; ARNOLD et al. 2009; HOFFMANN et al. 2011; STACK et al. 2011; FAST et al. 2013). Thereby, this GALT in the ileum was shown to function as the entry port for the agent after oral C-BSE exposure of cattle. Apart from those studies, data reflecting the early phase of C-BSE pathogenesis are limited.

Therefore, the first aim of this study was to track the initial phase of C-BSE infection, so as to understand the agent's early centripetal spread towards the brain (manuscripts I and III). In more detail, this thesis was focused on tracking the early BSE pathogenesis in the ileal Peyer's patch (manuscript I) and in nervous tissues in vicinity to the intestine (manuscript III). For this purpose, 20 unweaned Simmental calves were experimentally infected with classical BSE, while 2 additional animals served as negative controls. To ensure a 100 % attack rate (WELLS et al. 2007), the calves were orally challenged with a 100 g dose of a brainstem homogenate pool of clinically diseased cattle. Eighteen challenged calves were euthanised, necropsied and sampled at predetermined time points of 1 week as well as 2, 4, 6 and 8 mpi. As a working hypothesis based on the current state of knowledge (ARNOLD et al. 2007; HOFFMANN et al. 2007; ARNOLD et al. 2009; KAATZ et al. 2012), it was postulated that nervous tissues of the peripheral and central nervous system are most probably free of PrP^{BSE} and BSE prion infectivity at such early time points after oral infection. By performing the first experimental challenge of young unweaned calves, this thesis also looked for indications whether the BSE infection progress in cattle is influenced by the age of the infected animal (manuscripts I and III). The animals were inoculated at this young age of 4 to 6 weeks, in order to simulate the worst-case scenario of supposedly highest prion susceptibility of the animals. Young animals are generally considered to have a higher susceptibility to prion infection (BROWN et al. 2009; HUNTER et al. 2012; BROWN & MABBOTT 2014), and epidemiological data have led to the conclusion that calves up to 6 months had a higher infection risk during the BSE epidemic (ARNOLD & WILESMITH 2004). Using highly sensitive detection methods (IHC, PMCA and Tgbov XV mouse bioassay) (BUSCHMANN & GROSCHUP 2005; BALKEMA-

BUSCHMANN et al. 2011a; HOFFMANN et al. 2011) this study determined the earliest time point of prion detection in the ileal Peyer's patch (IPP) (manuscript I) and in the nervous system (manuscript III) of cattle within short time periods up to 8 months after challenge.

The second aim of this thesis was to explore the potential of the PMCA as an *in-vitro* alternative method to the mouse bioassay for the highly sensitive detection of C-BSE prions (manuscript II).

5.1 PMCA as a potential *in-vitro* alternative method to mouse bioassays and the limitations of the highly sensitive detection of BSE prions

During the course of this study, the analytical sensitivities of the Tgbov XV mouse bioassay and the used PMCA protocol were compared. In a sensitivity assay (manuscript II), PrP^{BSE} and BSE infectivity were detectable by PMCA and Tgbov XV mouse bioassay respectively up to a $10^{-8.3}$ dilution of the brainstem homogenate pool used for challenging the calves. Those findings confirm the comparable sensitivities reported for those two methods before by BALKEMA-BUSCHMANN et al. (2011a).

MURAYAMA et al. (2010) described the use of potassium dextran sulphate as a reaction enhancing additive to enable a PrP^{BSE} detection up to a $10^{-9}/10^{-10}$ dilution of a brain homogenate, which thus made the PMCA 10^5-fold more sensitive than a Tg4092 transgenic mouse bioassay used in that study. Tg4092 mice, also expressing bovine PrP, used in that study remained asymptomatic after inoculation of a 10^{-6} dilution of a BSE-positive spinal cord homogenate (YOSHIOKA et al. 2013). In comparison, the here reported solid detection of BSE infectivity up to a $10^{-6.9}$ dilution indicates a higher sensitivity of the Tgbov XV mouse bioassay. Moreover, the use of additives may cause non-specific PrP accumulation side effects (FRANZ et al. 2012).

Alternatively, cautiously increasing the number of performed PMCA amplification rounds can also enhance the sensitivity, but should also be restricted, as such an approach may favour spontaneous *de novo* generation of prions (SAÁ et al. 2006; FRANZ et al. 2012; MORALES et al. 2012) and therefore lead to false positive results. The overall good accordance between the results obtained for the IPP samples by transgenic mouse bioassay and 3 rounds of PMCA did not indicate a need for further improvement of the PMCA's sensitivity at first. For all other studies, the number of rounds was increased to 4 in order to achieve even a better sensitivity. This resulted in the detection of seeding activity up to a dilution of $10^{-8.3}$ of the positive pool, which was also the end-point of the transgenic mouse bioassay. Adopting more than 4 rounds was presumed to risk a spontaneous *de novo* generation, as observed by FRANZ et al. (2012) after 5 PMCA rounds. Finally, the outstanding sensitivity of the 4 rounds PMCA is illustrated by the detection of seeding activity in the splanchnic nerve of the positive control animal WAIT 04, while using only 3 rounds of amplification had failed to detect this.

It should however be emphasised that the implementation of the BSE-PMCA as a full replacement method of the mouse bioassay still has to be considered a long-term goal, as this still requires further improvement of the sensitivity and repeatability of this PMCA protocol. This is in contrast to the situation published for the hamster adapted laboratory scrapie strain 263K, where the detection of seeding activity using PMCA has been reported to be far more sensitive than bioassays in Syrian hamsters and bank voles (SAÁ et al. 2006; BOERNER et al. 2013; MORALES et al. 2013; CHIANINI et al. 2015). These results have led to the full replacement of bioassays by PMCA for the 263K scrapie strain. As discussed in the previous paragraph, establishing BSE-PMCA protocols with such high sensitivities is more complex, however.

The comparable analytical sensitivities reported here confirm the suitability of the BSE-PMCA to at least partially replace mouse bioassays for examining bovine tissue samples in future C-BSE pathogenesis studies. However during this study, BSE infectivity was also detected by mouse bioassay in the absence of PrP^{BSE} amplification by PMCA for some of the analysed tissue samples. Diluting the analyte tissue in 0.9% saline solution enabled using the same homogenate for analyses by both methods for a better comparison, and thereby excluded sampling artefacts. The samples were first tested by 3 rounds of PMCA, which in case of a positive reaction already represented the final result. If 3 rounds of amplification yielded a negative result, a fourth round was added to ensure the ultra-sensitive detection of low PrP^{BSE} amounts. For future studies, the same homogenate should be reassessed by bovine PrP transgenic mouse bioassay in case of a negative or inconclusive PMCA result. This approach can considerably reduce the number of bioassay mice needed to track minute amounts of prions in bovine samples in future studies. This leads to a considerable reduction of experimental animals used for such studies, since only one Tgbov XV brain was needed to initially screen 5 bovine samples by PMCA, while 40 mice were necessary to bioassay one sample with expectably low, if any, infectivity in this study. The approach described here also refines the remaining number of bioassays, as only a few of the mice are likely to develop clinical BSE signs after inoculation with extremely low titre homogenates (BUSCHMANN & GROSCHUP 2005; BALKEMA-BUSCHMANN et al. 2011a). Moreover, performing PMCA is faster and less costly than the bioassay.

Variable bioassay attack rates were observed in Tgbov XV mice inoculated with dilutions close to the end-point, as 0% and 50% of the mice were positive after challenge with dilutions of $10^{-7.6}$ and $10^{-8.3}$, respectively. Such highly diluted homogenates include very low to minute amounts of the agent. Subsequently, only a few, if any, mice may be challenged by a 30 µl dose of inoculum that actually contains infectivity, considering that the infectious particles may be contained as aggregates and not homogenously distributed within the inoculum, although it has been thoroughly homogenized and sonicated. The latter hypothesis may also account for the similar results by PMCA, when the $10^{-7.6}$ dilution was positive only in the fourth round and the $10^{-8.3}$ dilution in the third and fourth rounds, respectively.

The IHC is performed on sections from formalin-fixed paraffin-embedded samples, while the homogenates are prepared from frozen tissue samples for examination by protein biochemical

methods (PMCA) and the mouse bioassay. Consequently, tissue samples were cut in halves during necropsy. Asymmetry in agent distribution (GONZÁLEZ et al. 2012) may bring along that the locations in which the agent is present may be missed in some cases (CHIANINI et al. 2015). Thus, there is a remaining risk of false negative results by IHC or PMCA/bioassay, as the 2 halves display distinct locations of the same tissue containing possibly different amounts of the BSE agent. This consideration is in line with the current finding of different intensities of (+ and (+)++ positive) PMCA reactions obtained for different areas of the same spinal cord sample from calf WAIT 02. Moreover, this might be linked to certain cellular compartments involved in the agent replication within a tissue. By IHC, CHIANINI et al. (2015) observed that PrP^{Sc} was located in specific locations of the kidney and muscles from scrapie-affected sheep while large areas of these tissues were negative. As a single section would only include a particular location of a formalin-fixed sample, the serial section procedure (HOFFMANN et al. 2011; KAATZ et al., 2012; KAATZ 2014) was used here to examine 5 levels of a block with an overall depth of approximately 195 µm (Figure 9.1 in Chapter 9). Therefore, the used IHC protocol can also be considered a highly sensitive method with the additional advantage of studying PrP^{BSE} accumulation on a cellular level.

Altogether, the different methods can complement each other and thus increase the detection rate of positive samples. IHC was able to show the presence of PrP^{BSE} in individual ganglia cells in the caudal mesenteric ganglion of the positive control animals, while those samples were negative by PMCA. Vice versa, negative or inconclusive IHC results were verified by PMCA due to PrP^{BSE} amplification in low titre samples as shown by the results for splanchnic and vagal nerves from the positive controls. The same was observed for ileal Peyer's patch samples of 2 preclinically infected calves (WAIT 02 and WAIT 19), where the positive PMCA result was also in line with infectivity detection by mouse bioassay. As discussed above, the PMCA and Tgbov XV mouse bioassay provide the advantage of their higher sensitivities when compared to IHC. Hence, the assays chosen here are highly sensitive methods appropriate for the objective of tracking small amounts of BSE prions in tissues probably participating in the early C-BSE pathogenesis.

5.2 Age-dependent facilitated C-BSE prion uptake and replication in the ileal Peyer's patch of unweaned calves

The ileal Peyer's patch was demonstrated to function as the primary entry port for the C-BSE agent in the intestine of bovines after oral infection, as PrP^{BSE} and BSE infectivity were detectable in this location from 4 mpi (TERRY et al. 2003; ARNOLD et al. 2009; HOFFMANN et al. 2011; STACK et al. 2011; FAST et al. 2013). In those studies, cattle were orally challenged at 4 to 6 months of age (WELLS et al. 1996; HOFFMANN et al. 2007). The proportions of IHC positive IPP follicles were low at 4 and 6 mpi (HOFFMANN et al. 2011; STACK et al. 2011), while moderate infectivity loads were detectable already at 4 mpi (FAST et al. 2013). The ear-

lier time point of 2 mpi either revealed negative results by IHC and RIII wildtype mouse bioassay (WELLS et al. 1994; TERRY et al. 2003; ARNOLD et al. 2009) or it was not included in the analyses (HOFFMANN et al. 2011; FAST et al. 2013).

The current study documents the earliest time point of BSE prion amplification in bovines reported thus far. At 2 mpi, PrP^{BSE} was detectable using the highly sensitive PMCA (IPP of WAIT 15) and low infectivity loads (IPPs of WAIT 15 and 16) were shown by Tgbov XV mice, which are 10,000-fold more sensitive as compared to RIII mice (BUSCHMANN & GROSCHUP 2005). The calves in this study were orally challenged before weaning at 4 to 6 weeks of age, so that the young age upon challenge may have influenced the intestinal BSE pathogenesis. This is indicated by high infectivity titres and ++ to +++ positive PMCA amplification reactions in the IPPs of most calves from 4 mpi. Moreover, IHC analyses here already revealed moderate proportions of up to 1.18% PrP^{BSE}-positive IPP follicles for the majority of the calves from 4 mpi, as compared to earlier reported IHC results of up to 0.6% positive follicles in the IPPs from cattle at 4 and 8 mpi (HOFFMANN et al. 2011). Surprisingly, higher PrP^{BSE} amounts were observed in the IPP of 3 calves, with 2.15% (WAIT 06, 6 mpi), 4.26% (WAIT 20, 4 mpi) and 5.24% (WAIT 08, 6 mpi) positive follicles, as compared to the other animals in this experiment. These data suggest that, due to the young age, prion replication and accumulation was favoured in the IPP follicles of these calves in the intestinal phase of BSE pathogenesis.

An influence of the high (100 g) dose challenge can be excluded, as the current experiment was compared to data from cattle equally dosed in the earlier studies (TERRY et al. 2003; ARNOLD et al. 2009; HOFFMANN et al. 2011; STACK et al. 2011; FAST et al. 2013). The influence of the age on the intestinal pathogenesis is also indicated by studies in mice, where aged animals showed a reduced susceptibility to oral prion infection due to an inefficient prion uptake caused by a decreased density of functional mature M cells in the FAE (KOBAYASHI et al. 2013) and age-dependent reduction of FDCs impairing agent accumulation in lymphoid tissues (BROWN et al. 2009; BROWN & MABBOTT 2014). In the current study, a combination of these 2 mechanisms may have been the cause of intense prion replication in the IPP:

Firstly, the uptake of BSE prion proteins, as macromolecules, may have been increased in the intestinal mucosa of such young calves. ANO et al. (2008) showed that β-amyloid protein, another protein rich in β-sheet structure, was incorporated more easily in 2-week-old calves during the suckling period as compared to 6 months old calves. Facilitated macromolecule uptake in the intestine of young animals (UDALL et al. 1981; UDALL & WALKER 1982) has also been considered a reason for the enhanced susceptibility of unweaned lambs orally challenged with BSE (HUNTER et al. 2012).

Secondly, the early FDC-associated IHC staining patterns in the IPP follicles of some calves from four mpi suggest an age-dependent reduction of clearing processes by the TBMs. Such FDCs staining indicates the breakdown of an initially existing balance between prion degradation by macrophages and accumulation on FDCs (MABBOTT & BRUCE 2001; HOFFMANN et al. 2011). HOFFMANN et al. (2011) reported the earliest PrP^{BSE} accumulation on FDCs from 12 mpi in IPP follicles of older cattle. A favoured agent accumulation may depend on functional

FDCs (BROWN et al. 2009; BROWN & MABBOTT 2014) and on better developed FDC networks in the IPP, while the latter might have determined the enhanced susceptibility of young lambs compared to adult sheep (MARRUCHELLA et al. 2012).

These new data confirm the IPP as the entry port for the BSE agent as well as a site of initial prion propagation, which seems to occur already within 2 months after oral challenge. Moreover, a facilitated BSE prion uptake combined with an age-dependent reduction of clearance efforts seemed to trigger the detection of relatively large amounts of the agent at such early time points after oral infection.

5.3 The Role of the LRS in C-BSE pathogenesis

With the exception of the IPP, the LRS is rarely involved in the BSE pathogenesis in cattle (BUSCHMANN & GROSCHUP 2005; WELLS et al. 2005; ESPINOSA et al. 2007). In bovines, infections of the tonsils are thought to occur primarily by direct exposure during oral prion challenge and possibly also due to rumination (WELLS et al. 2005). A cattle bioassay only revealed infectivity in a pool of tonsil samples collected from 3 cattle at 10 mpi (WELLS et al. 2005). Low attack rates were also observed in boPrP-Tg110 mice after analysis of pooled tonsil samples collected between 20 and 33 mpi (ESPINOSA et al. 2007). However, in most cattle neither infectivity by bioassay (BUSCHMANN & GROSCHUP 2005) nor PrP^{BSE} by IHC (WELLS et al. 2005) or PMCA (FRANZ et al. 2012) was detected in the tonsils thus far. FRANZ et al. (2012) suggested that an only transient presence of BSE prions after oral challenge might be a possible reason for the negative PMCA results obtained for tonsils from cattle at 36 to 50 mpi. However, the detection of PrP^{BSE} at 46 mpi by IHC in the retropharyngeal tonsil (OKADA et al. 2011a) and at 57 mpi by PMCA in the palatine tonsil of orally challenged cattle (MURAYAMA et al. 2010) has been reported by others.

Also in this current study, all lingual tonsils sampled from the calves up to 8 mpi were negative by IHC and PMCA, while infectivity was detectable in a retropharyngeal lymph node from one calf at 6 mpi (WAIT 06; Table 9.2 in Chapter 9). Since the medial retropharyngeal lymph nodes receive tributaries from the pharynx and other lymph nodes of the head, infectivity in this location most likely originated from the oral prion exposure of this calf, while infection after rumination can be excluded in an unweaned calf. It does indeed seem possible that BSE prions may spread at a very low level to lymph nodes, as PMCA analyses showed PrP^{BSE} amplification in mesenterial lymph node samples from cattle at 36 mpi (FRANZ et al. 2012) and 57 mpi (MURAYAMA et al. 2010).

Nevertheless, the new data from calves up to 8 mpi indicate that the pharyngeal LRS components are not required for prion propagation in the early pathogenesis phase after oral infection. This is in line with the common view that BSE pathogenesis generally bypasses the LRS of cattle (BUSCHMANN & GROSCHUP 2005; WELLS et al. 2005; ESPINOSA et al. 2007; HOFFMANN et al. 2007; FRANZ et al. 2012).

5.4 Early occurrence of C-BSE neuroinvasion is independent of the age of the infected animal

The relatively extensive prion accumulation in the IPP of the calves raised the question whether neuroinvasion could already be ongoing at these early time points. Considering the results of 2 cattle C-BSE pathogenesis studies in the UK (ARNOLD et al. 2007, 2009) and Germany (HOFFMANN et al. 2007; KAATZ et al. 2012), it seemed extremely unlikely that BSE infectivity could be present in the nervous system of cattle within the first 8 months after an oral challenge. HOFFMANN et al. (2011) reported the first evidence of neuroinvasion in preclinical cattle from 16 mpi, when IHC detected PrP^{BSE} accumulation in the enteric nervous system (ENS) of the IPP.

In this current study, glial PrP^{BSE} staining was seen in a single submucosal plexus of the ENS of one calf (WAIT 16) sacrificed at 2 mpi. However, a single ENS positive result provides just a first indication of a beginning neuroinvasion. But at 8 mpi, infectivity and PMCA-detectable PrP^{BSE} in the nodal ganglion and thoracic spinal cord (WAIT 02) confirmed the successful centripetal spread in the initial phase of C-BSE pathogenesis. The detection of infectivity in the CNS at 8 mpi seems in line with infection of the ENS within 2 months after oral challenge.

The PrP^{BSE} accumulation in the ENS combined with negative IPP follicles of calf WAIT 16 may indicate a possible direct neuroinvasion in these young calves. Earlier studies considered that such observations resulted from the simultaneous exposure of ENS and LRS via nerve fibres present in the lamina propria mucosae and the suprafollicular dome (HEGGEBØ et al. 2003; JEFFREY et al. 2006; HOFFMANN et al. 2011). On the one hand, the lack of IHC-detectable PrP^{BSE} in the ENS plexuses of the other calves' IPP samples may support the idea of neuroinvasion directly through nerve fibres in the intestinal mucosa. On the other hand, PrP^{BSE} in the IPP follicles of WAIT 16 or in the ENS of the other animals could have been present in amounts below a threshold of IHC detection (JEFFREY et al. 2006). The presence of only small amounts of PrP^{BSE} seems plausible, as a + positive PMCA reaction revealed PrP^{BSE} amplification in the IHC negative IPP of 2 mpi calf WAIT 15. Thus, it cannot be excluded that PrP^{BSE} replication in the IPP follicles may have preceded infection of the ENS.

Nevertheless, PrP^{BSE} in the ENS and infectivity in the nervous system of these calves indicate the initiation of centripetal prion spread very early after oral BSE challenge. Such surprising results refuted the working hypothesis that nervous tissues of the PNS and CNS would most probably be free of PrP^{BSE} and BSE infectivity at early stages up to 8 months after oral infection. In an earlier study, infectivity was observed in peripheral nerves and ganglia (coeliac ganglion complex, splanchnic nerves and cranial cervical ganglion) as well as in the CNS (thoracic spinal cord) of cattle as early as 16 mpi (KAATZ et al. 2012). However, that study analysed only nervous tissue samples from cattle incubating at least 16 mpi (KAATZ et al. 2012), which seemed reasonable when examining a disease with such an exceptionally long mean incubation period (WELLS et al. 1987; WILESMITH et al. 1988; BRAUN et al. 1998; ARNOLD & WILESMITH 2004). Based on the results obtained so far in the current study, the brainstem

seems free of the agent up to 8 mpi. Albeit, the detection of infectivity in the thoracic spinal cord as well as in the parasympathetic nodal ganglion (located close to the brain) of calf WAIT 02 confirmed that neuroinvasion had occurred within a short incubation phase of only 8 months after oral infection. Moreover, these novel findings indicate that BSE prions may spread through the nervous system faster than hitherto presumed.

To evaluate the influence of the age on the progression of neuroinvasion, samples of the thoracic spinal cord from cattle challenged at 4 to 6 months of age (during the German BSE pathogenesis study) and sacrificed at 8 mpi (HOFFMANN et al. 2007; KAATZ et al. 2012) were analysed with the current PMCA protocol. Thereby, PrP^{BSE} was detected in the spinal cord of 2 out of the 4 examined cattle, which supported the results obtained for WAIT 02 (8 mpi). These findings, taken together, suggest that the speed of neuroinvasion in cattle does not vary largely with age.

According to earlier studies, prion spread via the coeliac mesenteric ganglion complex and subsequently through the sympathetic splanchnic nerve and the parasympathetic vagal nerve precedes accumulation in the thoracic spinal cord and nodal ganglion (HOFFMANN et al. 2007; KAATZ et al. 2012). Surprisingly, in the current study it was not possible to track any prions in PNS samples in vicinity to the intestine. So far, only negative results were obtained for the coeliac and caudal mesenteric ganglia as well as for the splanchnic and vagal nerves of all examined calves up to 8 mpi, including animal WAIT 02 where the spinal cord and the nodal ganglion were positive for PrP^{BSE} and BSE infectivity. One possible explanation for this finding is that prions might be in transit without actively replicating in nerves (MCBRIDE et al. 2001), which has been considered as a reason for negative nerve samples before (HEGGEBØ et al. 2003; HOFFMANN et al. 2007). Otherwise, a remaining risk of false negative results, for instance for the coeliac ganglion of WAIT 02, cannot be excluded, considering that different locations of the same sample may possibly contain different amounts of the BSE agent (as discussed in more detail in Section 5.1). By the time of writing this thesis, some of the bioassays and PMCA analyses were still ongoing. Hence, no final conclusions regarding the BSE pathogenesis in peripheral nervous tissues in vicinity to the intestine can be provided at this stage.

Although the BSE epidemic has mostly been overcome and regulations have partly been lifted, a number of consumer and public health protection measures are still in place to date. The current findings indicate that the IPP and nervous system of infected cattle might contain PrP^{BSE} and BSE infectivity and thus may provide a risk of exposure to humans. Regulation (EC) No 999/2001 (as of 05.2017) defines the current list of specified risk materials (SRM). Removing the skull including the brain and eyes as well as the spinal cord of animals over 12 months of age may remain an adequate consumer protection measure in case the slaughtered cattle were born in a country with a negligible BSE risk. Albeit, these data may hint that the removal of the spinal cord from cattle of all age groups should be considered in countries with a controlled or undermined risk. Furthermore, in case of cattle originating from these countries, the current SRM list additionally includes the last 4 metres of the small intestine, tonsils, caecum and mesentery (with the mesenteric ganglion complex, nerves and fat) from animals of all ages. The

current analyses suggest that the IPPs of BSE-infected calves may provide an exposure risk to humans, regardless whether BSE infectivity originates from newly propagated BSE prions (from about 2 mpi) or from residual inoculum (which is possibly still contained in the intestine at about 1 week post infection). These observations support the removal of the last 4 metres of the small intestine (distal ileum) at slaughter as an essential measure for consumer protection.

Moreover, different regulations are in place for pharmaceutical products and are regulated by the EU commission through the 'Note for guidance on minimising the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products' (EMA/410/01 rev.3). Accordingly, medicinal products shall not, or only in justified exceptions, be produced from high-infectivity tissues (category IA based on WHO tables, WHO 2010), while the tissues in these 3 major infectivity categories IA, IB, IC are grouped regardless of the origin of the animal, the stage of disease and thus the age of the animals (Note for guidance EMA/410/01 rev.3 (EUROPEAN COMMISSION 2011)). The here observed neuroinvasion in calves early after an oral BSE infection indicates that it seems advisable to maintain these consumer protection measures for the production of pharmaceutical products and meat, but that these measures shall be reviewed taking in account these new data obtained in the current study.

Finally, it should be highlighted that a high 100 g dose was used in this study in order to receive a complete attack rate in the challenged calves (WELLS et al. 2007). But, such a high exposure dose is highly unlikely under field conditions (ARNOLD et al. 2009), especially as natural BSE infections seem more and more improbable considering the successful control of the epidemic.

5.5 Indications for age-dependent intestinal pathogenesis but age-independent neuroinvasion of C-BSE in cattle

Thus far, the estimation of a higher infection risk of cattle up to 6 months of age was solely based on epidemiological data (ARNOLD & WILESMITH 2004). An experimental BSE challenge of sheep showed that unweaned lambs were more susceptible to the infection than older weaned lambs or adults (HUNTER et al. 2012). Here, the experimental BSE infection of unweaned calves provided insights into the age-dependent susceptibility of bovines to BSE.

Although this study did not perform a comparative challenge of unweaned and weaned calves, the results obtained for unweaned calves can be compared to those of an earlier BSE challenge of 4 to 6 months old cattle (HOFFMANN et al. 2007, 2011; KAATZ et al. 2012). The challenge inocula used in both studies displayed a similar infectivity titre, as determined by end-point titration in Tgbov XV mice: The inoculum used in this present work to challenge the unweaned calves had a LD_{50}-titer of $10^{-5.730}$ (95 % confidence interval, $10^{-6.569}$ – $10^{-4.891}$). In the earlier study, the challenge of 4 to 6 months old cattle was performed using an inoculum with a LD_{50}-titer of $10^{-5.417}$ (95 % confidence interval, $10^{-5.705}$ – $10^{-5.130}$) alias $10^{6.1}$ ID_{50}/g tissue (HOFFMANN et al. 2007).

The findings seem to indicate an elevated susceptibility of young calves to the infection of the IPP. A facilitated prion uptake combined with a decreased clearance seems to favour prion replication in the IPP of these calves (as discussed in Section 5.2). Likewise, the positive control animals showed the first clinical signs as early as 32 and 36 mpi (Table 9.3 in Chapter 9), which may indicate a shorter average incubation time as compared to the average incubation periods in earlier studies challenging older calves (ARNOLD et al. 2007; KAATZ et al. 2012). However, this assumption is still unproven, as two animals are not a sufficient number for it.

However, seeding activity was detectable at 8 mpi in the thoracic spinal cord of both age groups challenged before weaning (WAIT 02) and at 4-6 months of age (IT 20 and 39), respectively. This finding provided an indication that the progress of the BSE infection in the nervous system (neuroinvasion) is not influenced by the age of bovines.

Comparing the age-related variation in prion pathogenesis of cattle to that of sheep and mice, the difference may result from the overall number of FDCs providing prion replication in the LRS. Similar to the observations in the young calves in this study, the age-dependent enhancement of prion uptake may only be the first trigger of the higher susceptibility of young sheep and mice (HUNTER et al. 2012; KOBAYASHI et al. 2013; DONALDSON et al. 2016). From then on, prion replication on FDCs of the LRS follicles may play a considerable role for further progression of the infection (MABBOTT et al. 2003; GLAYSHER & MABBOTT 2007; MCCULLOCH et al. 2011). The age-dependent reduction of functional FDCs impaired the agent accumulation in lymphoid tissues and thus neuroinvasion after peripheral prion infection of aged mice (BROWN et al. 2009; BROWN & MABBOTT 2014). Similarly, IPPs of young lambs were shown to have better developed FDC networks than those of adult sheep, which might result in an enhanced susceptibility (MARRUCHELLA et al. 2012). This effect might not occur in bovines, as the LRS – except for the IPP – is rarely involved in cattle BSE pathogenesis (as discussed in Section 5.3) (BUSCHMANN & GROSCHUP 2005; WELLS et al. 2005; ESPINOSA et al. 2007).

Otherwise, these age-dependent differences in FDC development were seen in young animals as compared to adults (BROWN et al. 2009; MARRUCHELLA et al. 2012; BROWN & MABBOTT 2014). These circumstances may thus not apply for the bovines examined in both studies mentioned here. Although the animals challenged at 4 to 6 months of age were older than the unweaned calves, they were still not adult and the development of the FDC system and the IPP may still differ from that of adult cattle. However, the direct neuroinvasion may be independent of the FDC status or the development of FDC networks and could thus provide another explanation for the observations in these cattle.

Finally, even if prion uptake and replication seemed to be increased in the IPP of the unweaned calves, this study did not observe any hints for age-dependent differences in the progress of the C-BSE infection. The further analyses ongoing in this pathogenesis study (indicated in manuscript III) will provide deeper insights concerning the early centripetal spread of C-BSE during the first 8 months after oral infection.

Chapter 6 Summary

Early Pathogenesis of Classical Bovine Spongiform Encephalopathy in Young Calves

Ivett Ackermann

Classical bovine spongiform encephalopathy (C-BSE) is a fatal neurodegenerative disease in cattle. It belongs to the group of transmissible spongiform encephalopathies, or prion disease, which are caused by the conversion of the host-encoded cellular prion protein (PrP^{C}) to its pathological isoform PrP^{TSE}. After oral exposure to C-BSE, the ileal Peyer's patch (IPP) represents the entry port for the BSE agent. Then, the agent spreads centripetally from the gut to the central nervous system (CNS), utilizing primarily the autonomic nervous system. However, the timeline of this infection progression has remained widely undetermined so far, as previous studies focused on later time points after oral challenge of cattle at 4 to 6 months of age. There are strong indications that young animals have a higher risk of infection.

The aim of the present study was to determine the earliest time point at which newly formed PrP^{BSE} and BSE infectivity are detectable in the IPP and in the nervous system of cattle. Furthermore, this study aimed to evaluate the potential of the Protein Misfolding Cyclic Amplification (PMCA) as an *in-vitro* alternative method to mouse bioassays for the highly sensitive detection of BSE prions. For this, 20 unweaned calves were orally challenged with C-BSE and euthanised 1 week as well as 2, 4, 6 and 8 months post infection (mpi). The IPP as well as peripheral and central nervous tissues were examined for BSE infectivity by Tgbov XV mouse bioassay and for PrP^{BSE} by immunohistochemistry (IHC) and PMCA.

During the course of this study, the analytical sensitivities of these methods used were assessed. The transgenic Tgbov XV mouse bioassay and a PMCA protocol with 4 amplification rounds both detected BSE prions up to a $10^{-8.3}$ dilution of the brainstem homogenate used for challenge of the calves. The comparable sensitivities of PMCA and Tgbov XV mouse bioassay may allow the partial replacement and thereby reduction as well as refinement of bioassays aiming to prove the presence or absence of the BSE agent in bovine samples, which is relevant for future BSE pathogenesis studies. Thus, the PMCA was shown to be an optimal method for analysing tissues with even low titres of BSE prions. This finding proved that the assays chosen here are highly sensitive methods that are appropriate for tracking small amounts of the C-BSE agent in bovine tissues.

This study showed the presence of the BSE agent in the ileum of cattle earlier than hitherto reported. BSE prions were detected in the IPP as early as 2 mpi by Tgbov XV mouse bioassay and PMCA as well as from 4 mpi by IHC in follicular dendritic cells of the IPP follicles. From

4 mpi, high infectivity titres and ++ to +++ positive PMCA amplification reactions were revealed for the IPPs of most calves. These new data indicated that a facilitated BSE prion uptake combined with an age-dependent reduction of clearance efforts may have triggered the prion replication at such early time points after oral infection.

The here reported analyses refuted the hypothesis that nervous tissues of cattle are most probably free of PrP^{BSE} and BSE prion infectivity during the early phase after oral BSE infection. By IHC, PrP^{BSE} granules were seen in a submucosal plexus of the enteric nervous system of one calf at 2 mpi. Mouse bioassay and PMCA detected BSE prions in the nodal ganglion and the thoracic spinal cord of one calf at 8 mpi. These novel findings indicated that the centripetal prion spread is initiated almost immediately after oral BSE challenge. Subsequently, this study performed comparative PMCA analyses of thoracic spinal cord samples from cattle challenged at 4 to 6 months of age during an earlier pathogenesis study. Thereby, PrP^{BSE} amplification at 8 mpi in the thoracic spinal cord of older cattle confirmed the observation of early centripetal spread and suggested that the speed of neuroinvasion in cattle does not vary with age.

In conclusion, these new data confirmed the IPP as the entry port for the BSE agent and a location of initial prion propagation almost immediately after oral infection. Although the prion uptake seemed to be increased in the intestine of unweaned calves, this study did not observe any hints for age-dependent differences in the neuroinvasion or further progression of the C-BSE infection. The obtained data might be useful for risk assessment regarding consumer and public health protection measures. Moreover, the highly sensitive PMCA protocol allows reduction of mouse bioassays in future BSE pathogenesis studies.

Chapter 7 Zusammenfassung

Frühe Pathogenese der klassischen Bovinen Spongiformen Enzephalopathie bei jungen Kälbern

Ivett Ackermann

Die klassische Bovine Spongiforme Enzephalopathie (C-BSE) ist eine tödlich verlaufende neurodegenerative Erkrankung bei Rindern, welche zur Gruppe der Transmissiblen Spongiformen Enzephalopathien, auch Prionen-Erkrankungen genannt, gehört. Die Entstehung dieser Erkrankungen basiert auf der Konversion des körpereigenen zellulären Prion-Proteins (PrP^{C}) in seine pathologische Isoform PrP^{TSE}. Nach oraler Exposition mit C-BSE stellt die ileale Peyer'sche Platte (IPP) die Eingangspforte für das BSE-Agens dar. Danach breitet sich der Erreger zentripetal vom Darm, vorwiegend über das autonome Nervensystem, in Richtung des zentralen Nervensystems (ZNS) aus. Allerdings blieb der genaue zeitliche Verlauf dieses Infektionsfortschritts bisher überwiegend unbestimmt, da sich bisherige Studien auf spätere Zeitpunkte nach der oralen Infektion von 4 bis 6 Monate alten Rindern konzentrierten. Es gibt aber durchaus Hinweise darauf, dass Jungtiere empfänglicher für BSE sind.

Ziel dieser Studie war es, den frühestmöglichen Zeitpunkt zu bestimmen, zu dem neuentstandene BSE-Infektiosität und PrP^{BSE} in der IPP und dem Nervensystem von Rindern detektierbar sind. Zudem wurde in dieser Studie angestrebt, die Protein Misfolding Cyclic Amplification (PMCA) hinsichtlich ihres Potentials als *in-vitro*-Ersatzmethode zum Maus-Bioassay für eine hochsensitive Detektion von BSE-Prionen zu beurteilen. Dazu wurden 20 Saugkälber oral mit C-BSE infiziert und 1 Woche sowie 2, 4, 6 und 8 Monate post infectionem (MPI) euthanasiert. Die IPP sowie periphere und zentrale nervale Gewebe wurden mittels TgbovVX-Maus-Bioassay auf BSE-Infektiosität und mittels Immunhistochemie (IHC) und PMCA auf PrP^{BSE} untersucht.

Im Verlauf dieser Studie wurde die analytische Sensitivität der verwendeten Methoden verglichen. Dabei detektierten der transgene TgbovXV-Maus-Bioassay und das PMCA-Protokoll mit vier Amplifikationsrunden BSE-Prionen bis zu einer Verdünnung von $10^{-8.3}$ des Hirnstamm-Homogenats, welches für die Infektion der Kälber genutzt wurde. Die vergleichbare Sensitivität der PMCA zu der des TgbovXV-Maus-Bioassays ermöglicht nun dessen partiellen Ersatz und somit eine Reduzierung und Verbesserung von Bioassays, welche auf eine Überprüfung des Vorhandenseins von BSE-Prionen in Rinder-Gewebeproben abzielen. Dies wäre für zukünftige BSE-Pathogenese-Studien relevant. Es konnte so gezeigt werden, dass die PMCA grundsätzlich

geeignet ist, um Gewebe mit geringen BSE-Prionen-Gehalten zu untersuchen. Diese Erkenntnisse bestätigten die Eignung dieser hochsensitiven Methoden zum Nachweis kleinster BSE-Prionen-Konzentrationen in den Rinderproben in dieser Studie.

Dabei wurden BSE-Erreger im Ileum der Rinder bereits zu einem früheren als dem bisher bekannten Zeitpunkt gefunden. BSE-Prionen waren in der IPP bereits nach 2 Monaten *post infectionem* (mittels TgbovXV-Maus-Bioassay und PMCA) detektierbar und mittels IHC ab 4 MPI in follikulären dendritischen Zellen der IPP-Follikel nachweisbar. Für die IPPs der meisten Kälber ab 4 MPI waren hohe Mengen an BSE-Infektiosität und mittel- bis hochgradig (++ bis +++) positive PMCA-Amplifikationsreaktionen nachweisbar. Diese neuen Erkenntnisse deuten darauf hin, dass eine erleichterte BSE-Prionen-Aufnahme gemeinsam mit einer altersabhängig verbesserten Prionen-Replikation in der IPP zu solch frühen Zeitpunkten nach oraler Infektion vorliegen.

Die hier gezeigten Untersuchungen widerlegten die Hypothese, dass während der frühen Phase einer BSE-Infektion in Rindern, die nervalen Gewebe höchstwahrscheinlich frei von PrP^{BSE} und BSE-Prionen-Infektiosität sind. Mittels IHC wurden PrP^{BSE}-Granula in einem *Plexus submucosus* des enterischen Nervensystems eines Kalbes bereits nach 2 MPI detektiert. Maus-Bioassay und PMCA wiesen BSE-Prionen im *Ganglion nodosum* und im thorakalen Rückenmark eines Kalbes 8 Monate nach der Infektion nach. Die neuartigen Ergebnisse lassen darauf schließen, dass die zentripetale Prionen-Ausbreitung nahezu unmittelbar nach der oralen Infektion beginnt. Daraufhin wurden vergleichende PMCA-Analysen von Proben des thorakalen Rückenmarks älterer Tiere durchgeführt. Diese Tiere wurden im Rahmen einer früheren Pathogenese-Studie im Alter von 4 bis 6 Monaten mit klassischer BSE infiziert. Dabei wurde eine PrP^{BSE}-Amplifikation bereits nach 8 Monaten im thorakalen Rückenmark dieser später infizierten Tiere gefunden. Dies legt eine frühzeitig beginnende zentripetale Ausbreitung nahe. Zudem lässt dieses Ergebnis vermuten, dass die Geschwindigkeit der Neuroinvasion unabhängig vom Alter der Rinder zum Infektionszeitpunkt ist.

Schlussendlich bestätigen diese neuen Daten, dass die IPP die Eintrittspforte für das BSE-Agens und einen Ort der initialen Prionen-Vermehrung darstellt, welche nahezu direkt nach der Infektion stattfindet. Obwohl die Prionen-Aufnahme im Darm von Saugkälbern erhöht zu sein scheint, konnte diese Studie keine Hinweise auf altersabhängige Unterschiede in der Neuroinvasion bzw. weiteren Ausbreitung der C-BSE-Infektion feststellen. Die Daten ermöglichen verbesserte Risikobewertungen bezüglich des Verbraucherschutzes und des öffentlichen Gesundheitsschutzes. Außerdem erlaubt das hochsensitive PMCA-Protokoll die Reduzierung von Maus-Bioassays in zukünftigen BSE-Pathogenese-Studien.

Chapter 8 References

ABID, K., R. MORALES & C. SOTO (2010):
Cellular factors implicated in prion replication.
FEBS Lett 584, 2409-2414

ACKERMANN, I., A. BALKEMA-BUSCHMANN, R. ULRICH, K. TAUSCHER, J. C. SHAWULU, M. KELLER, O. I. FATOLA, P. BROWN & M. H. GROSCHUP (2017):
Detection of PrP^{BSE} and prion infectivity in the ileal Peyer's patch of young calves as early as 2 months after oral challenge with classical bovine spongiform encephalopathy.
Vet Res 48, 88

ACKERMANN, I., J. C. SHAWULU, M. KELLER, O. I. FATOLA, M. H. GROSCHUP & A. BALKEMA-BUSCHMANN (2018):
Exploring PMCA as a potential in-vitro alternative method to mouse bioassays for the highly sensitive detection of BSE prions.
Berl Münch Tierärztl Wochenschr OA, 18021

ÅKESSON, C. P., L. P. C. MC, A. ESPENES & M. ALEKSANDERSEN (2008):
Phenotypic characterisation of intestinal dendritic cells in sheep.
Dev Comp Immunol 32, 837-849

ÅKESSON, C. P., G. MCGOVERN, M. P. DAGLEISH, A. ESPENES, L. P. C. MC, T. LANDSVERK & M. JEFFREY (2011):
Exosome-producing follicle associated epithelium is not involved in uptake of PrP^{d} from the gut of sheep (Ovis aries): an ultrastructural study.
PLoS One 6, e22180

ÅKESSON, C. P., C. M. PRESS, M. A. TRANULIS, M. JEFFREY, M. ALEKSANDERSEN, T. LANDSVERK & A. ESPENES (2012):
Phenotypic characterization of cells participating in transport of prion protein aggregates across the intestinal mucosa of sheep.
Prion 6, 261-275

ANDRÉOLETTI, O., P. BERTHON, D. MARC, P. SARRADIN, J. GROSCLAUDE, L. VAN KEULEN, F. SCHELCHER, J. M. ELSEN & F. LANTIER (2000):
Early accumulation of PrP^{Sc} in gut-associated lymphoid and nervous tissues of susceptible sheep from a Romanov flock with natural scrapie.
J Gen Virol 81, 3115-3126

ANO, Y., H. NAKAYAMA, Y. SAKAI, A. SAKUDO, M. ENDO, S. EBISU, J. LI, K. UETSUKA, N. MANABE & T. ONODERA (2008):
Incorporation of beta-amyloid protein through the bovine ileal epithelium before and after weaning: model for orally transmitted amyloidoses.
Microbiol Immunol 52, 429-434

ARNOLD, J. E., C. TIPLER, L. LASZLO, J. HOPE, M. LANDON & R. J. MAYER (1995):
The abnormal isoform of the prion protein accumulates in late-endosome-like organelles in scrapie-infected mouse brain.
J Pathol 176, 403-411

ARNOLD, M. E., S. A. HAWKINS, R. GREEN, I. DEXTER & G. A. WELLS (2009):

Pathogenesis of experimental bovine spongiform encephalopathy (BSE): estimation of tissue infectivity according to incubation period.
Vet Res 40, 8

ARNOLD, M. E., J. B. RYAN, T. KONOLD, M. M. SIMMONS, Y. I. SPENCER, A. WEAR, M. CHAPLIN, M. STACK, S. CZUB, R. MUELLER, P. R. WEBB, A. DAVIS, J. SPIROPOULOS, J. HOLDAWAY, S. A. HAWKINS, A. R. AUSTIN & G. A. WELLS (2007):
Estimating the temporal relationship between PrP^{Sc} detection and incubation period in experimental bovine spongiform encephalopathy of cattle.
J Gen Virol 88, 3198-3208

ARNOLD, M. E. & J. W. WILESMITH (2004):
Estimation of the age-dependent risk of infection to BSE of dairy cattle in Great Britain.
Prev Vet Med 66, 35-47

ASANTE, E. A., J. M. LINEHAN, M. DESBRUSLAIS, S. JOINER, I. GOWLAND, A. L. WOOD, J. WELCH, A. F. HILL, S. E. LLOYD, J. D. WADSWORTH & J. COLLINGE (2002):
BSE prions propagate as either variant CJD-like or sporadic CJD-like prion strains in transgenic mice expressing human prion protein.
EMBO J 21, 6358-6366

ATARASHI, R., K. SATOH, K. SANO, T. FUSE, N. YAMAGUCHI, D. ISHIBASHI, T. MATSUBARA, T. NAKAGAKI, H. YAMANAKA, S. SHIRABE, M. YAMADA, H. MIZUSAWA, T. KITAMOTO, G. KLUG, A. MCGLADE, S. J. COLLINS & N. NISHIDA (2011):
Ultrasensitive human prion detection in cerebrospinal fluid by real-time quaking-induced conversion.
Nat Med 17, 175-178

ATARASHI, R., J. M. WILHAM, L. CHRISTENSEN, A. G. HUGHSON, R. A. MOORE, L. M. JOHNSON, H. A. ONWUBIKO, S. A. PRIOLA & B. CAUGHEY (2008):
Simplified ultrasensitive prion detection by recombinant PrP conversion with shaking.
Nat Methods 5, 211-212

BALDAUF, E., M. BEEKES & H. DIRINGER (1997):
Evidence for an alternative direct route of access for the scrapie agent to the brain bypassing the spinal cord.
J Gen Virol 78, 1187-1197

BALKEMA-BUSCHMANN, A., M. EIDEN, C. HOFFMANN, M. KAATZ, U. ZIEGLER, M. KELLER & M. H. GROSCHUP (2011a):
BSE infectivity in the absence of detectable PrP^{Sc} accumulation in the tongue and nasal mucosa of terminally diseased cattle.
J Gen Virol 92, 467-476

BALKEMA-BUSCHMANN, A., C. FAST, M. KAATZ, M. EIDEN, U. ZIEGLER, L. MCINTYRE, M. KELLER, B. HILLS & M. H. GROSCHUP (2011b):
Pathogenesis of classical and atypical BSE in cattle.
Prev Vet Med 102, 112-117

BALKEMA-BUSCHMANN, A., U. ZIEGLER, L. MCINTYRE, M. KELLER, C. HOFFMANN, R. ROGERS, B. HILLS & M. H. GROSCHUP (2011c):
Experimental challenge of cattle with German atypical bovine spongiform encephalopathy (BSE) isolates.
J Toxicol Environ Health A 74, 103-109

BANNACH, O., E. REINARTZ, F. HENKE, F. DRESSEN, A. OELSCHLEGEL, M. KAATZ, M. H. GROSCHUP, D. WILLBOLD, D. RIESNER & E. BIRKMANN (2013):
Analysis of prion protein aggregates in blood and brain from pre-clinical and clinical BSE cases.
Vet Microbiol 166, 102-108

BARON, T., A. G. BIACABE, J. N. ARSAC, S. BENESTAD & M. H. GROSCHUP (2007):
Atypical transmissible spongiform encephalopathies (TSEs) in ruminants.
Vaccine 25, 5625-5630

BARON, T., J. VULIN, A. G. BIACABE, L. LAKHDAR, J. VERCHERE, J. M. TORRES & A. BENCSIK (2011):
Emergence of classical BSE strain properties during serial passages of H-BSE in wild-type mice.
PLoS One 6, e15839

BARRON, R. M., S. L. CAMPBELL, D. KING, A. BELLON, K. E. CHAPMAN, R. A. WILLIAMSON & J. C. MANSON (2007):
High titers of transmissible spongiform encephalopathy infectivity associated with extremely low levels of PrP^{Sc} in vivo.
J Biol Chem 282, 35878-35886

BEEKES, M., E. BALDAUF & H. DIRINGER (1996):
Sequential appearance and accumulation of pathognomonic markers in the central nervous system of hamsters orally infected with scrapie.
J Gen Virol 77, 1925-1934

BEEKES, M. & P. A. MCBRIDE (2000):
Early accumulation of pathological PrP in the enteric nervous system and gut-associated lymphoid tissue of hamsters orally infected with scrapie.
Neurosci Lett 278, 181-184

BEEKES, M. & P. A. MCBRIDE (2007):
The spread of prions through the body in naturally acquired transmissible spongiform encephalopathies.
FEBS J 274, 588-605

BEEKES, M., P. A. MCBRIDE & E. BALDAUF (1998):
Cerebral targeting indicates vagal spread of infection in hamsters fed with scrapie.
J Gen Virol 79, 601-607

BENDHEIM, P. E., H. R. BROWN, R. D. RUDELLI, L. J. SCALA, N. L. GOLLER, G. Y. WEN, R. J. KASCSAK, N. R. CASHMAN & D. C. BOLTON (1992):
Nearly ubiquitous tissue distribution of the scrapie agent precursor protein.
Neurology 42, 149-156

BENNETT, K. M., E. A. PARNELL, C. SANSCARTIER, S. PARKS, G. CHEN, M. G. NAIR & D. D. LO (2016):
Induction of Colonic M Cells during Intestinal Inflammation.
Am J Pathol 186, 1166-1179

BÉRINGUE, V., O. ANDRÉOLETTI, A. LE DUR, R. ESSALMANI, J. L. VILOTTE, C. LACROUX, F. REINE, L. HERZOG, A. G. BIACABE, T. BARON, M. CARAMELLI, C. CASALONE & H. LAUDE (2007):
A bovine prion acquires an epidemic bovine spongiform encephalopathy strain-like phenotype on interspecies transmission.

J Neurosci 27, 6965-6971

BÉRINGUE, V., M. DEMOY, C. I. LASMÉZAS, B. GOURITIN, C. WEINGARTEN, J. P. DESLYS, J. P. ANDREUX, P. COUVREUR & D. DORMONT (2000):
Role of spleen macrophages in the clearance of scrapie agent early in pathogenesis.
J Pathol 190, 495-502

BIACABE, A. G., J. L. LAPLANCHE, S. RYDER & T. BARON (2004):
Distinct molecular phenotypes in bovine prion diseases.
EMBO Rep 5, 110-115

BIESCHKE, J., P. WEBER, N. SARAFOFF, M. BEEKES, A. GIESE & H. KRETZSCHMAR (2004):
Autocatalytic self-propagation of misfolded prion protein.
Proc Natl Acad Sci U S A 101, 12207-12211

BILLINIS, C., C. H. PANAGIOTIDIS, V. PSYCHAS, S. ARGYROUDIS, A. NICOLAOU, S. LEONTIDES, O. PAPADOPOULOS & T. SKLAVIADIS (2002):
Prion protein gene polymorphisms in natural goat scrapie.
J Gen Virol 83, 713-721

BOERNER, S., K. WAGENFÜHR, M. L. DAUS, A. THOMZIG & M. BEEKES (2013):
Towards further reduction and replacement of animal bioassays in prion research by cell and protein misfolding cyclic amplification assays.
Lab Anim 47, 106-115

BOLEA, R., C. HEDMAN, O. LÓPEZ-PÉREZ, B. MARÍN, E. VIDAL, M. PUMAROLA, F. CORBIÈRE, A. ROMERO, B. MORENO, I. MARTÍN-BURRIEL, O. ANDRÉOLETTI & J. J. BADIOLA (2017):
Experimental transmission to a calf of an isolate of Spanish classical scrapie.
J Gen Virol 98, 2628-2634

BOLTON, D. C., M. P. MCKINLEY & S. B. PRUSINER (1982):
Identification of a protein that purifies with the scrapie prion.
Science 218, 1309-1311

BORGHESI, C., M. J. TAUSSIG & C. NICOLETTI (1999):
Rapid appearance of M cells after microbial challenge is restricted at the periphery of the follicle-associated epithelium of Peyer's patch.
Lab Invest 79, 1393-1401

BOUJON, C., F. SERRA & T. SEUBERLICH (2016):
Atypical variants of bovine spongiform encephalopathy: rare diseases with consequences for BSE surveillance and control.
Schweiz Arch Tierheilkd 158, 171-177

BRADBURY, J. (1996):
Maternal transmission of BSE demonstrated in cattle.
Lancet 348, 393

BRADFORD, B. M., B. REIZIS & N. A. MABBOTT (2017):
Oral Prion Disease Pathogenesis Is Impeded in the Specific Absence of CXCR5-Expressing Dendritic Cells.
J Virol 91, e00124-17

BRADLEY, R. (1999):
BSE transmission studies with particular reference to blood.
Dev Biol Stand 99, 35-40

BRAUN, U. (2002):
Klinische Symptome und Diagnose von BSE.
Schweiz Arch Tierheilkd 144, 645-652

BRAUN, U., U. KIHM, N. PUSTERLA & M. SCHÖNMANN (1997):
Klinischer Untersuchungsgang bei Verdacht auf bovine spongiforme Enzephalopathie (BSE).
Schweiz Arch Tierheilkd 139, 35-41

BRAUN, U., E. SCHICKER, N. PUSTERLA & M. SCHÖNMANN (1998):
Klinische Befunde bei 50 Kühen mit boviner spongiformer Enzephalopathie (BSE).
Berl Münch Tierärztl Wochenschr 111, 27-32

BROWN, D. R., R. S. NICHOLAS & L. CANEVARI (2002):
Lack of prion protein expression results in a neuronal phenotype sensitive to stress.
J Neurosci Res 67, 211-224

BROWN, K. L. & N. A. MABBOTT (2014):
Evidence of subclinical prion disease in aged mice following exposure to bovine spongiform encephalopathy.
J Gen Virol 95, 231-243

BROWN, K. L., K. STEWART, D. L. RITCHIE, N. A. MABBOTT, A. WILLIAMS, H. FRASER, W. I. MORRISON & M. E. BRUCE (1999):
Scrapie replication in lymphoid tissues depends on prion protein-expressing follicular dendritic cells.
Nat Med 5, 1308-1312

BROWN, K. L., G. J. WATHNE, J. SALES, M. E. BRUCE & N. A. MABBOTT (2009):
The effects of host age on follicular dendritic cell status dramatically impair scrapie agent neuroinvasion in aged mice.
J Immunol 183, 5199-5207

BRUCE, M. E., R. G. WILL, J. W. IRONSIDE, I. MCCONNELL, D. DRUMMOND, A. SUTTIE, L. MCCARDLE, A. CHREE, J. HOPE, C. BIRKETT, S. COUSENS, H. FRASER & C. J. BOSTOCK (1997):
Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent.
Nature 389, 498-501

BÜELER, H., A. AGUZZI, A. SAILER, R. A. GREINER, P. AUTENRIED, M. AGUET & C. WEISSMANN (1993):
Mice devoid of PrP are resistant to scrapie.
Cell 73, 1339-1347

BUSCHMANN, A., A. GRETZSCHEL, A. G. BIACABE, K. SCHIEBEL, C. CORONA, C. HOFFMANN, M. EIDEN, T. BARON, C. CASALONE & M. H. GROSCHUP (2006):
Atypical BSE in Germany--proof of transmissibility and biochemical characterization.
Vet Microbiol 117, 103-116

BUSCHMANN, A. & M. H. GROSCHUP (2005):
Highly bovine spongiform encephalopathy-sensitive transgenic mice confirm the essential restriction of

infectivity to the nervous system in clinically diseased cattle.
J Infect Dis 192, 934-942

BUSCHMANN, A., E. PFAFF, K. REIFENBERG, H. M. MÜLLER & M. H. GROSCHUP (2000):
Detection of cattle-derived BSE prions using transgenic mice overexpressing bovine PrP^{C}.
Arch Virol Suppl 16, 75-86

CAPOBIANCO, R., C. CASALONE, S. SUARDI, M. MANGIERI, C. MICCOLO, L. LIMIDO, M. CATANIA, G. ROSSI, G. DI FEDE, G. GIACCONE, M. G. BRUZZONE, L. MINATI, C. CORONA, P. ACUTIS, D. GELMETTI, G. LOMBARDI, M. H. GROSCHUP, A. BUSCHMANN, G. ZANUSSO, S. MONACO, M. CARAMELLI & F. TAGLIAVINI (2007):
Conversion of the BASE prion strain into the BSE strain: the origin of BSE?
PLoS Pathog 3, e31

CAPUCCHIO, M. T., F. GUARDA, N. POZZATO, S. COPPOLINO, S. CARACAPPA & V. DI MARCO (2001):
Clinical signs and diagnosis of scrapie in Italy: a comparative study in sheep and goats.
J Vet Med A Physiol Pathol Clin Med 48, 23-31

CARLENS, O. (1928):
Studien über das lymphatische Gewebe des Darmkanals bei einigen Haustieren, mit besonderer Berücksichtigung der embryonalen Entwicklung, der Mengenverhältnisse und der Altersinvolution dieses Gewebes im Dünndarm des Rindes.
Z Anat Entwicklungsgesch 86, 393-493

CARP, R. I. & S. M. CALLAHAN (1982):
Effect of mouse peritoneal macrophages on scrapie infectivity during extended in vitro incubation.
Intervirology 17, 201-207

CASALONE, C., M. CARAMELLI, M. I. CRESCIO, Y. I. SPENCER & M. M. SIMMONS (2006):
BSE immunohistochemical patterns in the brainstem: a comparison between UK and Italian cases.
Acta Neuropathol 111, 444-449

CASALONE, C., G. ZANUSSO, P. ACUTIS, S. FERRARI, L. CAPUCCI, F. TAGLIAVINI, S. MONACO & M. CARAMELLI (2004):
Identification of a second bovine amyloidotic spongiform encephalopathy: molecular similarities with sporadic Creutzfeldt-Jakob disease.
Proc Natl Acad Sci U S A 101, 3065-3070

CASTILLA, J., A. GUTIÉRREZ ADÁN, A. BRUN, B. PINTADO, M. A. RAMÍREZ, B. PARRA, D. DOYLE, M. ROGERS, F. J. SALGUERO, C. SÁNCHEZ, J. M. SÁNCHEZ-VIZCAÍNO & J. M. TORRES (2003):
Early detection of PrP^{res} in BSE-infected bovine PrP transgenic mice.
Arch Virol 148, 677-691

CASTILLA, J., P. SAÁ, C. HETZ & C. SOTO (2005a):
In vitro generation of infectious scrapie prions.
Cell 121, 195-206

CASTILLA, J., P. SAÁ, R. MORALES, K. ABID, K. MAUNDRELL & C. SOTO (2006):
Protein misfolding cyclic amplification for diagnosis and prion propagation studies.
Methods Enzymol 412, 3-21

CASTILLA, J., P. SAÁ & C. SOTO (2005b):

Detection of prions in blood.
Nat Med 11, 982-985

CASTLE, A. R. & A. C. GILL (2017):
Physiological Functions of the Cellular Prion Protein.
Front Mol Biosci 4, 19

CAUGHEY, B., G. S. BARON, B. CHESEBRO & M. JEFFREY (2009):
Getting a grip on prions: oligomers, amyloids, and pathological membrane interactions.
Annu Rev Biochem 78, 177-204

CAUGHEY, B., R. E. RACE, D. ERNST, M. J. BUCHMEIER & B. CHESEBRO (1989):
Prion protein biosynthesis in scrapie-infected and uninfected neuroblastoma cells.
J Virol 63, 175-181

CHADI, S., R. YOUNG, S. LE GUILLOU, G. TILLY, F. BITTON, M. L. MARTIN-MAGNIETTE, L. SOUBIGOU-TACONNAT, S. BALZERGUE, M. VILOTTE, C. PEYRE, B. PASSET, V. BÉRINGUE, J. P. RENOU, F. LE PROVOST, H. LAUDE & J. L. VILOTTE (2010):
Brain transcriptional stability upon prion protein-encoding gene invalidation in zygotic or adult mouse.
BMC Genomics 11, 448

CHEN, B., R. MORALES, M. A. BARRIA & C. SOTO (2010):
Estimating prion concentration in fluids and tissues by quantitative PMCA.
Nat Methods 7, 519-520

CHIANINI, F., G. M. COSSEDDU, P. STEELE, S. HAMILTON, J. HAWTHORN, S. SÍSO, Y. PANG, J. FINLAYSON, S. L. EATON, H. W. REID, M. P. DAGLEISH, M. A. DI BARI, C. D'AGOSTINO, U. AGRIMI, L. TERRY & R. NONNO (2015):
Correlation between infectivity and disease associated prion protein in the nervous system and selected edible tissues of naturally affected scrapie sheep.
PLoS One 10, e0122785

CHIOCCHETTI, R., G. MAZZUOLI, V. ALBANESE, M. MAZZONI, P. CLAVENZANI, G. LALATTA-COSTERBOSA, M. L. LUCCHI, G. DI GUARDO, G. MARRUCHELLA & J. B. FURNESS (2008):
Anatomical evidence for ileal Peyer's patches innervation by enteric nervous system: a potential route for prion neuroinvasion?
Cell Tissue Res 332, 185-194

CLARK, W. W., J. L. HOURRIGAN & W. J. HADLOW (1995):
Encephalopathy in cattle experimentally infected with the scrapie agent.
Am J Vet Res 56, 606-612

CLAWSON, M. L., J. A. RICHT, T. BARON, A. G. BIACABE, S. CZUB, M. P. HEATON, T. P. SMITH & W. W. LAEGREID (2008):
Association of a bovine prion gene haplotype with atypical BSE.
PLoS One 3, e1830

CLOUSCARD, C., P. BEAUDRY, J. M. ELSEN, D. MILAN, M. DUSSAUCY, C. BOUNNEAU, F. SCHELCHER, J. CHATELAIN, J. M. LAUNAY & J. L. LAPLANCHE (1995):
Different allelic effects of the codons 136 and 171 of the prion protein gene in sheep with natural scrapie.
J Gen Virol 76, 2097-2101

COHEN, F. E., K. M. PAN, Z. HUANG, M. BALDWIN, R. J. FLETTERICK & S. B. PRUSINER

(1994):
Structural clues to prion replication.
Science 264, 530-531

COLBY, D. W. & S. B. PRUSINER (2011):
Prions.
Cold Spring Harb Perspect Biol 3, a006833

COLCHESTER, A. C. & N. T. COLCHESTER (2005):
The origin of bovine spongiform encephalopathy: the human prion disease hypothesis.
Lancet 366, 856-861

COMOY, E. E., C. CASALONE, N. LESCOUTRA-ETCHEGARAY, G. ZANUSSO, S. FREIRE, D. MARCÉ, F. AUVRÉ, M. M. RUCHOUX, S. FERRARI, S. MONACO, N. SALÈS, M. CARAMELLI, P. LEBOULCH, P. BROWN, C. I. LASMÉZAS & J. P. DESLYS (2008):
Atypical BSE (BASE) transmitted from asymptomatic aging cattle to a primate.
PLoS One 3, e3017

CORONA, C., E. V. COSTASSA, B. IULINI, M. CARAMELLI, E. BOZZETTA, M. MAZZA, R. DESIATO, G. RU & C. CASALONE (2017):
Phenotypical Variability in Bovine Spongiform Encephalopathy: Epidemiology, Pathogenesis, and Diagnosis of Classical and Atypical Forms.
Prog Mol Biol Transl Sci 150, 241-265

CUTLIP, R. C., J. M. MILLER, A. N. HAMIR, J. PETERS, M. M. ROBINSON, A. L. JENNY, H. D. LEHMKUHL, W. D. TAYLOR & F. D. BISPLINGHOFF (2001):
Resistance of cattle to scrapie by the oral route.
Can J Vet Res 65, 131-132

CUTLIP, R. C., J. M. MILLER, R. E. RACE, A. L. JENNY, J. B. KATZ, H. D. LEHMKUHL, B. M. DEBEY & M. M. ROBINSON (1994):
Intracerebral transmission of scrapie to cattle.
J Infect Dis 169, 814-820

DAWSON, M., G. A. WELLS & B. N. PARKER (1990):
Preliminary evidence of the experimental transmissibility of bovine spongiform encephalopathy to cattle.
Vet Rec 126, 112-113

DELEAULT, N. R., J. C. GEOGHEGAN, K. NISHINA, R. KASCSAK, R. A. WILLIAMSON & S. SUPATTAPONE (2005):
Protease-resistant prion protein amplification reconstituted with partially purified substrates and synthetic polyanions.
J Biol Chem 280, 26873-26879

DICKINSON, A. G. & G. W. OUTRAM (1988):
Genetic aspects of unconventional virus infections: the basis of the virino hypothesis.
Ciba Found Symp 135, 63-83

DIRINGER, H., M. BEEKES & U. OBERDIECK (1994):
The nature of the scrapie agent: the virus theory.
Ann N Y Acad Sci 724, 246-258

DIRINGER, H., J. ROEHMEL & M. BEEKES (1998):

Effect of repeated oral infection of hamsters with scrapie.
J Gen Virol 79, 609-612

DONALDSON, D. S., K. J. ELSE & N. A. MABBOTT (2015):
The Gut-Associated Lymphoid Tissues in the Small Intestine, Not the Large Intestine, Play a Major Role in Oral Prion Disease Pathogenesis.
J Virol 89, 9532-9547

DONALDSON, D. S., A. KOBAYASHI, H. OHNO, H. YAGITA, I. R. WILLIAMS & N. A. MABBOTT (2012):
M cell-depletion blocks oral prion disease pathogenesis.
Mucosal Immunol 5, 216-225

DONALDSON, D. S. & N. A. MABBOTT (2016):
The influence of the commensal and pathogenic gut microbiota on prion disease pathogenesis.
J Gen Virol 97, 1725-1738

DONALDSON, D. S., A. SEHGAL, D. RIOS, I. R. WILLIAMS & N. A. MABBOTT (2016):
Increased Abundance of M Cells in the Gut Epithelium Dramatically Enhances Oral Prion Disease Susceptibility.
PLoS Pathog 12, e1006075

DONNELLY, C. A., N. M. FERGUSON, A. C. GHANI, J. W. WILESMITH & R. M. ANDERSON (1997):
Analysis of dam-calf pairs of BSE cases: confirmation of a maternal risk enhancement.
Proc Biol Sci 264, 1647-1656

EDDY, R. G. (1995):
Origin of BSE.
Vet Rec 137, 648

EIGEN, M. (1996):
Prionics or the kinetic basis of prion diseases.
Biophysical chemistry 63, A1-18

ELOIT, M., K. ADJOU, M. COULPIER, J. J. FONTAINE, R. HAMEL, T. LILIN, S. MESSIAEN, O. ANDRÉOLETTI, T. BARON, A. BENCSIK, A. G. BIACABE, V. BÉRINGUE, H. LAUDE, A. LE DUR, J. L. VILOTTE, E. COMOY, J. P. DESLYS, J. GRASSI, S. SIMON, F. LANTIER & P. SARRADIN (2005):
BSE agent signatures in a goat.
Vet Rec 156, 523-524

ESPINOSA, J. C., M. MORALES, J. CASTILLA, M. ROGERS & J. M. TORRES (2007):
Progression of prion infectivity in asymptomatic cattle after oral bovine spongiform encephalopathy challenge.
J Gen Virol 88, 1379-1383

FAST, C., M. KELLER, A. BALKEMA-BUSCHMANN, B. HILLS & M. H. GROSCHUP (2013):
Complementary studies detecting classical bovine spongiform encephalopathy infectivity in jejunum, ileum and ileocaecal junction in incubating cattle.
Vet Res 44, 123

FELTEN, D. L., S. Y. FELTEN, S. L. CARLSON, J. A. OLSCHOWKA & S. LIVNAT (1985):
Noradrenergic and peptidergic innervation of lymphoid tissue.

J Immunol 135, 755s-765s

FERGUSON, N. M., C. A. DONNELLY, M. E. WOOLHOUSE & R. M. ANDERSON (1997):
The epidemiology of BSE in cattle herds in Great Britain. II. Model construction and analysis of transmission dynamics.
Philos Trans R Soc Lond B Biol Sci 352, 803-838

FOLLET, J., C. LEMAIRE-VIEILLE, F. BLANQUET-GROSSARD, V. PODEVIN-DIMSTER, S. LEHMANN, J. P. CHAUVIN, J. P. DECAVEL, R. VAREA, J. GRASSI, M. FONTÈS & J. Y. CESBRON (2002):
PrP expression and replication by Schwann cells: implications in prion spreading.
J Virol 76, 2434-2439

FORD, M. J., L. J. BURTON, R. J. MORRIS & S. M. HALL (2002):
Selective expression of prion protein in peripheral tissues of the adult mouse.
Neuroscience 113, 177-192

FOSTER, J. D., J. HOPE & H. FRASER (1993):
Transmission of bovine spongiform encephalopathy to sheep and goats.
Vet Rec 133, 339-341

FOSTER, J. D., D. PARNHAM, A. CHONG, W. GOLDMANN & N. HUNTER (2001):
Clinical signs, histopathology and genetics of experimental transmission of BSE and natural scrapie to sheep and goats.
Vet Rec 148, 165-171

FOSTER, N. & G. G. MACPHERSON (2010):
Murine cecal patch M cells transport infectious prions in vivo.
J Infect Dis 202, 1916-1919

FRANZ, M., M. EIDEN, A. BALKEMA-BUSCHMANN, J. GREENLEE, H. SCHÄTZL, C. FAST, J. RICHT, J. P. HILDEBRANDT & M. H. GROSCHUP (2012):
Detection of PrP^{Sc} in peripheral tissues of clinically affected cattle after oral challenge with bovine spongiform encephalopathy.
J Gen Virol 93, 2740-2748

FUKUDA, S., Y. IWAMARU, M. IMAMURA, K. MASUJIN, Y. SHIMIZU, Y. MATSUURA, Y. SHU, M. KURACHI, K. KASAI, Y. MURAYAMA, S. ONOE, K. HAGIWARA, T. SATA, S. MOHRI, T. YOKOYAMA & H. OKADA (2009):
Intraspecies transmission of L-type-like Bovine Spongiform Encephalopathy detected in Japan.
Microbiol Immunol 53, 704-707

GERDES, H. H. (2009):
Prions tunnel between cells.
Nat Cell Biol 11, 235-236

GIACHIN, G., P. T. MAI, T. H. TRAN, G. SALZANO, F. BENETTI, V. MIGLIORATI, A. ARCOVITO, S. DELLA LONGA, G. MANCINI, P. D'ANGELO & G. LEGNAME (2015):
The non-octarepeat copper binding site of the prion protein is a key regulator of prion conversion.
Sci Rep 5, 15253

GLATZEL, M. & A. AGUZZI (2000):
Peripheral pathogenesis of prion diseases.
Microbes Infect 2, 613-619

GLATZEL, M., F. L. HEPPNER, K. M. ALBERS & A. AGUZZI (2001):
Sympathetic innervation of lymphoreticular organs is rate limiting for prion neuroinvasion.
Neuron 31, 25-34

GLAYSHER, B. R. & N. A. MABBOTT (2007):
Role of the GALT in scrapie agent neuroinvasion from the intestine.
J Immunol 178, 3757-3766

GOLDMANN, W., N. HUNTER, G. BENSON, J. D. FOSTER & J. HOPE (1991a):
Different scrapie-associated fibril proteins (PrP) are encoded by lines of sheep selected for different alleles of the Sip gene.
J Gen Virol 72, 2411-2417

GOLDMANN, W., N. HUNTER, J. D. FOSTER, J. M. SALBAUM, K. BEYREUTHER & J. HOPE (1990):
Two alleles of a neural protein gene linked to scrapie in sheep.
Proc Natl Acad Sci U S A 87, 2476-2480

GOLDMANN, W., N. HUNTER, T. MARTIN, M. DAWSON & J. HOPE (1991b):
Different forms of the bovine PrP gene have five or six copies of a short, G-C-rich element within the protein-coding exon.
J Gen Virol 72, 201-204

GOLDMANN, W., T. MARTIN, J. FOSTER, S. HUGHES, G. SMITH, K. HUGHES, M. DAWSON & N. HUNTER (1996):
Novel polymorphisms in the caprine PrP gene: a codon 142 mutation associated with scrapie incubation period.
J Gen Virol 77, 2885-2891

GONZÁLEZ, L., L. THORNE, M. JEFFREY, S. MARTIN, J. SPIROPOULOS, K. E. BECK, R. W. LOCKEY, C. M. VICKERY, T. HOLDER & L. TERRY (2012):
Infectious titres of sheep scrapie and bovine spongiform encephalopathy agents cannot be accurately predicted from quantitative laboratory test results.
J Gen Virol 93, 2518-2527

GOUSSET, K., E. SCHIFF, C. LANGEVIN, Z. MARIJANOVIC, A. CAPUTO, D. T. BROWMAN, N. CHENOUARD, F. DE CHAUMONT, A. MARTINO, J. ENNINGA, J. C. OLIVO-MARIN, D. MÄNNEL & C. ZURZOLO (2009):
Prions hijack tunnelling nanotubes for intercellular spread.
Nat Cell Biol 11, 328-336

GOVAERTS, C., H. WILLE, S. B. PRUSINER & F. E. COHEN (2004):
Evidence for assembly of prions with left-handed beta-helices into trimers.
Proc Natl Acad Sci U S A 101, 8342-8347

GRAVENOR, M. B., N. STALLARD, R. CURNOW & A. R. MCLEAN (2003):
Repeated challenge with prion disease: the risk of infection and impact on incubation period.
Proc Natl Acad Sci U S A 100, 10960-10965

GRETZSCHEL, A., A. BUSCHMANN, M. EIDEN, U. ZIEGLER, G. LÜHKEN, G. ERHARDT & M. H. GROSCHUP (2005):
Strain typing of German transmissible spongiform encephalopathies field cases in small ruminants by biochemical methods.

J Vet Med B Infect Dis Vet Public Health 52, 55-63

GROSCHUP, M. H., M. BEEKES, P. A. MCBRIDE, M. HARDT, J. A. HAINFELLNER & H. BUDKA (1999):
Deposition of disease-associated prion protein involves the peripheral nervous system in experimental scrapie.
Acta Neuropathol 98, 453-457

HAASE, B., M. G. DOHERR, T. SEUBERLICH, C. DRÖGEMÜLLER, G. DOLF, P. NICKEN, K. SCHIEBEL, U. ZIEGLER, M. H. GROSCHUP, A. ZURBRIGGEN & T. LEEB (2007):
PRNP promoter polymorphisms are associated with BSE susceptibility in Swiss and German cattle.
BMC Genet 8, 15

HAFEZPARAST, M., S. BRANDNER, J. LINEHAN, J. E. MARTIN, J. COLLINGE & E. M. FISHER (2005):
Prion disease incubation time is not affected in mice heterozygous for a dynein mutation.
Biochem Biophys Res Commun 326, 18-22

HARDT, M., T. BARON & M. H. GROSCHUP (2000):
A comparative study of immunohistochemical methods for detecting abnormal prion protein with monoclonal and polyclonal antibodies.
J Comp Pathol 122, 43-53

HEALY, A. M., E. WEAVERS, M. MCELROY, M. GOMEZ-PARADA, J. D. COLLINS, E. O'DOHERTY, T. SWEENEY & M. L. DOHERTY (2003):
The clinical neurology of scrapie in Irish sheep.
J Vet Intern Med 17, 908-916

HEATON, M. P., J. W. KEELE, G. P. HARHAY, J. A. RICHT, M. KOOHMARAIE, T. L. WHEELER, S. D. SHACKELFORD, E. CASAS, D. A. KING, T. S. SONSTEGARD, C. P. VAN TASSELL, H. L. NEIBERGS, C. C. CHASE, JR., T. S. KALBFLEISCH, T. P. SMITH, M. L. CLAWSON & W. W. LAEGREID (2008):
Prevalence of the prion protein gene E211K variant in U.S. cattle.
BMC Vet Res 4, 25

HEGGEBØ, R., L. GONZÀLEZ, C. M. PRESS, G. GUNNES, A. ESPENES & M. JEFFREY (2003):
Disease-associated PrP in the enteric nervous system of scrapie-affected Suffolk sheep.
J Gen Virol 84, 1327-1338

HEGGEBØ, R., C. M. PRESS, G. GUNNES, L. GONZÁLEZ & M. JEFFREY (2002):
Distribution and accumulation of PrP in gut-associated and peripheral lymphoid tissue of scrapie-affected Suffolk sheep.
J Gen Virol 83, 479-489

HEGGEBØ, R., C. M. PRESS, G. GUNNES, K. I. LIE, M. A. TRANULIS, M. ULVUND, M. H. GROSCHUP & T. LANDSVERK (2000):
Distribution of prion protein in the ileal Peyer's patch of scrapie-free lambs and lambs naturally and experimentally exposed to the scrapie agent.
J Gen Virol 81, 2327-2337

HEIKENWALDER, M., N. ZELLER, H. SEEGER, M. PRINZ, P. C. KLÖHN, P. SCHWARZ, N. H. RUDDLE, C. WEISSMANN & A. AGUZZI (2005):
Chronic lymphocytic inflammation specifies the organ tropism of prions.

Science 307, 1107-1110

HENDERSON, D. M., K. A. DAVENPORT, N. J. HALEY, N. D. DENKERS, C. K. MATHIASON & E. A. HOOVER (2015):
Quantitative assessment of prion infectivity in tissues and body fluids by real-time quaking-induced conversion.
J Gen Virol 96, 210-219

HEPPNER, F. L., A. D. CHRIST, M. A. KLEIN, M. PRINZ, M. FRIED, J. P. KRAEHENBUHL & A. AGUZZI (2001)
Transepithelial prion transport by M cells.
Nat Med 7, 976–977

HERRMANN, L. M., W. P. CHEEVERS, W. C. DAVIS, D. P. KNOWLES & K. I. O'ROURKE (2003):
CD21-positive follicular dendritic cells: A possible source of PrP^{Sc} in lymph node macrophages of scrapie-infected sheep.
Am J Pathol 162, 1075-1081

HILL, A. F., M. DESBRUSLAIS, S. JOINER, K. C. SIDLE, I. GOWLAND, J. COLLINGE, L. J. DOEY & P. LANTOS (1997a):
The same prion strain causes vCJD and BSE.
Nature 389, 448-450, 526

HILL, A. F., M. ZEIDLER, J. IRONSIDE & J. COLLINGE (1997b):
Diagnosis of new variant Creutzfeldt-Jakob disease by tonsil biopsy.
Lancet 349, 99-100

HILTON, D. A., E. FATHERS, P. EDWARDS, J. W. IRONSIDE & J. ZAJICEK (1998):
Prion immunoreactivity in appendix before clinical onset of variant Creutzfeldt-Jakob disease.
Lancet 352, 703-704

HOFFMANN, C., M. EIDEN, M. KAATZ, M. KELLER, U. ZIEGLER, R. ROGERS, B. HILLS, A. BALKEMA-BUSCHMANN, L. VAN KEULEN, J. G. JACOBS & M. H. GROSCHUP (2011):
BSE infectivity in jejunum, ileum and ileocaecal junction of incubating cattle.
Vet Res 42, 21

HOFFMANN, C., U. ZIEGLER, A. BUSCHMANN, A. WEBER, L. KUPFER, A. OELSCHLEGEL, B. HAMMERSCHMIDT & M. H. GROSCHUP (2007):
Prions spread via the autonomic nervous system from the gut to the central nervous system in cattle incubating bovine spongiform encephalopathy.
J Gen Virol 88, 1048-1055

HOUSTON, E. F. & M. B. GRAVENOR (2003):
Clinical signs in sheep experimentally infected with scrapie and BSE.
Vet Rec 152, 333-334

HOUSTON, F., J. D. FOSTER, A. CHONG, N. HUNTER & C. J. BOSTOCK (2000):
Transmission of BSE by blood transfusion in sheep.
Lancet 356, 999-1000

HOUSTON, F., S. MCCUTCHEON, W. GOLDMANN, A. CHONG, J. FOSTER, S. SISÓ, L. GONZÁLEZ, M. JEFFREY & N. HUNTER (2008):
Prion diseases are efficiently transmitted by blood transfusion in sheep.
Blood 112, 4739-4745

HUANG, F. P., C. F. FARQUHAR, N. A. MABBOTT, M. E. BRUCE & G. G. MACPHERSON (2002):
Migrating intestinal dendritic cells transport PrP^{Sc} from the gut.
J Gen Virol 83, 267-271

HUNTER, N. (1997):
PrP genetics in sheep and the implications for scrapie and BSE.
Trends Microbiol 5, 331-334

HUNTER, N., W. GOLDMANN, G. SMITH & J. HOPE (1994):
The association of a codon 136 PrP gene variant with the occurrence of natural scrapie.
Arch Virol 137, 171-177

HUNTER, N., F. HOUSTON, J. FOSTER, W. GOLDMANN, D. DRUMMOND, D. PARNHAM, I. KENNEDY, A. GREEN, P. STEWART & A. CHONG (2012):
Susceptibility of young sheep to oral infection with bovine spongiform encephalopathy decreases significantly after weaning.
J Virol 86, 11856-11862

IWAMARU, Y., M. IMAMURA, Y. MATSUURA, K. MASUJIN, Y. SHIMIZU, Y. SHU, M. KURACHI, K. KASAI, Y. MURAYAMA, S. FUKUDA, S. ONOE, K. HAGIWARA, Y. YAMAKAWA, T. SATA, S. MOHRI, H. OKADA & T. YOKOYAMA (2010):
Accumulation of L-type bovine prions in peripheral nerve tissues.
Emerg Infect Dis 16, 1151-1154

JACOBS, J. G., J. P. LANGEVELD, A. G. BIACABE, P. L. ACUTIS, M. P. POLAK, D. GAVIER-WIDEN, A. BUSCHMANN, M. CARAMELLI, C. CASALONE, M. MAZZA, M. GROSCHUP, J. H. ERKENS, A. DAVIDSE, F. G. VAN ZIJDERVELD & T. BARON (2007):
Molecular discrimination of atypical bovine spongiform encephalopathy strains from a geographical region spanning a wide area in Europe.
J Clin Microbiol 45, 1821-1829

JARRETT, J. T. & P. T. LANSBURY, JR. (1993):
Seeding "one-dimensional crystallization" of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie?
Cell 73, 1055-1058

JEFFREY, M. & L. GONZÁLEZ (2004):
Pathology and pathogenesis of bovine spongiform encephalopathy and scrapie.
Curr Top Microbiol Immunol 284, 65-97

JEFFREY, M. & L. GONZÁLEZ (2007):
Classical sheep transmissible spongiform encephalopathies: pathogenesis, pathological phenotypes and clinical disease.
Neuropathol Appl Neurobiol 33, 373-394

JEFFREY, M., L. GONZÁLEZ, A. ESPENES, C. M. PRESS, S. MARTIN, M. CHAPLIN, L. DAVIS, T. LANDSVERK, C. MACALDOWIE, S. EATON & G. MCGOVERN (2006):
Transportation of prion protein across the intestinal mucosa of scrapie-susceptible and scrapie-resistant sheep.
J Pathol 209, 4-14

JEFFREY, M., C. M. GOODSIR, M. E. BRUCE, P. A. MCBRIDE & C. FARQUHAR (1994):
Morphogenesis of amyloid plaques in 87V murine scrapie.

Neuropathol Appl Neurobiol 20, 535-542

JEFFREY, M., G. MCGOVERN, S. SISÓ & L. GONZÁLEZ (2011):
Cellular and sub-cellular pathology of animal prion diseases: relationship between morphological changes, accumulation of abnormal prion protein and clinical disease.
Acta Neuropathol 121, 113-134

JOINER, S., E. A. ASANTE, J. M. LINEHAN, L. BROCK, S. BRANDNER, S. J. BELLWORTHY, M. M. SIMMONS, J. HOPE, J. COLLINGE & J. D. F. WADSWORTH (2018):
Experimental sheep BSE prions generate the vCJD phenotype when serially passaged in transgenic mice expressing human prion protein.
J Neurol Sci 386, 4-11

JONES, M., D. WIGHT, R. BARRON, M. JEFFREY, J. MANSON, C. PROWSE, J. W. IRONSIDE & M. W. HEAD (2009):
Molecular model of prion transmission to humans.
Emerg Infect Dis 15, 2013-2016

JULING, K., H. SCHWARZENBACHER, J. L. WILLIAMS & R. FRIES (2006):
A major genetic component of BSE susceptibility.
BMC Biol 4, 33

KAADEN, O. R., U. TRUYEN, M. H. GROSCHUP, A. UYSAL, E. KAISER, H. KRETZSCHMAR, T. BOGUMIL, J. POHLENZ, H. DIRINGER, P. STEINHAGEN & ET AL. (1994):
Bovine spongiform encephalopathy in Germany.
Zentralbl Veterinarmed B 41, 294-304

KAATZ, M. (2014):
Ausbreitung der klassischen BSE-Prion-Erreger vom Darm über das periphere Nervensystem zum Gehirn.
Hannover, tierärztl. Hochsch., Diss.

KAATZ, M., C. FAST, U. ZIEGLER, A. BALKEMA-BUSCHMANN, B. HAMMERSCHMIDT, M. KELLER, A. OELSCHLEGEL, L. MCINTYRE & M. H. GROSCHUP (2012):
Spread of classic BSE prions from the gut via the peripheral nervous system to the brain.
Am J Pathol 181, 515-524

KANEKO, K., L. ZULIANELLO, M. SCOTT, C. M. COOPER, A. C. WALLACE, T. L. JAMES, F. E. COHEN & S. B. PRUSINER (1997):
Evidence for protein X binding to a discontinuous epitope on the cellular prion protein during scrapie prion propagation.
Proc Natl Acad Sci U S A 94, 10069-10074

KIMBERLIN, R. H. (1992):
Bovine spongiform encephalopathy.
Rev Sci Tech 11, 347-489

KIMBERLIN, R. H., S. COLE & C. A. WALKER (1987):
Pathogenesis of scrapie is faster when infection is intraspinal instead of intracerebral.
Microb Pathog 2, 405-415

KIMBERLIN, R. H., S. M. HALL & C. A. WALKER (1983):
Pathogenesis of mouse scrapie. Evidence for direct neural spread of infection to the CNS after injection of sciatic nerve.

J Neurol Sci 61, 315-325

KIMBERLIN, R. H. & C. A. WALKER (1982):
Pathogenesis of mouse scrapie: patterns of agent replication in different parts of the CNS following intraperitoneal infection.
J R Soc Med 75, 618-624

KIMBERLIN, R. H. & C. A. WALKER (1989):
Pathogenesis of scrapie in mice after intragastric infection.
Virus Res 12, 213-220

KLINGEBORN, M., B. RACE, K. D. MEADE-WHITE, R. ROSENKE, J. F. STRIEBEL & B. CHESEBRO (2011):
Crucial role for prion protein membrane anchoring in the neuroinvasion and neural spread of prion infection.
J Virol 85, 1484-1494

KOBAYASHI, A., D. S. DONALDSON, C. ERRIDGE, T. KANAYA, I. R. WILLIAMS, H. OHNO, A. MAHAJAN & N. A. MABBOTT (2013):
The functional maturation of M cells is dramatically reduced in the Peyer's patches of aged mice.
Mucosal Immunology 6, 1027

KONG, Q., M. ZHENG, C. CASALONE, L. QING, S. HUANG, B. CHAKRABORTY, P. WANG, F. CHEN, I. CALI, C. CORONA, F. MARTUCCI, B. IULINI, P. ACUTIS, L. WANG, J. LIANG, M. WANG, X. LI, S. MONACO, G. ZANUSSO, W. Q. ZOU, M. CARAMELLI & P. GAMBETTI (2008):
Evaluation of the human transmission risk of an atypical bovine spongiform encephalopathy prion strain.
J Virol 82, 3697-3701

KONOLD, T., G. BONE, M. M. SIMMONS, G. DEXTER, S. J. MOORE & R. G. PETTITT (2007):
Scrapie in goats.
Vet Rec 161, 395-396

KONOLD, T., G. E. BONE, D. CLIFFORD, M. J. CHAPLIN, S. CAWTHRAW, M. J. STACK & M. M. SIMMONS (2012):
Experimental H-type and L-type bovine spongiform encephalopathy in cattle: observation of two clinical syndromes and diagnostic challenges.
BMC Vet Res 8, 22

KONOLD, T., G. E. BONE, L. J. PHELAN, M. M. SIMMONS, L. GONZÁLEZ, S. SISÓ, W. GOLDMANN, S. CAWTHRAW & S. A. HAWKINS (2010):
Monitoring of clinical signs in goats with transmissible spongiform encephalopathies.
BMC Vet Res 6, 13

KONOLD, T., Y. H. LEE, M. J. STACK, C. HORROCKS, R. B. GREEN, M. CHAPLIN, M. M. SIMMONS, S. A. HAWKINS, R. LOCKEY, J. SPIROPOULOS, J. W. WILESMITH & G. A. WELLS (2006):
Different prion disease phenotypes result from inoculation of cattle with two temporally separated sources of sheep scrapie from Great Britain.
BMC Vet Res 2, 31

KONOLD, T., R. NONNO, J. SPIROPOULOS, M. J. CHAPLIN, M. J. STACK, S. A. HAWKINS, S. CAWTHRAW, J. W. WILESMITH, G. A. WELLS, U. AGRIMI, M. A. DI BARI, O. ANDRÉOLETTI,

J. C. ESPINOSA, P. AGUILAR-CALVO & J. M. TORRES (2015):
Further characterisation of transmissible spongiform encephalopathy phenotypes after inoculation of cattle with two temporally separated sources of sheep scrapie from Great Britain.
BMC Res Notes 8, 312

KONOLD, T., J. SPIROPOULOS, M. J. CHAPLIN, M. J. STACK, S. A. HAWKINS, J. W. WILESMITH & G. A. WELLS (2013):
Unsuccessful oral transmission of scrapie from British sheep to cattle.
Vet Rec 173, 118

KOVACS, G. G., M. PUOPOLO, A. LADOGANA, M. POCCHIARI, H. BUDKA, C. VAN DUIJN, S. J. COLLINS, A. BOYD, A. GIULIVI, M. COULTHART, N. DELASNERIE-LAUPRETRE, J. P. BRANDEL, I. ZERR, H. A. KRETZSCHMAR, J. DE PEDRO-CUESTA, M. CALERO-LARA, M. GLATZEL, A. AGUZZI, M. BISHOP, R. KNIGHT, G. BELAY, R. WILL & E. MITROVA (2005):
Genetic prion disease: the EUROCJD experience.
Hum Genet 118, 166-174

KRATZEL, C., D. KRÜGER & M. BEEKES (2007a):
Prion propagation in a nerve conduit model containing segments devoid of axons.
J Gen Virol 88, 3479-3485

KRATZEL, C., J. MAI, K. MADELA, M. BEEKES & D. KRÜGER (2007b):
Propagation of scrapie in peripheral nerves after footpad infection in normal and neurotoxin exposed hamsters.
Vet Res 38, 127-139

KUCZIUS, T., I. HAIST & M. H. GROSCHUP (1998):
Molecular analysis of bovine spongiform encephalopathy and scrapie strain variation.
J Infect Dis 178, 693-699

KUJALA, P., C. R. RAYMOND, M. ROMEIJN, S. F. GODSAVE, S. I. VAN KASTEREN, H. WILLE, S. B. PRUSINER, N. A. MABBOTT & P. J. PETERS (2011):
Prion uptake in the gut: identification of the first uptake and replication sites.
PLoS Pathog 7, e1002449

KUNZI, V., M. GLATZEL, M. Y. NAKANO, U. F. GREBER, F. VAN LEUVEN & A. AGUZZI (2002):
Unhampered prion neuroinvasion despite impaired fast axonal transport in transgenic mice overexpressing four-repeat tau.
J Neurosci 22, 7471-7477

LAPLANCHE, J. L., J. CHATELAIN, D. WESTAWAY, S. THOMAS, M. DUSSAUCY, J. BRUGERE-PICOUX & J. M. LAUNAY (1993):
PrP polymorphisms associated with natural scrapie discovered by denaturing gradient gel electrophoresis.
Genomics 15, 30-37

LASMÉZAS, C. I., J. P. DESLYS, O. ROBAIN, A. JAEGLY, V. BÉRINGUE, J. M. PEYRIN, J. G. FOURNIER, J. J. HAUW, J. ROSSIER & D. DORMONT (1997):
Transmission of the BSE agent to mice in the absence of detectable abnormal prion protein.
Science 275, 402-405

LEGNAME, G., I. V. BASKAKOV, H. O. NGUYEN, D. RIESNER, F. E. COHEN, S. J. DEARMOND

& S. B. PRUSINER (2004):
Synthetic mammalian prions.
Science 305, 673-676

LIGIOS, C., M. G. CANCEDDA, A. CARTA, C. SANTUCCIU, C. MAESTRALE, F. DEMONTIS, M. SABA, C. PATTA, J. C. DEMARTINI, A. AGUZZI & C. J. SIGURDSON (2011):
Sheep with scrapie and mastitis transmit infectious prions through the milk.
J Virol 85, 1136-1139

LIGIOS, C., C. J. SIGURDSON, C. SANTUCCIU, G. CARCASSOLA, G. MANCO, M. BASAGNI, C. MAESTRALE, M. G. CANCEDDA, L. MADAU & A. AGUZZI (2005):
PrP^{Sc} in mammary glands of sheep affected by scrapie and mastitis.
Nat Med 11, 1137-1138

LOMBARDI, G., C. CASALONE, D. A. A, D. GELMETTI, G. TORCOLI, I. BARBIERI, C. CORONA, E. FASOLI, A. FARINAZZO, M. FIORINI, M. GELATI, B. IULINI, F. TAGLIAVINI, S. FERRARI, M. CARAMELLI, S. MONACO, L. CAPUCCI & G. ZANUSSO (2008):
Intraspecies transmission of BASE induces clinical dullness and amyotrophic changes.
PLoS Pathog 4, e1000075

MABBOTT, N. A. (2017):
Immunology of Prion Protein and Prions.
Prog Mol Biol Transl Sci 150, 203-240

MABBOTT, N. A. & M. E. BRUCE (2001):
The immunobiology of TSE diseases.
J Gen Virol 82, 2307-2318

MABBOTT, N. A. & G. G. MACPHERSON (2006):
Prions and their lethal journey to the brain.
Nat Rev Microbiol 4, 201-211

MABBOTT, N. A., J. YOUNG, I. MCCONNELL & M. E. BRUCE (2003):
Follicular dendritic cell dedifferentiation by treatment with an inhibitor of the lymphotoxin pathway dramatically reduces scrapie susceptibility.
J Virol 77, 6845-6854

MAESTRALE, C., G. DI GUARDO, M. G. CANCEDDA, G. MARRUCHELLA, M. MASIA, S. SECHI, S. MACCIOCU, C. SANTUCCIU, M. PETRUZZI & C. LIGIOS (2013):
A lympho-follicular microenvironment is required for pathological prion protein deposition in chronically inflamed tissues from scrapie-affected sheep.
PLoS One 8, e62830

MAIGNIEN, T., C. I. LASMÉZAS, V. BÉRINGUE, D. DORMONT & J. P. DESLYS (1999):
Pathogenesis of the oral route of infection of mice with scrapie and bovine spongiform encephalopathy agents.
J Gen Virol 80, 3035-3042

MAIGNIEN, T., M. SHAKWEH, P. CALVO, D. MARCÉ, N. SALÈS, E. FATTAL, J. P. DESLYS, P. COUVREUR & C. I. LASMÉZAS (2005):
Role of gut macrophages in mice orally contaminated with scrapie or BSE.
Int J Pharm 298, 293-304

MAKARAVA, N., G. G. KOVACS, O. BOCHAROVA, R. SAVTCHENKO, I. ALEXEEVA, H.

BUDKA, R. G. ROHWER & I. V. BASKAKOV (2010):
Recombinant prion protein induces a new transmissible prion disease in wild-type animals.
Acta Neuropathol 119, 177-187

MAKARAVA, N., G. G. KOVACS, R. SAVTCHENKO, I. ALEXEEVA, H. BUDKA, R. G. ROHWER & I. V. BASKAKOV (2011):
Genesis of mammalian prions: from non-infectious amyloid fibrils to a transmissible prion disease.
PLoS Pathog 7, e1002419

MANUELIDIS, L. (1994):
Dementias, neurodegeneration, and viral mechanisms of disease from the perspective of human transmissible encephalopathies.
Ann N Y Acad Sci 724, 259-281

MARRUCHELLA, G., C. LIGIOS, M. BAFFONI, M. G. CANCEDDA, F. DEMONTIS, G. DONATUCCI, R. CHIOCCHETTI, P. CLAVENZANI, G. LALATTA-COSTERBOSA & G. DI GUARDO (2009):
Ileal tract and Peyer's patch innervation in scrapie-free versus scrapie-affected ovines.
Arch Virol 154, 709-714

MARRUCHELLA, G., C. LIGIOS & G. DI GUARDO (2012):
Age, scrapie status, PrP genotype and follicular dendritic cells in ovine ileal Peyer's patches.
Res Vet Sci 93, 853-856

MARTIN, G. R., A. L. ALVAREZ, M. BASHASHATI, C. M. KEENAN, F. R. JIRIK & K. A. SHARKEY (2012):
Endogenous cellular prion protein regulates contractility of the mouse ileum.
Neurogastroenterol Motil 24, e412-424

MASUJIN, K., D. MATTHEWS, G. A. WELLS, S. MOHRI & T. YOKOYAMA (2007):
Prions in the peripheral nerves of bovine spongiform encephalopathy-affected cattle.
J Gen Virol 88, 1850-1858

MCBRIDE, P. A. & M. BEEKES (1999):
Pathological PrP is abundant in sympathetic and sensory ganglia of hamsters fed with scrapie.
Neurosci Lett 265, 135-138

MCBRIDE, P. A., W. J. SCHULZ-SCHAEFFER, M. DONALDSON, M. BRUCE, H. DIRINGER, H. A. KRETZSCHMAR & M. BEEKES (2001):
Early spread of scrapie from the gastrointestinal tract to the central nervous system involves autonomic fibers of the splanchnic and vagus nerves.
J Virol 75, 9320-9327

MCCULLOCH, L., K. L. BROWN, B. M. BRADFORD, J. HOPKINS, M. BAILEY, K. RAJEWSKY, J. C. MANSON & N. A. MABBOTT (2011):
Follicular dendritic cell-specific prion protein (PrP) expression alone is sufficient to sustain prion infection in the spleen.
PLoS Pathog 7, e1002402

MCLEAN, A. R. & C. J. BOSTOCK (2000):
Scrapie infections initiated at varying doses: an analysis of 117 titration experiments.
Philos Trans R Soc Lond B Biol Sci 355, 1043-1050

MEYER, R. K., M. P. MCKINLEY, K. A. BOWMAN, M. B. BRAUNFELD, R. A. BARRY & S. B.

PRUSINER (1986):
Separation and properties of cellular and scrapie prion proteins.
Proc Natl Acad Sci U S A 83, 2310-2314

MILLER, J. M., A. L. JENNY, W. D. TAYLOR, R. F. MARSH, R. RUBENSTEIN & R. E. RACE (1993):
Immunohistochemical detection of prion protein in sheep with scrapie.
J Vet Diagn Invest 5, 309-316

MOK, S. W., R. L. PROIA, V. BRINKMANN & N. A. MABBOTT (2012):
B cell-specific S1PR1 deficiency blocks prion dissemination between secondary lymphoid organs.
J Immunol 188, 5032-5040

MORALES, R., C. DURAN-ANIOTZ, R. DIAZ-ESPINOZA, M. V. CAMACHO & C. SOTO (2012):
Protein misfolding cyclic amplification of infectious prions.
Nat Protoc 7, 1397-1409

MORALES, R., S. PRITZKOW, P. P. HU, C. DURAN-ANIOTZ & C. SOTO (2013):
Lack of prion transmission by sexual or parental routes in experimentally infected hamsters.
Prion 7, 412-419

MURAYAMA, Y., M. YOSHIOKA, K. MASUJIN, H. OKADA, Y. IWAMARU, M. IMAMURA, Y. MATSUURA, S. FUKUDA, S. ONOE, T. YOKOYAMA & S. MOHRI (2010):
Sulfated dextrans enhance in vitro amplification of bovine spongiform encephalopathy PrP^{Sc} and enable ultrasensitive detection of bovine PrP^{Sc}.
PLoS One 5, e13152

MURDOCH, B. M., M. L. CLAWSON, S. YUE, U. BASU, S. MCKAY, M. SETTLES, R. CAPOFERRI, W. W. LAEGREID, J. L. WILLIAMS & S. S. MOORE (2010):
PRNP haplotype associated with classical BSE incidence in European Holstein cattle.
PLoS One 5, e12786

NICHOLSON, E. M., B. W. BRUNELLE, J. A. RICHT, M. E. KEHRLI, JR. & J. J. GREENLEE (2008):
Identification of a heritable polymorphism in bovine PRNP associated with genetic transmissible spongiform encephalopathy: evidence of heritable BSE.
PLoS One 3, e2912

O'ROURKE, K. I., G. R. HOLYOAK, W. W. CLARK, J. R. MICKELSON, S. WANG, R. P. MELCO, T. E. BESSER & W. C. FOOTE (1997):
PrP genotypes and experimental scrapie in orally inoculated Suffolk sheep in the United States.
J Gen Virol 78, 975-978

OESCH, B., D. WESTAWAY, M. WÄLCHLI, M. P. MCKINLEY, S. B. KENT, R. AEBERSOLD, R. A. BARRY, P. TEMPST, D. B. TEPLOW, L. E. HOOD, S. B. PRUSINER & C. WEISSMANN (1985):
A cellular gene encodes scrapie PrP 27-30 protein.
Cell 40, 735-746

OKADA, H., Y. IWAMARU, M. IMAMURA, K. MASUJIN, Y. MATSUURA, Y. MURAYAMA, S. MOHRI & T. YOKOYAMA (2011a):
Detection of disease-associated prion protein in the posterior portion of the small intestine involving the continuous Peyer's patch in cattle orally infected with bovine spongiform encephalopathy agent.
Transbound Emerg Dis 58, 333-343

OKADA, H., Y. IWAMARU, M. IMAMURA, K. MASUJIN, Y. MATSUURA, Y. SHIMIZU, K. KASAI, S. MOHRI, T. YOKOYAMA & S. CZUB (2011b):
Experimental H-type bovine spongiform encephalopathy characterized by plaques and glial- and stellate-type prion protein deposits.
Vet Res 42, 79

OKADA, H., Y. IWAMARU, M. IMAMURA, K. MIYAZAWA, Y. MATSUURA, K. MASUJIN, Y. MURAYAMA & T. YOKOYAMA (2017):
Oral Transmission of L-Type Bovine Spongiform Encephalopathy Agent among Cattle.
Emerg Infect Dis 23, 284-287

OKADA, H., Y. IWAMARU, T. YOKOYAMA & S. MOHRI (2013):
Immunohistochemical detection of disease-associated prion protein in the peripheral nervous system in experimental H-type bovine spongiform encephalopathy.
Vet Pathol 50, 659-663

OKADA, H., K. MIYAZAWA, S. FUKUDA, Y. IWAMARU, M. IMAMURA, K. MASUJIN, Y. MATSUURA, T. FUJII, K. FUJII, S. KAGEYAMA, M. YOSHIOKA, Y. MURAYAMA & T. YOKOYAMA (2014):
The presence of disease-associated prion protein in skeletal muscle of cattle infected with classical bovine spongiform encephalopathy.
J Vet Med Sci 76, 103-107

ORGEL, L. E. (1996):
Prion replication and secondary nucleation.
Chem Biol 3, 413-414

PADILLA, D., V. BÉRINGUE, J. C. ESPINOSA, O. ANDRÉOLETTI, E. JAUMAIN, F. REINE, L. HERZOG, A. GUTIERREZ-ADAN, B. PINTADO, H. LAUDE & J. M. TORRES (2011):
Sheep and goat BSE propagate more efficiently than cattle BSE in human PrP transgenic mice.
PLoS Pathog 7, e1001319

PAMMER, J., H. S. CROSS, Y. FROBERT, E. TSCHACHLER & G. OBERHUBER (2000):
The pattern of prion-related protein expression in the gastrointestinal tract.
Virchows Arch 436, 466-472

PAN, K. M., M. BALDWIN, J. NGUYEN, M. GASSET, A. SERBAN, D. GROTH, I. MEHLHORN, Z. HUANG, R. J. FLETTERICK, F. E. COHEN & S. B. PRUSINER (1993):
Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins.
Proc Natl Acad Sci U S A 90, 10962-10966

PARRY, H. B. (1983):
Scrapie disease in sheep: historical, clinical, epidemiological, pathological, and practical aspects of the natural disease / H.B. Parry ; edited by D.R. Oppenheimer.
Academic Press, London; New York

PRINZ, M., M. HEIKENWALDER, T. JUNT, P. SCHWARZ, M. GLATZEL, F. L. HEPPNER, Y. X. FU, M. LIPP & A. AGUZZI (2003a):
Positioning of follicular dendritic cells within the spleen controls prion neuroinvasion.
Nature 425, 957-962

PRINZ, M., G. HUBER, A. J. MACPHERSON, F. L. HEPPNER, M. GLATZEL, H. P. EUGSTER, N. WAGNER & A. AGUZZI (2003b):

Oral prion infection requires normal numbers of Peyer's patches but not of enteric lymphocytes.
Am J Pathol 162, 1103-1111

PRUSINER, S. B. (1982):
Novel proteinaceous infectious particles cause scrapie.
Science 216, 136-144

PRUSINER, S. B. (1997):
Prion diseases and the BSE crisis.
Science 278, 245-251

PRUSINER, S. B. (1998):
Prions.
Proc Natl Acad Sci U S A 95, 13363-13383

PRUSINER, S. B., D. GROTH, A. SERBAN, R. KOEHLER, D. FOSTER, M. TORCHIA, D. BURTON, S. L. YANG & S. J. DEARMOND (1993):
Ablation of the prion protein (PrP) gene in mice prevents scrapie and facilitates production of anti-PrP antibodies.
Proc Natl Acad Sci U S A 90, 10608-10612

PRUSINER, S. B., M. P. MCKINLEY, K. A. BOWMAN, D. C. BOLTON, P. E. BENDHEIM, D. F. GROTH & G. G. GLENNER (1983):
Scrapie prions aggregate to form amyloid-like birefringent rods.
Cell 35, 349-358

RAYMOND, C. R., P. AUCOUTURIER & N. A. MABBOTT (2007):
In vivo depletion of CD11c+ cells impairs scrapie agent neuroinvasion from the intestine.
J Immunol 179, 7758-7766

RESCIGNO, M., M. URBANO, B. VALZASINA, M. FRANCOLINI, G. ROTTA, R. BONASIO, F. GRANUCCI, J. P. KRAEHENBUHL & P. RICCIARDI-CASTAGNOLI (2001):
Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria.
Nat Immunol 2, 361-367

RICHT, J. A. & S. M. HALL (2008):
BSE case associated with prion protein gene mutation.
PLoS Pathog 4, e1000156

ROBINSON, M. M., W. J. HADLOW, D. P. KNOWLES, T. P. HUFF, P. A. LACY, R. F. MARSH & J. R. GORHAM (1995):
Experimental infection of cattle with the agents of transmissible mink encephalopathy and scrapie.
J Comp Pathol 113, 241-251

SAÁ, P., J. CASTILLA & C. SOTO (2006):
Ultra-efficient replication of infectious prions by automated protein misfolding cyclic amplification.
J Biol Chem 281, 35245-35252

SAÁ, P. & L. CERVENAKOVA (2015):
Protein misfolding cyclic amplification (PMCA): Current status and future directions.
Virus Res 207, 47-61

SABA, R. & S. A. BOOTH (2013):
The genetics of susceptibility to variant Creutzfeldt-Jakob disease.

Public Health Genomics 16, 17-24

SABORÍO, G. P., B. PERMANNE & C. SOTO (2001):
Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding.
Nature 411, 810-813

SABORÍO, G. P., C. SOTO, R. J. KASCSAK, E. LEVY, R. KASCSAK, D. A. HARRIS & B. FRANGIONE (1999):
Cell-lysate conversion of prion protein into its protease-resistant isoform suggests the participation of a cellular chaperone.
Biochem Biophys Res Commun 258, 470-475

SAFAR, J. G., M. SCOTT, J. MONAGHAN, C. DEERING, S. DIDORENKO, J. VERGARA, H. BALL, G. LEGNAME, E. LECLERC, L. SOLFOROSI, H. SERBAN, D. GROTH, D. R. BURTON, S. B. PRUSINER & R. A. WILLIAMSON (2002):
Measuring prions causing bovine spongiform encephalopathy or chronic wasting disease by immunoassays and transgenic mice.
Nat Biotechnol 20, 1147-1150

SANDER, P., H. HAMANN, C. DRÖGEMÜLLER, K. KASHKEVICH, K. SCHIEBEL & T. LEEB (2005):
Bovine prion protein gene (PRNP) promoter polymorphisms modulate PRNP expression and may be responsible for differences in bovine spongiform encephalopathy susceptibility.
J Biol Chem 280, 37408-37414

SANDER, P., H. HAMANN, I. PFEIFFER, W. WEMHEUER, B. BRENIG, M. H. GROSCHUP, U. ZIEGLER, O. DISTL & T. LEEB (2004):
Analysis of sequence variability of the bovine prion protein gene (PRNP) in German cattle breeds.
Neurogenetics 5, 19-25

SASSA, Y., Y. INOSHIMA & N. ISHIGURO (2010):
Bovine macrophage degradation of scrapie and BSE PrP^{Sc}.
Vet Immunol Immunopathol 133, 33-39

SCOTT, M. R., J. SAFAR, G. TELLING, O. NGUYEN, D. GROTH, M. TORCHIA, R. KOEHLER, P. TREMBLAY, D. WALTHER, F. E. COHEN, S. J. DEARMOND & S. B. PRUSINER (1997):
Identification of a prion protein epitope modulating transmission of bovine spongiform encephalopathy prions to transgenic mice.
Proc Natl Acad Sci U S A 94, 14279-14284

SEEGER, H., M. HEIKENWALDER, N. ZELLER, J. KRANICH, P. SCHWARZ, A. GASPERT, B. SEIFERT, G. MIELE & A. AGUZZI (2005):
Coincident scrapie infection and nephritis lead to urinary prion excretion.
Science 310, 324-326

SHEARIN, H. & R. A. BESSEN (2014):
Axonal and transynaptic spread of prions.
J Virol 88, 8640-8655

SIGURDSON, C. J., M. HEIKENWALDER, G. MANCO, M. BARTHEL, P. SCHWARZ, B. STECHER, N. J. KRAUTLER, W. D. HARDT, B. SEIFERT, A. J. MACPHERSON, I. CORTHESY & A. AGUZZI (2009):
Bacterial colitis increases susceptibility to oral prion disease.

J Infect Dis 199, 243-252

SIGURDSON, C. J., E. S. WILLIAMS, M. W. MILLER, T. R. SPRAKER, K. I. O'ROURKE & E. A. HOOVER (1999):
Oral transmission and early lymphoid tropism of chronic wasting disease PrP^{res} in mule deer fawns (Odocoileus hemionus).
J Gen Virol 80, 2757-2764

SIMMONS, M. M., P. HARRIS, M. JEFFREY, S. C. MEEK, I. W. BLAMIRE & G. A. WELLS (1996):
BSE in Great Britain: consistency of the neurohistopathological findings in two random annual samples of clinically suspect cases.
Vet Rec 138, 175-177

SIMMONS, M. M., J. SPIROPOULOS, P. R. WEBB, Y. I. SPENCER, S. CZUB, R. MUELLER, A. DAVIS, M. E. ARNOLD, S. MARSH, S. A. HAWKINS, J. A. COOPER, T. KONOLD & G. A. WELLS (2011):
Experimental classical bovine spongiform encephalopathy: definition and progression of neural PrP immunolabeling in relation to diagnosis and disease controls.
Vet Pathol 48, 948-963

SÖDERHOLM, J. D., C. STREUTKER, P. C. YANG, C. PATERSON, P. K. SINGH, D. M. MCKAY, P. M. SHERMAN, K. CROITORU & M. H. PERDUE (2004):
Increased epithelial uptake of protein antigens in the ileum of Crohn's disease mediated by tumour necrosis factor alpha.
Gut 53, 1817-1824

SOHN, H. J., Y. H. LEE, R. B. GREEN, Y. I. SPENCER, S. A. HAWKINS, M. J. STACK, T. KONOLD, G. A. WELLS, D. MATTHEWS, I. S. CHO & Y. S. JOO (2009):
Bone marrow infectivity in cattle exposed to the bovine spongiform encephalopathy agent.
Vet Rec 164, 272-273

SOTO, C. (2011):
Prion hypothesis: the end of the controversy?
Trends Biochem Sci 36, 151-158

SPIROPOULOS, J., R. LOCKEY, R. E. SALLIS, L. A. TERRY, L. THORNE, T. M. HOLDER, K. E. BECK & M. M. SIMMONS (2011):
Isolation of prion with BSE properties from farmed goat.
Emerg Infect Dis 17, 2253-2261

ST ROSE, S. G., N. HUNTER, L. MATTHEWS, J. D. FOSTER, M. E. CHASE-TOPPING, L. E. KRUUK, D. J. SHAW, S. M. RHIND, R. G. WILL & M. E. WOOLHOUSE (2006):
Comparative evidence for a link between Peyer's patch development and susceptibility to transmissible spongiform encephalopathies.
BMC Infect Dis 6, 5

STACK, M. J., M. J. CHAPLIN & J. CLARK (2002):
Differentiation of prion protein glycoforms from naturally occurring sheep scrapie, sheep-passaged scrapie strains (CH1641 and SSBP1), bovine spongiform encephalopathy (BSE) cases and Romney and Cheviot breed sheep experimentally inoculated with BSE using two monoclonal antibodies.
Acta Neuropathol 104, 279-286

STACK, M. J., M. J. CHAPLIN, L. A. DAVIS, S. EVERITT, M. M. SIMMONS, O. WINDL, J. HOPE

& P. BURKE (2013):
Four BSE cases with an L-BSE molecular profile in cattle from Great Britain.
Vet Rec 172, 70

STACK, M. J., S. J. MOORE, A. VIDAL-DIEZ, M. E. ARNOLD, E. M. JONES, Y. I. SPENCER, P. WEBB, J. SPIROPOULOS, L. POWELL, P. BELLERBY, L. THURSTON, J. COOPER, M. J. CHAPLIN, L. A. DAVIS, S. EVERITT, R. FOCOSI-SNYMAN, S. A. HAWKINS, M. M. SIMMONS & G. A. WELLS (2011):
Experimental bovine spongiform encephalopathy: detection of PrP^{Sc} in the small intestine relative to exposure dose and age.
J Comp Pathol 145, 289-301

STAHL, N., D. R. BORCHELT, K. HSIAO & S. B. PRUSINER (1987):
Scrapie prion protein contains a phosphatidylinositol glycolipid.
Cell 51, 229-240

SUARDI, S., C. VIMERCATI, C. CASALONE, D. GELMETTI, C. CORONA, B. IULINI, M. MAZZA, G. LOMBARDI, F. MODA, M. RUGGERONE, I. CAMPAGNANI, E. PICCOLI, M. CATANIA, M. H. GROSCHUP, A. BALKEMA-BUSCHMANN, M. CARAMELLI, S. MONACO, G. ZANUSSO & F. TAGLIAVINI (2012):
Infectivity in skeletal muscle of cattle with atypical bovine spongiform encephalopathy.
PLoS One 7, e31449

SUZUKI, K., I. GRIGOROVA, T. G. PHAN, L. M. KELLY & J. G. CYSTER (2009):
Visualizing B cell capture of cognate antigen from follicular dendritic cells.
J Exp Med 206, 1485-1493

TAHOUN, A., S. MAHAJAN, E. PAXTON, G. MALTERER, D. S. DONALDSON, D. WANG, A. TAN, T. L. GILLESPIE, M. O'SHEA, A. J. ROE, D. J. SHAW, D. L. GALLY, A. LENGELING, N. A. MABBOTT, J. HAAS & A. MAHAJAN (2012):
Salmonella transforms follicle-associated epithelial cells into M cells to promote intestinal invasion.
Cell Host Microbe 12, 645-656

TAKAKURA, I., K. MIYAZAWA, T. KANAYA, W. ITANI, K. WATANABE, S. OHWADA, H. WATANABE, T. HONDO, M. T. ROSE, T. MORI, S. SAKAGUCHI, N. NISHIDA, S. KATAMINE, T. YAMAGUCHI & H. ASO (2011):
Orally administered prion protein is incorporated by m cells and spreads into lymphoid tissues with macrophages in prion protein knockout mice.
Am J Pathol 179, 1301-1309

TELLING, G. C., M. SCOTT, J. MASTRIANNI, R. GABIZON, M. TORCHIA, F. E. COHEN, S. J. DEARMOND & S. B. PRUSINER (1995):
Prion propagation in mice expressing human and chimeric PrP transgenes implicates the interaction of cellular PrP with another protein.
Cell 83, 79-90

TERRY, L. A., S. MARSH, S. J. RYDER, S. A. HAWKINS, G. A. WELLS & Y. I. SPENCER (2003):
Detection of disease-specific PrP in the distal ileum of cattle exposed orally to the agent of bovine spongiform encephalopathy.
Vet Rec 152, 387-392

TOBLER, I., S. E. GAUS, T. DEBOER, P. ACHERMANN, M. FISCHER, T. RULICKE, M. MOSER, B. OESCH, P. A. MCBRIDE & J. C. MANSON (1996):

Altered circadian activity rhythms and sleep in mice devoid of prion protein.
Nature 380, 639-642

TORRES, J. M., O. ANDRÉOLETTI, C. LACROUX, I. PRIETO, P. LORENZO, M. LARSKA, T. BARON & J. C. ESPINOSA (2011):
Classical bovine spongiform encephalopathy by transmission of H-type prion in homologous prion protein context.
Emerg Infect Dis 17, 1636-1644

TURNER, M., A. GULBRANSON-JUDGE, M. E. QUINN, A. E. WALTERS, I. C. MACLENNAN & V. L. TYBULEWICZ (1997):
Syk tyrosine kinase is required for the positive selection of immature B cells into the recirculating B cell pool.
J Exp Med 186, 2013-2021

UDALL, J. N., K. PANG, L. FRITZE, R. KLEINMAN & W. A. WALKER (1981):
Development of gastrointestinal mucosal barrier. I. The effect of age on intestinal permeability to macromolecules.
Pediatr Res 15, 241-244

UDALL, J. N. & W. A. WALKER (1982):
The physiologic and pathologic basis for the transport of macromolecules across the intestinal tract.
J Pediatr Gastroenterol Nutr 1, 295-301

VALLERON, A. J., P. Y. BOËLLE, R. WILL & J. Y. CESBRON (2001):
Estimation of epidemic size and incubation time based on age characteristics of vCJD in the United Kingdom.
Science 294, 1726-1728

VAN KEULEN, L. J., A. BOSSERS & F. VAN ZIJDERVELD (2008):
TSE pathogenesis in cattle and sheep.
Vet Res 39, 24

VAN KEULEN, L. J., B. E. SCHREUDER, R. H. MELOEN, G. MOOIJ-HARKES, M. E. VROMANS & J. P. LANGEVELD (1996):
Immunohistochemical detection of prion protein in lymphoid tissues of sheep with natural scrapie.
J Clin Microbiol 34, 1228-1231

VAN KEULEN, L. J., B. E. SCHREUDER, M. E. VROMANS, J. P. LANGEVELD & M. A. SMITS (2000):
Pathogenesis of natural scrapie in sheep.
Arch Virol Suppl 16, 57-71

VAN KEULEN, L. J., M. E. VROMANS & F. G. VAN ZIJDERVELD (2002):
Early and late pathogenesis of natural scrapie infection in sheep.
APMIS 110, 23-32

WANG, F., X. WANG, C. G. YUAN & J. MA (2010):
Generating a prion with bacterially expressed recombinant prion protein.
Science 327, 1132-1135

WATTS, J. C. & S. B. PRUSINER (2014):
Mouse models for studying the formation and propagation of prions.
J Biol Chem 289, 19841-19849

WELLS, G. A., M. DAWSON, S. A. HAWKINS, R. B. GREEN, I. DEXTER, M. E. FRANCIS, M. M. SIMMONS, A. R. AUSTIN & M. W. HORIGAN (1994):
Infectivity in the ileum of cattle challenged orally with bovine spongiform encephalopathy.
Vet Rec 135, 40-41

WELLS, G. A., S. A. HAWKINS, R. B. GREEN, A. R. AUSTIN, I. DEXTER, Y. I. SPENCER, M. J. CHAPLIN, M. J. STACK & M. DAWSON (1998):
Preliminary observations on the pathogenesis of experimental bovine spongiform encephalopathy (BSE): an update.
Vet Rec 142, 103-106

WELLS, G. A., S. A. HAWKINS, R. B. GREEN, Y. I. SPENCER, I. DEXTER & M. DAWSON (1999):
Limited detection of sternal bone marrow infectivity in the clinical phase of experimental bovine spongiform encephalopathy (BSE).
Vet Rec 144, 292-294

WELLS, G. A., T. KONOLD, M. E. ARNOLD, A. R. AUSTIN, S. A. HAWKINS, M. STACK, M. M. SIMMONS, Y. H. LEE, D. GAVIER-WIDEN, M. DAWSON & J. W. WILESMITH (2007):
Bovine spongiform encephalopathy: the effect of oral exposure dose on attack rate and incubation period in cattle.
J Gen Virol 88, 1363-1373

WELLS, G. A., A. C. SCOTT, C. T. JOHNSON, R. F. GUNNING, R. D. HANCOCK, M. JEFFREY, M. DAWSON & R. BRADLEY (1987):
A novel progressive spongiform encephalopathy in cattle.
Vet Rec 121, 419-420

WELLS, G. A., J. SPIROPOULOS, S. A. HAWKINS & S. J. RYDER (2005):
Pathogenesis of experimental bovine spongiform encephalopathy: preclinical infectivity in tonsil and observations on the distribution of lingual tonsil in slaughtered cattle.
Vet Rec 156, 401-407

WELLS, G. A. & J. W. WILESMITH (1995):
The neuropathology and epidemiology of bovine spongiform encephalopathy.
Brain Pathol 5, 91-103

WELLS, G. A., J. W. WILESMITH & I. S. MCGILL (1991):
Bovine spongiform encephalopathy: a neuropathological perspective.
Brain Pathol 1, 69-78

WELLS, G. A. H., M. DAWSON, S. A. C. HAWKINS, A. R. AUSTIN, R. B. GREEN, I. DEXTER, M. W. HORIGAN & M. M. SIMMONS (1996):
Preliminary Observations on the Pathogenesis of Experimental Bovine Spongiform Encephalopathy.
In: Bovine Spongiform Encephalopathy: The BSE Dilemma
Springer New York, New York, NY, pp. 28-44

WESTAWAY, D., V. ZULIANI, C. M. COOPER, M. DA COSTA, S. NEUMAN, A. L. JENNY, L. DETWILER & S. B. PRUSINER (1994):
Homozygosity for prion protein alleles encoding glutamine-171 renders sheep susceptible to natural scrapie.
Genes Dev 8, 959-969

WILESMITH, J. W. (1993):

Epidemiology of bovine spongiform encephalopathy and related diseases.
Arch Virol Suppl 7, 245-254

WILESMITH, J. W. & J. B. RYAN (1997):
Absence of BSE in the offspring of pedigree suckler cows affected by BSE in Great Britain.
Vet Rec 141, 250-251

WILESMITH, J. W., J. B. RYAN & M. J. ATKINSON (1991):
Bovine spongiform encephalopathy: epidemiological studies on the origin.
Vet Rec 128, 199-203

WILESMITH, J. W., G. A. WELLS, M. P. CRANWELL & J. B. RYAN (1988):
Bovine spongiform encephalopathy: epidemiological studies.
Vet Rec 123, 638-644

WILESMITH, J. W., G. A. WELLS, J. B. RYAN, D. GAVIER-WIDEN & M. M. SIMMONS (1997):
A cohort study to examine maternally-associated risk factors for bovine spongiform encephalopathy.
Vet Rec 141, 239-243

WILHAM, J. M., C. D. ORRÚ, R. A. BESSEN, R. ATARASHI, K. SANO, B. RACE, K. D. MEADE-WHITE, L. M. TAUBNER, A. TIMMES & B. CAUGHEY (2010):
Rapid end-point quantitation of prion seeding activity with sensitivity comparable to bioassays.
PLoS Pathog 6, e1001217

WILL, R. G., J. W. IRONSIDE, M. ZEIDLER, S. N. COUSENS, K. ESTIBEIRO, A. ALPEROVITCH, S. POSER, M. POCCHIARI, A. HOFMAN & P. G. SMITH (1996):
A new variant of Creutzfeldt-Jakob disease in the UK.
Lancet 347, 921-925

WILSON, G. A., S. M. NAKADA, T. K. BOLLINGER, M. J. PYBUS, E. H. MERRILL & D. W. COLTMAN (2009):
Polymorphisms at the PRNP gene influence susceptibility to chronic wasting disease in two species of deer (Odocoileus Spp.) in western Canada.
J Toxicol Environ Health A 72, 1025-1029

WOOD, J. N., S. H. DONE, G. C. PRITCHARD & M. J. WOOLDRIDGE (1992):
Natural scrapie in goats: case histories and clinical signs.
Vet Rec 131, 66-68

WOPFNER, F., G. WEIDENHÖFER, R. SCHNEIDER, A. VON BRUNN, S. GILCH, T. F. SCHWARZ, T. WERNER & H. M. SCHÄTZL (1999):
Analysis of 27 mammalian and 9 avian PrPs reveals high conservation of flexible regions of the prion protein.
J Mol Biol 289, 1163-1178

YOSHIOKA, M., Y. MATSUURA, H. OKADA, N. SHIMOZAKI, T. YAMAMURA, Y. MURAYAMA, T. YOKOYAMA & S. MOHRI (2013):
Rapid assessment of bovine spongiform encephalopathy prion inactivation by heat treatment in yellow grease produced in the industrial manufacturing process of meat and bone meals.
BMC Vet Res 9, 134

ZABEL, M. D. & A. C. AVERY (2015):
Prions--not your immunologist's pathogen.
PLoS Pathog 11, e1004624

Legislation and Regulation:

EUROPEAN COMMISSION (2011):
Note for guidance on minimising the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products (EMA/410/01 rev.3)
(Official Journal of the European Union C 73/1 as of 05.03.2011)

EUROPEAN COMMISSION (2009):
2009/719/EC: Commission Decision of 28 September 2009 authorising certain Member States to revise their annual BSE monitoring programmes (notified under document C(2009) 6979)
(Official Journal of the European Union L 256/35 as of 29.09.2009)

EUROPEAN COMMISSION (2005):
Commission Regulation (EC) No 214/2005 of 9 February 2005 amending Annex III to Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards monitoring of transmissible spongiform encephalopathies in caprine animals
(Official Journal of the European Union L 37/9 as of 10.02.2005)

EUROPEAN PARLIAMENT, COUNCIL OF THE EUROPEAN UNION (2001):
Regulation (EC) No 999/2001 of the European Parliament and of the Council of 22 May 2001 laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies (in the consolidated version as of 17.05.2017) (*last amended by Commission Regulation (EU) 2018/221 of 15 February 2018 L 43/6)*
(Official Journal of the European Union L 147, 31/05/2001 P. 0001 – 0040)

Resources from the internet:

EUROPEAN PHARMACOPEDIA (Ph. Eur.) 9th Edition:
EP monograph 5.2.8. "MINIMISING THE RISK OF TRANSMITTING ANIMAL SPONGIFORM ENCEPHALOPATHY AGENTS VIA HUMAN AND VETERINARY MEDICINAL PRODUCTS"
http://www.bda.bg/images/stories/documents/analiz/pharmacopoeia/EP%20chapter_528_TSE.pdf
https://www.edqm.eu/en/certification-background-77.html

RUSSEL, W. M. S. & BURCH R. L. (1959) (as reprinted 1992):
The principles of humane experimental technique.
Wheathampstead (UK): Universities Federation for Animal Welfare.
http://altweb.jhsph.edu/pubs/books/humane_exp/het-toc

WORLD HEALTH ORGANISATION (WHO) (2010):
WHO tables on tissue infectivity distribution in transmissible spongiform encephalopathies (Updated 2010).
Geneva: WHO Press.
http://www.who.int/bloodproducts/tablestissueinfectivity.pdf

WORLD ORGANISATION FOR ANIMAL HEALTH (OIE) (2008):
'Manual of Diagnostic Tests and Vaccines for Terrestrial Animals' Chapter 2.4.5. BSE (in the adopted version of May 2016)
http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.04.05_BSE.pdf

Chapter 9 Annex

Table 9.1 Lists of all tissues grouped by organ systems sampled from each experimental calf during necropsy

Samples of the head	**Samples of the gastrointestinal tract**
Ggl. cervicale craniale	Truncus vagalis dorsalis
Ggl. nodosum	Truncus vagalis ventralis
Ggl. trigeminale	Lnn. jejunales
Gl. mandibularis	Lnn. caecales
Gl. parotis	Rumen
Lingua / Glossa	Abomasum
Lnn. retropharyngei	Jejunum
M. masseter	Ileal Peyer's patch
N. accessorius (cranial nerv XI)	Jejunal Peyer's patch
N. facialis	Papilla ilealis (ileocaecal junction)
N. glossopharyngeus (cranial nerv IX)	Colon proximalis
N. opticus	Colon mediales
Tonsilla lingualis	Rectum
	Caecum
	Faeces
Samples of the animal body	
Cor (Septum interventriculare)	N. medianus
Ggl. mesentericum caudale	N. radialis
Ggl. stellatum	N. saphenus
Ggll. aorticorenalia	N. splanchnicus major
Gl. suprarenalis	N. tibialis
Hepar	N. vagus (Pars thoracica)
Lien	Oesophagus
Lnn. bronchomediastinales	Ovaria
Lnn. iliaci mediales	Pancreas
Lnn. mammarii/ Lnn. inguinales superficiales	Plexus brachialis
Lnn. poplitei	Pulmones
M. biceps brachii	Ren
M. longissimus dorsi	Truncus sympathicus (Pars thoracica, including Ggll. paravertebralia)
M. psoas major	Truncus vagosympathicus
M. semitendinosus	Urina
Mamma	Uterus
Medulla ossis femoris	Vesica fellea
N. ischiadicus	

Legend: Ggl./Ggll.: Ganglion/Ganglia, Gl.: Gandula, Ln./Lnn: Lymphonodus/Lymphonodi, M. = Musculus, N.= Nervus.

Table 9.1 *Continued*

Samples of the central nervous system	
Brain [#]	**Spinal cord** *
Cerebellum	C 1 – 2
Mesencephalon	C 2 – 3
Pons	C 4 – 5
Medulla oblongata (cranial part)	C 6 – 7
Obex	Th 1 – 2
Medulla oblongata (caudal part)	Th 3 – 4
Rhinencephalon	Th 5 – 6
Cortex frontalis	Th 7 – 8
Cortex parietalis	Dorsal root ganglia C 1 – C 6
Cortex occipitalis	Dorsal root ganglia Th 1 – Th 8
Corpus striatum	Th 9 – 10
Thalamus	Th 10 – 11
	Th 12 – L 1
	L 2 – L 3
	L 3 – L 4
	L 5 – L 6
	Cauda equina
	Liquor cerebrospinalis

Legend: [#] Each brain part was sampled on either side and then left and right part each were sampled for histology and biochemical analysis, thus one part was fixed in 4 % neutral buffered formaldehyde and the other one was frozen to be stored at –20°C and –70°C;

***** parts of spinal cord are given according to the level of the *Nervi (Nn.) spinales*, C: *Nn. cervicales* (on the level of the *Vertebrae cervicales*), Th: *Nn. thoracales* (on the level of the *Vertebrae thoracales*), L: *Nn. lumbales* (on the level of the *Vertebrae lumbales*)

Table 9.2 Results for lingual tonsil samples obtained by IHC and PMCA as well as bioassay results for retropharyngeal lymph nodes

Months p. i.	Animal–ID	lingual tonsil		retropharyngeal lymph nodes #
		IHC *	PMCA	mouse bioassay
0 (n = 2) (= 1 week p.i.)	WAIT 17	0/2279	neg.	n. d.
	WAIT 18	0/1690	neg.	n. d.
2 (n = 2)	WAIT 15	0/3013	neg.	n. d.
	WAIT 16	0/1302	neg.	n. d.
4 (n = 6)	WAIT 11	0/1287	neg.	n. d.
	WAIT 12	0/1956	neg.	n. d.
	WAIT 13	0/1864	neg.	n. d.
	WAIT 14	0/2984	neg.	n. d.
	WAIT 19	0/1557	neg.	n. d.
	WAIT 20	0/2463	neg.	n. d.
6 (n = 6)	WAIT 05	0/1431	neg.	(~) 0/40, neg. (13), > 568
	WAIT 06	0/1900	neg.	‡ 6/40, 484 ± 18
	WAIT 07	0/1498	neg.	(~) 0/38, neg. (10), > 568
	WAIT 08	0/2653	neg.	0/39, > 730
	WAIT 09	0/1784	neg.	(~) 0/38, neg. (11), > 564
	WAIT 10	0/1211	neg.	(~) 0/40, neg. (17), > 539
8 (n = 2)	WAIT 02	0/1814	neg.	0/38, > 730
	WAIT 03	0/1374	neg.	(~) 0/39, neg. (17), > 505
35 (n = 1)	WAIT 04	0/2385	neg.	n. d.
36 (n = 1)	WAIT 01	0/1966	neg.	n. d.
negative controls	WAKT 01	0/1270	neg.	n. d.
	WAKT 02	0/1440	neg.	n. d.

Legend: p. i.: post infection; # samples of retropharyngeal lymph nodes instead of tonsils were tested by bioassay, as experience from earlier experiments has shown that toxins possibly contained in tonsils can risk the health of the inoculated mice;
IHC: immunohistochemistry, ***** given as number of positive / total number of lymph follicles;
PMCA: protein misfolding cyclic amplification, neg.: negative;
bioassay in Tgbov XV mice: n.d.: not done; positive / inoculated mice and mean incubation time in days ± standard error of the mean (SEM) (‡ bioassay is ongoing) or survival time of oldest negative mice, (~) bioassay is ongoing: positive / inoculated mice, actual result (with number of tested mice), survival time of oldest negative mouse

Table 9.3 Detailed compilation of results obtained during neurological examination of positive control animals over time starting from 24 mpi (which are evaluated in manuscript I)

months p.i.	animal-ID	*optic stimuli*				*tactile stimuli*		*acoustic stimulus*	*body condition**	*behaviour in motion*			*Scores*
		approach of animal	menace response	reaction to flash light	boots test ‡	sensibility of the head	broom test #	loud clapping		gait	behaviour	bar test ^	
		1 2 3	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3	1 2 3 4 5	1 2 3 4	1 2 3 4	1 2 3 4	
24	WAIT 01	x	x	x	x	x	x	x	x	x	x	x	13
	WAIT 04	x	x	x	x	x	x x	x	x	x	x	x	12.5
25	WAIT 01	x	x	x	x	x	x	x x	x	x	x	x	12.5
	WAIT 04	x	x	x	x	x	x	x	x	x	x	x	12
26	WAIT 01	x	x	x	x	x	x	x	x	x	x	x	12
	WAIT 04	x	x	x	x	x	x	x	x	x	x	x	12
27	WAIT 01	x	x	x	x	x	x	x	x	x	x	x	12
	WAIT 04	x	x	x	x	x	x	x	x	x	x	x	12
28	WAIT 01	x	x	x	x	x	x	x	x x	x	x	x	12.5
	WAIT 04	x	x	x	x	x	x	x	x	x	x	x	12
29	WAIT 01	x	x	x	x	x	x	x	x x	x	x	x	12.5
	WAIT 04	x	x	x	x	x	x	x	x x	x	x	x	12.5
30	WAIT 01	x	x	x	x	x	x	x	x x	x	x	x	12.5
	WAIT 04	x	x	x	x	x	x	x	x	x	x	x	12
31	WAIT 01	x	x	x	x	x	x	x	x x	x	x	x x	13
	WAIT 04	x	x	x	x	x	x	x	x x	x	x	x x	13
32	WAIT 01	x	x	x	x	x	x	x	x x	x x	x	x	13
	WAIT 04	x	x	x	x	x	x	x x	x	x x	x	x	15
33	WAIT 01	x	x	x	x	x	x	x	x x	x x	x	x	13
	WAIT 04	x	x	x	x	x	x	x x	x x	x x	x	x x	16
34	WAIT 01	x	x	x	x	x	x	x	x x	x x	x	x	13
	WAIT 04	x	x x	x x	x	x x	x x	x x	x x	x	x x	x x	23
35	WAIT 01	x	x	x	x	x	x	x	x	x	x	x	14
	WAIT 04	x	x	x x	x x	x x	x	x x	x x	x	x x	x	24
36	WAIT 01	x	x	x x	x x	x x	x	x	x	x x	x x	x	16.5

Legend: Only reproducible reactions (more than three times consecutively reactions or changes differing from normal behaviour) were evaluated as BSE-specific. The detailed evaluation criteria were described before by KAATZ (2014). In short, overreactions were rated due to intensity in low (2), moderate (2-3) and intense (3-4), while species-specific reactions were classifed as normal (1). * body condition was scored as follows: increased (1), normal (2), reduced (3-4), emanciation (5); WAIT 04 showed mild symptoms suspicious for BSE starting 32 mpi, followed by a progressive development of the disease (status 3, definitive BSE). WAIT 01 started showing mild changes of the gait from 35 mpi, while a clear overreaction to tactible stimuli was seen 36 mpi (status 2, probable BSE). ‡ moving ones boot in the feeding fence in the animals field of vision; # tests reaction to tactile stimulus of the hindlimb (by brushing each hindlimb with a broom); ^ tests behaviour while stepping over obstacles on the ground (using a bar placed on the floor)

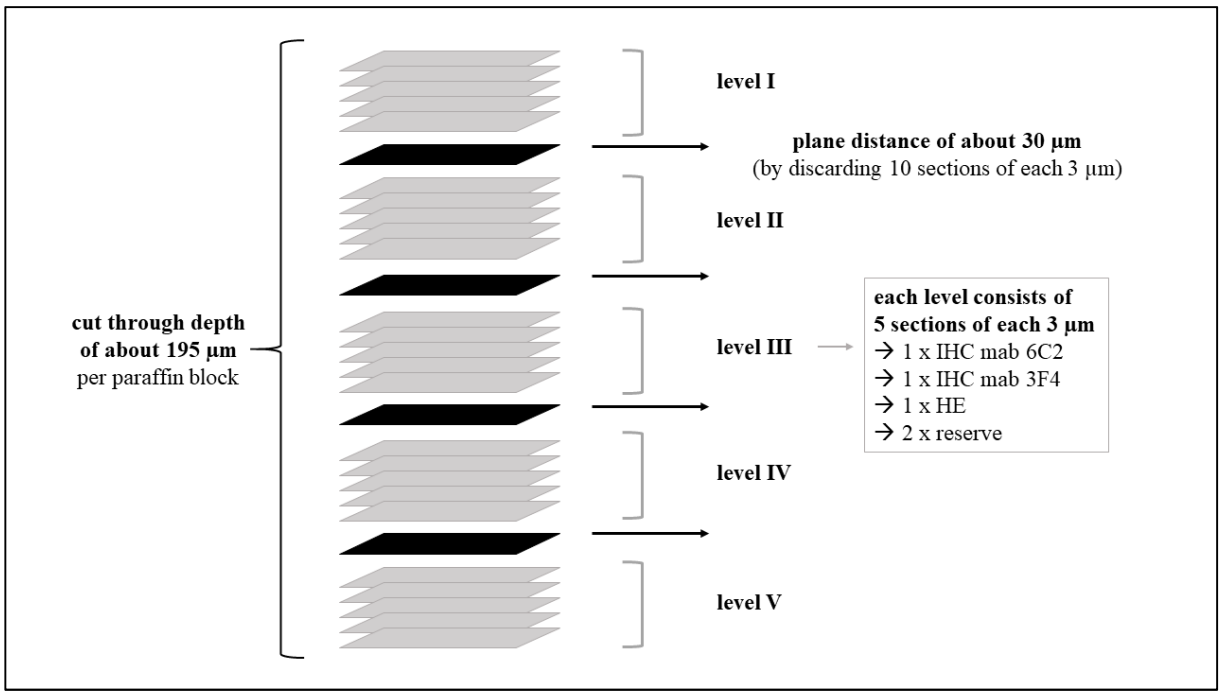

Figure 9.1 Schematic illustration of the serial cutting technique used to prepare sections for histological analyses (modified according to KAATZ (2014)). To attain a great depth of approximately 195 µm per block, a serial cutting technique according to HOFFMANN et al. (2011) and KAATZ (2014) was used, which allowed an examination of 5 different levels per block with a plane distance of about 30 µm each. In case of positive control animal samples firstly just one level of the block was analysed and in case of a negative results the remaining 4 levels were examined in addition.

Chapter 10 Acknowledgements

First of all, I would like to express my sincere gratitude to Prof. Dr. Martin H. Groschup and PD Dr. Anne Balkema-Buschmann for the academic supervision, the scientific encouragement and the outstanding support throughout the course of my thesis. I am grateful for the opportunity to work at the Institute of Novel and Emerging Infectious Diseases of the Friedrich-Loeffler-Institute and for being part of this fascinating project. Thank you for the numerous discussions, for your constructive suggestions as well as for revising my thesis and manuscripts. Your optimism and your trust in me and my work during the last years, encouraged my confidence to follow through with this thesis.

I am deeply thankful to my lab colleagues, especially Julia Herger, Daniel Balkema, Dr. Kerstin Fischer and Vinicius Reis for sharing your experience and knowledge, for your help whenever needed and the support during the last years. It was a pleasure to work with you!

Animal experiments require the dedicated efforts of a lot of people. Therefore, I would also like to thank all my colleagues at the INNT and all animal keepers, for your help and support especially during the times of necropsies. Moreover, I am grateful to Bärbel Hammerschmidt for her support and sharing her experience as well as to James C. Shawulu, Olanrewaju I. Fatola and Lukas Steinke for their enthusiastic help with the care for the mice. Excellent care for the calves was provided by Stefanie Marzahl, Ben Schiller and Volker Netz. Thank you very much indeed!

I am sincerely thankful to Dr. Kerstin Tauscher and PD Dr. Reiner Ulrich for the help with performing the necropsies, all the interesting discussions and valuable advices in terms of pathology as well as for the encouraging words during the last years. Thank you for sharing your knowledge with me!

Moreover, I would like to thank Prof. Dr. Florian Stintzing and Dr. Jürgen Schilk for the constructive discussions during the project meetings and the appreciation of my work, which always gave me an additional motivation for my commitment to this interesting project. I like to express my thanks to the Scientific Advisory Group (SAG), especially to Dr. Stefanie Czub and Dr. Paul Brown, for their scientific advices regarding the project and the manuscripts.

Special thanks to Melanie, Isolde, Claudia, Kerstin, Vinicius, Franziska, Karina, Judith, Jule and Laura who supported me with encouragement and optimism throughout the last years.

Thank you to my dear sister Jenny for always being there and for your writing tips and proofreading whenever needed.

Thanks Christoph for listening and for supporting me with your tacit understanding, your friendship and love.

Zu guter Letzt, aber von ganzem Herzen, danke ich meinen Eltern, die mich mit ihrer bedingungslosen Liebe und Unterstützung auf meinem Weg begleitet haben und mir so den Rückhalt gaben, um diese Arbeit fertigzustellen.