ENVIRONMENTAL PRION TRANSMISSION: IDENTIFYING NOVEL DISEASE DISSEMINATION ROUTES

by

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DEDICATION

To my Mother and Father,
For instilling the belief and drive to pursue my life's goals.

And to my Brother,
Whose free spirit continually inspires mine.

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ABBREVIATIONS

BH	brain homogenate
BSE	bovine spongiform encephalopathy
CJD	Creutzfeldt-Jakob disease
CWD	chronic wasting disease
DPI	days post infection
DY	“drowsy” strain of hamster passaged transmissible mink encephalopathy
EDTA	ethylene diamine tetraacetic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HY	“hyper” strain of hamster passaged transmissible mink encephalopathy
i.c.	intracerebral
iCJD	iatrogenic Creutzfeldt-Jakob disease
IgG	immunoglobulin G
i.p.	intraperitoneal
kDa	kilodalton
LS	Linsmaier and Skoog plant growth medium
MS	Murashige and Skoog plant growth medium
MWCO	molecular weight cutoff
NBH	normal brain homogenate
NMR	nuclear magnetic resonance spectroscopy
ORF	open reading frame

PBS	phosphate buffered saline
PMCA	protein misfolding cyclic amplification
PMCAb	protein misfolding cyclic amplification with beads
p.o.	peroral
PrP	total prion protein, without distinction for different isoforms
PrP^{C}	normal cellular prion protein
PrP^{TSE}	disease-associated misfolded prion protein; a.k.a. PrP^{Sc}, PrP^{d}, $PrP^{BSE,CJD,CWD,etc.}$
PrP^{res}	proteinase K-resistant core of the prion protein, which consists of the C-terminal two thirds of the protein; may or may not be identical to PrP^{TSE}
RML	Rocky Mountain Laboratories strain of mouse passaged sheep scrapie (a.k.a. “Chandler” strain)
RT	room temperature
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sPMCA	serial protein misfolding cyclic amplification
SRM	Specified risk material
TBS	Tris-buffered saline
TME	transmissible mink encephalopathy
TSE	transmissible spongiform encephalopathy
vCJD	variant Creutzfeldt-Jakob disease
WT	wild-type

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Prion diseases (transmissible spongiform encephalopathies, TSEs) are infectious neurodegenerative disorders that affect a variety of mammals. Scrapie and CWD are unique among TSEs in that they exhibit indirect horizontal transmission through contaminated environments. While it is well-established that both CWD and scrapie prions are extraordinarily stable in environmental settings and an environmental reservoir of infectivity contributes to the maintenance of these diseases, routes of natural transmission remain to be clarified. In this work, we identify two novel routes of environmentally relevant prion transmission: 1. interspecies transmission of CWD and scrapie to a native North American rodent species, the meadow vole (*Microtus pennsylvanicus*), and 2. uptake and transmission of prions by plants.

Using animal bioassays, we demonstrate that meadow voles are susceptible to CWD by oral and intraperitoneal challenge. Oral challenge of meadow voles with CWD resulted in subclinical infection in primary passage animals, but manifested as clinical prion disease upon

subpassage. These data support a role for meadow voles in environmental CWD transmission, possibly as vector, reservoir or bridge species. Additionally, serial subpassage of CWD isolates from white-tailed deer expressing different genotypes with regard to the prion protein (either 96GG or 96 GS) gave rise to differential strain selection in meadow voles, providing evidence to support the concept that at least two strains of CWD circulate in naturally-infected cervid populations in North America and that meadow voles are useful tools for CWD strain typing. We found meadow voles to be poorly susceptible to sheep scrapie as compared to CWD, suggesting this species is unlikely to acquire scrapie on the landscape and serve as a disease reservoir.

An environmental reservoir of infectivity contributes to natural transmission of prions diseases in deer, elk, and sheep and previous research has implicated soil. Thus, we tested and found evidence to support the hypothesis that plants take up prion protein and transmit infectivity to mammalian hosts. Importantly, mice were found to be orally susceptible to prion contaminated plants in rodent bioassay experiments, suggesting that contaminated plants may represent a previously unrecognized risk of human, domestic animal and wildlife exposure to CWD and scrapie agents.

CHAPTER ONE:

Introduction

Transmissible spongiform encephalopathies: overview and significance

Prion diseases, also known as transmissible spongiform encephalopathies (TSEs) form a biologically unique group of infectious fatal neurodegenerative disorders that afflict a variety of mammalian species (Table 1-1). Despite their low incidence and limited infectiousness as compared with many bacterial and viral diseases, TSEs are capable of causing epidemics of incurable, deadly disease in humans and animals, such as bovine spongiform encephalopathy (BSE), variant Creutzfeldt-Jakob disease (vCJD), and chronic wasting disease (CWD), that have attracted enormous attention due to their devastating impacts on public health, agriculture, and ecology.

TSEs have been recognized for hundreds of years, even though the most widely accepted identification and description of the etiological agent was made merely 30 years ago. Scrapie, the archetypal prion disease, has been known in Europe since the early 18th century among sheep farmers and breeders who observed and recorded recumbency, persistent scratching, loss of appetite, and wasting of affected animals during a prolonged clinical phase, ultimately resulting in death, and described it as a "scraping" or "scratching" disease (Fast and Groschup, 2013; Schneider et al., 2008). Early reports of scrapie also recognized that scrapie-free flocks developed the disease upon introduction of affected animals, suggesting transmissibility. Transmission was confirmed in experiments carried out in 1936 in which brain and spinal cord tissue from scrapie diseased sheep was inoculated into healthy sheep that subsequently developed scrapie after incubation periods of up to two years (Cuille and Chelle, 1936; Cuille and Chelle, 1938a; Cuille and Chelle, 1938b; Cuille and Chelle, 1939).

When human TSEs, such as Creutzfeldt-Jakob disease (CJD) and Gerstmann-Straussler-Scheinker syndrome (GSS) were first described in the 1920s and 1930s, these diseases were not thought to be transmissible (Creutzfeldt, 1920; Gerstmann et al., 1936; Richardson and Masters, 1995). In 1959, veterinary pathologist, William Hadlow, recognized close epizootiological, etiological, clinical, and pathological similarities between scrapie and kuru in humans (Hadlow, 1959), an incurable neurodegenerative disorder endemic to tribal regions of Papua New Guinea (Gajdusek and Zigas, 1957). Hadlow's observations led to the successful transmission of kuru, and later CJD, from humans to chimpanzees and other primates by Gajdusek and colleagues (Gajdusek et al., 1966; Gajdusek and Gibbs, 1971). These findings were the first to identify that the TSE diseases of animals and humans belonged to the same group and that transmissibility was one of the defining hallmarks of TSEs, resulting in a research boom that continues to this day into the identification of the causative agent and its pathogenesis.

Research interest in TSEs was accelerated, and met with international public and political interest, with the outbreak of bovine spongiform encephalopathy (BSE), or "mad cow" disease, in 1985 in the United Kingdom (U.K.) (Smith and Bradley, 2003). Not only did BSE represent a newly emerging TSE, but it also demonstrated, for the first time in recorded history of prion diseases, that TSEs were capable of "jumping" the species barrier to infect humans and other animals (Belay and Schonberger, 2005; Bruce et al., 1997; Will et al., 1996). Overall, the BSE epidemic of the 1980s and 1990s involved an estimated one million infected cattle, with a cumulative gross budgetary cost to the U.K. estimated at $5 billion (U.S. dollars) between 1996 and 2000 (USDA Economic Research Service, 2001). Additionally, zoonosis of BSE caused a new variant of Creutzfeldt-Jakob disease (vCJD) in humans following consumption of BSE-

contaminated food products, which, as of November 2013, has resulted in 228 cases of reported vCJD worldwide (Bruce et al., 1997; The University of Edinburgh, 2013). Progression of the U.K. BSE outbreak, especially in light of scientific evidence supporting its transmission to humans, resulted in the implementation of several important preventative public health measures. These measures included a 1989 specified risk material ban for human food, a 1996 prohibition of the processing of cattle ≥ 30 months old for human food, and a total ban on the feeding of mammalian protein to any farmed animals (Smith and Bradley, 2003). Improved BSE surveillance, particularly in the European Union, contributed to the rapid increase in the number of countries with confirmed BSE. Many other countries within Europe had imported cattle and meat-and-bone meal from the U.K.; by 2002, BSE among domestic cattle had been detected in 19 other European countries, and, for the first time outside of Europe, in Japan and Isreal (Belay and Schonberger, 2005). Canada, Mexico, and the United States implemented bans on importation of cattle and cattle products from countries known to have BSE or be at risk of BSE and on ruminant feeds. Despite these precautions, multiple indigenous and imported cases of BSE have been reported in the U.S. and Canada since 1989 (World Organization for Animal Health, 2013).

While the number of food-borne vCJD cases reported worldwide each year is currently in decline, prevalence in the U.K. is still uncertain, and concern remains over secondary spread of the agent via blood products and individuals potentially harboring subclinical infection. Three clinical cases of transfusion-transmitted vCJD and one asymptomatic infection have been reported in the U.K. (Llewelyn et al., 2004; Peden et al., 2004; UK Health Protection Agency, 2006; UK Health Protection Agency, 2007). All transmissions followed transfusions of red blood

cells from donors who later became ill with vCJD. One additional presumptive case of vCJD was reported in a patient with hemophilia and a long history of treatment with U.K.-sourced human plasma-derived growth factor VII (Peden et al., 2010). Additionally, a recent survey of appendix tissue in the U.K. estimated that as many as 1 in 2,000 people had accumulation of abnormal prion protein in lymphoid follicles (UK Health Protection Agency, 2012). These individuals are currently asymptomatic, but might be incubating vCJD; they may either remain in a silent carrier state and never develop clinical vCJD, or eventually progress to clinical disease. Multiple cases of iatrogenic transmission of non-variant CJD have been associated with corneal and dura mater grafts, contaminated neurosurgical equipment, and receipt of pituitary-derived human growth hormone from infected donors (Brown et al., 2012). Thus, despite the decline in number of food-borne vCJD cases and low prevalence of CJD, these diseases still threaten public health.

Since the first reports of scrapie were recorded in the early-to-mid 1700s, we have struggled with TSEs and we continue to face new agents and new problems associated with them. The 20th century saw the emergence of chronic wasting disease (CWD), the first and only known TSE to affect free-ranging wildlife species (deer, elk, and moose species, or cervids) (Gilch et al., 2011). Since its initial detection in Colorado and Wyoming in the 1960s, CWD has spread across North America in epizootic fashion, with devastating effects on both wild and farmed cervid populations and the hunting economies that depend upon them, as well as cascading ecological consequences (Bishop, 2004; Petigara et al., 2011; Seidel and Koontz, 2004). Although the zoonotic potential of CWD is considered low, recent breakthroughs in our basic understanding of prion biology, including the existence of various prion strains (Collinge

and Clarke, 2007) and the potential for agent evolution (Li et al., 2010) hinder a definitive conclusion. In recent years our knowledge of TSE diversity expanded with identification of so-called atypical forms of prion diseases in sheep (atypical/Nor98 scrapie) and cattle (BSE-H and BSE-L) (Tranulis et al., 2011). These maladies resemble sporadic or genetic human TSEs in etiology and display discordant pathobiological and phenotypic features as compared to their classical infectious counterparts, and evade current diagnostic and detection methods. We have only begun to understand the disease risks and outcomes associated with these novel TSEs, including possible epidemiological links between atypical animal and human TSEs.

Despite over a century of research on TSEs, their etiology remains enigmatic and there are no prophylaxes, therapies, or cures for these invariably fatal diseases. These facts highlight how vastly incomplete our knowledge of even fundamental prion biology currently stands, notwithstanding the remarkable progress that has been made in prion science over the last few decades. Development of novel approaches to combat prion diseases clearly requires advancement of the current state of knowledge on prion etiology, pathogenesis, transmission, evolution, diagnosis, prevention, therapy, and epidemiology. Investment in TSE research may well extend beyond prion diseases themselves as recent research has identified striking similarities in the protein misfolding mechanisms underlying prion diseases and proteinopathies such as amyloid A amyloidosis, Alzheimer's disease and other tauopathies (Novak et al., 2011; Reiniger et al., 2011). Thus, TSE research may result, in principle, in a paradigm for a broader spectrum of protein misfolding and aggregation disorders than is currently recognized.

Scrapie and chronic wasting disease

Scrapie is a naturally occurring TSE that affects sheep, goats, and moufflons nearly worldwide. In Europe, the disease has been known since the early 18th century and is currently endemic in almost all member states of the European Union (EU) as well as Norway, Iceland and Switzerland (Fast and Groschup, 2013). Since its initial detection in the United States in 1947, sheep scrapie has been detected in all but three states (Alaska, Hawaii, and Florida) with a current nationwide prevalence of 0.03% (Alan Huddleston, personal communication) and is estimated to cost American sheep producers $10 – 20 million per year (USDA APHIS, 2010). Within the last fifty years, depopulation and selective breeding efforts have limited spread and frequency of outbreaks and have eliminated scrapie from Australia and New Zealand (Detwiler and Baylis, 2003). Although no evidence exists supporting scrapie transmission to humans, sheep and goat tissues containing the highest levels of infectivity (including the spleen and ileum of sheep and goats of all ages, and skull, brain, eyes, tonsils and spinal cord for animals over 12 months) are designated as "specified risk material (SRM)" and excluded from human consumption as a precautionary risk reduction measure in the EU (European Parliament, 2001).

Clinically, scrapie is characterized by slow, gradual progression of neurologic and dermatologic abnormalities. Onset of clinical signs may occur several months to years after exposure. Early clinical features include generalized wasting and loss of body mass, tremors, and behavioral changes (Fast and Groschup, 2013). Pruritis may develop, and infected sheep frequently rub themselves against objects and compulsively chew at their coat. Clinical signs gradually progress, with development of ataxia, recumbency, and occasionally blindness. Death

generally follows within two to four weeks (Williams, 2003). Classic scrapie is diagnosed on the basis of characteristic histopathologic changes within the CNS or results of immunodiagnostic testing of brain or lymphoid tissues for the scrapie agent. In live sheep, lymphoid tissue is often obtained via biopsy of the third eyelid or rectum (Raeber and Oesch, 2006). The scrapie agent may be detectable in many tissues and body fluids, including spleen, tonsils, rectum, tongue, retinas, CSF, and blood, prior to the appearance of both clinical signs and deposition of the scrapie agent in nervous tissue, thereby possibly allowing for preclinical screening of herds (Williams, 2003). About 3-5% of animals per affected flock may die annually and an increase of up to 20% in annual mortality rates has also been noted in some flocks (Jeffrey and Gonzalez, 2007; Maddison et al., 2010; Novakofski et al., 2005; O'Rourke et al., 2011). In 1998, the atypical form of scrapie, termed Nor98, was discovered for the first time in Norwegian sheep, although retrospective investigation revealed that cases had been documented as early as the 1980s (Benestad et al., 2003). Interestingly, atypical scrapie cases were detected in sheep displaying genotypes known to confer high resistance to classical scrapie (Tranulis et al., 1999). Clinical symptoms, histopathology, and agent characteristics of atypical scrapie were different from those of classical scrapie (Benestad et al., 2008; Buschmann et al., 2004; Onnasch et al., 2004; Orge et al., 2010; Wemheuer et al., 2009a; Wemheuer et al., 2009b). A surprisingly uniform prevalence of atypical scrapie (about 6-8 cases per 10,000 tested animals) found across geographically disparate flocks contrasted with the more variable and clustered occurrence of classical scrapie, suggesting a sporadic etiology for atypical scrapie as compared with the transmissible nature of classical scrapie (Fediaevsky et al., 2008).

Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy (TSE; prion disease) of cervids (i.e., deer, elk, moose), and the only TSE known to affect free-ranging wildlife species (Baeten et al., 2007; Williams, 2005). First discovered in Colorado in the 1960s (Williams and Young, 1980), CWD has since been identified in free-ranging and captive cervids in 22 U.S. states, 2 Canadian provinces, and South Korea (Kim et al., 2005; Sohn et al., 2002; USGS National Wildlife Health Center, 2013) (Figure 1-1). Along with the expansion of its known geographical distribution, CWD's prevalence in North America is increasing in areas currently experiencing epizootics, reported as upwards of 80% in some captive herds (Keane et al., 2008; Miller and Williams, 2003; Williams and Young, 1980) and >50% in the free-ranging South Converse mule deer herd in Wyoming (Wyoming Game and Fish Department, 2011) .

Clinical features of CWD are qualitatively similar to sheep scrapie or cattle BSE; the salient features of CWD in adult cervids is weight loss and behavioral changes that typically span weeks or months (Williams et al., 2002; Williams and Young, 1980; Williams and Young, 1982; Williams and Young, 1992). In addition to these general features, additional signs have been observed inconsistently in some cases, including: ataxia and head tremors, excessive salivation due to difficulty swallowing, esophageal dilation and regurgitation and aspiration pneumonia. Terminal physiologic and behavioral alterations can include polydipsia and polyuria, syncope, periods of lack of awareness, fixed stare, altered gait or stance, and hyperexcitability when handled (Williams, 2005). Average incubation periods for CWD range from two to four years and duration of clinical disease is extremely variable, with death typically occurring within four months, although few animals may survive as long as a year (Williams, 2005).

The appearance of CWD in wild cervids presents significant challenges to disease control or eradication due to 1) the extensive geographic range of North American deer and elk, 2) the logistical difficulty in applying ante-mortem diagnostic tests such as tonsil biopsy (Wolfe et al., 2002) and 3) the inability to rid the environment of potential prion contaminated excreta. While apparent elimination of CWD in free-ranging cervids was achieved in New York upon early, intensive depopulation and surveillance efforts following the detection of two positive free-ranging deer (New York State Department of Environmental Conservation and Cornell University Animal Health Diagnostic Center, 2013), it appears that once the disease has become endemic in a region, the lack of effective prophylaxes, therapeutics, and management strategies make eradication currently infeasible. Management approaches in CWD-enzootic states focus on depopulation or reduction of suspect herds in an effort to limit the spread of CWD to non-affected herds (Wisconsin Department of Natural Resources, 2010). While the economic consequences of CWD to hunting, agricultural and tourism industries are already being felt across North America (Bishop, 2004; Petigara et al., 2011; Seidel and Koontz, 2004), the long-term effects of CWD on cervid populations and ecosystems remain unclear as the disease continues to spread and prevalence increases. Epidemiologic modeling suggests disease could have severe effects on free-ranging deer populations, including potential population collapse (Almberg et al., 2011; Wasserberg et al., 2009), and in several high prevalence study areas in Colorado and Wyoming, biologists have witnessed cervid population declines of up to 30 – 50% (Miller et al., 2008; Wyoming Game and Fish Department, 2011).

Additional aspects of scrapie and CWD biology, including nature of the infectious agent, genetics, transmissibility, host range, species barrier and strains are described within the following sections.

Clinical and pathological characteristics of TSEs

Neuropathology played a central role in the early definition of prion diseases and has remained an important tool in TSE diagnosis and pathogenic understanding. Prion infection is characterized by a number of pathogenic events that occur in the central nervous system of the host. At clinical stage, the classical neuropathological triad of changes that occur include vacuolation of central nervous system (CNS) tissue, resulting in a "sponge-like" appearance under microscopic examination (hence the term "spongiform encephalopathy"), the chronic proliferation of astrocytes and microglia, and neuronal loss (Aguzzi et al., 2007; Budka, 2003; Collinge, 2001; Prusiner, 1998). These characteristic changes may also be accompanied by the accumulation of aggregates of abnormal prion protein in infected brain and lymphoid tissues that can be observed *in situ* by immunohistochemistry (Figure 1-2). Immunohistochemical detection of abnormal prion protein has been used as a surrogate marker for TSE infectivity, and has become the most important diagnostic marker in neuropathological confirmation of prion disease in infected hosts (Kubler et al., 2003). The overriding outcome of prion diseases is progressive neurodegeneration, but the means by which prions induce neuronal loss and damage the CNS remain unknown. One hypothesis is that neurodegeneration stems from loss of normal prion protein function (Aguzzi and Falsig, 2012). Another posits that the abnormal prion protein, or an

intermediate species produced during prion replication, has neurotoxic properties (Collinge and Clarke, 2007).

Clinical manifestations of prion disease arise after an abnormally long incubation period (spanning months to decades, depending on the host species), and include multiple effects of progressive physical and cognitive impairment, ultimately leading to death of the affected individual. Clinical effects of disease in animals include motor system dysfunction (e.g. ataxia, myoclonus), severe cognitive impairment (e.g. dementia), behavioral changes (e.g. loss of motivation, depression), and general wasting (Detwiler, 1992; Miller and Williams, 2004; Wadsworth et al., 2003).

TSE agent and prion protein

The physical nature of the etiological prion agent has been a historically debated topic and remains controversial today. Several theories have been proposed to describe the TSE agent's characteristics, and range from viruses (Cuille and Chelle, 1938a; Manuledis, 1996; Rohwer, 1984a; Rohwer, 1984b; Rohwer, 1991; Sigurdsson, 1954) to membrane fragments (Gibbons and Hunter, 1967; Hunter et al., 1968) to polysaccharides (Adams and Caspary, 1968; Alper et al., 1967) to lipids (Alper et al., 1978) to proteins (Griffith, 1967; Pattison and Jones, 1967). The transmissibility of the agent, retention of its infectivity after filtration, and the long incubation period prior to disease onset led to the early concept of a "slow virus," or possibly a viroid (Diener, 1972; Sigurdsson, 1954). However, no viral particles or disease-specific nucleic acids were identified in association with TSE infection (Alper et al., 1966; Pattison and Jones, 1967). Resistance of the TSE agent to ionizing and UV irradiation (Alper, 1993; Alper et al.,

1978; Bellinger-Kawahara et al., 1987a; Bellinger-Kawahara et al., 1987b), formalin fixation (Pattison, 1965), nucleases (Hunter and Millson, 1964; Prusiner et al., 1980b), and standard sterilization and disinfection agents (Brown et al., 2004; Hunter and Millson, 1964; Taylor, 1999; Taylor, 2000) and its inactivation by procedures that modify proteins (Prusiner et al., 1980a) supported the hypothesis that the agent could be a protein with self-replicating properties (Griffith, 1967). Subsequent studies by Stanley Prusiner *et al.* led to the purification of the scrapie agent and establishment of the requirement of protein for infectivity, and, ultimately, proposal of the "prion hypothesis, " which stated that "prions are small proteinaceous particles which are resistant to inactivation by most procedures that modify nucleic acids" (Prusiner, 1982; Prusiner et al., 1982). The discovery and characterization of the disease-associated prion protein (PrP^{TSE}) suggested that PrP^{TSE} may be a major component of the infectious agent, if not the agent itself (Bolton et al., 1987). Subsequent studies demonstrated that PrP^{TSE}, rather than being a novel protein, was a conformational variant of a normal brain protein, PrP^{C} (Westaway et al., 1987).

The cellular form of the prion protein (PrP^{C}) is a highly conserved, host-encoded sialoglycoprotein expressed primarily on the cell surface of neurons within the CNS (Stahl et al., 1987), but also on a number of other cell types, including lymphocytes (Cashman et al., 1990) and follicular dendritic cells (Mcbride et al., 1992). PrP^{C} is encoded by the *PRNP* gene, which is located on chromosome 20 with an open reading frame encoding 253 amino acids, in humans (schematically represented in Figure 1-3). The first 22 amino acids are a secretory signal peptide that is cleaved upon entry into the endoplasmic reticulum. Likewise, the *C*-terminal 23 amino acids are cleaved upon the addition of a glycosylphosphatidylinositol (GPI) anchor. The *N*-

terminal portion of PrP is unstructured, but contains two defined, conserved regions. The first consists of a segment of five octapeptide repeats with the consensus sequence PHGGGWGQ, which bind transition metals, including copper and zinc (Brown, 2001a; Brown et al., 1997). A second conserved *N*-terminal region lies downstream of the octapeptide repeats and contains a highly hydrophobic profile, which is thought to make up the hydrophobic core of the prion protein and possibly function as a transmembrane region (Aguzzi and Heikenwalder, 2006). The structured *C*-terminal region of the protein consists of three α-helices and two small anti-parallel β-sheets. The second β-sheet and the second α-helix are connected by a large loop with varying structural properties among species that is hypothesized to play a role in cross-species transmission of prion diseases (Sigurdson et al., 2010). Two *N*-linked glycosylation sites at Asn 180 and Asn 196 and a single disulfide bridge are also prominent features of the *C*-terminal region of PrP^{C} (Riek et al., 1996). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of denatured PrP reveals three bands representing di-, mono-, and nonglycosylated protein (Figure 1-4). Although the three-dimensional structure of PrP^{C} has been elucidated by nuclear magnetic resonance spectroscopy (NMR) (Figure 1-4), the avid aggregation and insolubility of PrP^{TSE} have hindered its structural determination by NMR spectroscopy or X-ray crystallography (Wuthrich and Riek, 2001). Circular dichroism and infrared spectroscopy, and more recently, mass spectrometry coupled with hydrogen-deuterium exchange studies indicate that, relative to PrP^{C}, PrP^{TSE} contains a higher β-strand and little-to-no α-helical content (Caughey et al., 1991; Smirnovas et al., 2011). Crystallography and electron microscopy data suggest that PrP^{TSE} forms structured trimers with diameters of ~10 nm that aggregate into fibrils (typically 50 – 300 nm long) (Smith et al., 2011). Recent studies indicate that smaller subfibrillar

aggregates of 14 to 28 PrP^{TSE} molecules possess the highest specific activity (Silveira et al., 2005).

PrP^{TSE} possesses the same primary sequence and covalent post-translational modifications as does PrP^{C}; the only difference detected to date between the two isoforms is the secondary and tertiary folding of the protein. These structural differences between PrP^{C} and PrP^{TSE} are thought to confer unique biological and physicochemical properties upon each isoform (Smith et al., 2011). PrP^{TSE} is largely insoluble in water and detergents while PrP^{C} is fully soluble. PrP^{TSE} and prion infectivity exhibit extraordinary resistance to inactivation by ultraviolet and ionizing radiation, exposure to heat treatments, chemical disinfectants, and proteases, while PrP^{C} is labile to all of them (Smith et al., 2011). Treatment of PrP^{TSE} with proteinase K (PK) cleaves the *N*-terminal ~90 amino acids, leaving a truncated form of the protein designated PrP^{res} (or PrP27-30) (Figure 1-4). PrP^{res} is a reliable biomarker for TSE infection and has remained the gold standard for TSE diagnosis since its initial description approximately thirty years ago (Raeber and Oesch, 2006). Finally and importantly, PrP^{TSE} is associated with prion infectivity and disease while PrP^{C} is a non-infectious, functional protein in eukaryotic cells (Aguzzi and Falsig, 2012).

The normal function of the cellular prion protein (PrP^{C}) is still uncertain. *Prnp* knockout mice show no abnormalities in development or behavior (Bueler et al., 1992); their only overt phenotype is resistance to developing prion disease upon challenge (Bueler et al., 1993), further supporting the "protein-only" hypothesis of prion etiology. Yet, it seems unlikely that a protein conserved in mammals, birds (Gabriel et al., 1992), reptiles (Simonic et al., 2000), amphibians (Strumbo et al., 2001), and fish (Suzuki et al., 2002) is nonessential or exists for the sole reason

of bestowing susceptibility to prion diseases. PrP^C has been implicated in a number of cellular processes, including normal synaptic function (Baumann et al., 2007; Bremer et al., 2010; Carleton et al., 2001; Mallucci et al., 2002), neuroprotection mediated by anti-apoptotic and anti-oxidative mechanisms (Brown et al., 1999; Roucou and LeBlanc, 2005), neurite outgrowth (Kanaani et al., 2005; Lopes et al., 2005; Santuccione et al., 2005), metal binding (Brown, 2001a; Brown et al., 1997), and circadian rhythms (Lasmezas, 2003; Martins et al., 2002), among others.

The fundamental event in prion disease pathogenesis is thought to be the conversion of PrP^C to PrP^{TSE}. Two models have been developed to describe this process; both involve direct interaction of PrP^C with PrP^{TSE}, with PrP^{TSE} conferring its abnormal conformation to the normal PrP^C molecule. In the "template-directed conversion" model, monomeric PrP^{TSE} interacts with monomeric PrP^C, forming a heterodimer. Refolding of PrP^C occurs, resulting in a PrP^{TSE} homodimer, which then dissociates to form two PrP^{TSE} molecules. This cycle is repeated resulting in an increase in both infectious titer and PrP^{TSE} (Figure 1-5). Alternatively, the "seeded nucleation" model proposes that PrP^C and PrP^{TSE} exist in a reversible thermodynamic equilibrium, and only if several monomeric PrP^{TSE} molecules are mounted in a highly ordered seed can more monomeric PrP^C be recruited and eventually aggregated to form amyloid. Fragmentation of PrP^{TSE} aggregates increases the number of PrP^{TSE} seeds able to recruit and convert additional PrP^C (reviewed in Aguzzi and Sigurdson, 2004). Advances in cell-free conversion (Kocisko et al., 1994) and protein misfolding cyclic amplification (PMCA) technology (Saborio et al., 2001) have allowed for the detailed study of prion conversion *in vitro*. Much of this work supports the seeded nucleation model of prion protein conversion.

Prion strains

According to the protein-only hypothesis, prions propagate in the absence of nucleic acid by transmission of an altered folding conformation onto the normal host version of the protein (Prusiner, 1982). Even though prions are thought to be devoid of nucleic acid, they share with nucleic-acid based pathogens the ability to maintain and replicate strain information and exhibit strain diversity. The prion strain concept is perhaps one of the most puzzling in all of prion biology and, as an incompletely understood concept, is an active area of current research.

The first indication of the existence of prion strains resulted from a 1961 study carried out by Pattison and Millson in which goats were infected with the same inoculum (a brain pool from three scrapie-diseased sheep) and yet developed two distinct clinicopathological syndromes, termed "scratchy" and "drowsy" to reflect each variant's overt clinical manifestation (Pattison and Millson, 1961a; Pattison and Millson, 1961b). Animals affected with the "scratchy" variant of goat-passaged scrapie will rub against objects, scraping off their wool while animals affected with the "drowsy" variant will appear chronically lethargic and will stagger while walking. The authors of the study suggested that the observed divergence in clinical syndromes may reflect infection by unique strains of the scrapie agent. Because the outbred nature of most large mammalian hosts used in experimental studies provides the confounding factor that differences in disease outcomes could be the result of differences in the genetic background of the host, much of the prion strain isolation and characterization work to date has been carried out in genetically defined rodent hosts. Passage of the "drowsy" goat strain in C57BL/6 mice resulted in the isolation of the "Chandler" strain, from which the Rocky Mountain

Laboratories (RML) strain was also derived (Chandler, 1961). Overall, over 20 distinct strains have been extensively characterized and studied in experimental mouse models of scrapie (Bruce, 2003). From these studies were identified the *in vivo* characteristics that can be used to characterize and differentiate prion strains. These include incubation period, clinical signs of disease, and profile of histological damage in the brain (Bessen and Marsh, 1992b; Bruce, 1993; Fraser, 1993). The most commonly used is incubation period, which corresponds to the time elapsed between experimental inoculation of the infectious agent and clinical onset of the disease. If experimental variables are kept constant, each TSE strain produces a characteristic and highly reproducible incubation period in a given host (Bruce, 2003). Different strains also induce reproducible patterns of histopathological change in the CNS of the infected host. These differences are characterized mainly by the distribution and patterns of PrP^{TSE} deposition and the degree of vacuolization, or spongiform change, in specific, defined brain regions (Bruce et al., 2002; Bruce et al., 1989; Lasmezas et al., 1996). Brain vacuolization can be quantified using a standardized procedure for vacuolization scoring, called lesion profiling, in which nine gray matter and three white matter brain regions are analyzed and scored according to the density of vacuolization in each region (Fraser and Dickinson, 1973). This procedure produces a histopathological "signature" for a given TSE agent in a given host that is used in defining prion strains. In fact, the finding that BSE isolates from cattle and vCJD isolates from humans produced nearly identical lesion profiles in genetically identical mice was central to the determination of the former being the likely cause of the latter (Bruce et al., 1997).

In striking parallel to the scratchy and drowsy goat-passaged scrapie variants, similar "hyper" (HY) and "drowsy" (DY) phenotypes were observed upon experimental transmission of

Syrian hamsters with transmissible mink encephalopathy (TME), a rare foodborne prion disease of unknown origin (Bessen and Marsh, 1992b). Studies of the HY and DY strains led to the seminal discovery that, in addition to the previously identified *in vivo* strain defining parameters, there also exist distinct agent-specific biochemical properties that can be used to characterize and differentiate prion strains (Bessen and Marsh, 1992a). Hamsters infected with HY agent display an overt hyperexcitability clinical phenotype while DY infected hamsters display an increasingly lethargic phenotype with slowed movements and loss of coordination. HY and DY agent produced stable incubation periods of 65 ± 1 and 168 ± 2 days, respectively. Hamsters clinically affected with HY have brain titers of $10^{9.5}$ LD_{50}/g whereas DY animals, at end-stage, have brain titers of $10^{7.5}$ LD_{50}/g. Similar to mouse TSE strains, HY- and DY- affected animals differ with respect to histopathology. Additionally, HY and DY strains can be readily distinguished biochemically with the PK-resistant forms of PrP^{HY} and PrP^{DY} differing in size by 1-2 kDa. PrP^{HY} and PrP^{DY} also have marked differences in their sensitivities to PK digestion. Structural data using infrared spectroscopy (Caughey et al., 1998) or conformation-dependent immunoassay (Safar et al., 1998) have now demonstrated that PrP^{res} from HY and DY are indeed conformationally different, supporting the hypothesis that prion self-propagation may proceed via distinct three-dimensional structures, which may underlie the molecular basis for TSE strains.

Polymorphisms in the prion protein gene, *Prnp,* can influence prion strain selection. In mice, only two alleles of *Prnp* have been identified, encoding proteins that differ by two amino acids at codons 108 and 189 (Bruce, 2003). When mice possessing each of these genotypes are infected with a single TSE strain, the differences in PrP genotype can make a difference of hundreds of days to the incubation period. Genes other than *Prnp* can influence prion incubation

period, but usually to a lesser extent. PrP polymorphisms are not unique to mice. In sheep, three polymorphic codons in *Prnp* have been identified that give rise to amino acid changes in PrP: codons 136 (valine (V) or alanine (A)), 154 (histidine (H) or arginine (R)), and 171 (glutamine (Q) or histidine (H)) are associated with either susceptibility or resistance to scrapie (Baylis and Goldmann, 2004; Detwiler and Baylis, 2003). Sheep with the VRQ/VRQ, ARH/VRQ, and ARQ/VRQ alleles are most susceptible to scrapie whereas animals encoding ARR/ARR, ARR/ARH and ARQ/ARH alleles are most resistant (Tranulis, 2002). Selective breeding programs have been implemented in numerous countries to promote resistance genotypes in sheep flocks toward the eradication of scrapie (Detwiler, 1992; Detwiler and Baylis, 2003). A total of 16 polymorphic codons in *Prnp* have been reported in cervid species and coding variations in elk, white-tailed deer, and mule deer are associated with the rate of CWD infection or disease progression with no evidence for a completely resistant genotype (Robinson et al., 2012b). Genetics studies in free-ranging and captive white-tailed deer have consistently reported lower CWD infection rates in 96GS animals compared with 96GG (wild-type). Lower CWD infection has also been suggested for minor alleles 95H and 116G, however these alleles occur at very low frequencies, thus current trends are based on observational studies only and may not reflect true population trends (Robinson et al., 2012b). Epidemiological models for wild and captive animals indicate 96GG deer are infected at three-to-four times the rate of animals with at least one copy of 96S (Keane et al., 2008; Robinson et al., 2012a). Additionally, controlled experiments using high dosage oral inoculations in white-tailed deer have demonstrated that CWD-infected 96GS animals succumbed to disease with an average

incubation time of 956 ± 107 days as compared to 96GG animals with an average incubation period of 693 ± 27 days (Johnson et al., 2011).

Species barrier and generation of new prion strains

The primary source of prion strain diversity arises from interspecies infection (Bruce et al., 1994; Bruce et al., 1989; Foster et al., 1994; Hill and Collinge, 2004; Muramoto et al., 1992). Transmission of prion diseases between different mammalian species is typically far less efficient than within species because interspecies transmission is affected by the prion species barrier (reviewed in Hill and Collinge, 2004). The species barrier gives rise to infrequent transmission and prolonged incubation periods prior to neurodegenerative disease onset following initial prion inoculation. It is occasionally of sufficient magnitude to substantially block TSE transmission, or prevent many of the inoculated animals from developing disease during their natural lifetime (Hill et al., 2000). Often, the species barrier can be overcome by serial subpassage of a prion agent in a new host, during which incubation times shorten and attack rates increase; this is referred to as adaptation. Differences in amino acid sequences of PrP in donor and recipient animals are a strong constraint to the species barrier, however these differences alone do not fully account for the species barrier effect. For example, even though both CJD and vCJD prions have identical human primary structure, transmission of classical CJD prions to conventional mice is difficult or fails whereas transmission of vCJD occurs much more readily (Collinge et al., 1995; Hill et al., 1997). In contrast, transmission of classical CJD prions to transgenic humanized mice proceeds efficiently while vCJD transmits inefficiently. Thus, two strains propagated in the same host may have completely different transmission

barriers to another species, suggesting that 1) primary PrP sequence is not the sole determinant of the species barrier, and 2) a prion's strain characteristics (i.e. three-dimensional conformation, glycoform ratios) may be fundamental to its ability to infect a new species.

The "mad cow" disease epidemic of the 1980s and 1990s in the UK established BSE as undoubtedly the most notorious prion disease of our time. As the widely accepted cause of vCJD in humans, BSE established that the species barrier may not protect against cross-species prion transmission events (Bruce et al., 1997; Hill et al., 1997). BSE has not only been transmitted to humans. The extensive use of bovine-derived material for feeding other animals led to the generation of new TSEs in exotic felines and ungulates, non-human primates, and domestic cats, demonstrating this strain as highly promiscuous in cross species transmission (Bons et al., 1999; Collee, 1997a; Collee, 1997b; Kirkwood and Cunningham, 1994; Pearson et al., 1992; Taylor and Woodgate, 1997). Although the properties of the BSE strain remained remarkably consistent upon passage through various species, recent identification of atypical BSE strains, BSE-L and BSE-H (with BSE-H currently suspected as the possible progenitor to classical BSE), have brought into question the role for prion strains in cross species transmission (Tranulis et al., 2011). Even though mammalian PrP genes are highly conserved and the close similarity of PrP primary structures and three-dimensional folding is likely central to the ability of prions to cross-infect between species, a large number of PrP^{TSE} types or strains are seen in the spectrum of mammalian prion diseases (Collinge and Clarke, 2007). Under the conformational selection model of prion transmission, only a subset of the full range of prion strains are thermodynamically preferred (with respect to conformation) in a given host, and host PrP^{C} primary structure influences which strains are ultimately selected. In this model, the species

barrier, or transmission barrier, is determined by the degree of overlap between the subset of PrP^{TSE} strains allowed or preferred by PrP^{C} in the donor and host species (Collinge, 2010; Collinge and Clarke, 2007). In the example of BSE, this strain may represent a thermodynamically highly favored PrP^{TSE} conformation that is readily imprinted on PrP^{C} from a range of different species, accounting for the high promiscuity of this strain in mammals.

Unlike BSE, some prion strains do not "breed true" upon serial subpassage in a new host and a new, distinct strain is instead propagated. This phenomenon is referred to as strain mutation and is thought to be caused by one of two possible scenarios: 1) a strain can exist as a molecular clone and strain mutation involves generation of a distinct PrP^{TSE} type, or 2) strains consist of a mixture of PrP^{TSE} types, or "quasispecies", containing a dominant PrP^{TSE} type that is detected using standard techniques and preferentially propagated by its usual host and from which a less populous subspecies may be selected upon transmission to an alternative host (Collinge, 2010; Collinge and Clarke, 2007).

So far, BSE is the only non-human prion described to be transmissible to humans. Despite the fact that people have been caring for and consuming sheep potentially infected with scrapie for centuries, no correlation has been found between patients suffering from CJD and sheep husbandry or consumption. Scrapie transmissibility experiments in transgenic animal models expressing chimeric human/mouse PrP support this assumption (Gombojav et al., 2003). The zoonotic potential of CWD is perhaps least well-understood. Concern over the possibility for human infection by CWD is fueled by the increasing geographic distribution and prevalence of the disease among cervids across North America (Figure 1-1), as well as the presumption that a large number of hunters in the U.S. and Canada have been in contact with or consumed CWD-

infected meat (Belay et al., 2001). Thus far, no clinical or epidemiological evidence linking CWD exposed humans and CJD patients have been found (Belay et al., 2004). CWD transmission to transgenic humanized mice and to rhesus macaques failed to result in TSE, suggesting a strong species barrier exists to CWD in humans (Race et al., 2009). However, recent studies transmitting various isolates of CWD into rodents, including work presented in Chapter 1 of this thesis, strongly supports the existence of at least two, if not more, CWD strains circulating among naturally-infected cervids on the landscape (Angers et al., 2010; Raymond et al., 2007). At this time, the disease risks and outcomes, including zoonotic potential, associated with these different CWD strains have not been characterized.

Prion persistence and transmission in the environment

Transmissibility of prion diseases makes them fundamentally different from other neurodegenerative diseases. Host-to-host transmission was confirmed in 1936 by inoculating brain and spinal cord tissue from scrapie infected sheep into healthy sheep, which subsequently developed scrapie (Cuille and Chelle, 1936). Since then, experimental infections of various TSEs have determined that intracerebral injection is the most efficient route of inducing prion disease within a susceptible host; intraocular, intraspinal, intraperitoneal, and subcutaneous injections as well as scarification and peroral exposure are less efficient (Weissmann et al., 2002). In laboratory rodent models, TSE transmission via the oral route of exposure is much less efficient than intracerebral inoculation by a factor of $\sim 10^5$, yet non-experimental transmission of non-genetic TSEs appears to occur largely via the oral route (i.e. ingestion of infected materials) (Kimberlin et al., 1983). The kuru epidemic that developed in the first half of the twentieth

century in Papua New Guinea was propagated by ritualistic cannibalism (Gajdusek and Zigas, 1957). BSE transmits through consumption of bovine protein-contaminated feed (Morley et al., 2003). Human vCJD is thought to be a direct consequence of consumption of BSE-contaminated foodstuffs (Brown, 2001b). Other routes of TSE transmission are possible. Surgical procedures and blood transfusions have resulted in multiple iatrogenic cases of CJD in humans (Brown et al., 2000).

Unlike human prion diseases, which are largely sporadic or heritable in etiology, animal prion diseases mainly occur as infectious disorders. Among animal TSEs, scrapie and CWD are unique due to their facile host-to-host transmission under natural (free ranging wild populations) or near natural (extensive husbandry) conditions that can result in enzootic infections within susceptible populations (Gough and Maddison, 2010). In scrapie, maternal (vertical) transmission is thought to contribute to disease spread (Detwiler and Baylis, 2003), while CWD is mainly spread horizontally through direct contact or shared environments (Miller and Williams, 2003). Scrapie and CWD prions are shed from infected animals in both preclinical and clinical stages of disease in a wide range of tissues, including milk, saliva, urine, feces, blood, parturient materials, milk, and antler velvet (Angers et al., 2009; Gonzalez-Romero et al., 2008; Gregori et al., 2008b; Haley et al., 2009; Maddison et al., 2009; Maddison et al., 2010; Mathiason et al., 2006; Murayama et al., 2007; Safar et al., 2008; Tamguney et al., 2009). Horizontal transmission is likely mediated by direct contact with these tissues and fluids.

A number of studies have demonstrated the ability for both CWD and scrapie to be horizontally transmitted in situations where animal-to-animal contact is avoided, indicating the presence of environmental vectors or reservoirs. In support of this, studies carried out by Miller

and colleagues found that a CWD-infected carcass left in a pasture for two years was sufficient to transmit the disease to healthy deer (Miller et al., 2004). Additionally, exposure of naïve deer to pasture previously inhabited by infected deer also led to CWD transmission, as did cohabitation of naïve and infected deer (Miller et al., 2004). Healthy deer exposed to water, feed buckets, and bedding used by CWD-infected deer resulted in transmission of disease (Mathiason et al., 2009). Environmental scrapie transmission has also been recorded. In one study, the scrapie agent remained infectious after burial in garden soil for three years (Brown and Gajdusek, 1991). In another study, a scrapie-infected pasture and sheep house were decontaminated and left fallow; when healthy sheep were introduced onto the premises 16 years later, they contracted scrapie, suggesting the scrapie agent remained infectious in a farm setting after 16 years (Georgsson et al., 2006). These findings of environmental CWD and scrapie transmission are consistent with accounts of disease epidemiology and mathematical models for both scrapie and CWD spread (Almberg et al., 2011; Detwiler and Baylis, 2003; Matthews et al., 1999; Woolhouse et al., 1998). It has been hypothesized that soil serves as a plausible reservoir for CWD and scrapie infectivity in the environment as the shedding of prions from infected animals onto soil, the burial of farm animals or the natural deposition of wild animal carcasses may contribute to prion presence in the soil. Various studies have demonstrated the binding and persistence of PrP^{TSE} to soil and soil minerals (Cooke et al., 2007; Johnson et al., 2006; Leita et al., 2006; Ma et al., 2007; Saunders et al., 2009a; Saunders et al., 2009b). Importantly, PrP^{TSE} remains infectious in bioassays when bound to soil minerals, some of which actually significantly enhance oral infectivity as compared to non-bound PrP^{TSE} (Johnson et al., 2007). While the soil reservoir hypothesis is supported by existing evidence, no study to date has

detected prion protein or prion infectivity from naturally occurring soils in CWD or scrapie infected areas. Figure 1-6 outlines horizontal transmission of CWD in cervids and also highlights a role for possible environmental locations of concentrated prion infectivity, also known as "hotspots." These are hypothesized to occur at areas of communal activity where shedding of prions in saliva, urine, feces, or other fluids/tissues occurs. Animal mortality sites, where highly infectious CNS tissue would enter the environment, mineral licks, and scrapes are possible hotspots (Schramm et al., 2006). In a study of deer carcass decomposition in Wisconsin, carcasses persisted for 18 – 101 days depending on season and were visited by deer (Jennelle et al., 2009a). Additionally, cervid carcasses are visited by various scavenger species, such as raccoons, opossums, coyotes, vultures, and crows, which could consume and transport CWD-infected tissue and increase CWD spread (Jennelle et al., 2009b). Predators may also contribute to the spread of the CWD agent and transmission (Miller et al., 2008).

Voles as candidates for cross-species CWD and scrapie transmission

The disease transmission threat posed by CWD to other wildlife species remains unknown. CWD infectivity is shed in secretions, excreta, and the tissues of dead cervids, providing potential sources for environmental contamination (Angers et al., 2006; Angers et al., 2009; Haley et al., 2009; Mathiason et al., 2006; Miller et al., 2004; Safar et al., 2008; Tamguney et al., 2009). This, coupled with the extraordinary environmental stability of the etiological prion agent likely results in prion accumulation in disease endemic regions. Other wildlife species are inevitably exposed to infectious material in the environment. While CWD appears easily transmissible among cervids, natural transmission to other wildlife species has not yet been

documented. Experimental intracerebral challenge of raccoons (*Procyon lotor*) with CWD failed to result in TSE (Hamir et al., 2003), although both mink and ferrets developed disease post i.c. challenge (Harrington et al., 2008; Sigurdson et al., 2008). American crows (*Corvus brachyrhynchos*) force-fed prions were capable of passing infectivity in their feces, as demonstrated by rodent bioassay (VerCauteren et al., 2012). Field studies examining CWD prevalence among mountain lion killed mule deer suggested that these predators selectively prey on CWD infected animals (Miller et al., 2008), demonstrating yet another route of wildlife exposure to CWD.

Rodents sympatric with current CWD epizootics in North America are candidates for possible cross-species transmission of CWD in the wild due to their widespread distribution, relative abundance and opportunistic carcass scavenging behavior (Ebensperger et al., 2000; Heisey et al., 2010; Litvin et al., 1977; Riewe, 1973; Thomas, 1988; Zimmerman, 1965). Additionally, rodents serve as important food sources for higher trophic level predator and scavenger species, and small rodent tissue enters the domestic livestock and human food chain by accidental inclusion in grain and forage (Meerburg, 2006; Meerburg et al., 2004; Meerburg et al., 2009), providing additional possible routes of disease transmission to other wildlife, domestic animals, and humans. Finally, rodents are known vector, reservoir, and bridge species for various viral, bacterial, and parasitic diseases (Centers for Disease Control and Prevention, 2013). Previous work carried out in our laboratory by Heisey *et al*. demonstrated efficient i.c. transmission of white-tailed deer CWD to four species of native North American rodents: meadow voles (*Microtus pennsylvanicus*), red-backed voles (*Myodes gapperi*), white-footed mice (*Peromyscus leucopus*), and deer mice (*Peromyscus maniculatus*) (Heisey et al., 2010).

Meadow voles and red-backed voles proved to be the most susceptible to CWD, displaying high attack rates and reaching end-stage disease the soonest.

These data are consistent with a growing body of evidence identifying voles as wild species unusually susceptible to a wide variety of TSEs, including CWD and scrapie, CJD, and rodent-adapted TSEs *in vivo,* with evidence of little-to-no species barrier (Di Bari et al., 2008; Di Bari et al., 2013; Heisey et al., 2010; Kurt et al., 2011; Nonno et al., 2006). In fact, voles appear to be as susceptible to CWD and CJD as transgenic mice engineered to express the cervid or human PrP gene, respectively (Seelig et al., 2010; Telling et al., 1994). The reason for such high promiscuity exhibited by voles for various TSEs is not understood, but one suggestion is that the vole PrP sequence, despite sharing relatively little sequence homology with PrP^{TSE} of many mammalian TSEs, may be particularly prone to adopting a wide range of PrP conformations, thus allowing particularly efficient adaptation and/or replication of prions (Agrimi et al., 2008). The resolved NMR structure for bank vole PrP suggested that the unique susceptibility of this species to TSEs arises from a cervid-like β2-α2 structured loop in the C-terminal globular domain of the prion protein, resulting from an asparagine amino acid at position 170 (Christen et al., 2008). Residue 170 was further implicated as a determinant of PrP susceptibility to CWD: species that express asparagine at position 170, including prairie voles, were found to support amplification of PrP^{CWD} while those that express serine at position 170 generally did not amplify PrP^{CWD} in trans-species in vitro amplification assays (Kurt et al., 2009). However, red-backed voles, which are closely related to meadow voles and bank voles (*Myodes glareolus*), have a serine at position 170, consistent with species that do not support CWD amplification (Kurt et al., 2009) and are highly susceptible to CWD via intracerebral challenge (Heisey et al., 2010).

These observations, in addition to the relatively low sequence homology (~87%) shared between meadow vole and cervid PrP sequences, suggest that the sequence compatibility does not play the chief role in CWD permissiveness in voles.

Role for plants in environmental prion transmission

In considering natural CWD and scrapie transmission, it is notable that healthy animals can contract disease from indirect sources years after infected animals, carcasses, or excrement once contaminated an area, implying the existence of an environmental reservoir of infectivity. As discussed above, soil has been hypothesized to be a reservoir of scrapie and CWD infectivity (Schramm et al., 2006). With CWD, prions shed from deer saliva or excrement are likely to enter soil environments, decomposing infected animals leave agent directly on the soil surface and hunters field-dressing deer deposit potentially infectious viscera on the landscape. The persistence of prions in soil has been examined and data suggest that prions in soil environments are stable and viable. Brown and Gajdusek buried scrapie-infected brains contained in petri dishes in garden soil for up to three years. Following internment, the soil was assayed for scrapie infectivity; the authors found limited degradation (Brown and Gajdusek, 1991). Seidel *et al.* simulated the contamination of soil with scrapie agent using outdoor lysometers and measured prion protein and infectivity levels for time points up to 29 months (Seidel et al., 2007). In both studies, brain material was subject to microbial decomposition, but prion protein and scrapie infectivity persisted. Other work has revealed that prions bind to soil and soil minerals and soil-borne prions can remain infectious (Johnson et al., 2006). Furthermore, via the oral route of inoculation, soil-bound prions are more likely to cause disease than unbound prions (Johnson et

al., 2007). Deer consumption of soil at licks, scrapes and as a contaminant with food is a likely means whereby environmental CWD transmission occurs. The persistence of prions in soil environments requires that they withstand a variety of assaults, including freeze-thaw cycles, bacterial and fungal enzymes and transport or breakdown by plants.

Plants can absorb a variety of substances from soil including water, metals and inorganic and organic compounds. The uptake of proteins by plants was first noted in 1909 and studies performed in the 1960s and 70s indicated that proteins are absorbed by numerous species of plants and absorbed proteins exert biological effects on recipient plants (Drew et al., 1970; Jones, 1909; McLaren et al., 1960; Seear et al., 1968; Sung, 1974). Notably, some plants rely on organic nitrogen sources, which include proteins, for growth. Two publications by Nishizawa and Mori clearly demonstrate that hemoglobin can serve as a sole nitrogen source for rice and barley growth and provide evidence indicating protein uptake into roots by endocytic pathways (Nishizawa and Mori, 1977; Nishizawa and Mori, 1980a). Further work has clarified the use of organic nitrogen sources in plants; numerous species, including pearl millet, sorghum, birch trees, barley and rice are able to utilize exogenous proteins for nitrogen (Okamoto and Okada, 2004; Yamagata and Ae, 1999). Metabolism of organic nitrogen sources by root-associated microbes is not requisite to utilization of organic nitrogen as Chapin *et al* found that a non-mycorrhizal sedge, cottongrass, preferentially grows using amino acids as a sole source of nitrogen (Chapin, 1993). More recent work by Paungfoo-Lonhienne *et al*. demonstrated that both woody healthland plant *Hakea actites* and the herbaceous model plant *Arabidopsis thaliana,* non-mycorrhizal species, can use BSA as a nitrogen source for growth without assistance from other organisms (Paungfoo-Lonhienne et al., 2008). Additionally, the authors found evidence for

uptake of whole BSA or GFP in root cells of both plant species and hypothesized an endocytosis-mediated mechanism. Root tissues of crop plants exposed to biotinylated proteins present in soil amended with organic nitrogen sources stain positive when histologic sections are exposed to labeled avidin (Koga, 2006), and evidence has been published demonstrating presence of the insecticidal Cry1Ab protein in aerial tissues of crops grown on fields previously used to grow BT corn (Icoz et al., 2009). Taken together, these reports support the concept that plants absorb proteins and suggest that prions may be absorbed by plants, affecting the fate of CWD agent in the environment.

Implications of thesis research to prion biology

Horizontal transmission of the agent causing CWD and scrapie is a major mechanism of natural transmission, and undoubtedly contributes to the abilities of these animal TSEs to establish endemic infections within susceptible populations and cause widespread epizootics of disease. Scrapie and CWD are unique among prion diseases in that they may be transmitted indirectly through contaminated environments. While it is well-established that both CWD and scrapie prions are extraordinarily stable in environmental settings, maintaining infectivity for years to decades (Brown and Gajdusek, 1991; Georgsson et al., 2006; Miller et al., 2004; Seidel et al., 2007), and available evidence indicates that an environmental reservoir of infectivity, possibly soil, contributes to the maintenance of these diseases in affected populations (Schramm et al., 2006), routes of natural transmission remain to be clarified. The overriding goal of my thesis research is to contribute to the knowledge deficit regarding environmental CWD and scrapie transmission by identifying new potential routes of disease dissemination. In the following chapters, I present evidence supporting the identification of two novel routes of environmentally relevant prion transmission: 1. cross-species transmission of CWD and scrapie to a native North American rodent species, the meadow vole (*Microtus pennsylvanicus*), and 2. the uptake and transmission of prions and prion infectivity by plants.

While interspecies transmission of CWD to non-cervids has not been observed under natural conditions, CWD has been experimentally transmitted, via intracerebral inoculation, to cattle (Greenlee et al., 2012; Hamir et al., 2001; Hamir et al., 2011; Hamir et al., 2005), sheep (Hamir et al., 2006a), goats (Williams and Young, 1992), cats (Mathiason et al., 2013), mink (Harrington et al., 2008), ferrets (Sigurdson et al., 2008), voles (Heisey et al., 2010), mice

(Raymond et al., 2007), and squirrel monkeys (Race et al., 2009), raising concern over the cross-species potential of this emerging TSE. In Chapter 1, we identify meadow voles, a rodent species sympatric with current CWD epizootics across North America, as peripherally susceptible to CWD, highlighting the possibility that these animals may serve as vector, reservoir, or bridge species for the disease in the environment. To our knowledge, this is the first demonstration of successful heterologous peripheral transmission of CWD to a non-cervid wild species. Unexpectedly, CWD challenges in meadow voles resulted in an additional finding: evidence supporting the existence of multiple CWD strains, with possible roles for cervid *Prnp* genotype and route of inoculation in CWD strain selection. This finding is congruent with previous studies suggesting more than one CWD strain circulates among free-ranging cervids (Angers et al., 2010; Raymond et al., 2007) and extends previous research by identifying unique biochemical agent-specific characteristics, in addition to TSE-induced host responses, by which unique CWD strains may be differentiated. Our findings also highlight meadow voles as useful strain typing tools and small animal models for future CWD research. In Chapter 2 we tested the ability of meadow voles to support sheep scrapie infection via intracerebral inoculation as an initial investigation into the potential for voles to facilitate environmental scrapie transmission. We found that meadow voles were not as susceptible to sheep scrapie as they were to CWD, indicating that they are unlikely to be involved in environmental scrapie transmission.

In Chapter 3, we investigated plant uptake and transmission of infectious prions. In CWD infected animals, lymphoid organs and salivary glands contain high levels of infectivity (Sigurdson and Aguzzi, 2007) and the infectious agent is excreted in saliva, feces, and urine (Haley et al., 2009; Mathiason et al., 2006; Safar et al., 2008; Tamguney et al., 2009). Once

deposited in the environment, TSE infectivity may accumulate in soil and other components of the natural environment. Considering a number of recent studies implicating soil as a natural reservoir for environmental TSEs (Cooke et al., 2007; Johnson et al., 2007; Johnson et al., 2006; Leita et al., 2006; Ma et al., 2007), we hypothesized that plants are able to take up prions and transmit infectivity to mammalian hosts. We tested this by growing the model plant *Arabidopsis thaliana* and a variety of crop species in various agar and hydroponic media containing infectious prions and subsequently injecting or feeding leaf and stem tissues to mice to determine infectivity. We found that plants are able to take up and maintain prion infectivity sufficient to cause TSE by both intracerebral and oral transmission routes.

Overall, this work has implications for environmental prion transmission not only to other wildlife, but also potentially to domestic animals and even humans. Voles are important food sources for many predator and scavenger species on the landscape, providing a possible route for cross-species TSE exposure. Also, accidental inclusion in grain harvest and infestation of grain stocks by rodents provides possible routes by which rodent-borne TSEs may enter the domestic and human food stocks. Additionally, prion contaminated plants may represent a previously unrecognized risk of TSE exposure via plant-derived food sources. New foci of CWD continue to be identified across North America, and while most seem to be related to increased surveillance and spread of the disease as a result of natural migration of deer and elk or translocation of infected cervids by humans, there do exist unexplained cases, like the 2012 detection of a single CWD-positive white-tailed deer in northwestern Wisconsin, ~250 miles from the known endemic region in the southern part of the state (Wisconsin Department of Natural Resources, 2013a). Whether novel transmission routes like those presented in this thesis

may be responsible for CWD transmission on the landscape remains to be determined. Future research will focus on determining the susceptibility of North American vole species to additional TSEs, characterizing the novel CWD strains we isolated, including determination of disease outcomes and risks (especially zoonotic potential), and determining the mechanisms underlying prion uptake and translocation by plants. Conclusions and future directions of this work are fully discussed in Chapter 5.

Figures, Tables, and Legends

Figure 1-1. Distribution of CWD in North America

Since its initial identification in captive cervids in the 1960s and free-ranging cervids in the 1980s, both in the Colorado/Wyoming area, CWD has spread to 22 U.S. states, 2 Canadian provinces, and South Korea (not shown on map), as of May 2013. Map is from (USGS National Wildlife Health Center, 2013).

Figure 1-1

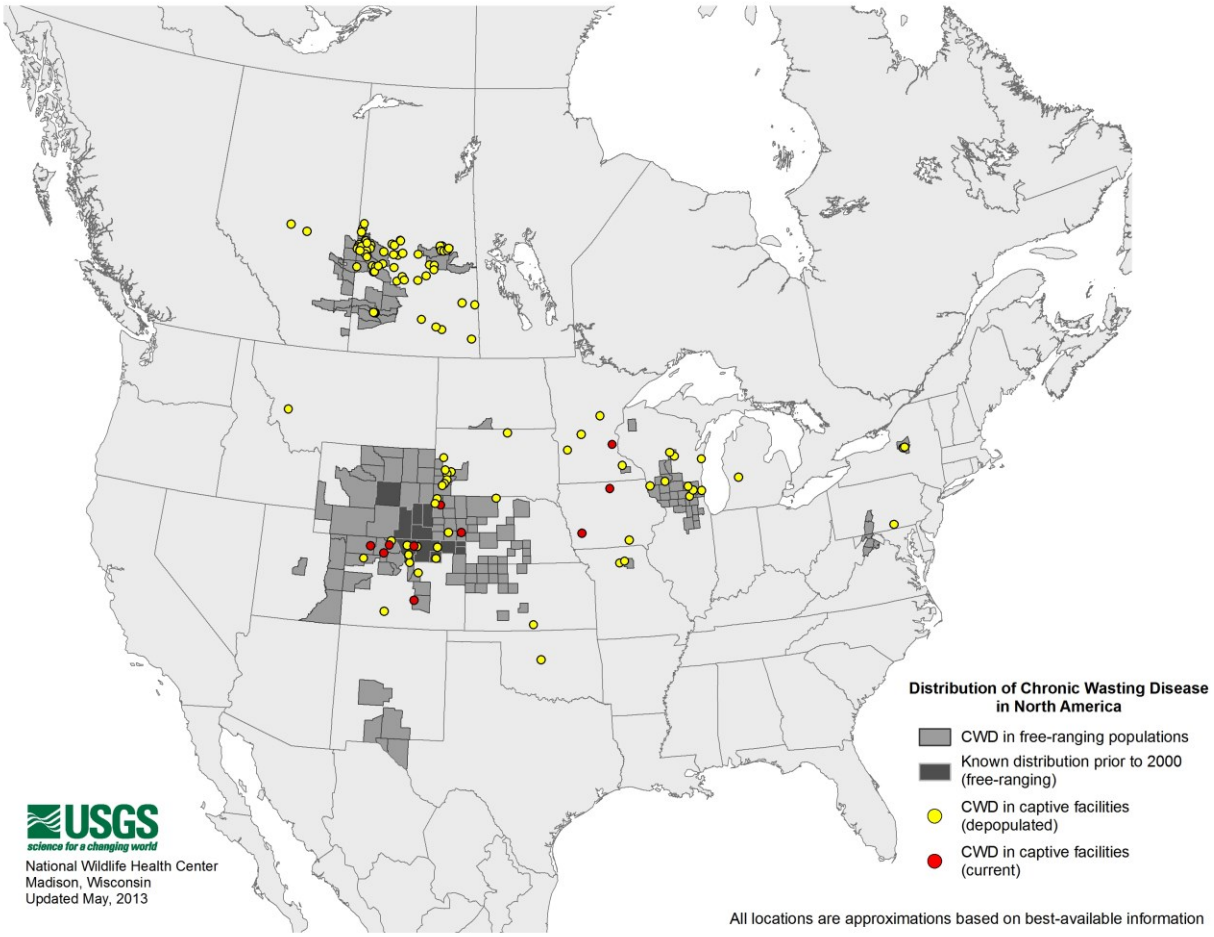

Distribution of Chronic Wasting Disease in North America
CWD in free-ranging populations
Known distribution prior to 2000 (free-ranging)
CWD in captive facilities (depopulated)
CWD in captive facilities (current)
USGS
science for a changing world
National Wildlife Health Center
Madison, Wisconsin
Updated May, 2013
All locations are approximations based on best-available information

Figure 1-2. Prion protein immunostaining of infected and non-infected brain tissue.

Healthy, non-infected meadow vole brain tissue (A) is compared with TSE-infected meadow vole brain, which display both spongiosis (B) and accumulation of PrP^{TSE} (red staining in both B and C) in the cerebral cortex. Slides developed with anti-PrP monoclonal antibody SAF 83 and counterstained with hematoxylin. Scale bar indicates 10 μm.

Figure 1-2

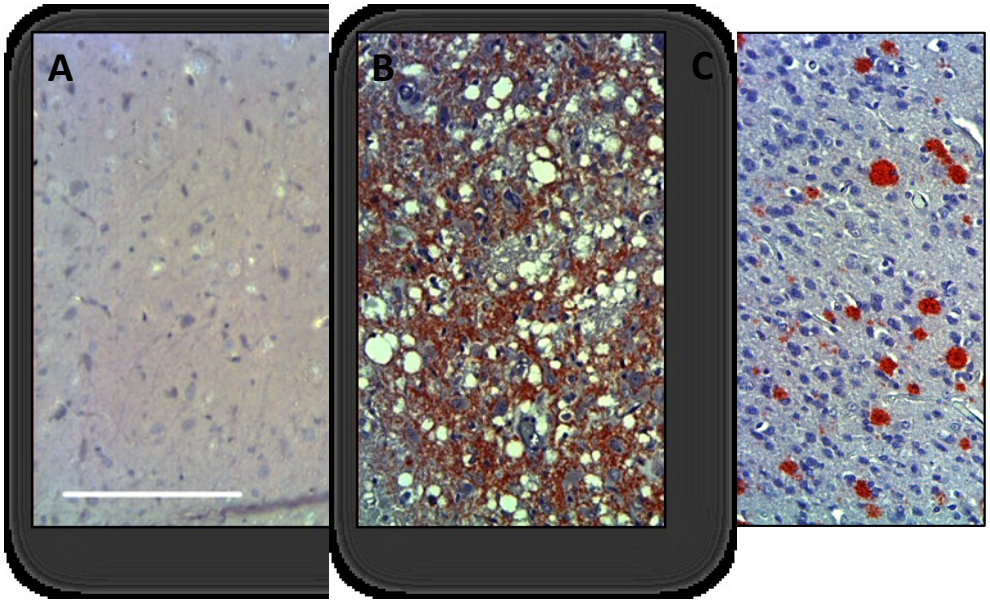
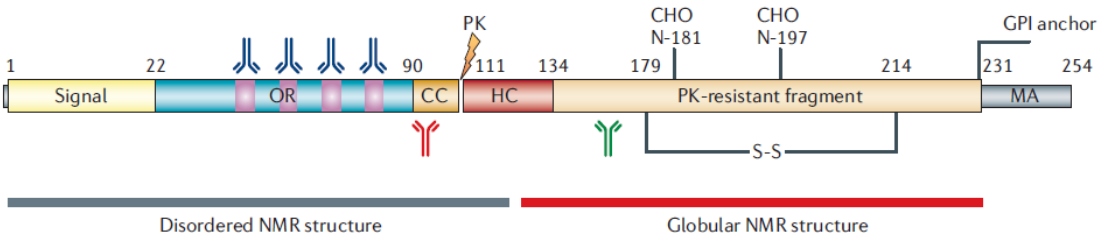


Figure 1-3. Structural features of the cellular prion protein (PrP^C).

(A) An outline of the primary structure of PrP^C, including post-translational modifications. A secretory signal peptide resides at the extreme N-terminus. The numbers describe the position of respective amino acids. CC (orange) defines the charged cluster. HC (red) defines the "hydrophobic core." S-S indicates the single disulfide bridge. OR, octarepeat region. The PK resistant core of PrP^{TSE} is depicted in gold and the approximate cutting site of PK within PrP^{TSE} is indicated by the lightning bolt symbol. MA denotes the membrane anchor region. Several antibody epitopes are also indicated. (B) Tertiary structure of PrP^C inserted into a lipid bilayer, as deduced from NMR spectroscopy, including the "unstructured" N-terminal tail (grey) and the glycosylphosphatidyl inositol (GPI) anchor. The α2-β2 loop is indicated by the black arrow. Image adapted from (Aguzzi and Heikenwalder, 2006).

Figure 1-3

A


PK
CHO
N-181
CHO
N-197
GPI anchor
1
22
90
111
134
179
214
231
254
Signal
OR
CC
HC
PK-resistant fragment
MA
S-S
Disordered NMR structure
Globular NMR structure

B

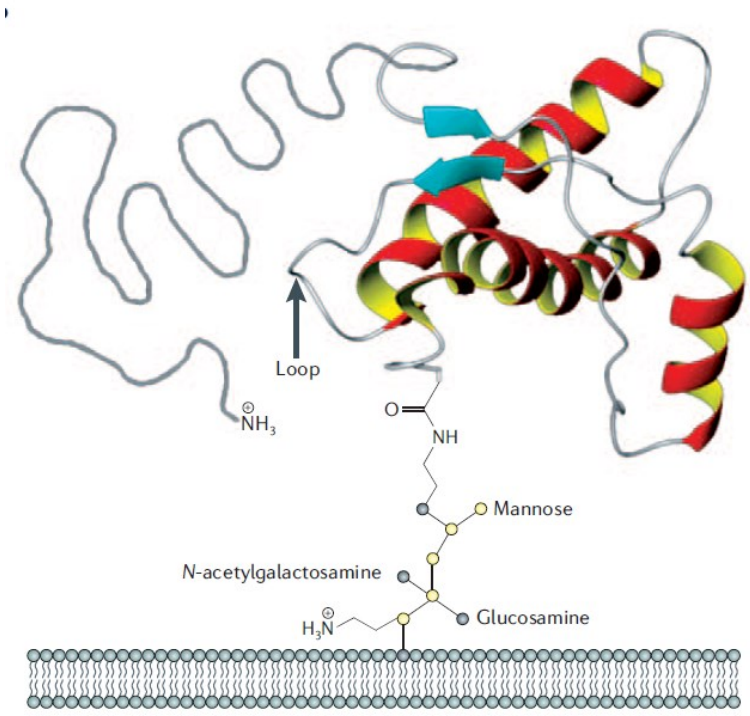
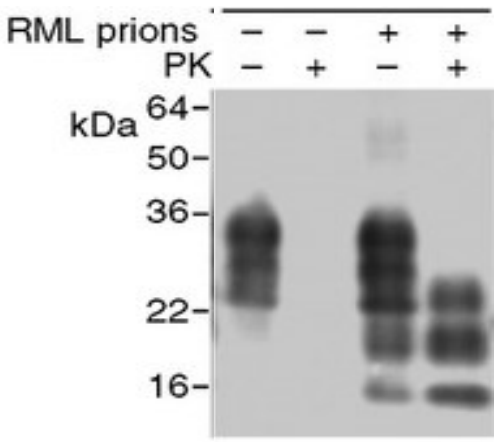

Loop
NH3
O
NH
Mannose
N-acetylgalactosamine
H3N
Glucosamine

Figure 1-4. Prion protein isoforms.

(A) Immunoblot of 10% w/v brain homogenates in PBS from non-infected (lanes 1 and 2) and prion-infected (lanes 3 and 4) mouse brain homogenates. Samples in lanes 2 and 4 were digested with 50 μg/mL PK for 30 min at 37ºC, completely hydrolyzing PrP^{C}. Proteinase digestion cleaves ~67 amino acids from the amino terminus of PrP^{TSE} to generate PrP^{res}, or PrP 27 – 30 (lane 4). Blot developed with anti-PrP monoclonal antibody $ICSM_{35}$. Image adapted from (Goold et al., 2011). (B) Bar diagrams of the *Prnp* gene and PrP isoforms. The *Prnp* ORF encodes a protein of 254 residues, which is shortened to 209 residues during posttranslational processing. PrP^{TSE} is an alternate conformation of PrP^{C} with identical primary structure. Limited proteolysis of PrP^{TSE} cleaves the amino terminus and produces PrP^{res}, or PrP (27 – 30), composed of approximately 142 residues. Figure adapted from (Colby and Prusiner, 2011).

Figure 1-4

A


RML prions – – + +
PK – + – +
kDa
64–
50–
36–
22–
16–

B

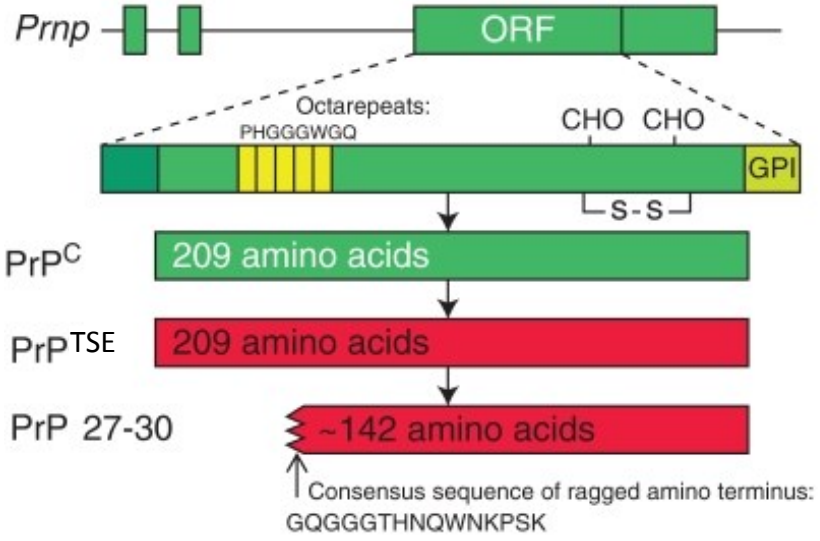
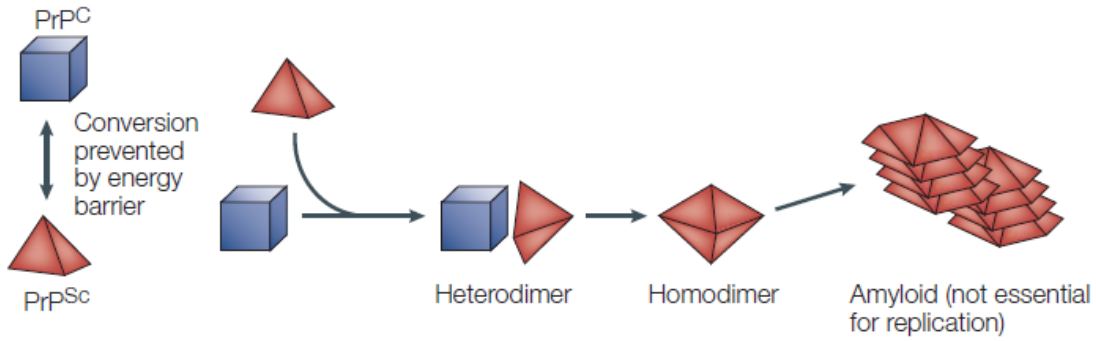

Prnp
ORF
Octarepeats:
PHGGGWGQ
CHO CHO
GPI
S-S
PrP^C
209 amino acids
PrP^TSE
209 amino acids
PrP 27-30
~142 amino acids
Consensus sequence of ragged amino terminus:
GQGGGTHNQWNKPSK

Figure 1-5. Models for conversion of PrP^{C} to PrP^{TSE}.

(A) Template-assisted conversion or refolding model proposed by (Prusiner, 1991). (B) Nucleation-dependent or "seeding" model proposed by (Jarrett and Lansbury, 1993). The seeding model is more consistent with the aggregated nature of PrP^{TSE} and is supported by studies using *in vitro* conversion assays. Figure adapted from (Aguzzi and Heikenwalder, 2006).

Figure 1-5

A


PrPC
Conversion prevented by energy barrier
PrPSc
Heterodimer
Homodimer
Amyloid (not essential for replication)

B

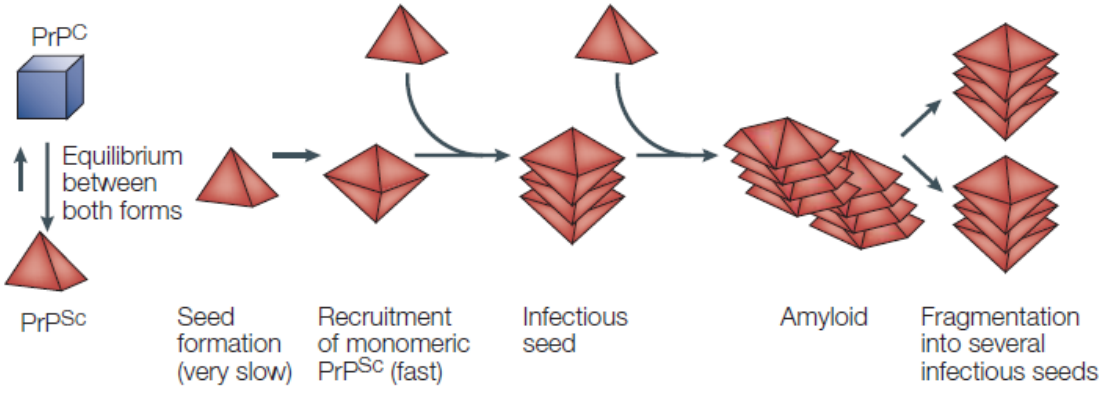

PrPC
Equilibrium between both forms
PrPSc
Seed formation (very slow)
Recruitment of monomeric PrPSc (fast)
Infectious seed
Amyloid
Fragmentation into several infectious seeds

Figure 1-6. Potential routes of horizontal transmission of cervid chronic wasting disease. Environmental CWD transmission is thought to occur either directly, via animal-to-animal contact through a bodily fluid like saliva, or indirectly via contaminated environmental fomites or areas. "Hotspots" are hypothesized areas of communal activity where shedding of prions in saliva, urine, feces, or other fluids/tissues occurs. Animal mortality sites, where highly infectious CNS tissue would enter the environment, mineral licks, and scrapes are possible hotspots. Diagram adapted from (Schramm et al., 2006).

Figure 1-6

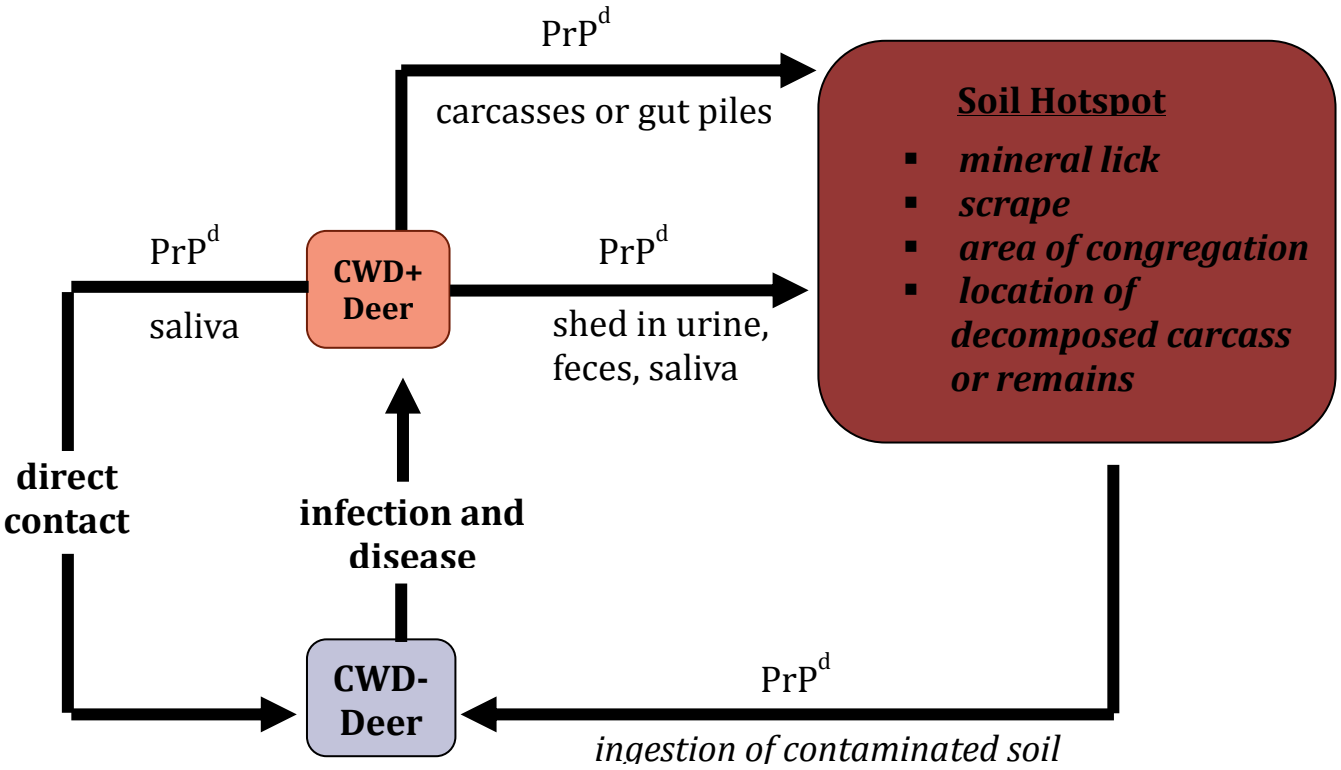

PrP^d
carcasses or gut piles
Soil Hotspot
mineral lick
scrape
area of congregation
location of decomposed carcass or remains
PrP^d
saliva
CWD+ Deer
PrP^d
shed in urine, feces, saliva
direct contact
infection and disease
CWD- Deer
PrP^d
ingestion of contaminated soil

Table 1-1. Spectrum of prion diseases

Disease	Nat'l host	Region(s)	Total (+) cases	Incidence rate*	Ref.
Scrapie	Sheep, goats	Worldwide	–	1,000 – 45,000 (3-5% of affected flock)	Imran *et al.* 2011
Bovine spongiform encephalopathy (BSE)	Cattle	United Kingdom	184,618	1.5	OIE
		Europe (-UK)	5,946	0.0 – 5.8	OIE
		Japan	36	0.0	OIE
		Canada	20	0.2	OIE
		United States	2	0.0	OIE
Chronic wasting disease (CWD)	Cervids	North America	~6,000	–	Saunders *et al.* 2012
		South Korea	10	–	Kim *et al.* 2005
Transmissible mink encephalopathy (TME)	Mink	United States (WI, MN, ID)	5[#]	–	Tatzeldt (ed.) 2011
		Canada, Finland, E. Germany, USSR	<12[#]	–	Liberski *et al.* 2009
Creutzfeldt Jakob disease (CJD)	Humans	Worldwide	– (sCJD) 222 (vCJD) – (fCJD) 405 (iCJD)	0.5 – 1.5 (sCJD) – 5–15% of all CJD cases (fCJD)	NCJDRSU Brown *et al.* 2006
Fatal familial insomnia	Humans	Worldwide	~100	–	Imran *et al.* 2011
Gerstmann Straussler-Scheinker syndrome(GSS)	Humans	Worldwide	–	0.001	Imran *et al.* 2011

*Cases per 1,000,000 individuals/year #Total number of outbreaks

CHAPTER TWO:

Transmission of Chronic Wasting Disease to Meadow Voles (*Microtus pennsylvanicus*) Results in Multiple Strains

In preparation to be submitted as:

Carlson, C.M., J.R. Schneider, J.K. Wiepz, C.L. Meyerett-Reid, M.D. Zabel, J.A. Pedersen, D.M. Heisey, and C.J. Johnson.

Transmission of Chronic Wasting Disease to Meadow Voles (*Microtus pennsylvanicus*) Results in Multiple Strains

To the journal:

Proceedings of the National Academy of Sciences U S A

Abstract

The disease transmission threat posed by chronic wasting disease (CWD) to other wildlife species remains unknown. CWD infectivity is shed in secretions, excreta, and the tissues of dead cervids, providing potential sources for environmental contamination. Additionally, studies have shown the etiological prion agent is able to persist in an infectious state on the landscape for years, likely resulting in environmental prion accumulation in disease endemic regions. Other wildlife species are inevitably exposed to infectious material in the environment. In this study, we investigated the transmission and adaptation of white-tailed deer CWD in the meadow vole (*Microtus pennsylvanicus*), a native North American rodent sympatric with current CWD epizootics. Expanding upon our previous research that established meadow voles as highly susceptible to CWD via intracerebral inoculation, we demonstrate here that they are also susceptible via peripheral challenge, albeit with lower attack rates and longer incubation periods. Interestingly, oral challenge of meadow voles with CWD resulted in subclinical infection in primary passage animals, but manifested as clinical prion disease upon intracerebral subpassage. Additionally, serial subpassage in meadow voles of CWD from white-tailed deer homozygous for glycine at position 96 (96GG) of the prion protein resulted in the selection of a single prion strain that was characterized by homogeneity in resulting incubation periods, abnormal prion protein (PrP^d) glycoform ratios, lesion profiles and PrP^d deposition patterns. In contrast, serial subpassage of CWD from heterozygous *PRNP* $Q^{95}G^{96}Q^{226}$/ $Q^{95}S^{96}Q^{226}$ genotype deer resulted in the initial manifestation of four unique disease phenotypes upon first passage that ultimately resulted in selection of a single strain by third passage that was distinct from the homozygous *PRNP* $Q^{95}G^{96}Q^{226}$ CWD-derived strain. These data establish that meadow voles are permissive

to CWD via exposure routes consistent with their exposure to the agent on the landscape, suggesting this species may serve as an environmental reservoir for CWD in disease-endemic regions. Additionally, these data are consistent with the hypothesis that at least two strains of CWD circulate in naturally-infected cervid populations in North America and provide evidence that meadow voles are a useful tool for CWD strain typing.

Introduction

Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy (TSE; prion disease) of cervids (i.e., deer, elk, moose), and the only TSE known to affect free-ranging wildlife species (Baeten et al., 2007; Williams, 2005). First discovered in Colorado in the 1960s (Williams and Young, 1980), CWD has since been identified in free-ranging and captive cervids in 22 U.S. states, 2 Canadian provinces, and South Korea (Kim et al., 2005; Sohn et al., 2002; USGS National Wildlife Health Center, 2013). Along with the expansion of its known geographical distribution, CWD's prevalence in North America is increasing in areas currently experiencing epizootics, reported as upwards of 80% in some captive herds (Keane et al., 2008; Miller and Williams, 2003; Williams and Young, 1980) and >50% in the free-ranging South Converse mule deer herd in Wyoming (Wyoming Game and Fish Department, 2011) .

Like other TSEs, CWD is thought to be caused by an abnormal isoform of the host prion protein (PrP). During infection, the normal cellular form of PrP (PrP^{C}) is misfolded into an aggregated, disease-associated conformer (PrP^{d}) that is partially resistant to degradation by proteinase K (PK) and many other treatments that inactivate conventional pathogens (Taylor, 2000). PK treatment of PrP^{d} results in cleavage of the N-terminal ~90 amino acids from the protein, leaving the protease resistant core, PrP^{res}, which is a common diagnostic indicator of TSE infection and presents as a classic tri-banded pattern by immunoblot analysis, representing PrP's three main glycosylation states: unglycosylated (~30 kDa), monoglycosylated (~25 kDa), and diglycosylated forms (~19 – 21 kDa) (Kubler et al., 2003). Accumulation of PrP^{d} in the brain and other central nervous system tissues is a hallmark of prion disease and generally correlates with prion infectivity in diseased brain tissues (Bolton et al., 1982; Prusiner, 1998).

While the mechanisms of CWD transmission are poorly understood, horizontal transmission is known to occur between infected and susceptible hosts either directly via animal-to-animal contact (Mathiason et al., 2006; Miller and Williams, 2003) or indirectly via environmental CWD contamination (Mathiason et al., 2009; Miller et al., 2004). Secretions, excreta, and the carcasses of infected animals potentially release CWD infectivity into the environment (Schramm et al., 2006) and, following release, the agent remains infectious to cervids for years (Miller et al., 2004). The increasing prevalence and geographic distribution of CWD, coupled with environmental CWD transmission, raise concerns about cross-species transmission of the disease. Other wildlife and humans are inevitably exposed to infectious material by consumption and handling of infected animals or through the environment, but the consequences of these exposures are currently unknown.

While interspecies transmission of CWD to non-cervids has not been observed under natural conditions, CWD has been experimentally transmitted, via intracerebral inoculation, to cattle (Greenlee et al., 2012; Hamir et al., 2001; Hamir et al., 2011; Hamir et al., 2005; Hamir et al., 2006b; Hamir et al., 2007), sheep (Hamir et al., 2006a), goats (Williams et al., 2002), cats (Mathiason et al., 2013), mink (Harrington et al., 2008), ferrets (Bartz et al., 1998), voles (Di Bari et al., 2013; Heisey et al., 2010; Kurt et al., 2011), mice (Raymond et al., 2007) and squirrel monkeys (Marsh et al., 2005). However, no experimental transmission study to date has demonstrated successful heterologous oral transmission of CWD, indicating the presence of a strong species barrier. Interspecies transmission of prions is often inefficient, characterized by low attack rates and prolonged incubation periods preceding clinical TSE, as compared with those of intraspecies transmission; these features are thought to comprise the prion species

barrier, which can be overcome by serial subpassage of the agent, reflecting prion adaptation to its new host (Aguzzi et al., 2007). Prion species barriers are thought to be most strongly dictated by differences in PrP primary sequence between donor and recipient hosts (minimal amino acid divergences can greatly impact prion transmission efficiency), but can also be influenced by transmission route and prion strain type (Aguzzi et al., 2007).

The prion strain concept is unique in biology and was developed upon the observation that genetically identical animals infected with the same prion isolate could develop very different pathologies, the clinical and biochemical outcomes of which can be maintained through multiple subpassages (Bruce, 2003). Prion strains can be classified by differences in these clinical and biochemical parameters, which include incubation periods, clinical disease presentation, distribution and intensity of spongiosis and PrP^d in brain tissue, resistance of PrP^d to denaturation by proteases and chaotropes, ratio and electrophoretic migration of glycosylated PrP^d species, and PrP^d structure. As "protein-only" agents devoid of nucleic acid, changes in which underlie the classical definition of strains that we apply to conventional pathogens, prion strain identity is thought to be enciphered in the three-dimensional conformation of PrP^d (Collinge and Clarke, 2007).

We previously demonstrated meadow voles, a native North American rodent species sympatric with current CWD epizootics, as highly susceptible hosts for white-tailed deer CWD via intracerebral (i.c.) transmission (Heisey et al., 2010). In this study, we investigated the cross-species transmission potential of white-tailed deer CWD into meadow voles by intraperitoneal (i.p.) and oral transmission routes. In addition, we examined CWD adaptation and strain selection in this species by serial subpassage of the agent and analysis of the resulting survival

time, neuropathology, and biochemical features of PrP^d. We found meadow voles to be susceptible to CWD via peripheral routes of infection and evidence for the selection of at least two CWD-derived strains in this species.

Results

Primary intracerebral transmission of white-tailed deer CWD to meadow voles results in four distinct PrP^{res} glycoform variants.

In previous work, we i.c. challenged four native North American rodent species with white-tailed deer CWD agent and found meadow voles to be the most susceptible, as evidenced by the shortest survival time and complete attack rate (Heisey et al., 2010). As a follow-up to our initial findings, here we performed i.c. inoculations of meadow voles with white-tailed deer CWD isolates representing two prion protein genotypes: the wild type homozygous PrP $Q^{95}G^{96}Q^{226}$ genotype (96GG) and the heterozygous PrP $Q^{95}G^{96}Q^{226}$/ $Q^{95}S^{96}Q^{226}$ genotype (96GS). Upon primary passage, all challenged voles displayed high attack rates, with 96GG challenged animals living ~30 days longer (284 ± 22 days post infection, dpi; median survival time ± 95% confidence interval) than 96 GS challenged animals (256 ± 19 dpi) (Table 2-1). All CWD isolates induced similar clinical outcomes in voles, with disease onset characterized by poor balance and thinning of the waist that progressed to hyperactivity and repetitive circling behavior in some animals; in later stages of disease, animals discontinued normal burrowing behavior and displayed lethargy and severe ataxia of hind legs that caused them to repeatedly fall into a supine position, which often required great difficulty to overcome.

Presence of PrP^{res} was confirmed in brain tissue of all clinically positive voles by immunoblotting, which revealed the presence of four unique phenotypes in 96 GS challenged voles, defined by differences in the electrophoretic migration of the PK-cleaved nonglycosylated band (Fig. 2-1A). “Type 1” vole-passaged CWD was characterized by a slow migrating

nonglycosylated band that resolved at 18 kDa (displayed by 2 of 13 total 96GS CWD challenged voles), consistent with that of the original white-tailed deer inocula (Fig. 2-1B). "Type 2" vole-passaged CWD displayed an intermediate-migrating band that resolved at 17 kDa (3/13 voles), "type 3" displayed a fast-migrating band at 16 kDa (6/13 voles), and "type 4" displayed a double-banded phenotype, with the upper and lower bands resolving at 18 and 16 kDa, respectively (1/13 voles; for another 1/13 voles, no PrP^{res} was ever detected). In contrast, all 17 voles challenged with 96GG CWD agent consistently displayed only the type 3 variant. All four vole-passaged CWD types maintained the ordering of PrP^{res} glycoform proportions as diglycosylated > monoglycosylated > nonglycosylated, consistent with that of the original deer inocula (Fig. 2-1). The deer isolates used for vole inocula displayed no conspicuous differences within or between 96GG and 96GS groups with regard to glycoform proportions and electrophoretic migration of the PK-cleaved nonglycosylated band, which resolved at 18 kDa, consistent with that of the type 1 variant (Fig. 2-1B). Voles challenged with CWD-negative deer inocula remained healthy until euthanasia at 523 – 746 dpi, at which time they were also confirmed negative for PrP^{d} by immunohistochemistry (IHC) and/or immunoblot (data not shown). These data demonstrate that cross-species i.c. transmission of white-tailed deer CWD to meadow voles results in the emergence of at least four biochemically distinguishable PrP^{res} phenotypes that reflect the co-existence of multiple PrP^{d} conformers and may suggest the existence of CWD strains.

Types 1 – 4 vole-passaged CWD correspond to four unique neuropathological profiles in infected meadow voles

To further characterize vole-passaged CWD types 1 – 4 according to classic prion strain typing methods, we performed brain lesion profiling on i.c. challenged meadow voles. Resulting patterns of spongiform degeneration were found to be distinct among infected voles representing each of the four biochemically defined vole-passaged CWD types described above (Fig. 2-2A). Type 1 animals displayed overall heavy spongiosis throughout all brain regions examined, with the greatest involvement observed in the medulla, cerebellum, thalamus and cerebral cortex; type 2 animals displayed less intense spongiosis in hindbrain regions and in the hippocampus as compared with type 1 animals, but maintained heavy involvement in the thalamus and forebrain regions; type 3 animals displayed a similar pattern of spongiosis as type 1 animals in hindbrain regions, but more closely resembled type 2 animals in forebrain regions; type 4 animals showed very little spongiosis across all brain regions examined, with the exception of heavier involvement in the septal nuclei of the paraterminal body.

Immunohistochemical analysis for PrP^d in infected meadow vole brains also revealed four unique deposition patterns that corresponded to types 1 – 4 vole-passaged CWD (Fig. 2-2B). The dorsal medulla, cerebellum, thalamus, and cerebral cortex were the most consistently involved areas, and also displayed the greatest variation between types. In type 1 infected animals there was a lack of PrP^d staining in both the medulla and the cerebral cortex, which contrasted with intense granular staining throughout the thalamus and inconsistent granular staining of varying intensity restricted to specific areas of the granular layer and white matter of the cortex of the cerebellum (Fig 2-2B, upper left panel). In contrast to all the other types, type 2-

infected vole brains were characterized by a conspicuous lack of PrP^d staining in nearly all brain regions, except for a very light, diffuse staining of the thalamus (Fig. 2-2B, upper right panel). Type 3 infected animals displayed a unique, heavy perineuronal PrP^d staining in the medulla that was not observed in any other tissue type in type 3 infected voles, or in voles infected with any of the other three vole-passaged CWD types (Fig. 2-2B, lower left panel). Additionally, type 3 infected animals displayed inconsistently distributed aggregates of PrP^d restricted to the granular layer and cortex of the cerebellum and plaque-like deposits in both the thalamus and cerebellar cortex. Finally, type 4 infected voles displayed a heavy, granular staining across the entire dorsal medulla, tiny, focal deposits in the thalamus, and a lack of staining in both the cerebellum and cerebral cortex (Fig. 2-2B, lower right panel). Together, these data demonstrate that the biochemically distinct types 1 – 4 vole-passaged CWD agents are each capable of causing unique neuropathological disease outcomes in the infected meadow vole host.

Serial subpassage of white-tailed deer CWD in meadow voles results in strain selection

To determine whether transmission of white-tailed deer CWD into meadow voles resulted in prion strain selection, we performed serial subpassages of both 96GG and 96GS CWD agent. For 96GG challenged voles, attack rates remained 100% throughout six total subpassages and survival times appeared to stabilize by third passage around 124 ± 9 dpi (median survival time ± 95% confidence interval; Fig. 2-3A and Table 2-1). Only the type 3 phenotype was observed throughout all subpassages (Fig. 2-3A). For 96GS challenged voles, attack rates remained at nearly 100% for all six subpassages; passage one and passage four each

resulted in one nonclinical vole for which PrP^{res} in brain tissue could not be confirmed and we conjecture these two animals represent failed inoculations that did not achieve intracranial penetration (Fig 2-3A and Table 2-1). As mentioned previously, primary passage of 96GS white-tailed deer CWD into meadow voles resulted in the selection of types 1 – 4 vole-passaged CWD with survival times ranging between 209 – 309 dpi (Fig. 2-3A). Brain tissue from one primary passage vole representing each of the four types was used as inoculum for i.c. challenges of second passage voles. Interestingly, all second passage voles that were challenged with type 1 (six total voles) and one of two voles challenged with type 4 inoculum manifested a type 3 phenotype (62 ± 10 dpi) upon developing clinical TSE; the second vole challenged with type 4 inoculum maintained the double-banded type 4 phenotype (118 dpi). Second passage voles that received type 2 inoculum developed a type 1 (three voles; 111 ± 7 dpi) or type 4 phenotype (1 vole; 111 dpi); two voles challenged with type 2 inoculum have yet to develop clinical signs of disease at 215 dpi (as of 1 December 2013). Voles receiving type 3 inoculum (two total voles) developed a type 1 phenotype (168 ± 61 dpi) upon second passage. These results prompted us to carry out further serial subpassages to determine whether these fluctuating phenotypes would eventually stabilize. Continued subpassage of the first-passaged type 2 inoculum resulted in the stable selection of only type 1 agent throughout passages 3 through 6 (Fig. 2-3A), and accompanying stabilized survival times that reached 85 ± 5 dpi by passage six (Table 2-1). As of 1 December 2013, subpassages three through six of first-passage types 1 and 3 agent are currently in progress. The phenotypic instability observed in initial passages of white-tailed deer CWD in meadow voles is similar to that observed in other cross-species prion transmission

events and reflects CWD agent adaptation via selection for PrP^{d} conformers that are compatible for conversion of meadow vole PrP^{C}.

Additional phenotypic variation was observed upon subpassage of the double-banded type 4 isolate that emerged upon second passage of type 2 inoculum; a third passage of a dilution series of the type 4 agent (10-fold dilutions ranging from 0.001 - 10% w/v infected vole brain homogenate in PBS) was carried out in meadow voles (Fig. 3B). Subpassage of all dilutions between 0.001% - 1% resulted in the selection of type 1 vole-passaged CWD, while the 10% dilution resulted in selection of both type 1 (three voles) and type 4 (two voles) phenotypes. These data indicate that the double banded phenotype can be propagated upon serial subpassage of high titer inoculum in meadow voles, but upon approaching a limiting dilution, type 1 is consistently selected. Interestingly, continued passage of the first-passage type 4 isolate resulted in selection of both type 3 and type 4 phenotypes upon second passage, suggesting that the type 4 variant may exist as one of a mixture of species that is selected upon CWD transmission to meadow voles, but also that all isolates that we are able to classify as "type 4" may not be comprised of identical subpopulations of PrP^{d} conformers.

Collectively, these data demonstrate that serial subpassage of 96GS and 96GG white-tailed deer CWD in meadow voles results in the stable selection of at least two separate vole-passaged CWD agents (type 1 and type 3, respectively), characterized by differences in biochemical features of the agent and clinical outcomes in the host, and thus providing conclusive evidence for CWD strain selection in this species.

Type 3 vole-passaged CWD lesion profile compares with that of Tg(cerPrP1536$^{+/-}$) mouse-passaged D10 mule deer CWD

We compared our meadow vole passaged CWD lesion profiles to lesion profiles we previously generated from brains of transgenic mice encoding cervidized PrP [Tg(cerPrP)1536$^{+/-}$] that had been inoculated with either the D10 isolate of mule deer (*Odocoileus hemionus hemionus*) CWD (Browning et al., 2004), or D10 amplified *in vitro* by serial protein misfolding cyclic amplification (sPMCA) (Meyerett et al., 2008). Figure 2-4 shows that the type 3 lesion profile for CWD-challenged meadow voles was comparable to that of sPMCA amplified D10 (amp D10 p1) in Tg(cerPrP)1536$^{+/-}$ mice (with a notable difference in lesion severity in the cerebellar cortex) yet unique from the lesion profile of Tg(cerPrP)1536$^{+/-}$ mice directly inoculated with the original, non-amplified D10 CWD inoculum (D10 p1). Second passage of non-amplified D10 in Tg(cerPrP)1536$^{+/-}$ mice (D10 p2) produced a lesion profile more closely resembling that of type 3 vole-adapted CWD than first passage. Additionally, the lesion profile for type 4 meadow vole passaged CWD displayed some similarity with that of bank voles (*Myodes glareolus*) inoculated with homozygous A^{136}R^{154}Q^{171} sheep scrapie (Di Bari et al., 2008) and cervid CWD (Di Bari et al., 2013) in regions 1 – 6 (comparisons not shown). Type 1 - 4 lesion profiles were dissimilar to those of *tga20* mice (transgenic mice overexpressing murine PrP) inoculated with mule deer CWD (Sigurdson et al., 2006).

Figure 2-4 also compares the lesion profile for the type 3 isolate obtained from 96GG challenged meadow voles with that of the type 3 isolate obtained from 96GS challenged meadow

voles. The two profiles are nearly identical, providing further evidence supporting the type 3 variant as a stable TSE strain.

Meadow voles are susceptible to CWD via peripheral transmission routes

To determine whether meadow voles were susceptible to white-tailed deer CWD via routes consistent with their exposure in the environment, we performed oral and intraperitoneal challenges with 96GG and 96GS CWD isolates (Fig. 2-5). Intraperitoneal challenge of meadow voles with 96GS CWD resulted in the development of clinical TSE in 2/10 challenged animals (at 466 and 519 dpi), suggesting that peripheral exposure of meadow voles to CWD could result in disease, but that a greater transmission barrier had to be overcome as compared to that for i.c. exposed meadow voles. Serial i.c. subpassage of brain homogenate from the two clinically affected meadow voles resulted in a 100% attack rate, and a switch from a type 2 phenotype to a type 3 phenotype with a reduction in survival time to 156 ± 9 dpi (media survival time ± 95% confidence interval), suggesting a role for the route of transmission in CWD strain selection in this species. Intraperitoneal challenges of meadow voles with 96GG CWD are currently in progress with no animals yet presenting clinical disease at 471 dpi (as of 1 December 2013).

All meadow voles orally challenged with CWD failed to develop clinical TSE, and following termination of the experiment at 785 dpi, no PrP^{res} could be detected in brain tissue of these animals, suggesting the existence of a complete transmission barrier to oral CWD exposure in this species (Fig. 2-5). However, brain tissue from non-clinical, orally-challenged meadow voles was used as inoculum for a second i.c. passage set of voles, which resulted in the development of clinical disease in three second passage animals at 217 ± 50 dpi that were all

challenged with the same first passage inoculum originating from a single meadow vole that had been orally challenged with 96GS CWD. Second passage i.c. challenges of meadow voles with both 96GG and 96GS derived inocula are still underway, with no clinical signs yet observed in animals subpassaged with 96GG derived tissue; experimental animals are currently at 942 - 971 dpi (as of 1 December 2013). Acknowledging that caution should be applied in drawing conclusions from limited observations, these data suggest that the possibility for meadow voles to harbor a subclinical or latent TSE as a result of oral exposure to white-tailed deer CWD exists.

Discussion

More than three-quarters of emerging infectious diseases that affect humans have a zoonotic origin, the majority of which originate in wildlife (Daszak et al., 2000; Taylor et al., 2001). The ability of pathogens to "jump" from one species to another is a major cause of new, emerging diseases. Chronic wasting disease is an emerging infectious prion disease of cervids, and although the zoonotic potential of CWD is considered low, an incomplete understanding of CWD strain diversity and the potential for agent evolution hinders a definitive conclusion. Even less is currently known about the consequences of environmental exposure of wildlife species to CWD prions.

To begin addressing this unknown, we challenged meadow voles with white-tailed deer CWD via peripheral routes consistent with likely environmental routes of CWD exposure in this species. Meadow voles provided ideal candidates for this study for a number of reasons. First, their natural habitat range overlaps with areas of current CWD epizootics (Heisey et al., 2010) and they exhibit opportunistic carcass scavenging and cannibalism behaviors (Ebensperger et al., 2000; Litvin et al., 1977; Riewe, 1973; Thomas, 1988; Zimmerman, 1965), making their environmental exposure to the agent on the landscape highly likely. Voles also serve as an important food source for higher trophic level predator and scavenger species and small rodent tissue enters the domestic livestock and human food chain by accidental inclusion in grain and forage (Meerburg, 2006; Meerburg et al., 2004; Meerburg et al., 2009), providing additional possible routes of disease transmission to other wildlife, domestic animals and humans. Finally, rodents are known vector, reservoir, and bridge species for various infectious diseases. Importantly, we had previously established that meadow voles were the most susceptible of four

native North American rodent species intracerebrally challenged with white-tailed deer CWD, demonstrating the highest attack rates and shortest survival times (Heisey et al., 2010). In this study, we found evidence to support that meadow voles are susceptible to white-tailed deer CWD by peripheral exposure routes, and data to suggest that they are additionally capable of harboring subclinical prion disease following consumption of CWD infected tissues. Additionally, we found evidence for the selection of at least two distinct CWD strains in meadow voles, characterized by unique PrP^{res} electrophoretic mobility profiles, PrP^{d} deposition patterns and lesion profiles.

Strain diversity has been identified in scrapie, BSE, and CJD and mounting evidence supports the existence of strains in CWD. Early studies identified differences in PrP^{res} glycoform ratios (Race et al., 2002) and PrP^{d} conformation (Safar et al., 2002) among different species of CWD-infected cervids, but differences in amino acid sequence of PrP between these species have confounded the conclusion that these differences actually represent unique CWD strains. Classically, isolation of a prion strain was achieved upon the consistent observation of identical clinical (e.g. incubation period, neuropathological features, clinical manifestations) and biochemical properties (e.g. PrP^{d} glycosylation, electrophoretic mobility of PrP^{res}, resistance to inactivation by heat or chemicals, etc.) resulting from serial subpassage in genetically defined hosts (Bruce, 2003). More recently, in two separate studies carrying out transmission and subpassage of white-tailed deer, mule deer and elk CWD isolates in either Syrian golden hamsters or transgenic mice expressing hamster PrP^{C}, or transgenic mice expressing cervid PrP^{C}, at least two unique clinical outcomes resulted, characterized by marked differences in rodent incubation period and neuropathological patterns, suggesting the existence of at least two distinct

CWD strains (Angers et al., 2010; Raymond et al., 2007). However, neither study was able to distinguish the differential outcomes of their transmission and passage studies by differences in biochemical properties intrinsic to the TSE agent; in both cases, discrimination was based upon clinical outcomes, which result from agent-host interactions and thus can be influenced by host effects. In this work, we demonstrate, for the first time, the isolation of at least two unique CWD strains, distinguishable by differences in both PrP^{res} fragment size and neuropathological outcomes in the host.

The differences in phenotypic variation observed in 96GG challenged meadow voles, which consistently resulted in a single strain outcome, as compared with 96GS challenged meadow voles, which displayed at least four variants, implies that CWD strain diversity lies within the inoculum, and that the cervid host PrP genotype may influence strain constitution in white-tailed deer. In support of this, the prion protein from 20 meadow voles used in this study was sequenced and no polymorphisms were identified, arguing against a role for vole PrP genotype in strain selection in these experiments. However, we cannot rule out the possibility for influence from exogenous genetic influences in the meadow vole host, due to their outbred nature. This idea is consistent with the findings of Angers *et al*., who consistently obtained mixed CWD strain responses from deer isolates versus the relatively uniform manifestation of one or the other of two identified strains in elk isolates inoculated into genetically defined cervidized transgenic mice, reflecting "strain constitutions in the natural host, rather than adaptation and divergence of progenitor strains in recipient mice" (Angers et al., 2010). Ultimately, many more than the three 96GG and three 96GS white-tailed deer CWD isolates used in this study would need to be strain typed in meadow voles (or transgenic mice expressing

meadow vole PrP^C) before a correlation can be drawn between deer PrP genotype and strain constitution. If a correlation does exist, it brings forth the concept that 96GS genotype white-tailed deer, capable of harboring multiple CWD conformers, may serve as a "mixing vessel" for various CWD strains that may be circulating among free-ranging cervids on the landscape. Additionally, the 96GS genotype has been demonstrated to confer a relative resistance to CWD over the wild type 96GG genotype in that 96GS animals live, on average, 200+ days longer than 96GG animals (Johnson et al., 2011), implying that CWD-infected 96GS white-tailed deer are capable of shedding infectious material into the environment for prolonged periods of time. The concept that 96GS white-tailed deer may be able to support greater CWD strain diversity warrants further investigation as it may provide guidance to management of CWD on the landscape by identifying those animals that pose the greatest risk for CWD transmission, contamination, and dissemination in the environment. CWD strain characterization in 96SS genotype white-tailed deer, thought to be most CWD resistant, would be of additional interest and is currently underway in our laboratory.

The ability of both type 1 and type 3 meadow vole-passaged CWD to imprint their properties on new meadow vole hosts over six serial subpassages provides strong evidence supporting their definition as strains. Interestingly, the lesion profile for meadow vole-passaged type 3 variant closely resembled that of *in vivo* passaged or *in vitro* amplified mule deer D10 CWD, which was isolated from a captive doe from Colorado (Fig. 4) (Browning et al., 2004), suggesting type 3 may represent a widespread CWD strain across disparate geographical locations and host species. Further serial subpassages will be required to determine whether vole-passaged CWD types 2 and 4 also represent stable conformers in the meadow vole host, and

thus are able to persist as strains. It is interesting to consider the phenotypic variation observed in 96GS challenged meadow voles and what it may indicate regarding prion strain constitution. To determine if the double-banded phenotype of type 4 vole-passaged CWD that emerged upon second passage of type 2 inoculum represented a mixture of coexisting type 1 and type 3 variants, or a unique variant separate from the others, we carried out a third passage of a dilution series of the type 4 agent (10-fold dilutions ranging from 0.001 - 10% w/v infected vole brain homogenate in PBS) in meadow voles. We obtained type 1 variants for all dilutions except the 10% inoculum cohort, which displayed both type 1 and type 4 variants upon third passage. Together with the unique lesion profile and PrP^d deposition patterns observed for the type 4 variant, it is tempting to speculate that these data suggest that type 4 exists as one of a mixture of species that is selected upon CWD transmission into meadow voles, and that the type 1 variant may be selectionally preferred in this host. This would be consistent with the conformation selection model of prion strains (Collinge, 2010). However, continued passage of another type 4 isolate from first passage resulted in selection of both type 3 and type 4 phenotypes upon second passage. These observations suggest that not all type 4 isolates are created equal, and may be comprised of distinct "quasi-species" of PrP^d conformers, the true diversity of which have, until now, been undetectable by CWD strain typing efforts. That each one of the four vole-passaged CWD types obtained upon primary passage displayed phenotype switching upon second passage suggests that overall CWD strain diversity may be greater than previously hypothesized and highlights meadow voles as a valuable CWD strain typing tool.

The results of peripheral challenges of meadow voles with white-tailed deer CWD indicates that prion strain selection may be additionally influenced by route-of-transmission. Of

the meadow voles i.p. challenged with 96GS CWD, 2/10 total animals developed clinical TSE and displayed the type 2 phenotype. Upon serial i.c. subpassage of those two clinical animals, all voles developed type 3 vole-passaged CWD. As of 1 December 2013 (at 471 dpi), no 96GG i.p. challenged meadow voles have developed clinical TSE; these challenges are ongoing. Should all 96GG challenged meadow voles fail to develop clinical TSE, it would demonstrate that 96GS and 96GG CWD have different cross-species transmission characteristics. In support of this, only 96GS orally challenged meadow voles have been found to harbor subclinical TSE; to date (942 - 971 dpi), no 96GG orally challenged or subpassaged voles have been confirmed positive for clinical or subclinical TSE. These findings are significant, because not only are they the first to demonstrate oral susceptibility of a non-cervid wildlife species to CWD, but they additionally suggest that 96GG and 96GS white-tailed deer CWD have different cross-species transmission characteristics. Of immediate priority is the extended characterization of each of the vole-passaged CWD types identified in this study, especially determination of host range and zoonotic potential. Challenge experiments investigating the transmission of each variant in transgenic mice expressing human PrP have been initiated.

Figures, Tables, and Legends

Figure 2-1. Four unique PrP^{res} phenotypes are obtained upon primary transmission of white-tailed deer CWD to meadow voles.

(A) Immunoblot of proteinase K-resistant PrP^{d} (PrP^{res}) from meadow voles infected with white-tailed deer CWD, displaying four resulting electrophoretic mobility patterns with regard to the non-glycosylated band: type 1 = slow migrating non-glycosylated band (18 kDa), type 2 = intermediate migrating (17 kDa), type 3 = fast migrating (16 kDa), type 4 = double banded (with one resolving at 16 kDa and one at 18 kDa). (B) Immunoblot comparison of 96GG and 96GS white-tailed deer inocula with type 1 and type 3 vole-passaged CWD electrophoretic mobility profiles. Membranes were probed with mAb SAF 83 for vole PrP and BAR 224 for deer PrP.

Figure 2-1

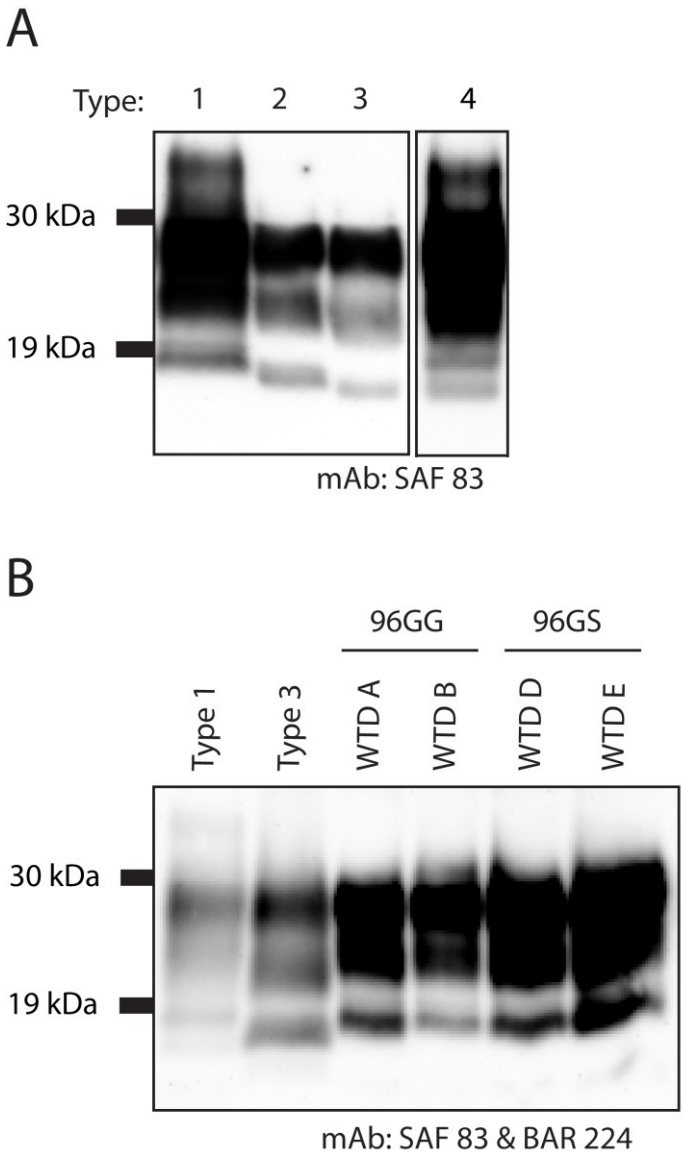

A
Type: 1 2 3 4
30 kDa
19 kDa
mAb: SAF 83
B
96GG
96GS
Type 1
Type 3
WTD A
WTD B
WTD D
WTD E
30 kDa
19 kDa
mAb: SAF 83 & BAR 224

Figure 2-2. Unique TSE-associated neuropathology profiles from brains of CWD-infected meadow voles correspond to PrP^{res} types 1 - 4.

(A) Lesion profiles for types 1 – 4 vole-passaged CWD were generated from scoring nine neuroanatomic brain regions for severity of vacuolization in H&E-stained medial sagittal brain sections; all lesion profiles are representative of a single 1st passage 96GS white-tailed deer CWD infected meadow vole, except the type 3 profile for which two infected meadow voles are represented. Brain scoring regions are: 1 – dorsal medulla, 2 – cerebellar cortex, 3 – superior colliculus, 4 – hypothalamus, 5 – thalamus, 6 – hippocampus, 7 – septal nuclei of the paraterminal body, 8 – cerebral cortex dorsal to the corpus callosum, 9 – cerebral cortex anterior to 7. (B) PrP^{d} deposition patterns for types 1 – 4 vole-passaged CWD were generated by immunohistochemical staining for PrP^{d} in medial sagittal sections of brain from clinical meadow voles using mAb SAF 83. Panels display stained sections from first passage voles challenged with CWD agent from 96GS deer only and contain the following brain regions: A – dorsal medulla, B – cerebellum, C – thalamus, D – cerebral cortex. Scale bars in the upper left panel are equal to 200 μm and are applicable to the identical brain regions in the remaining panels.

Figure 2-2

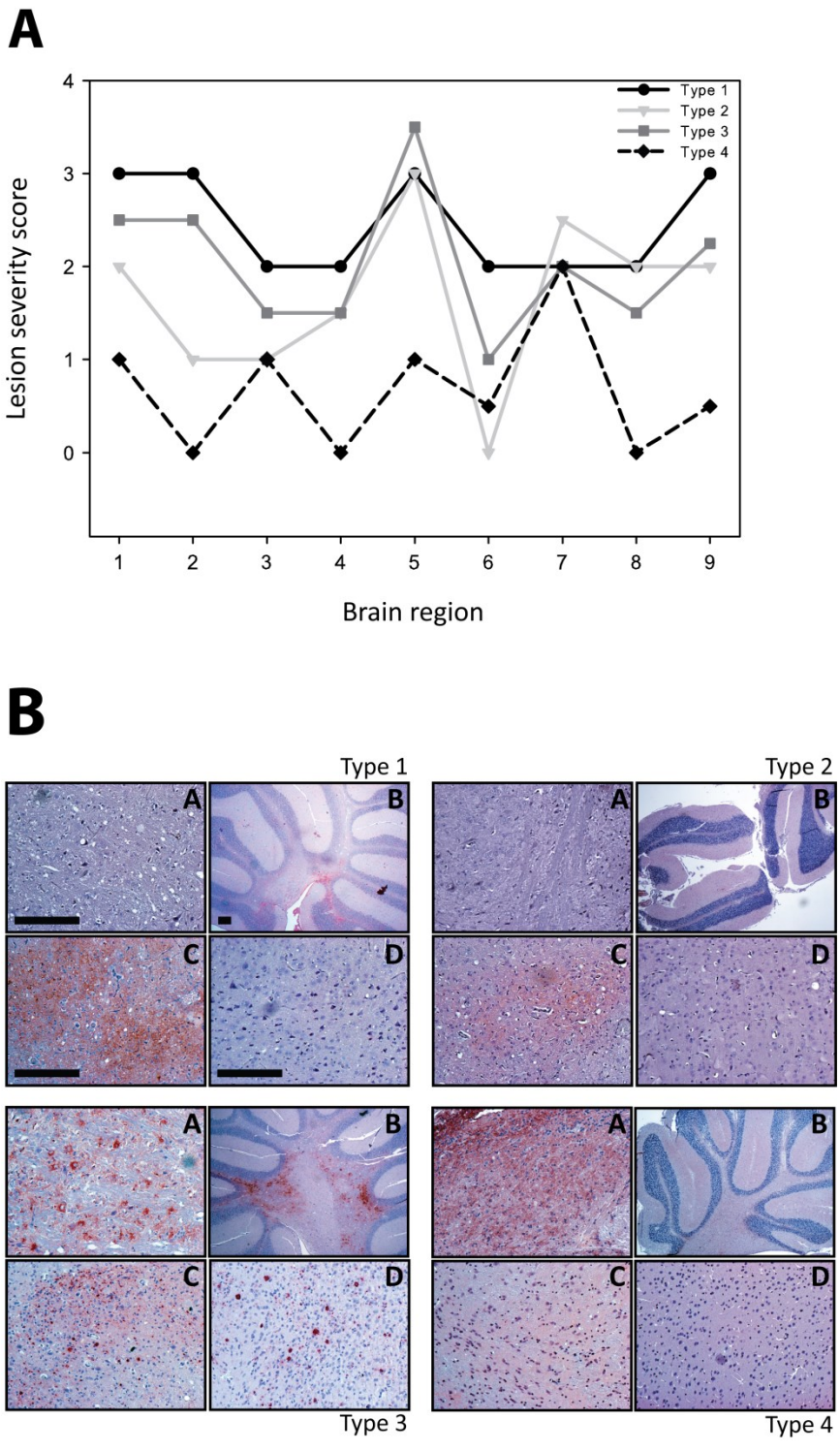

A
Type 1
Type 2
Type 3
Type 4
Lesion severity score
0
1
2
3
4
1
2
3
4
5
6
7
8
9
Brain region
B
Type 1
Type 2
Type 3
Type 4
A
B
C
D

Figure 2-3. Serial subpassage of white-tailed deer CWD in meadow voles results in strain selection.

(A) Circles in shades of pink and shades of grey represent voles challenged with 96GG and 96GS white-tailed deer CWD, respectively. Circle fill color/pattern reflects vole-passaged CWD phenotype as determined by electrophoretic mobility of PrP^{res} and patterns of neuropathology. Circles outlined in bright green represent those voles that were selected to have their brain tissue serve as inoculum for the next serial subpassage. Empty circles represent voles for which no PrP^{res} could be detected in brain tissue via either immunoblotting or IHC analysis. (B) Dilution series subpassage of a type 4 vole-passaged 96GS CWD isolate. Circles (voles) shown in passage 1 and 2 represent a subset of those displayed in 96GS passage history in Fig. 3A and, upon second passage, a type 4 isolate was selected for subpassage (in contrast to the second passage type 1 isolate selected in Fig. 3A). Serial 10-fold dilutions ranging from 0.001 – 10% w/v infected type 4 vole brain homogenate in PBS were used as inocula to challenge third passage voles. Circle fill color/pattern reflects vole-passaged CWD phenotype as in Fig. 3A.

Figure 2-3

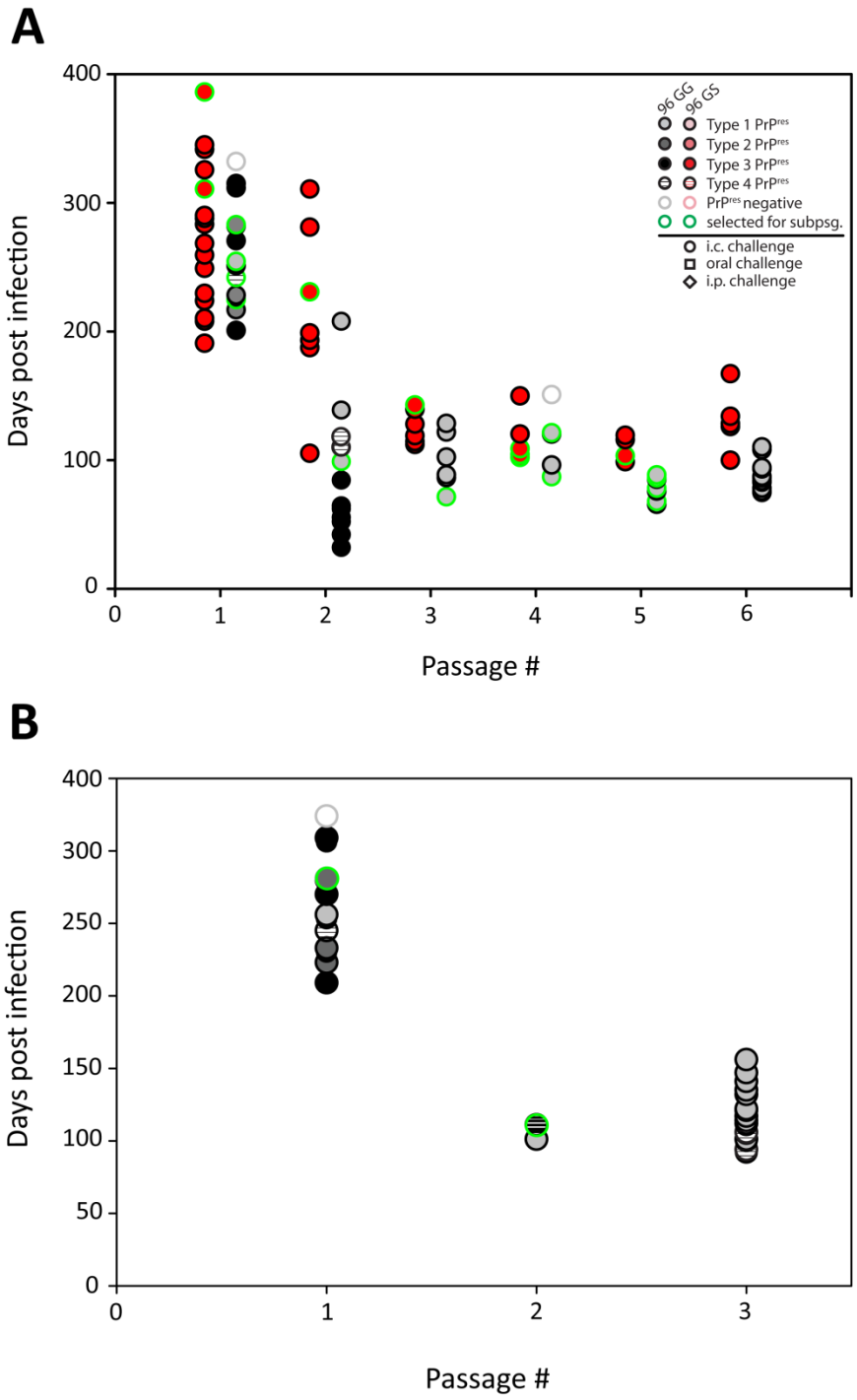

A
Days post infection
Passage #
96 GG
96 GS
Type 1 PrPres
Type 2 PrPres
Type 3 PrPres
Type 4 PrPres
PrPres negative
selected for subpsg.
i.c. challenge
oral challenge
i.p. challenge
B
Days post infection
Passage #

Figure 2-4. Comparison of lesion profiles for vole-passaged CWD type 3 and *in vivo* or *in vitro* passaged D10 mule deer CWD in Tg(cerPrP)1536$^{+/-}$.

Lesion profiles for 96GS and 96GG CWD-derived type 3 (dark and light red lines, respectively) are compared with those of *in vitro* sPMCA adapted D10 CWD passaged into Tg(cerPrP)1536$^{+/-}$ mouse substrate (amp D10 p1) and primary (D10 p1) and secondary (D10 p2) *in vivo* passage of D10 in Tg(cerPrP)1536$^{+/-}$ mice. Brain scoring regions are identical to those used in Fig. 2-2A. Note that brain region 7 refers to the septal nuclei of the paraterminal body for vole-passaged CWD lesion profiles, and to the caudate putamen for D10 CWD lesion profiles (Meyerett et al., 2008).

Figure 2-4

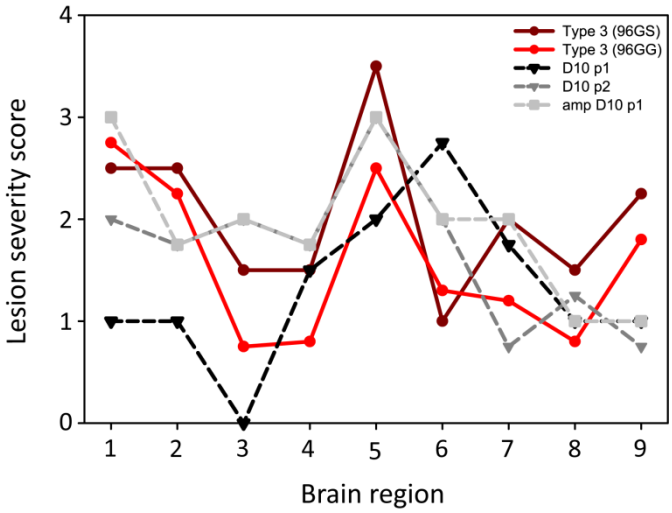

Type 3 (96GS)
Type 3 (96GG)
D10 p1
D10 p2
amp D10 p1
Lesion severity score
Brain region
0
1
2
3
4
1
2
3
4
5
6
7
8
9

Figure 2-5. Peripheral transmission and subpassage of white-tailed deer CWD in meadow voles.

Squares, diamonds, and circles represent voles orally, i.p., or i.c. challenged, respectively, with 96GS or 96GG white-tailed deer CWD or vole-passaged white-tailed deer CWD; shape fill color/pattern reflects vole-passaged CWD phenotype with regard to electrophoretic mobility of PrP^{res} and patterns of neuropathology, the identification of which is provided in the key on the upper right-hand corner of the figure. Shapes outlined in bright green represent those voles that were selected to have their brain tissue serve as inoculum for the next serial subpassage. Empty shapes represent voles for which no PrP^{res} could be detected in brain tissue via either immunoblotting or IHC analysis. Asterisks represent experiments still in progress, containing challenged voles that are still alive and TSE-free.

Figure 2-5

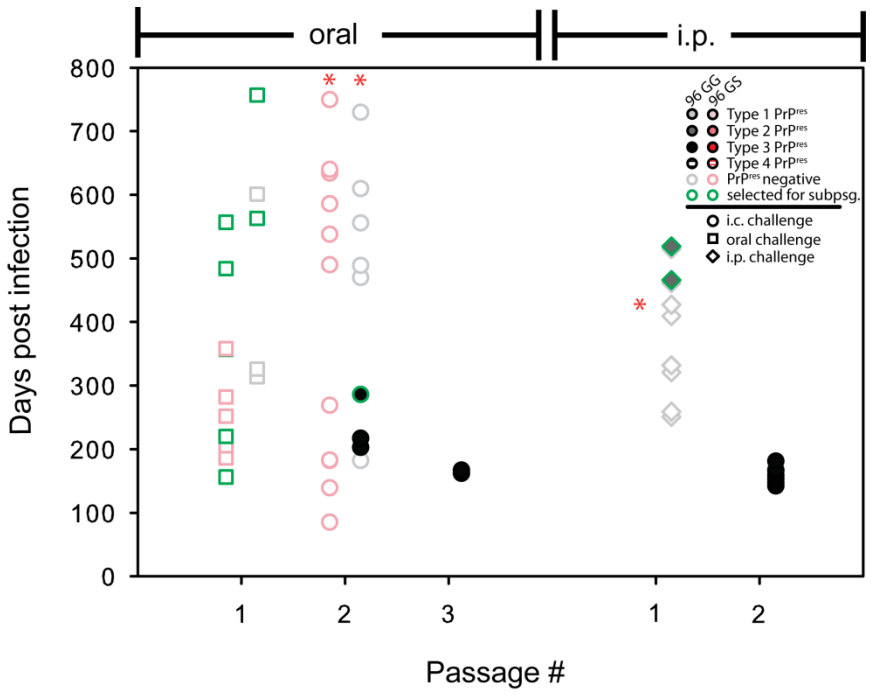

oral
i.p.
Days post infection
800
700
600
500
400
300
200
100
0
1
2
3
1
2
Passage #
96 GG
96 GS
Type 1 PrPres
Type 2 PrPres
Type 3 PrPres
Type 4 PrPres
PrPres negative
selected for subpsg.
i.c. challenge
oral challenge
i.p. challenge

Figure 2-6. Hematoxylin and eosin-stained sagittal brain sections display CWD lesions of six degrees of severity in meadow voles.

(A) score 0: no vacuoles or vacuolation comparable to the same region in uninfected, healthy brain, (B) score 1: a few vacuoles, widely and unevenly scattered, (C) score 2: a few vacuoles evenly scattered, (D) score 3: moderate numbers of vacuoles, evenly scattered, (E) score 4: many vacuoles with some confluence, (F) score 5: dense vacuolation with most of the field confluent. Scale bar = 10 μm.

Figure 2-6

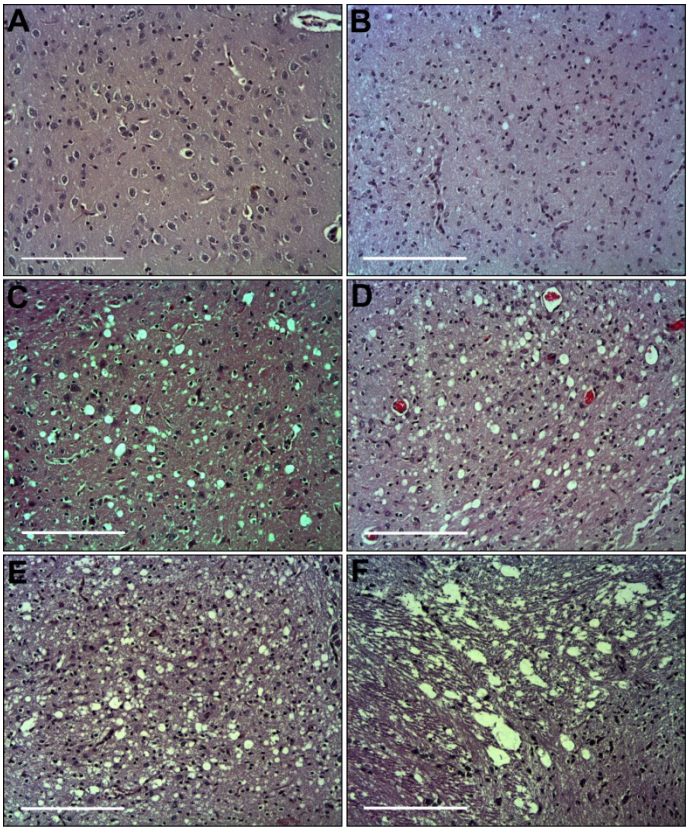


Figure 2-7. Immunohistochemistry stain sagittal brain sections display PrPd deposition staining of five degrees of intensity in meadow voles.

(A) score 0: no staining, (B) score 1: weakly positive staining, (C) score 2: moderately positive staining, (D) score 3: strongly positive staining, (E) score 4: intensely positive staining. Scale bar = 10 μm.

Figure 2-7

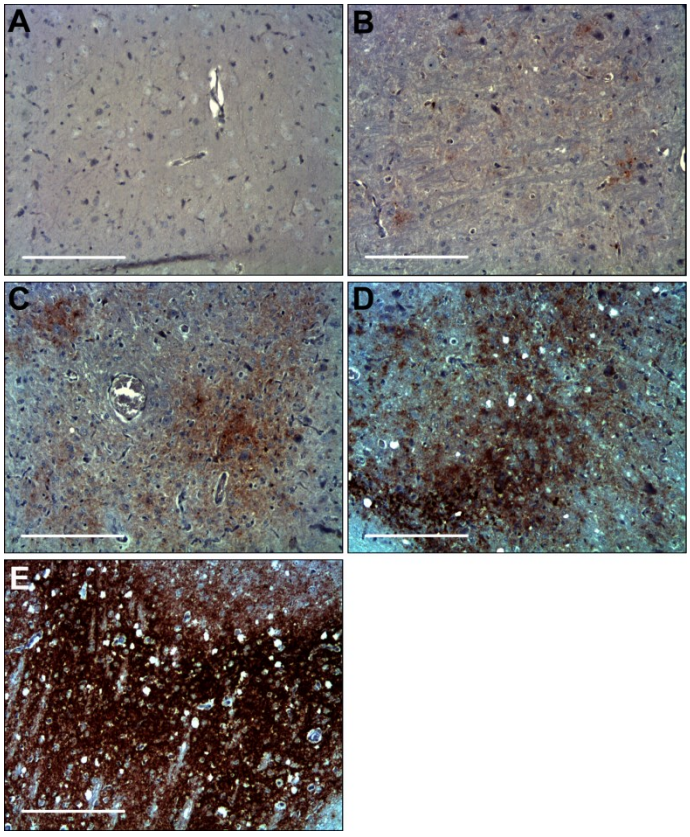


Table 2-1. Survival times of meadow voles infected with serially-passaged CWD agent

Table 2-1. Survival times of meadow voles infected with serially-passaged CWD agent[a]

		96GG lineage		96GS lineage	
Challenge	**Inoculum**	**Median Survival Time, d (95% CI)**	**Clinical Disease, n/n_o**	**Median Survival Time, d (95% CI)**	**Clinical Disease, n/n_o**
Intracerebral					
passage 1	cervid CWD vole-passaged	284 (262 - 306)	17/17	256 (237 - 275)	12/13
passage 2	CWD vole-passaged	195 (161 - 229)	9/9	95 (54 - 136)	14/14
passage 3	CWD vole-passaged	124 (115 - 133)	6/6	96 (81 - 111)	6/6
passage 4	CWD vole-passaged	111 (96 - 126)	5/5	120 (101 - 139)	4/5
passage 5	CWD vole-passaged	115 (109 - 121)	6/6	81 (77 - 85)	10/10
passage 6	CWD	129 (114 - 144)	6/6	85 (80 - 90)	14/14
Oral					
passage 1	cervid CWD vole-passaged	252 (175 - 329)	0/11	566 (396 - 730)	0/5
passage 2*	CWD vole-passaged	365 (275 - 455)	0/24†	470 (339 - 601)	3/13†
passage 3*	CWD	N/A	N/A	165 (160 - 170)	2/2
Intraperitonial					
passage 1	cervid CWD vole-passaged	*ongoing*	0/8†	418 (356 - 479)	2/10
passage 2*	CWD	N/A	N/A	156 (147 - 165)	8/8

[a]Survival time reported as median time from inoculation to death, calculated using Kaplan-Meier statistics

*Denotes subpassage challenge was administered intracerebrally

†Experimental challenge group still underway; survival times and attack rates calculated as of 10 Nov 2013

d = days; CI = confidence interval; n = number of voles with neurological signs of TSE (confirmed PrP^d-positive by WB and/or IHC); n_o = number of voles inoculated, voles with intercurrent illness excluded; N/A = not attempted

Material and Methods

Animals

Meadow voles were raised from wild-caught progenitors in a colony maintained at the USGS National Wildlife Health Center (Madison, WI, USA). All animals were handled in strict accordance with good animal practice, as defined by the NIH Office of Laboratory Animal Welfare, and all live animal work was reviewed and approved by the USGS National Wildlife Health Center Animal Care and Use Committee (approvals: EP040614-R1, EP130802-R1, EP080715-A2).

Study samples and preparation

Six isolates of white-tailed deer (*Odocoileus virginianus borealis*) CWD agent were used in this study: three represented wild-type homozygous PrP $Q^{95}G^{96}Q^{226}$ genotype deer, two of which were hunter harvested animals from the CWD management zone in southwestern Wisconsin (animals A and B) (Wisconsin Department of Natural Resources, 2013b) and one of which was experimentally challenged with white-tailed deer CWD (animal C) (Johnson et al., 2011); the other three isolates represented heterozygous PrP $Q^{95}G^{96}Q^{226}$/ $Q^{95}S^{96}Q^{226}$ genotype hunter-harvested deer from the CWD management zone in southwestern Wisconsin (animals D, E, F). A 10% w/v homogenate (in phosphate-buffered saline, PBS) of CWD positive deer obex tissue was used for the primary challenge (i.e. first passage) inocula into meadow voles via intracerebral (i.c.) and intraperitoneal (i.p.) routes; a 20% (w/v in PBS) obex homogenate was used for primary oral challenges in meadow voles. Each inoculum was prepared from a single deer source, not pooled. Identically prepared obex homogenate from a single CWD-negative

white-tailed deer harvested outside the WI CWD management zone was used as negative control inoculum for primary challenges. For subsequent passages in voles (all second through sixth passages were carried out via i.c. challenge), a 10% w/v homogenate (in PBS) of whole brain tissue from PrP^d-positive voles was used as challenge inocula for the next passage set of voles. Inocula were prepared from several individual representative voles from each passage and were not pooled. A 10% w/v homogenate (in PBS) of whole brain tissue from a single PrP^d-negative vole was used as control inoculum.

Serial passage of CWD in voles

Vole-adapted CWD agent was generated by serial experimental infection of meadow voles with the initial cervid CWD agent described above. Voles were anesthetized with isofluorane and inoculated via i.c., i.p., or oral routes with 20, 50, or 60 μL of inoculum, respectively. Orally challenged voles received one dose per day for five consecutive days while i.c. and i.p. challenged animals were dosed only once. Animals were monitored at least weekly in the early stages of infection, and up to daily upon onset of clinical signs. Any voles with intercurrent illnesses were excluded from data analysis; all others were euthanized when they exhibited significant clinical impairment and neurological signs of disease consistent with TSE (including circling, lethargy, poor balance, thinning of the waist, and lack of motivation to burrow and/or acquire food and water). Survival time was defined as the number of days from inoculation to euthanization. Brains were harvested immediately post-mortem and divided sagittally, with one half prepared for evaluation by Western blotting and the other half fixed in 10% neutral-buffered formalin for histopathology and immunohistochemistry (IHC); all

challenged animals in each passage group were tested for the presence of PrP^d in brain tissue by Western blotting, IHC, or both.

Western blotting

Brain tissue was initially prepared as a 20% w/v homogenate in PBS and subsequently diluted 1:1 in PBS. Homogenates were then digested with proteinase K (PK; 50 μg/mL final concentration; Sigma-Aldrich, St. Louis, MO, USA) for 1 hr at 37 °C, followed by the addition of Pefabloc SC (2 mM final concentration; Roche Diagnostics, Indianapolis, IN, USA) for 30 min at room temperature to halt reactions. SDS-PAGE and Western blotting were carried out as previously described (Heisey et al., 2010) using primary monoclonal antibodies (MAb) SAF 83 and BAR 224 (Cayman Chemical Company, Ann Arbor, MI) for vole and deer samples, respectively, and secondary goat anti-mouse immunoglobulin G conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), both diluted 1:10,000 in 5% milk in Tris-buffered saline plus 0.5% Tween 20 (TTBS). Immunoreactivity was detected using an enhanced chemiluminescent detection system (Thermo Fisher Scientific, Inc., Rockford, IL, USA) in a UVP EC3 Imaging System (UVP Bioimaging Systems, Upland, CA, USA). Densitometric analyses were performed using VisionWorks LS software version 606a (UVP Bioimaging Systems, Upland, CA, USA).

Histopathology and immunohistochemistry

Whole vole brains or hemispheres were fixed in 10% phosphate-buffered formalin for ≥48 hr, embedded in paraffin, sectioned to 5 μm, and mounted onto positively charged glass

slides. Tissue sections were deparaffinized and rehydrated through a graded ethanol series. For hematoxylin and eosin (H&E)-stained tissues: sections were submerged in Harris hematoxylin (Thermo Fisher Scientific, Inc, Rockford, IL, USA) for 3 min, washed with deionized water, submerged in eosin Y stain (Ricca Chemical Company, Arlington, TX, USA) for 1 min, washed, dehydrated, and coverslipped. Immunostaining for PrP^d followed a previously described procedure (Hoefert et al., 2004). Briefly, following rehydration, endogenous peroxidase activity was quenched by incubation in 0.3% hydrogen peroxide for 30 min. Antigen retrieval was achieved by autoclaving tissues in 100 mM sodium citrate buffer, pH 6.0, for 30 min, followed by submersion in 88% formic acid for 10 min and then in 4 M guanidine isothiocyanate for 2 hr. Tissues were then blocked with 1.5% v/v normal goat serum (Vector Laboratories, Burlingame, CA, USA) for 1 hr and stained overnight at 4°C using primary MAb SAF83 (1:5,000 final dilution), followed by incubation with biotinylated horse anti-mouse immunoglobulin G (1:200 final dilution) for 30 min. Detection was completed using horseradish peroxidase-mediated hydrogen peroxide immunostaining (NovaRed; Vector Laboratories, Burlingame, CA, USA). Sections were counterstained with Harris hematoxylin, dehydrated, and coverslipped. All tissues were imaged using a Leica DM 2000 light microscope equipped with a Leica DFC280 camera and visualized using the Leica Application Suite, version 2.8.1 (Leica Microsystems, Ltd., Switzerland).

Lesion profiling and PrPd deposition patterning

Brain lesion profiling was performed using the method of Fraser and Dickenson (Fraser and Dickinson, 1968), with slight modifications. Briefly, nine neuroanatomic regions were

scored in H&E-stained medial sagittal brain sections from at least three voles for each passage group: 1 – dorsal medulla, 2 – cerebellar cortex, 3 – superior colliculus, 4 – hypothalamus, 5 – thalamus, 6 – hippocampus, 7 – septal nuclei of the paraterminal body, 8 – cerebral cortex anterior to 7, and 9 – cerebral cortex dorsal to the corpus callosum. Two investigators blindly scored each region for intensity of vacuolation using the following severity scale: (0) no vacuoles or vacuolation comparable to the same region in uninfected, healthy brain, (1) a few vacuoles, widely and unevenly scattered, (2) a few vacuoles evenly scattered, (3) moderate numbers of vacuoles, evenly scattered, (4) many vacuoles with some confluence, and (5) dense vacuolation with most of the field confluent; representative images of each severity rating are provided in Figure 2-6. The average of the three scores constitutes the severity score for each region and these scores were used to construct lesion profiles of spongiform degeneration in CWD-challenged voles. Additionally, PrP^{d} deposition patterns were recorded by i) identifying the localization and pattern of PrP^{d} expression within each of the nine above-listed brain regions and corresponding subregions and ii) scoring intensity of PrP^{d} immunostaining, using a semi-quantitative five-point scale: (0) no staining; (1) weakly positive staining; (2) moderately positive staining; (3) strongly positive staining; (4) intensely positive staining. At least three brain sections from each of three to four voles were stained for every passage. Representative images of each severity rating are provided in Figure 2-7.

Acknowledgments

We thank Jennifer Brunner and the animal care staff at the USGS National Wildlife Health Center for excellent animal care, Dr. Julie Langenberg (Wisconsin Department of Natural Resources) for the CWD isolates, Dr. Delwyn Keane, Ben Johnson, and David LaBeause (Wisconsin Veterinary Diagnostic Laboratory) for tissue preparation and use of histology equipment, Bryan Richards (USGS National Wildlife Health Center) and Dr. Chad Johnson (University of Wisconsin) for helpful discussions, and Justin Binfet (Wyoming Game and Fish Department) for providing epidemiological information on CWD in Wyoming's South Converse mule deer herd. This work was funded by the USGS Wildlife, Terrestrial and Endangered Resources program and through USGS Research Work Order G09AC00441. Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the U. S. Government.

CHAPTER THREE:

Experimental Infection of Meadow Voles (*Microtus pennsylvanicus*) with Sheep Scrapie

in revision as:

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Experimental Infection of Meadow Voles (*Microtus pennsylvanicus*) with Sheep Scrapie

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Abstract

Meadow voles (*Microtus pennsylvanicus*) are permissive to chronic wasting disease (CWD) infection, but their susceptibility to other transmissible spongiform encephalopathies (TSEs) is poorly characterized. In this study, we intracerebrally challenged six meadow voles with two isolates of sheep scrapie. Three meadow voles acquired a TSE following scrapie challenge and an extended incubation period. The glycoform profile of proteinase K-resistant prion protein in scrapie-sick voles remained similar to the sheep inocula, but differed from that of voles clinically-affected by CWD. Lesion profiles and disease-associated prion protein deposition were generally similar in all scrapie-affected voles, except for the hippocampus, where PrP^{Sc} staining varied markedly among the animals. Our results demonstrate that meadow voles can acquire a TSE following intracerebral scrapie challenge, but the incomplete attack rate and lengthy incubation period suggests there is little risk of scrapie transmission to meadow voles on the landscape.

Introduction

Sheep scrapie is a member of a group of diseases referred to as transmissible spongiform encephalopathies (TSEs, prion diseases). Other TSEs include bovine spongiform encephalopathy (BSE), chronic wasting disease (CWD) and Creutzfeldt-Jakob disease (CJD). Neuropathological hallmarks of TSEs are spongiosis and neuronal loss in the central nervous system (CNS), which lead to dysfunction and death (Budka, 2003). These processes are thought to result from conformational conversion of the normal host-encoded prion protein (PrP^{C}) to an abnormal, disease-associated isoform (PrP^{Sc}) that resists degradation and accumulates in insoluble protein aggregates in infected nervous tissues (Prusiner, 1998).

Studies of scrapie, as the archetypal prion disease, have provided fundamental knowledge on the role of host genetics in susceptibility to prion disease, the distribution of infectivity in host tissues and the occurrence of different prion strains (Fast and Groschup, 2013). Research on these topics has relied heavily on bioassays in rodent species, especially mice, due to the impracticality and cost of using of large animals in protracted challenge studies. The ability to generate genetically-defined rodent hosts and the finding that scrapie strain diversity can be maintained and propagated following serial subpassage in inbred mice are also useful advantages to using mice in scrapie research (Bruce et al., 1991; Dickinson and Fraser, 1977).

Rodent models of scrapie are however hindered by some critical limitations including lengthy incubation periods (1 – 2 years) and inefficient or unsuccessful transmission of a number of natural scrapie isolates to mice. (Bruce et al., 2002; Piening et al., 2006). Transgenic mice overexpressing ovine PrP^{C} have been developed to overcome some of these obstacles and serve as competent hosts for scrapie isolates from sheep expressing different genotypes of the prion

protein gene (*Prnp*) (Groschup and Buschmann, 2008). Recently, voles have been identified as rodent hosts uniquely susceptible to a wide range of different TSEs including scrapie, CWD, CJD, and rodent-adapted TSEs (Agrimi et al., 2008; Di Bari et al., 2008; Di Bari et al., 2013; Heisey et al., 2010; Kurt et al., 2011; Nonno et al., 2006). Early challenge studies showed that field voles (*Microtus agrestis*) were more susceptible to mouse and rat-passaged scrapie than were gerbils, guinea pigs, and Chinese or golden hamsters (Chandler, 1971; Chandler and Turfrey, 1972). More recently, Agrimi and colleagues demonstrated fast (145 – 200 day) and efficient transmission of sheep and goat scrapie isolates to European bank voles (*Myodes glareolus*), including isolates that failed to transmit to laboratory mice or hamsters (Di Bari et al., 2008; Piening et al., 2006). Our group has identified two North American vole species, meadow voles (*Microtus pennsylvanicus*) and red-backed voles (*Myodes gapperi*), that are highly permissive to CWD in experimental challenges (Heisey et al., 2010). In the present study, we investigate the susceptibility of meadow voles to sheep scrapie to: 1) begin to characterize the spectrum of prion disease phenotypes in meadow voles; 2) investigate meadow voles as a potential rodent model for use in scrapie research; and, 3) begin to address the concern that free-ranging voles could serve as an environmental reservoir for scrapie infectivity.

Results

Meadow voles were intracerebrally challenged with either sheep scrapie isolate 1 (voles A, B and C) or isolate 2 (voles D, E and F) (Table 3-1). Only one of the three voles challenged with scrapie isolate 1 (vole A) developed clinical signs of a TSE and was euthanized 593 days post-inoculation (dpi). Voles B and C lived for 571 and 653 dpi, respectively, and did not display clinical signs. In the group of animals challenged with scrapie isolate 2, voles D and E showed clinical signs of disease and were euthanized at 354 and 483 dpi, respectively. Vole F suffered an intercurrent death at 304 dpi, a timepoint that may not have been sufficient to observe clinical signs of TSE onset. Clinical disease manifestations were unremarkable for a rodent TSE and included initial ataxia followed by a state of excessive lethargy where animals were non-responsive to physical stimuli and lacked motivation to burrow and acquire food or water. No notable differences in clinical disease signs were observed for sick animals challenged with either scrapie isolate.

Proteinase-K-resistant prion protein (PrP^{res}) is a marker for PrP^{Sc} and TSE infection. We found that only animals which displayed clinical signs were PrP^{res}-positive (Figure 3-1A). Two asparagine-linked sites in the PrP molecule are variably glycosylated. PrP^{res} can be found in un-, mono-, or diglycosylated forms (Somerville, 1999). The ratio among these three forms of PrP^{res} corresponds to factors controlled by both host and TSE agent. We observed that meadow vole-passaged scrapie maintained the general glycosylation site occupancy ratio ("glycoform profile") of the ovine inocula: diglycosylated > monoglycosylated > unglycosylated (Figure 3-1). On average, the proportion of diglycosylated PrP was reduced and unglycosylated PrP appeared to be increased in the vole-passaged scrapie samples, relative to the ovine inocula. The

monoglycosylated form remained similar across all samples. None of these differences between the glycoform profiles of vole-passaged scrapie and the ovine inocula, however, were found to be statistically significant at a confidence level of 95% (Figure 3-1B). We did find that the glycoform profile for meadow vole-passaged scrapie differed significantly from those we previously reported for clinically-ill meadow voles that had been challenged with white-tailed deer CWD (Figure 3-1B) (Heisey et al., 2010). Acknowledging the limited data set with which we are working, our results suggest that passage of scrapie in meadow voles generally maintains the glycoform characteristics of the original sheep inocula and PrP^{res} glycoform ratios in meadow vole-passaged scrapie differ from those in meadow vole passaged CWD.

Quantitation of spongiosis in specific neuroanatomic areas of TSE-infected brains produces a lesion profile that is characteristic of each host-TSE combination and can be used to discriminate TSE strains (Fraser and Dickinson, 1968). Lesion profiles for TSE-positive, scrapie-challenged meadow voles had regions of both similarity and differences to each other (Figure 3-2). In all three animals, pronounced lesion density was consistently found in the medulla (region 1), cerebellum (region 2), and thalamus (region 5). The degree of spongiosis was somewhat variable in the superior colliculus (region 3) and hippocampus (region 6). Lesion profiles for meadow vole-passaged sheep scrapie were distinct from those previously published for bank vole-passaged sheep scrapie (Di Bari et al., 2008), ovinized transgenic mouse-passaged sheep scrapie (Beck et al., 2013), and scrapie in sheep (Ligios et al., 2002).

To further characterize scrapie-induced neuropathology in meadow voles, we investigated PrP^{Sc} deposition in the brains of infected animals. Table 3-1 summarizes the distribution and amount of PrP^{Sc} staining. A consistent granular pattern of staining that

sometimes coalesced into focal aggregates was observed throughout multiple gray matter regions of brains of all three TSE-positive voles. Affected areas include the dorsal medulla, cerebellar cortex, superior colliculus, thalamus, septal nuclei, and cerebral cortex. Representative images of each brain region are displayed in Figure 3-3. The thalamus consistently displayed the heaviest staining of all brain regions observed (Figure 3-3H) while staining was conspicuously absent from the hypothalamus (Figure 3-3G). The intensity of PrP^{Sc} staining in these regions appeared to roughly correspond with the density of spongiosis, however that relationship did not hold true for all brain regions examined (*e.g.,* cerebellum, dorsal medulla for vole E). Unlike the other brain regions examined, the hippocampus displayed a unique PrP^{Sc} immunostaining pattern for each of the three TSE-positive voles, with a multifocal stellate staining throughout multiple layers of the hippocampus of vole A (Figure 3-3M), no hippocampal staining in vole D (Figure 3-3N), and dense, granular staining largely restricted to the strata oriens, pyrimidale, and radiatum of the CA1, 2 and 3 regions of the hippocampus and the molecular and granular layers of the dentate gyrus of vole E (Figure 3-3O).

Discussion

Overall, meadow voles displayed incomplete attack rates (three sick animals of six challenged) and lengthy survival times, ranging 354 – 593 dpi, following challenge with scrapie from homozygous *Prnp* $V^{136}R^{154}Q^{171}$ genotype sheep. Our data demonstrate the presence of an interspecies transmission barrier between meadow voles and sheep scrapie. The variability of lesion profiles and survival times for scrapie-challenged voles in this study may hint at heterogeneity within the sheep isolates used, but definitive evidence supporting the existence of multiple strains in our challenge studies was not identified. The variation observed in lesion profiles for the three TSE-positive voles may alternatively reflect the non-adapted state of the scrapie agent in meadow voles. Similar variation has been observed upon primary passage of scrapie isolates to laboratory mice in classical scrapie strain-typing studies (Bruce, 2003). Subsequent passages of these TSE agents in genetically-identical mice selects for "pure" strains with stabilized incubation periods and convergent lesion profiles (Bruce, 2003).

Glycoform analysis revealed that passage of scrapie in meadow voles retained the overall biochemical profile of the ovine inocula (Figure 3-1). The PrP^{res} glycosylation site occupancy ratios for white-tailed deer CWD passaged in meadow voles differ significantly from those for scrapie in meadow voles. (Figure 3-1B)(Heisey et al., 2010). The glycoform profile data, in combination with differences in attack rate between CWD and scrapie passaged in meadow voles, suggest that each of these TSEs produce a different strain in meadow voles. Additional subpassages of scrapie in meadow voles may select for pure strains and is an interesting future direction.

Immunostaining of PrP^{Sc} in scrapie-positive meadow voles indicated a consistent granular deposition pattern across nearly all brain regions examined with some animal-to-animal variation (Table 3-1, Figure 3-3). The hippocampus, however, was an exception in that it displayed completely different PrP^{Sc} staining patterns for all three TSE-positive meadow voles. In contrast to these results, identical brain regions were reported to contain similar amounts of PrP^{Sc} immunolabeling in scrapie-infected bank voles (Di Bari et al., 2008). Differences in the staining in our study may reflect variation in the strains affecting each vole or the outbred nature of both meadow voles and bank voles used in these studies. Overall, the distribution of PrP^{Sc} staining in scrapie-positive meadow voles was remarkably similar to that of published results for scrapie-challenged bank voles despite that our two studies used scrapie isolates of different sheep genotypes. The similarity in our results could suggest that both species of voles select for similarly-compatible strains or conformers of PrP^{Sc} that may be commonly found in scrapie-infected sheep. Also interesting in light of our results in meadow voles are the completely dissimilar PrP^{Sc} distributions and staining patterns for scrapie-challenged ovinized Tg338 mice, in which labeling is characterized by plaques, intraneuronal PrP^{Sc} deposition and involvement of the hypothalamus (Beck et al., 2013).

Compared to bank voles, meadow voles displayed much longer survival times and lower attack rates when challenged with sheep scrapie. Some of these differences may be due to differences in host genetics or scrapie isolates. Our results suggest that meadow voles are not as susceptible to sheep scrapie as are bank voles or transgenic ovinized mice. Similarly, our results argue that meadow voles are unlikely to acquire scrapie under natural conditions and serve as a disease reservoir.

Figures, Tables, and Legends

Figure 3-1. Meadow vole-passaged scrapie retains biochemical profile of ovine inocula. (A) Immunoblotting confirms the presence of proteinase K-resistant prion protein in the brains of sheep used for vole challenges and three (voles A, D, and E) of six challenged meadow voles. (B) Triplots of glyocoform ratios for sheep scrapie, cervid CWD and their meadow vole-passaged counterparts. Colored dots represent geometric centers for the groups identified in the key. Colored outlines represent 95% confidence intervals for each group. CWD data are from Heisey *et al.* 2010.

Figure 3-1

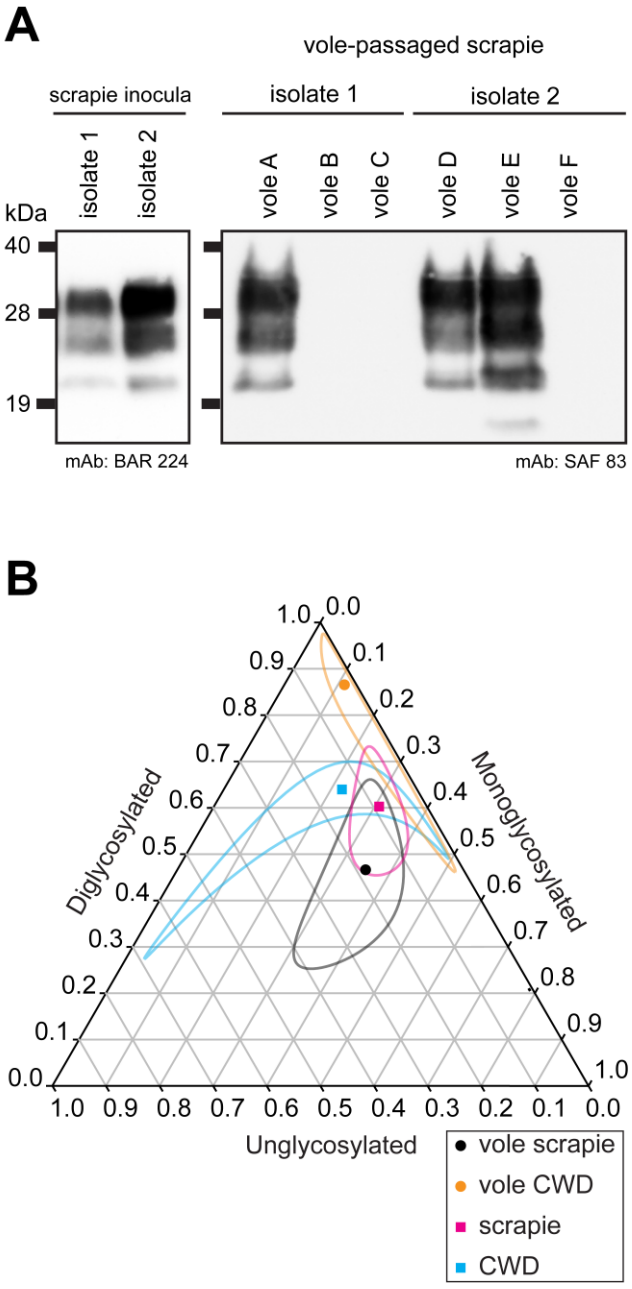
A
vole-passaged scrapie
scrapie inocula
isolate 1
isolate 2
isolate 1
isolate 2
vole A
vole B
vole C
vole D
vole E
vole F
kDa
40
28
19
mAb: BAR 224
mAb: SAF 83
B
Diglycosylated
Monoglycosylated
Unglycosylated
vole scrapie
vole CWD
scrapie
CWD

Figure 3-2. Lesion profiles from brains of scrapie-challenged meadow voles.

Neuroanatomic brain regions were scored for severity of vacuolation in clinically-diseased meadow voles. Regions were: 1 – dorsal medulla, 2 – cerebellar cortex, 3 – superior colliculus, 4 – hypothalamus, 5 – thalamus, 6 – hippocampus, 7 – septal nuclei of the paraterminal body, 8 – cerebral cortex anterior to 7, and 9 – cerebral cortex dorsal to the corpus callosum. Each line depicts the lesion profile for a single animal ± standard deviations of lesion severity scores reported by two independent scorers.

Figure 3-2

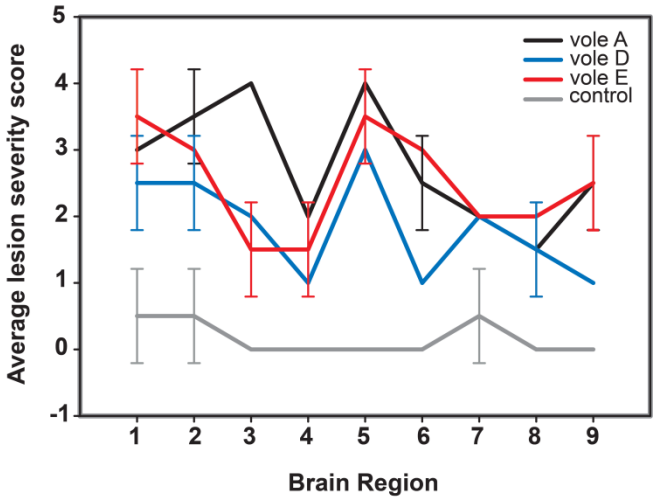
Average lesion severity score
5
4
3
2
1
0
-1
1
2
3
4
5
6
7
8
9
Brain Region
vole A
vole D
vole E
control

Figure 3-3. Immunohistochemical detection of PrP^{Sc} in meadow vole brains.

Sagital sections of brains from clinically-diseased and uninfected meadow voles were immunostained for PrP^{Sc} using mAb SAF 83. Representative images for the following brain regions for infected and control voles are shown. (A) infected and (D) uninfected dorsal medulla. (B) infected and (E) uninfected cerebellum (arrow indicates location of inset image from the granular layer). (C) infected and (F) uninfected superior colliculus. (G) infected and (J) uninfected hypothalamus. (H) infected and (K) uninfected thalamus. (I) infected and (L) uninfected cerebral cortex. Marked differences in PrP^{Sc} deposition were observed in the hippocampus of individual scrapie-challenged voles: vole A (M), vole D (N), and vole E (O). Uninfected voles showed no PrP^{Sc} staining in the hippocampus (not shown). Scale bars = 500 μm and are equally applicable to both images in each infected-noninfected set.

Figure 3-3

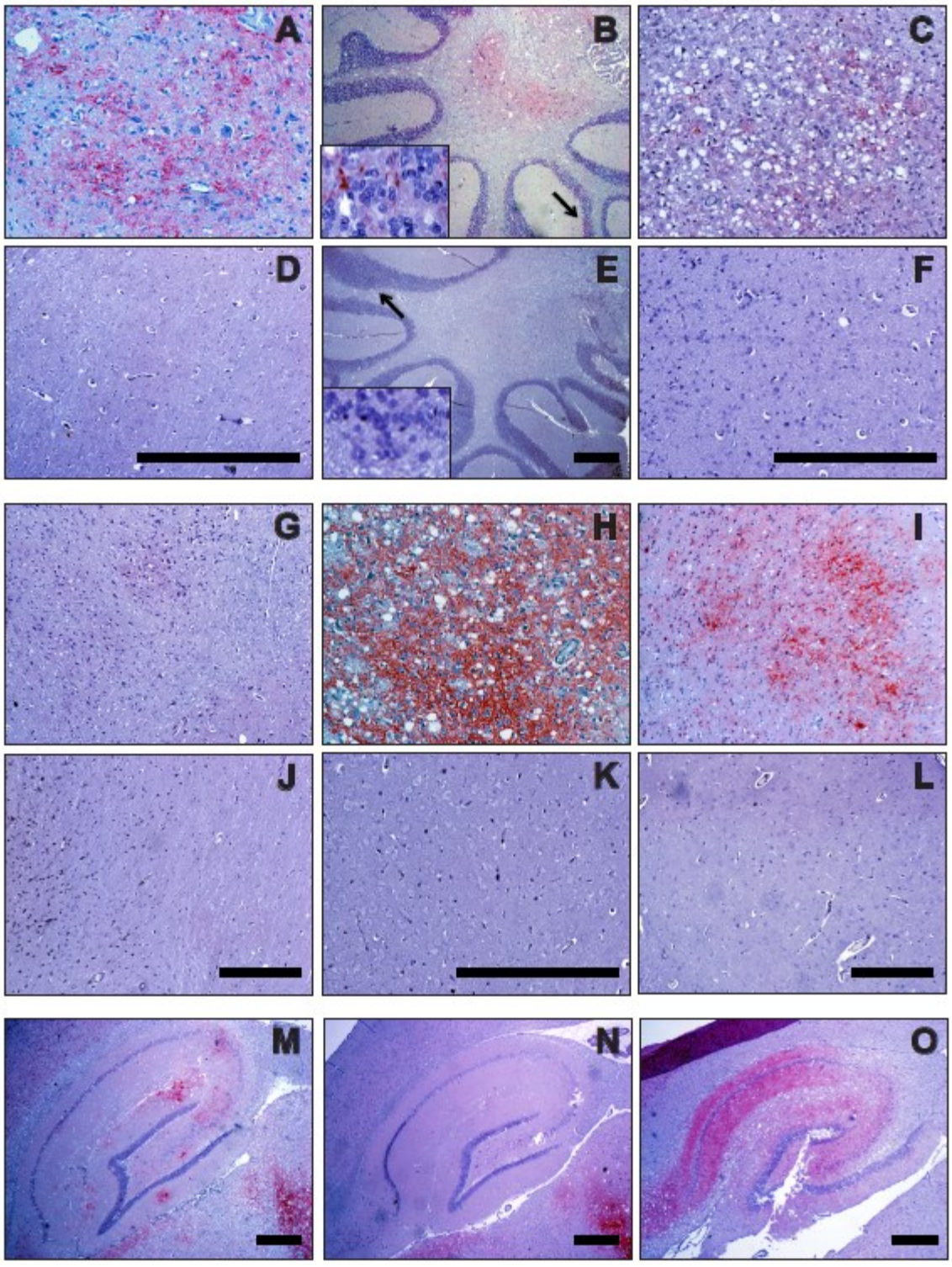

Table 3-1. Survival times of meadow voles infected with scrapie agent

Table 3-1. Survival times of meadow voles infected with scrapie agent[a]

Inoculum	Vole	Survival time, *dpi*	Clinical signs	PrP^{res} in brain
Sheep scrapie,				
isolate 1	A	593	+	+
	B	571	–	–
	C	653	–	–
Sheep scrapie,				
isolate 2	D	354	+	+
	E	483	+	+
	F	304	–	–

[a] Survival time reported as time from inoculation to death

Clinical signs and brain PrP^{res}: +, positive; –, negative

Table 3-2. Average PrP deposition patterns for scrapie-challenged meadow voles

Table 3-2. Average PrP deposition patterns for scrapie-challenged meadow voles

Brain region	PrP deposition score	PrP deposition pattern
Brainstem		
Dorsal medulla	+	granular
Cerebellum		
Molecular layer	-	-
Purkinje layer	-	-
Granular tracts	+	focal granular deposits
White matter tracts	+	granular, diffuse
Superior colliculus	+	granular
Hypothalamus	-	-
Thalamus	+++	dense, granular
	vole A/vole D/vole E	
Hippocampus	- / + / ++	Variable (Figure 3M, N & O)
Septal nuclei	++	granular
Cerebral cortex	++	granular; small aggregates

–, no staining; +, weakly positive; ++, moderately positive; +++, strongly positive; ++++, intensely positive

Materials and Methods

Animals

Meadow voles were raised from wild-caught progenitors in a colony maintained at the USGS National Wildlife Health Center (Madison, WI, USA). All animals were handled in strict accordance with good animal practice, as defined by the NIH Office of Laboratory Animal Welfare, and all live animal work was reviewed and approved by the USGS National Wildlife Health Center Animal Care and Use Committee protocol #EP080715-A2.

Study sample preparation and vole inoculations

Two isolates of sheep scrapie agent from brains of clinically-positive, homozygous *Prnp* V136R154Q171 genotype (Genbank accession number: CAA04276.1) sheep maintained at the University of Idaho Caine Veterinary Teaching Center (Caldwell, Idaho, USA) were used in this study. Six 4-to-8-week-old meadow voles were anesthetized with isofluorane and inoculated intracerebrally (i.c.) with 20 µL of a 10% w/v brain homogenate in PBS made from one or the other scrapie isolate; each inoculum was prepared from a single source, not pooled. Animals were monitored daily for disease progression. Any voles with intercurrent illnesses were excluded from data analysis; all others were euthanized when they exhibited significant clinical impairment and neurological signs of disease consistent with TSE (including circling, lethargy, poor balance, thinning of the waist, and lack of motivation to burrow and/or acquire food and water). Survival time was defined as the number of days from inoculation to euthanization. Brains were harvested immediately post-mortem and divided sagittally, with one half prepared for evaluation by Western blotting and the other half fixed in 10% neutral-buffered formalin for

histopathology and immunohistochemistry (IHC); all challenged animals in each passage group were tested for the presence of PrP^{Sc} in brain tissue by Western blotting, IHC, or both.

Western blotting

Brain tissue was initially prepared as a 20% w/v homogenate in PBS and subsequently diluted 1:1 in PBS. Homogenates were then digested with proteinase K (PK; 50 μg/mL final concentration; Sigma-Aldrich, St. Louis, MO, USA) for 1 hr at 37 °C, followed by the addition of Pefabloc SC (2 mM final concentration; Roche Diagnostics, Indianapolis, IN, USA) for 30 min at room temperature to halt reactions. SDS-PAGE and Western blotting were carried out as previously described (Heisey et al., 2010) using primary monoclonal antibodies (MAb) SAF 83 and BAR 224 (Cayman Chemical Company, Ann Arbor, MI) for vole and sheep samples, respectively, and secondary goat anti-mouse immunoglobulin G conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), both diluted 1:10,000 in 5% milk in Tris-buffered saline plus 0.5% Tween 20 (TTBS). Immunoreactivity was detected using an enhanced chemiluminescent detection system (Thermo Fisher Scientific, Inc., Rockford, IL, USA) in a UVP EC3 Imaging System (UVP Bioimaging Systems, Upland, CA, USA). Densitometric analyses were performed using VisionWorks LS software version 606a (UVP Bioimaging Systems, Upland, CA, USA).

Histopathology and immunohistochemistry

Whole vole brains or hemispheres were fixed in 10% phosphate-buffered formalin for ≥48 hr, embedded in paraffin, sectioned to 5 μm, and mounted onto positively charged glass

slides. Tissue sections were deparaffinized and rehydrated through a graded ethanol series. For hematoxylin and eosin (H&E)-stained tissues: sections were submerged in Harris hematoxylin (Thermo Fisher Scientific, Inc, Rockford, IL, USA) for 3 min, washed with deionized water, submerged in eosin Y stain (Ricca Chemical Company, Arlington, TX, USA) for 1 min, washed, dehydrated, and coverslipped. Immunostaining for PrP^{Sc} followed a previously described procedure (Hoefert et al., 2004). Briefly, following rehydration, endogenous peroxidase activity was quenched by incubation in 0.3% hydrogen peroxide for 30 min. Antigen retrieval was achieved by autoclaving tissues in 100 mM sodium citrate buffer, pH 6.0, for 30 min, followed by submersion in 88% formic acid for 10 min and then in 4 M guanidine isothiocyanate for 2 hr. Tissues were then blocked with 1.5% v/v normal goat serum (Vector Laboratories, Burlingame, CA, USA) for 1 hr and stained overnight at 4°C using primary MAb SAF83 (1:5,000 final dilution), followed by incubation with biotinylated horse anti-mouse immunoglobulin G (1:200 final dilution) for 30 min. Detection was completed using horseradish peroxidase-mediated hydrogen peroxide immunostaining (NovaRed; Vector Laboratories, Burlingame, CA, USA). Sections were counterstained with Harris hematoxylin, dehydrated, and coverslipped. All tissues were imaged using a Leica DM 2000 light microscope equipped with a Leica DFC280 camera and visualized using the Leica Application Suite, version 2.8.1 (Leica Microsystems, Ltd., Switzerland).

Lesion profiling and PrP^{Sc} deposition patterning

Brain lesion profiling was performed using the method of Fraser and Dickenson (Fraser and Dickinson, 1968), with slight modifications. Briefly, nine neuroanatomic regions were

scored in H&E-stained medial sagittal brain sections from at least three voles for each passage group: 1 – dorsal medulla, 2 – cerebellar cortex, 3 – superior colliculus, 4 – hypothalamus, 5 – thalamus, 6 – hippocampus, 7 – septal nuclei of the paraterminal body, 8 – cerebral cortex anterior to 7, and 9 – cerebral cortex dorsal to the corpus callosum. Two investigators blindly scored each region for intensity of vacuolation using the following severity scale: (0) no vacuoles or vacuolation comparable to the same region in uninfected, healthy brain, (1) a few vacuoles, widely and unevenly scattered, (2) a few vacuoles evenly scattered, (3) moderate numbers of vacuoles, evenly scattered, (4) many vacuoles with some confluence, and (5) dense vacuolation with most of the field confluent; representative images of each severity rating are provided in Figure 2-6. The average of the three scores constitutes the severity score for each region and these scores were used to construct lesion profiles of spongiform degeneration in CWD-challenged voles. Additionally, PrP^{Sc} deposition patterns were recorded by i) identifying the localization and pattern of PrP^{Sc} expression within each of the nine above-listed brain regions and corresponding subregions and ii) scoring intensity of PrP^{Sc} immunostaining, using a semi-quantitative five-point scale: (0) no staining; (1) weakly positive staining; (2) moderately positive staining; (3) strongly positive staining; (4) intensely positive staining. At least three brain sections from each of three to four voles were stained for every passage. Representative images of each severity rating are provided in Figure 2-7.

Acknowledgements

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CHAPTER FOUR:

Plants as a Transmission Route for Infectious Prions

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Plants as a Transmission Route for Infectious Prions

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Abstract

As unconventional infectious agents remarkably resistant to inactivation and capable of maintaining infectivity for years to decades, prions pose a significant environmental contamination problem now and into the future. Prion protein binds strongly to soil particles and some soils increase oral prion infectivity, suggesting soil as a likely reservoir for prion diseases that have an environmental component to their transmission, such as sheep scrapie or cervid chronic wasting disease. Overall, routes of environmental prion transmission are not well understood. In this study, we demonstrate that infectious prions are taken up by various plant species and that infectivity is maintained in leaf and stem tissues as determined by bioassay in mice. Furthermore, mice fed prion-contaminated plants also developed prion disease, suggesting that environmental prion contamination of plants may represent a previously unrecognized risk for human, domestic animal and wildlife species exposure to chronic wasting disease and scrapie agents.

Introduction

Prions are proteinaceous infectious agents that cause incurable, invariably fatal neurodegenerative disease (prion disease or transmissible spongiform encephalopathy, TSE) in mammalian hosts, including human Creutzfeldt-Jakob disease, bovine spongiform encephalopathy, sheep and goat scrapie, and chronic wasting disease (CWD) of cervids (deer, elk, moose species). Prions are composed primarily, if not exclusively, of a misfolded host protein: the TSE-associated prion protein (PrP^{TSE}) (Prusiner, 1998). Whereas the cellular prion protein (PrP^{C}), the precursor to PrP^{TSE}, is labile, PrP^{TSE} and TSE infectivity are remarkably resistant to degradation; the fraction of PrP^{TSE} that specifically resists proteinase K (PK) digestion is denoted PrP^{res} (Bolton et al., 1982). Both PrP^{TSE} and TSE infectivity accumulate in a variety of host tissues and can be shed from infected cervids and sheep. Shedding of CWD and scrapie infectivity permits transmission of these diseases to naïve hosts via both direct (animal-to-animal) and indirect (environmentally- mediated) modes of transmission (Mathiason et al., 2009; Mathiason et al., 2006; Miller and Williams, 2003; Miller et al., 2004). Secretions, excreta, and the carcasses of dead, infected animals all release infectivity into the environment and numerous studies have found that prions are stable in the environment for years (Brown and Gajdusek, 1991; Georgsson et al., 2006; Miller et al., 2004; Seidel et al., 2007). Infected deer release large amounts of CWD infectivity months prior to the onset of clinical disease (Gregori et al., 2008b; Mathiason et al., 2006; Miller et al., 2004; Tamguney et al., 2009). Soil is the most likely reservoir of prions in the environment and our previous studies have shown that prions bind to soil and remain infectious (Johnson et al., 2006). Notably, we have also found that soil

minerals and other microparticles increase the oral transmissibility of prions (Johnson et al., 2007).

In environments likely to be contaminated with CWD, vegetation is ubiquitous. Plants absorb a variety of substances from soil, ranging from nutrients to contaminants. In the soil of natural ecosystems, nitrogen occurs predominantly as amino acids and/or protein and the uptake of proteins by plants was actively studied in the 1960s and 1970s to address organic nitrogen metabolism (Nasholm et al., 2009; Read and Perez-Moreno, 2003; Tabatabai and Senwo, 1998). In two seminal studies, Nishizawa and Mori clearly demonstrated that hemoglobin can serve as a sole nitrogen source for rice and barley growth and provided evidence indicating protein uptake into roots by endocytic pathways (Nishizawa and Mori, 1977; Nishizawa and Mori, 1980a). Several other *in vitro* studies have demonstrated uptake of bovine serum albumen (Paungfoo-Lonhienne et al., 2008), lysozyme (McLaren et al., 1960), ribonuclease (Bhide and Brachet, 1960; Brachet, 1954), ovalbumin (Starkey, 1958), Cry1Ab protein (Icoz et al., 2009), and green fluorescent protein (GFP) (Paungfoo-Lonhienne et al., 2008) by plant roots. When grown in soil amended with biotinylated proteins, crop plant root tissue sections stain positive with avidin (Koga, 2006). Taken together, these reports support the concept that plants absorb proteins. To determine plant uptake and transmission of infectious prion protein, we exposed the model plant *Arabidopsis thaliana* and several crop species to prion-contaminated hydroponic, agar, and soil media and tested for the presence of prion protein and infectivity by confocal laser scanning microscopy (CLSM), protein misfolding cyclic amplification with beads (PMCAb) and rodent bioassay.

Results

Root cells take up prion protein

We first investigated the internalization of whole protein by roots by incubating *A. thaliana* roots in a hydroponic solution containing green fluorescent protein (GFP). After 24 h incubation, GFP was observed inside root hairs by CLSM (Fig. 4-1A and B), providing evidence of intact protein entry into root cells. Plants were next exposed to fluorescently-tagged PrP^{res} that had been enriched using a novel prion preparation that avoids processes that hyper-aggregate PrP and better mimics prions in the environment, as compared to currently available prion purification methods (method described in full in materials and methods). After 24 hr incubation, fluorescently tagged PrP^{res} was observed adhered to the outer surfaces (Fig. 4-1C and D) and inside intact root hairs (Fig. 4-1E and F) of *A. thaliana*, alfalfa (*Medicago sativa*) (Fig. 4-1I and J), barley (*Hordeum vulgare*) (Fig. 4-1K and L), and tomato (*Solanum lycopersicum*) (Fig. 4-1M and N), demonstrating that plants with different root structures and specifications, specifically non-mycorrhizal *Arabidopsis* and mycorrhizal crop plants, were able to uptake whole prion protein. PrP^{res}-associated fluorescence was also observed in cross-sections of *A. thaliana* stems (Fig. 4-1G and H), in regions consistent with the location of vascular structures, suggesting prion protein transport to aerial tissues. Detection of fluorescently-tagged PrP^{res} in *Arabidopsis* stem tissues was infrequent and consistently presented as few scattered focal points of fluorescence associated with epidermal, cortex, or vascular structures; fluorescence was never observed in the pith. This low abundance of PrP^{res} in stem tissues, as compared to relatively abundant staining in roots may suggest plant turnover of PrP^{res} in roots, or sequestration in other tissues.

PMCAb detection of prion protein in aerial plant tissues

To confirm whether prion protein entered aerial plant tissues, combined stem and leaf homogenates from plants hydroponically grown on prion containing media were subjected to serial PMCAb to amplify and detect prion protein taken up in tissues. Plants were grown using an adaptation of the Ice-Cap liquid culture method developed by Krysan et al. (Clark and Krysan, 2007), which allowed only roots to directly contact prion-containing hydroponic media and prevented cross-contamination of aerial plant tissues (described in materials and methods). PMCAb is an exquisitely sensitive prion amplification and detection method (detection limit as low as 26 molecules or 1.3×10^{-9} ng PrP^{TSE}) that is conceptually similar to the polymerase chain reaction (PCR) for nucleic acids, but exploits the ability of PrP^{TSE} to catalyze the misfolding of PrP^{C} to amplify PrP^{TSE} *in vitro* (Gonzalez-Montalban et al., 2011; Saa et al., 2005; Saa et al., 2006). PMCAb generated detectable levels of PrP^{res} following three 96-cycle rounds of amplification of source plant tissue from *A. thaliana* (Fig. 2A), corn (*Zea mays*) (Fig 2B), and alfalfa (*Medicago* sativa) (Fig. 2C). Using the quantitative PMCA method of Chen et al. (Chen et al., 2010), we estimated the concentration of PrP^{TSE} in each of these plant species. For corn, PMCAb generated detectable levels of PrP^{res} in two 96-cycle rounds from a 10^{-1} dilution of a 50% plant homogenate, equivalent to 129.1 fg/mL PrP^{TSE} in the original plant homogenate. PrP^{res} in both *Arabidopsis* and alfalfa were detectable after three rounds of PMCAb, equivalent to 0.4 fg/mL PrP^{TSE} in the original plant homogenate. These PrP^{TSE} concentrations may reflect overestimates of the true PrP^{TSE} content in plant homogenate as they are calculated from standard curves generated from PMCAb amplification of serial dilutions of 10% hyper-infected hamster brain homogenate, for which the concentration of PrP^{TSE} (~6 μg/mL) and PMCAb

amplification efficiency may not be similar to that of prion-contaminated plant homogenate (Gregori et al., 2008a).

Prions taken up by plants remain infectious to mammalian hosts

It is hypothesized that plants internalize amino acids, peptides, and proteins as a source of nitrogen, and studies have shown that, once inside the plant, nitrogen from these organic forms becomes transferred largely into amino acids required for plant metabolism and transferred throughout root and shoot tissues (Nasholm et al., 2009). Thus, assimilation of organic nitrogen by plants would require catabolism of acquired proteins and peptides. If plants are able to catabolize infectious prion protein, a reduction or loss of infectivity would be expected, as degradation and denaturation renders the protein non-infectious (Caughey et al., 1997). We therefore tested whether PrP^{TSE} taken up by *Arabidopsis thaliana* plants 1) was transported into shoot tissues and 2) remained infectious by intracerebrally inoculating CD1 Swiss mice with homogenates made from stem and leaf tissues of *Arabidopsis* plants grown on prion-containing agar or liquid medium. Hydroponically grown *Arabidopsis* displayed higher infectivity in mice as compared with agar grown *Arabidopsis* (Fig. 4-3), as evidenced by earlier clinical disease onset (median incubation period ± 95% CI = 215±17 dpi for hydroponically grown stems and 243±39 dpi for hydroponically grown leaves vs. 284±53 dpi for agar grown stems and 338±0 dpi for agar grown leaves) and higher attack rates (number clinically diseased mice (n)/number of total mice challenged (n_0) = 16/20 for hydroponically grown stems and 13/18 for hydroponically grown leaves vs. 7/21 for agar grown stems and 1/12 for agar grown leaves). Control mice challenged with non-prion contaminated *Arabidopsis* tissue remained asymptomatic throughout

the course of the experiment (data not shown). These data suggest that prion uptake from a solid-state medium (agar) is less efficient than from a liquid medium, and, importantly, in both culture systems, prion infectivity is taken up by roots of *Arabidopsis* plants and transported to aerial tissues where it maintains the ability to cause prion disease in mammalian hosts.

Consumption of prion-contaminated plant tissues causes transmissible spongiform encephalopathy

To examine the effect of plant tissue on the oral transmissibility of prions, RML prions in the form of infected mouse brain homogenate were spiked with *A. thaliana* homogenate and administered to mice by oral gavage; controls included RML prions lacking plant spike. Four doses of RML brain homogenate (10%, 1%, 0.1%, 0.01%) were assayed. Diminished gastrointestinal bioavailability was expected to be evidenced by significant lengthening of incubation period, reduced disease penetrance, or both. Compared to plant-free controls, oral administration of RML mixed with *A. thaliana* shoot tissue homogenate decreased disease penetrance and lengthened incubation times at all doses. To quantify the contributions to changes in relative risk of prion dose and agent interaction with plant tissue, we constructed a multivariate Cox proportional hazards model with two covariates: $\log_{10}PrP^{TSE}$ dose and plant presence (Table 4-1). Each $\log_{10}$ increase in PrP^{TSE} dose multiplies the relative risk by a factor of 1.43 (i.e. a 10-fold increase in dose increases the risk of infection by approximately one-and-one-half times). Notably, addition of *A. thaliana* tissue to RML agent multiplies the relative risk by a factor of 0.18, thus reducing overall infective titer significantly. In other words, the relative risk

of infection by RML is multiplied by a factor of 5.5 in the absence of plant tissue, as compared to the presence of plant tissue.

We next examined the oral infectivity of plants grown on prion-containing media using two different oral inoculation methods. First, mice were orally gavaged with homogenates of stem and leaf tissue from *A. thaliana* hydroponically grown on media containing RML-infected or non-infected (controls) mouse brain homogenate. Mice received either a single dose of plant homogenate (equivalent to ~100 μg dry weight of *A. thaliana* tissue) or one dose per day for five consecutive days. For the second method of inoculation, mice were free-fed raw plant tissue from alfalfa, barley, or corn that had been hydroponically grown on media containing RML-infected or non-infected (controls) mouse brain homogenate. Each mouse received an identical dose of alfalfa, barley, or corn (0.14 g) every day for 30 days. As of 1 December 2013, these oral challenge studies are ongoing with the findings to date summarized in table 4-1. Of the 28 mice orally gavaged with a single dose of RML grown *Arabidopsis*, six animals to date have developed clinical TSE, confirmed by detection of PrP^{res} by immunoblot (data not shown), with a current median survival time ± 95% CI = 455 ± 24 dpi. Of the 32 mice orally gavaged with five consecutive doses of RML grown *Arabidopsis,* a single animal to date has developed clinical TSE at 371 dpi. Four of 26 total mice orally free-fed RML-contaminated maize shoot tissues have developed clinical TSE at 406 ± 47 dpi. Control mice challenged with non-prion contaminated plant shoot tissue have thus far remained asymptomatic throughout the course of the experiment. These data establish that prions taken up by *Arabidopsis* and maize remain infectious via the oral route of exposure, and that agent interaction with plant tissue decreases disease penetrance, reducing the efficiency of oral transmission.

Discussion

Collectively, these data establish that infectious prions are internalized by plants via roots, and are transported and maintained within above-ground plant tissues in sufficient quantity to cause disease in mammalian hosts by consumption, thereby implicating plants as a novel potential transmission route for prions in the environment. While extrapolation of these results to environmental conditions must be made with care, the specific finding of prion uptake by various widely-consumed crop species, including alfalfa, barley, corn and tomato, directly highlights the potential for a previously unrecognized food safety risk for prion exposure that extends beyond wildlife, and into domestic animals and humans, for whom these crops are primarily produced.

Interestingly, the interaction between plant tissue and prions significantly reduced the relative risk of infection (Table 4-1). Despite this, *Arabidopsis* and maize plants harbored sufficient infectivity to cause clinical TSE in mice via both i.c. and oral exposure routes. The concentration of PrP^{TSE} estimated in *A. thaliana,* maize and alfalfa plants ranged between 0.4 – 129.1 fg/mL of a 50% w/v plant homogenate, and crude infectivity estimates based on the i.c. challenge data suggest that each hydroponically-grown *A. thaliana* plant (16 mg wet weight) contained >1 i.c. LD_{50} of infectivity while each agar-grown *A. thaliana* plant contained <1 i.c. LD_{50}. The reason for reduced oral transmissibility associated with prion interaction with *A. thaliana* tissue remains to be elucidated. One possibility is that *Arabidopsis* plants may contain proteases capable of degrading infectious PrP^{TSE}. The *Arabidopsis* genome encodes 828 proteases, which function both within plant tissues and as secreted enzymes in metabolism and nutrient acquisition (Godlewski and Adamczyk, 2007; Hamilton et al., 2003; Paungfoo-Lonhienne et al., 2008; Paungfoo-Lonhienne et al., 2010; Tornero et al., 1996). It is possible that

one or a combination of plant proteases is capable of degrading PrP^{TSE} and associated infectivity. Prion binding to indigestible fiber components of plant tissue, including cellulose, chitin, lignin, among others, which are not broken down in the monogastric rodent gut, may facilitate clearance through the gastrointestinal tract and reduce translocation of infectious agent from the gut lumen into the body.

The amount of prion protein and infectivity taken up and harbored by plants used in this study may be higher than that taken up by plants in the environment due to the simplified plant culture systems we employed. It is possible that in the soils of natural ecosystems, microorganisms and soil-inhabiting invertebrates, as well as other factors, may reduce the amount of infectious prion available for plant uptake in a natural prion contamination site. CWD-infected deer shed large amounts of infectivity; in a recent study, it was demonstrated that the total titer shed from CWD-infected mule deer during its lifespan may be approximately equal to the total titer contained in an infected carcass (Tamguney et al., 2009). While it is known that CWD and scrapie prions are stable in the environment for years to decades (Brown and Gajdusek, 1991; Georgsson et al., 2006; Miller et al., 2004), and that soil serves as a likely reservoir for environmentally shed prions (Johnson et al., 2007; Johnson et al., 2006), there is little known about the long-term fate of prions in soil and whether their uptake by plants would be plausible in environmental settings. Currently, we are testing the uptake of prions from four different soil types (Ottawa sand, Bluestem clay, Plainfield soil, and potting soil) by *A. thaliana* in an attempt to begin to address some of these unknowns.

The mechanism by which plants are able to take up whole prion protein remains to be determined. While historically thought to be inaccessible to plants, a number of studies

demonstrate that organic forms of nitrogen in the soil, including whole proteins, are taken up by plants and catabolized (Nasholm et al., 2009; Paungfoo-Lonhienne et al., 2008; Paungfoo-Lonhienne et al., 2010), likely as an alternative or supplement to more readily accessible inorganic nitrogen (ammonia and nitrates), when these sources become depleted. Our observations of prion protein uptake primarily by root hairs is consistent with previous studies demonstrating protein uptake in plants and hypothesizing an endocytosis-mediated mechanism of uptake (Nishizawa and Mori, 1977; Nishizawa and Mori, 1980b; Paungfoo-Lonhienne et al., 2008). The major role for roots hairs is to increase the overall absorbing surface area of roots and to take up water and nutrients, as well as contribute to rapid cellular responses to intrinsic and extrinsic cues (Foreman and Dolan, 2001; Gahoonia and Nielsen, 1998; Greulach and Adams, 1967; Parker et al., 2000). To achieve this, plasma membrane recycling via endocytosis and exocytosis occurs with such frequency in root hairs that the entire surface area of the plasma membrane can turn over on an hourly basis, making root hairs key environmental sampling structures for the plant (Samaj et al., 2004; Samaj et al., 2005). Another possibility includes entry through natural breaks in roots caused by lateral root formation or breakage of root hairs (Agrios, 2005; Melotto et al., 2008).

In conclusion, our results provide compelling evidence in support of the hypothesis that plants serve as a currently unrecognized environmental transmission route for prion disease. While these studies found that the relative risk of prion infection was reduced in the presence of plant tissue, the mechanism by which infectivity is effectively reduced in the mammalian host and kinetics of prion persistence within plant tissues remain to be determined. Of additional interest is investigating whether plant uptake alters prions in other ways, e.g. altered strain

properties or host ranges. Although natural occurrences of cross-species transmission of CWD and scrapie prions have yet to be reported, the exposure risk from prion contaminated crops to humans, domesticated animals and other wildlife species warrants the investigation of zoonotic and other cross-species transmission potentials.

Figures, Tables, and Legends

Figure 4-1. Uptake of GFP and Alexa-fluor 488-tagged PrP^{res} by roots and stems of various plants.

Plants were grown for 1-2 weeks on 0.5× MS or LS agar medium and then transferred into a 50 μg/mL GFP or 5% or 0.5% Alexa-Fluor 488-tagged PrP^{res}-enriched RML-infected mouse brain homogenate for 24 hr, roots rinsed extensively and imaged by CLSM. Fluorescence was detected inside root hairs of *A. thaliana* incubated with GFP (B) and fluorescently-tagged PrP^{res} (F), as well as inside root hairs of the following plants incubated with tagged PrP^{res}: alfalfa (J), barley (L), tomato (N). PrP^{res}-associated fluorescence was also observed adhered to the surface of root hairs (D) and inside stem tissues (H) of *A. thaliana.* No fluorescence was observed in corresponding control plants that were exposed to non-GFP or non-prion containing hydroponic solutions and likewise visualized by CLSM (A, C, E, G, I, K, M). Scale bar = 20 μm.

Figure 4-1

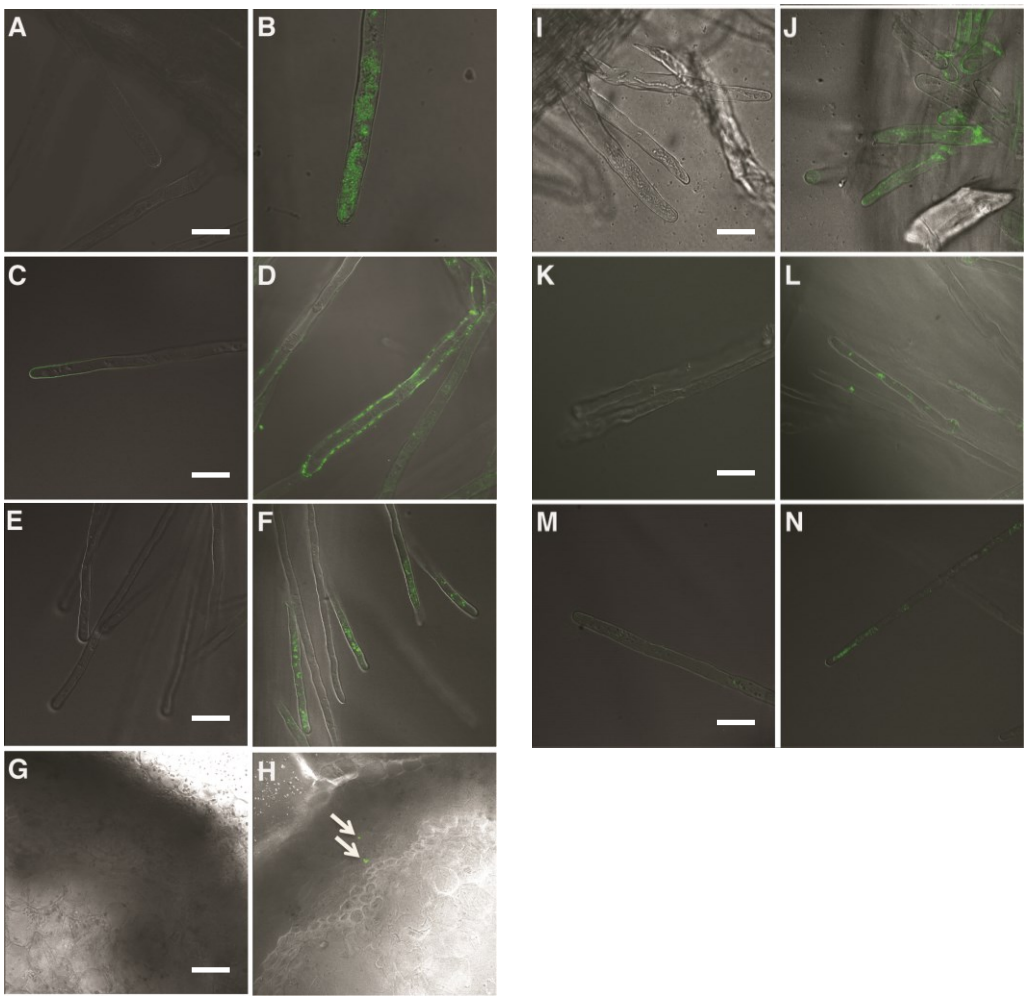

Figure 4-2. PMCAb amplification of PrP^{res} in prion contaminated plant tissues.

Plants were grown for 4 – 50 d on 0.1 – 2.0% HY-containing hydroponic media using the Ice-Cap method (Clark and Krysan, 2007) and aerial tissues harvested and homogenized. Ten μL seeds of 50% plant homogenates were used to seed 90 μL hamster NBH and subjected to three 96-cycle rounds of PMCAb. Results from the third round only are shown. PrP^{res}-positive bands were detected in prion-contaminated *A. thaliana* (A), corn (B), and alfalfa (C) (prion-contaminated plants denoted as "HY PX", where each unique number assigned to "X" refers to a unique plant). PMCAb controls included 1) various ten-fold dilutions of HY-positive 10% brain homogenate (dilution factors listed above each panel in the figure) that were used to seed hamster NBH (in the same 10:90 μL ratio as used for plant reactions) and 2) 100 μL hamster NBH to control for *de novo* prion synthesis during PMCAb (labeled "NBH" on figure panels) that were PMCAb assayed simultaneously with plant reactions. No PrP^{res} was detected in any samples assayed in which plants grown on non-prion infected media were used to seed PMCAb reactions (non-prion contaminated plants denoted as "PX" where each unique number assigned to "X" refers to a unique plant).

Figure 4-2

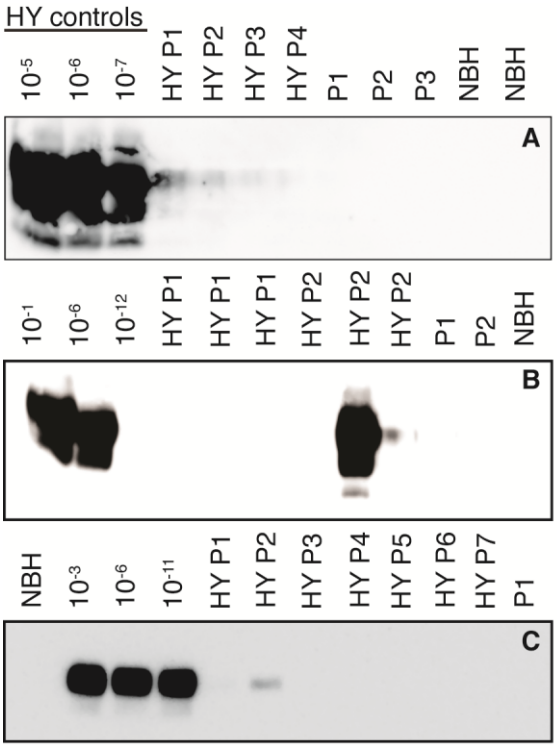
HY controls
10-5
10-6
10-7
HY P1
HY P2
HY P3
HY P4
P1
P2
P3
NBH
NBH
A
10-1
10-6
10-12
HY P1
HY P1
HY P1
HY P2
HY P2
HY P2
P1
P2
NBH
B
NBH
10-3
10-6
10-11
HY P1
HY P2
HY P3
HY P4
HY P5
HY P6
HY P7
P1
C

Figure 4-3. Intracerebral transmission of aerial tissues from *A. thaliana* grown on prion-contaminated media to mice results in TSE.

CD1 mice were intracerebrally inoculated with 20 μL of stem (17% w/v in mouse NBH) and leaf (56% w/v in mouse NBH) homogenate from *A. thaliana* grown either hydroponically (red lines) or on agar (black lines) containing RML prions. Data are represented as Kaplan-Meier survival curves; n = 19, 20 for hydroponically grown leaves and stems, respectively and n = 11, 21 for agar-grown leaves and stems, respectively. Mice receiving homogenate from *A. thaliana* grown on non-infected media remain asymptomatic throughout the experiment (data not displayed).

Figure 4-3

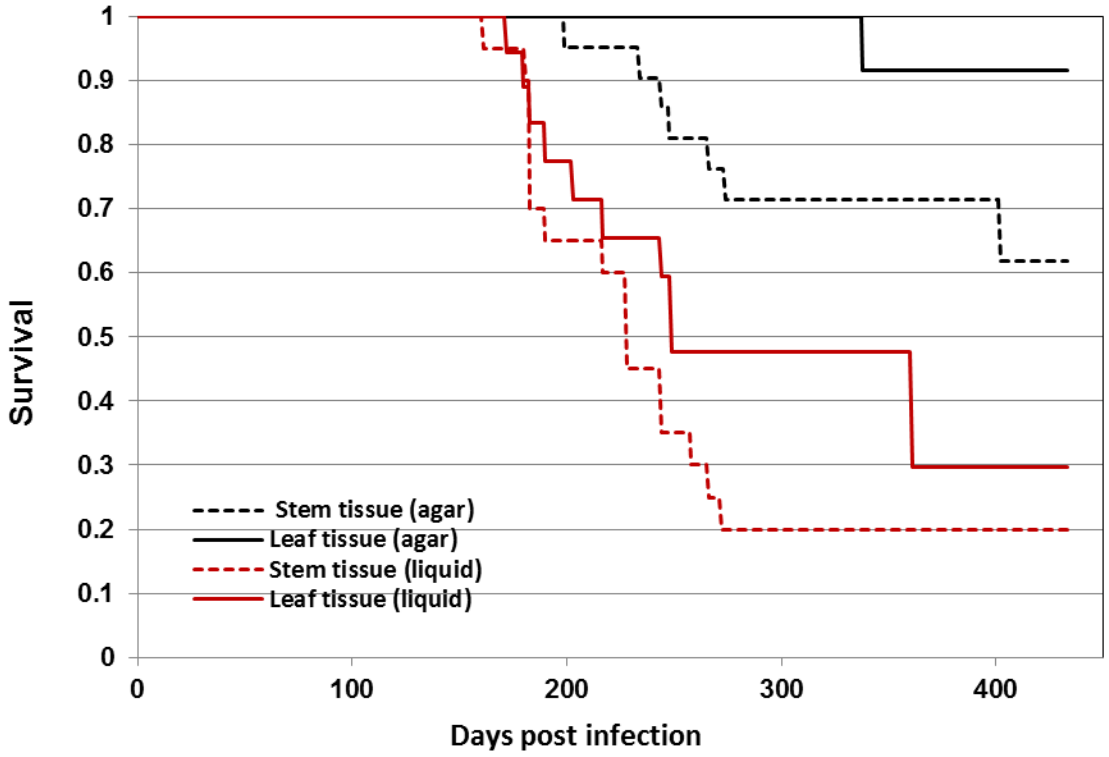
Survival
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
100
200
300
400
Days post infection
Stem tissue (agar)
Leaf tissue (agar)
Stem tissue (liquid)
Leaf tissue (liquid)

Figure 4-4. Concurrent peroral administration of *A. thaliana* and PrP^{TSE} reduces disease penetrance at agent doses that typically produce clinical symptoms.

Arial plant tissue decreases disease penetrance and lengthens incubation periods associated with oral exposure of 10, 1, 0.1, 0.01% RML-infected mouse brain homogenate. CD1 mice were orally gavaged with 150 µL of 10, 1, 0.1, or 0.01% RML infected mouse brain homogenate (lines in shades of grey) or RML-infected brain homogenate spiked with 100 mg (dry weight) of combined stem and leaf homogenate from axenically grown *A. thaliana* plants. Data are presented as Kaplan-Meier survival curves; $n = 8$ for all treatment groups except the 10% RML and 10% RML + plant groups for which $n = 4$.

Figure 4-4

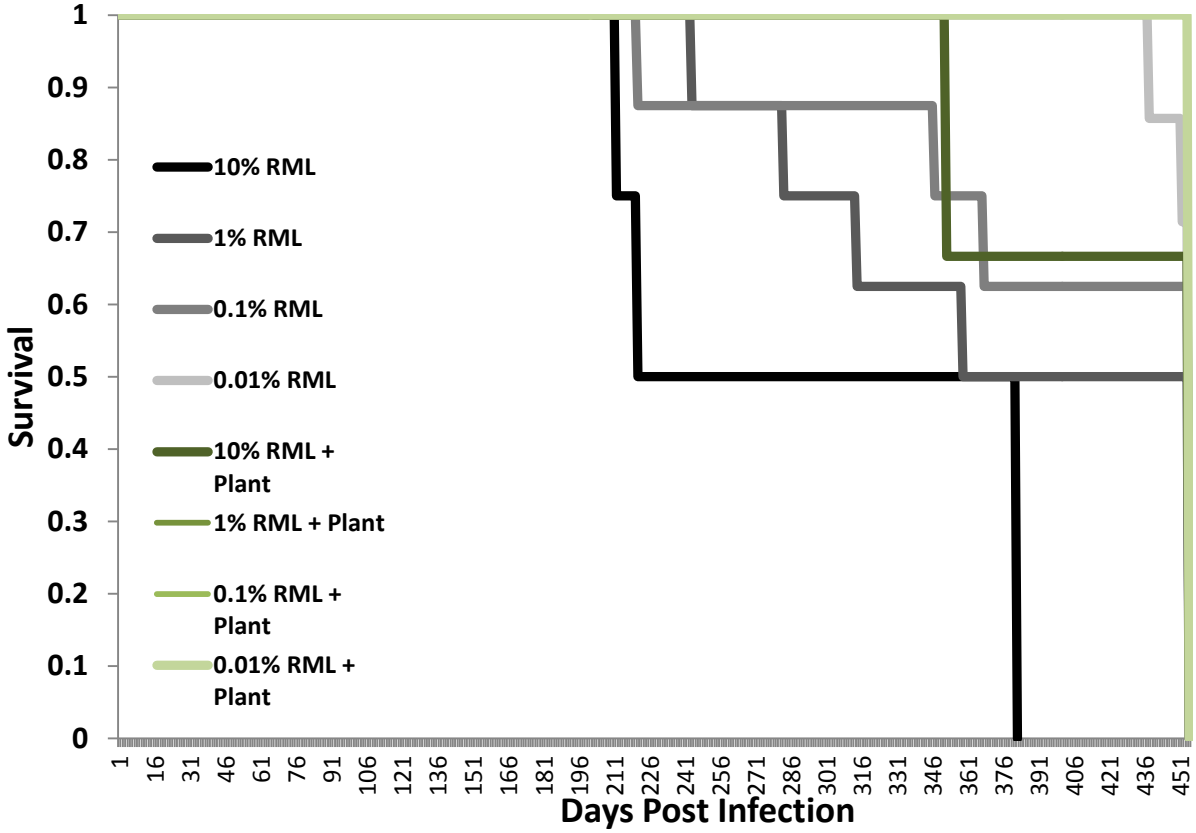
Survival
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10% RML
1% RML
0.1% RML
0.01% RML
10% RML + Plant
1% RML + Plant
0.1% RML + Plant
0.01% RML + Plant
1
16
31
46
61
76
91
106
121
136
151
166
181
196
211
226
241
256
271
286
301
316
331
346
361
376
391
406
421
436
451
Days Post Infection

Table 4-1

Table 4-1. Estimated hazard ratios due to prion dose and plant addition

Predictor	Estimated Hazard Ratio	95% CI	p-Value
RML BH (per $\log_{10}$ increase)	1.43	1.13, 1.80	0.0025
RML BH + absence versus presence of plant tissue	5.49	1.55, 19.4	0.0083

Table 4-2

Table 4-1. Survival times of mice orally challenged with prion contaminated plants[α†]

Inoculum	# doses	Median survival time, d (95% CI)	Clinical Disease, n/n_o
RML *Arabidopsis*	1	455 (431 - 479)	6/28
non-infected *Arabidopsis*	1	—	0/8
RML *Arabidopsis*	5	371 (–)	1/32
non-infected *Arabidopsis*	5	—	0/8
RML alfalfa	1	—	0/32
non-infected alfalfa	1	—	0/8
RML barley	1	—	0/28
non-infected barley	1	—	0/8
RML maize	1	406 (359 - 453)	4/26
non-infected maize	1	—	0/8

[α]Survival time reported as median time from inoculation to death, calculated using Kaplan-Meier statistics

†All oral experimental challenges currently in progress; median survival times and attack rates calculated as of 1 December 2013

d = days; CI = confidence interval, *n* = number of mice with neurological signs of TSE (confirmed PrP^{TSE} positive by immunoblotting); n_o = number of mice inoculated, mice with intercurrent illness excluded

Materials and Methods

Animals

All animal work was conducted at the National Wildlife Health Center in accordance with the following institutional animal care and use committee approved protocols: #EP080716-A3 and #EP090616B. Syrian golden hamsters and CD1 Swiss mice were purchased from Harlan Laboratories (Madison, WI).

Source of prion protein and brains

Hamster PrP^{TSE} was generated by the experimental infection of Syrian golden hamsters with the hyper (HY) strain of hamster-passaged transmissible mink encephalopathy (TME) agent (Bessen and Marsh, 1992b). Mouse PrP^{TSE} was generated by the experimental infection of CD-1 Swiss mice with the Rocky Mountain Laboratories (Chandler) strain of mouse-passaged sheep scrapie (Chandler, 1961). Brains were harvested from animals displaying clinical signs of disease consistent with TSE and stored at -80°C until used. Non-infected brains were from animals never experimentally challenged or exposed to areas where prion-challenged animals were housed.

Enrichment of the disease-associated prion protein by sequential enzymatic degradation of transmissible spongiform encephalopathy-affected brain tissue

Brain tissue was processed according to a novel prion enrichment method we developed for this study that more closely mimics environmental degradation of prion-infected tissues than currently available protocols (Bolton et al., 1991; Raymond and Chabry, 2004). Prion-infected or non-infected brain tissue was made into a 10 – 20% w/v homogenate in either deionized H_2O,

1X PBS, or 10 mM HEPES, pH 7.0. Brain homogenates were then subjected to a series of enzyme treatments aimed at degrading the major classes of biomolecules found in mammalian tissue and enriching PrP^{res} (all enzyme stocks were prepared and stored as per manufacturer's instructions): First, 10 µg/mL each of DNAse I (Worthington Biochemical Corp. #LS002139, lot S0N12281A) and RNAse A (AppliChem #A2760, lot 8M003055) were simultaneously added to HY-infected or non-infected (controls) BHs and incubated for 24 hr at room temperature (RT) on an end-over-end mixer. Following nuclease treatment, 100 µg/mL of AY30 lipase (Acros Organics #2947, lot A0269035) was added to BHs and incubated for 24 hr in an identical fashion. Finally, 50 µg/mL equivalent of PK-agarose (Sigma #P9290, lot 037K5159) was added to BHs and incubated 2 – 24 hr on an end-over-end mixer at RT. PK was subsequently removed from samples by low speed centrifugation (≤ 0.2 RCF) and separation of BH preparations from PK-agarose pellets by pipette transfer to a sterile microcentrifuge tube.

Immediately following enzymatic digestion, lipid extraction was performed on BHs using three parts Freon 113 (1,1,2-trichloro-1,2,2-trifluoroethane; ChemNet #CN1099.9) mixed with two parts BH and vigorous shaking on a tabletop shaker for 30 min at RT. The aqueous upper phase containing PrP^{res} was separated from the denser Freon 113 phase by pipette transfer to a sterile microcentrifuge tube. BHs were then dialyzed using Slide-A-Lyzer G2 dialysis cassettes (10 kDa MWCO; Thermo Scientific #66380) against three changes per day of ultra-pure, deionized water (pH 7.0) over five days at 4°C to remove small molecular weight contaminants and products of the enzymatic digestion process. Dialyzed BH samples were diluted to a 4% working concentration for all subsequent analyses, unless otherwise stated and stored at -80ºC

until needed. All experiments in this study use PrP^{res} prepared by this method, unless specifically stated otherwise.

Plant growth and culture conditions

Arabidopsis thaliana ecotype Columbia [Col-0] was used in this study (kind gift from the laboratory of Dr. Patrick Masson, University of Wisconsin - Madison). To meet the specific requirements of individual experiments, various plant growth and culture methods were employed, described as follows:

Vertical plate agar culture: surface-sterilized seeds were grown on square (100mm x 15mm), gridded petri dishes containing 45 mL of nitrogen-free 0.5× Murashige and Skoog (MS) (Murashige and Skoog, 1962) or 0.5× Linsmaier and Skoog (LS) (Linsmaier and Skoog, 1965) basal salt solution (Caisson Labs, North Logan, UT) supplemented with 15 $g \cdot L^{-1}$ sucrose, 0.4% phytoagar (MIDSCI, St. Louis, MO), and 2.5 mM MES buffer, pH 5.7 (Thermo Fisher Scientific, Inc., Waltham, MA). Plated seeds were incubated at 4°C for four days, then transferred to a growth rack (room temperature, 16 h/8 h day/night, 150 $\mu mol/m^2$ sec^{-1} light intensity) for growth in a vertical position.

Horizontal plate agar culture: Plate and seed preparation and growth conditions for horizontal agar culture were identical to those for vertical plate agar culture (above), with the exception that plants were grown in a horizontal position. Additionally, for prion uptake experiments, brain homogenate (BH) resulting from the novel prion enrichment process described above and equivalent to one RML-infected or non-infected mouse brain per plate (average wet weight =

0.4783 g, average infectivity = ~7 $\log_{10}$ i.c. ID_{50} units/g brain tissue) was added to cooled, yet molten, agar just prior to pouring. Once the BH containing agar layer had set, 10 mL of sterile agar (identical composition to that used in vertical plate agar culture above), containing no BH, was poured on top as a "buffer layer." Seeds were sown atop the buffer layer of agar, which allowed germinating plant roots to grow down into the BH agar layer, but prevented direct contact between plant aerial tissues and BH containing agar, thereby preventing accidental cross-contamination of aerial plant tissues harvested for prion uptake analyses.

6-well plate liquid culture: Arabidopsis thaliana plants were initially germinated and grown on sterile MS agar via either the vertical or horizontal plate methods described above. At four weeks growth, plants were transferred to a hydroponic culture system in which roots were exposed to plant buffer solution (20 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM MES, pH 5.7) contained within the wells of a 6-well plate (5 mL of buffer solution per well) and aerial tissues were suspended above each of the wells and out of direct contact with the buffer solution below by a parafilm bridge. For prion uptake experiments, either RML-infected or non-infected mouse BH resulting from the novel prion enrichment process described above was added to plant buffer solution at a final concentration of 1 – 5% (w/v) BH. Plants grown by this method were maintained in a humidified chamber constructed by placing the 6-well plate containing plants and media on top of an empty pipette tip box in a deep glass dish filled with 1 – 2 inches of tap water and enclosing the entire system in plastic wrap for the duration of plant growth. Cultures were aerated daily by gentle manual shaking.

Ice-Cap liquid culture: Most of the plants used in these studies were grown using the Ice-Cap high throughput plant growth and tissue sampling system developed by Patrick Krysan and

colleagues (Clark and Krysan, 2007; Krysan, 2004; Su et al., 2011). Briefly, surface-sterilized plant seeds were sown onto the surface of sterile agar plugs (agar composition identical to that used in vertical plate agar culture above; 200 - 400 μL agar per well, depending on plant species) in 96-well unfritted deep well plates (Fisher catalog no. 278012). Once seeded, plates were covered with a clear plastic lid (e.g. lids taken from Falcon Microtest flat bottom 96-well polystyrene plates; Falcon catalog no. 351172), wrapped in foil and placed at 4°C for four days to synchronize germination. Following the 4°C incubation, foil was removed and seedling plates were transferred to a growth rack (room temperature, constant fluorescent light, 150 μmol/m^2 sec^{-1} light intensity) for 3 – 5 d, or until seedlings germinated. Once germination occurred, seedling plates were transferred to the Ice-Cap continuous watering system developed by and maintained in the laboratory of Dr. Patrick Krysan at the University of Wisconsin – Madison (Clark and Krysan, 2007). Unlike the classical Ice-Cap approach described by Krysan *et al*., our seedling plates were floated in the water-filled upper metal pan reservoir without lower root plates and plants allowed to grow at room temperature under constant light until root tissue was observed emerging from the holes in the bottom of each well of the 96-well seedling plates. Seedling plates were then removed from the continuous watering system and each placed onto a tight-fitting, inverted pipette tip box lid that contained 30 – 50 mL of 0.1% - 2% (w/v) prion-infected or non-infected BH in plant buffer solution, such that only emerging plant roots on the bottom of seedling plates came into direct contact with BH-containing liquid media in the reservoir. Media-containing reservoirs were adhered to seedling plates with tape and plants were grown on a growth rack (room temperature, 16 h/8 h day/night, 150 μmol/m^2 sec^{-1} light intensity) and cultures were aerated daily by gentle manual shaking.

The 96-well plate Ice-Cap method described above worked well for growth of *Arabidopsis thaliana*, alfalfa (*Medicago sativa*), and barley (*Hordeum vulgare*), but was not large enough to accommodate corn (*Zea mays*) plants. Corn plants were grown in a modified, scaled-up version of the Ice-Cap method in which pre-soaked and sterilized corn seeds were sown on the surface of agar-filled 50-mL conical tubes that had their bottoms cut off after agar had solidified. Seeded topless and bottomless conical tubes were placed into sterile 250-mL glass Erlenmeyer flasks containing 200 mL 0.1% (w/v) prion infected or non-infected BH in plant buffer solution such that only plant roots emerging from the bottom of conical tubes came into direct contact with BH containing media in the flask. Conical tubes were secured to Erlenmeyer flasks using parafilm and plants grown on a growth rack (room temperature, 16 h/8 h day/night, 150 μmol/m^2 sec^{-1} light intensity); cultures were aerated daily by gentle manual shaking.

Soil culture: HY-infected or non-infected hamster BH resulting from the novel prion enrichment method described in above was dialyzed against a 1:1 mixture of tap and distilled water and added to a final concentration of 2% (w/v) to 15 mL of each of the following soils in glass vials: potting soil (Miracle-Gro® Organic Choice Potting Mix), Plainfield A soil (kind gift of Dr. Matilde Urrutia, University of Wisconsin - Madison), Bluestem clay (kind gift of Dr. Joel Pedersen, University of Wisconsin – Madison), and Ottawa sand (Fisher catalog no. S23-3). Sterilized *A. thaliana* seeds were sown on the surface of agar-filled 5-mL pipette tips (Gilson reference no. F161571) that had their tops trimmed to approximately 1 cm above the agar fill line and bottoms cut off after agar had solidified. Seeded and trimmed agar tips were seated into the open tops of glass vials containing soil-BH mixtures such that germinating plant roots would grow downward through the agar plug and eventually into the soil medium below. Seeded agar

tips were secured to glass vials with parafilm, placed into a humidified chamber and grown on a growth rack (room temperature, 16 h/8 h day/night, 150 μmol/m^2 sec^{-1} light intensity).

Prion uptake by plant roots

For confocal microscopy detection of fluorescently-tagged PrP^{res}: *A. thaliana*, alfalfa, barley, and tomato (*Solanum lycopersicum*) were grown by the vertical or horizontal plate agar method (described above) for 1 – 2 weeks. Seedling roots were then incubated for 24 hr in either 1) 50 μg/mL GFP (Millipore, Billerica, MA) in plant buffer solution or 2) 5 or 0.5% (w/v) RML-infected or non-infected mouse brain homogenate that had been processed by the novel prion enrichment protocol described above and fluorescently tagged using Alexa Fluor® 488 carboxylic acid, succinimidyl ester (Life Technologies, Grand Island, NY) as per manufacturer's directions. Following incubation, plant roots were removed from solution, surface rinsed extensively with plant buffer solution, and imaged using a confocal microscope (see confocal laser scanning microscopy below).

For PMCAb detection of PrP^{res}: All plants used in these experiments were grown using the Ice-Cap or soil culture methods (described above). Duration of growth on prion-containing media (or non-infected media for controls) varied by plant species: 4 d for Ice-Cap grown and 22 d for soil grown *A. thaliana*, 25 d for Ice-Cap grown alfalfa, 25 d for Ice-Cap grown barley, 50 d for Ice-Cap grown corn. Following these incubation periods, aerial tissues of plants were harvested by cutting them away from the top surface of the agar plugs in 96-well plates (or 5 mL pipette tips for soil-grown *A. thaliana* or 50 mL conical tubes for corn plants) using scissors or blades never

before used for prion work. Stem and leaf tissues were stored -80°C until assayed (see protein misfolding cyclic amplification with beads section below).

For PrP^{TSE} and infectivity detection by mouse bioassay (intracerebral challenge): Only *A. thaliana* were used in these experiments, and all were grown using the horizontal plate agar culture and 6-well plate liquid culture methods (described above). Agar grown *Arabidopsis* were harvested after four weeks of growth on horizontal plate culture. Hydroponically-grown Arabidopsis were germinated on vertical or horizontal agar plates for four weeks and then transferred to the 6-well plate liquid culture system and allowed to grow on prion-containing media (or non-infected media for controls) for four days. Following this incubation, aerial tissues were harvested by cutting them away from roots, above the parafilm bridge using scissors or blades never before used for prion work. Stem and leaf tissues were stored at -80°C until assayed (see intracerebral mouse bioassay below).

For PrP^{TSE} and infectivity detection by mouse bioassay (oral challenge): All plants used in these experiments were grown, harvested, and stored identically to those used for PMCAb experiments (described above).

Confocal laser scanning microscopy (CLSM)

Intact roots or stem cross-sections from 1 – 2 week old seedlings were mounted between two 35×50-1 mm sheets of Fisherfinest® coverglass (Fisher, Pittsburgh, PA) with a couple of drops of plant buffer solution. A Nikon A1R-A1 (Nikon Instruments, Inc., Melville, NY) confocal laser scanning microscope was used with 10× and 20× dry and 60× and 100× oil-

immersion objectives. GFP and Alexa Fluor® 488 carboxylic acid, succinimidyl ester (Life Technologies, Grand Island, NY) were visualized by excitation with an argon laser at 488 nm and detection with a 500 – 550 nm band-path filter.

Protein misfolding cyclic amplification with beads (PMCAb)

Non-infected Golden Syrian hamsters, age 3 – 4 weeks, were euthanized by CO_2 asphyxiation and immediately perfused with Dulbecco's phosphate buffered saline (DPBS) without Ca^{2+} or Mg^{2+} (Thermo Scientific #SH30028.02) amended with 5 mM EDTA. Brains were then rapidly removed, flash frozen in liquid nitrogen, and stored at -80°C until use. Brains were homogenized on ice to 10% (w/v) using either a pestle grinder with disposable attachment (Fisher catalog no. 03-392-106) or a Bullet Blender Storm 24 bead beater (Next Advance, Averill Park, NY) in ice-cold PMCA conversion buffer (Ca^{2+}- and Mg^{2+}-free DPBS supplemented with 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, and 1 tablet Roche Complete EDTA-free protease inhibitors cocktail (Fisher catalog no. 50-720-4069) per 50 mL conversion buffer). Normal brain homogenates (NBH) were clarified by centrifugation (2 min, 2,000×g). Supernatant was transferred to pre-chilled microcentrifuge tubes, flash frozen in liquid nitrogen, and stored at -80°C until use.

Control or spike seeds for PMCAb were prepared from brain tissue from clinically-affected HY-positive hamsters by 1:1 dilution of 20% (w/v) BH in PBS with the conversion buffer described above. The resulting 10% BH was serially diluted 10-fold in NBH to generate dilution series ranging from 10^{-2} to 10^{-13}. Plant seeds for PMCAb were prepared from aerial tissues harvested from Ice-Cap or soil culture grown plants. Stem and/or leaf tissues were

homogenized to 30 – 50% (w/v) homogenates in conversion buffer by flash freezing tissues with liquid nitrogen and grinding with mortar and pestle and subsequently homogenizing using the Bullet Blender Storm 24 bead beater.

Control or plant seeds (10 μL) were used to seed 90 μL NBH in 0.2 mL thin-walled PCR tubes with two 2.38 mm Teflon® beads (McMaster-Carr, #9660K12). Experimental tubes were placed in a rack in a Misonix S-4000 microplate horn, and the reservoir was filled with 300 mL water. PMCAb conditions consisted of 96 cycles and each cycle consisted of 10 sec sonication at 80% of maximum power followed by 29 min 50 sec incubation at 37°C. For serial PMCAb, at the conclusion of one 96-cycle round of PMCAb, 10 μL of each dilution was transferred to 90 μL fresh NBH and subjected to another 96-cycle round of PMCAb.

Detection of PrP^{res} by immunoblotting

For PMCAb and bioassay samples, 20 μL of each PMCAb reaction or 10% (w/v in PBS) BH from brains of clinically diseased rodents, respectively, was transferred into a thin-walled PCR tube and treated with 100 μg mL^{-1} PK (Mo Bio Laboratories, Inc, Carlsbad, CA) and 4% (w/v) SDS in PBS and incubated for 1 hr at 37°C with shaking (1000 rpm). Following PK digestion, samples were treated with additions of 4× LDS sample buffer and 10× NuPAGE reducing agent (Life Technologies, Grand Island, NY), each to final concentrations of 1×, and were then heated for 5 min at 95°C. Thirty μL of each sample were resolved on 12% NuPAGE Bis-Tris gels (Life Technologies, Grand Island, NY) for 50 min at 200V and then transferred to a polyvinyl difluoride (PVDF) membrane for 60 min at 25V. PVDF membranes were blocked for 1 hr in 5% (w/v) powdered milk in Tris-buffered saline plus Tween-20 (TBST), followed by

application of the primary antibody—SAF-83 for mouse samples (Cayman Chemical Company, Ann Arbor, Michigan) and 3F4 for hamster samples (Millipore, Billerica, MA)—diluted 1:10,000 in 5% powdered milk in TBST, for 1 hr. PVDF membranes were then rised 3× with TBST and incubated for 1 hr with the secondary sheep anti-mouse immunoglobulin G conjugated with horseradish peroxidase (1:10,000 dilution in 5% milk-TBST). Immunoreactivity was detected using an enhanced chemiluminescent detection system (Thermo Scientific, Rockford, IL) in a EC3 imaging system (UVP Bioimaging Systems, Upland, California). Densitometry was performed using VisionWorks LS software version 6.6a (UVP Bioimaging Systems, Upland, California).

Intracerebral (i.c.) challenge mouse bioassay

Following harvest, *A. thaliana* grown by either the horizontal plate agar culture or 6-well plate liquid culture methods (described above) were separated into stem and leaf tissues and homogenized to 17% (w/v) and 56% (w/v) plant homogenates, respectively, in 10% (w/v in PBS) non-infected mouse BH, pH 7.4. Twenty μL of plant homogenate was intracerebrally inoculated into anesthetized 4 – 6 week old male CD1 mice. Mice were monitored daily for disease progression and euthanized upon development of clinical signs consistent with a TSE (including kyphosis, lethargy, poor balance and lack of motivation to acquire food and water). Brains from clinically diseased animals were harvested immediately post-mortem and divided sagittally with one half prepared for evaluation by immunoblot and the other half fixed in 10% neutral-buffered formalin for histopathology and immunohistochemistry (IHC). All challenged animals were tested for the presence of PrP^{TSE} in brain tissue by immunoblotting and/or IHC.

Survival time was defined as the number of days from inoculation (dpi) to euthanization. Animals that succumbed to intercurrent illness within the first 10 days post inoculation were excluded from data analysis.

Oral challenge mouse bioassay

Oral gavage: *A. thaliana* plants grown by the Ice-Cap method (described above) were homogenized into a 54% (w/v in PBS) homogenate and 200 μL orally gavaged into 4 – 6 week old male CD1 mice as either a single dose (each mouse received the equivalent of ~100 mg dry weight plant tissue) or one dose per day for five consecutive days. Simultaneously, a dilution series was also carried out in which identically prepared non-infected *A. thaliana* homogenates were spiked with 0.1% - 10% (w/v in PBS) RML-infected mouse BH and compared against an identical dilution series without plant homogenate to test the effect of plant tissue on RML infectivity in mice.

Free feeding: Arial tissues from alfalfa, barley and corn grown by the Ice-Cap method (as described above) were chopped into 0.5 in square (or smaller) tissue pieces and 0.14 g plant tissue/mouse was free fed to male CD1 mice every day for 30 days (this amount is equivalent to a single male CD1 mouse eating all arial tissues from a 6-week old maize plant).

Following oral challenges, mice were monitored daily for disease progression and euthanized upon development of clinical signs consistent with a TSE. Brain tissue was harvested, stored and analyzed as described above for i.c. challenged mice.

CHAPTER FIVE:

Conclusions and Future Directions

Conclusions

The goal of this project was to further our current understanding of environmentally-mediated transmission of natural animal prion diseases. The research presented in this thesis identifies two new potential transmission routes by which CWD and scrapie may disseminate on the landscape, and provides new insights into the fundamental nature of the CWD agent.

The research presented in Chapter 2 established that meadow voles (*Microtus pennsylvanicus*) are susceptible to white-tailed deer CWD agent by peripheral inoculation routes, consistent with their likely routes of exposure in the environment. This finding is consistent with previous work demonstrating bank voles (*Myodes glareolus*) (Di Bari et al., 2013), prairie voles (*Microtus ochrogaster*) (Kurt et al., 2011), and red-backed voles (*Myodes gapperi*) (Heisey et al., 2010) as susceptible to CWD, but the first to demonstrate peripheral susceptibility among these species, or any non-cervid wild species. While intracerebral challenge of meadow voles with CWD resulted in complete attack rates and efficient disease progression, intraperitoneal challenge was less efficient, with 20% of challenged animals developing clinical TSE. Interestingly, oral challenge of meadow voles failed to result in clinical TSE, but serial intracerebral subpassage of apparently non-clinical vole brain tissue produced clinical disease in naïve voles, indicating that meadow voles orally exposed to CWD are capable of harboring a subclinical TSE. These data support a role for meadow voles in environmental transmission of CWD, possibly as reservoirs for the disease on the landscape, or even vector or bridge species to other wildlife, domestic animals, and even humans. Also presented in Chapter 2 are experiments identifying the existence of at least two new CWD strains. Serial subpassage of CWD in meadow voles via intracerebral challenge resulted in the selection of, initially, four novel vole-passaged

CWD phenotypes that stabilized to two phenotypes by third passage. Unique PrP^{res} electrophoretic migration patterns, lesion profiles and PrP^{TSE} brain deposition patterns were identified for each of the four vole-passaged CWD variants, and supported definition of the two stabilized variants as novel strains. These data are the most conclusive presented to date supporting the existence of CWD strains, and are congruent with previous studies that suggested CWD strain variation in free-ranging cervids (Angers et al., 2010; Raymond et al., 2007). Additionally, we observed differential strain selection in meadow voles challenged with 96GG (wild-type) versus 96GS *Prnp* genotype white-tailed deer CWD, suggesting a role for deer genotype in CWD strain selection. Additional challenges including a larger pool of CWD isolates from various genotypes of cervids is needed to determine whether this relationship is correlative and is further discussed in the next section titled "Future Directions."

In Chapter 3, I investigated meadow vole susceptibility to sheep scrapie, using experimental approaches similar to those employed in Chapter 2 for CWD. In this study, meadow voles were challenged intracerebrally as an initial determination of their ability to support a scrapie-induced TSE. We found meadow voles to be poorly susceptible to sheep scrapie, exhibiting a 50% attack rate, suggesting this species is not as susceptible to scrapie as they are to white-tailed deer CWD, and thus are unlikely to acquire scrapie on the landscape and serve as a disease reservoir. We also provide a descriptive analysis of scrapie-induced TSE in meadow voles through glycoform analysis, lesion profiling, and PrP^{TSE} deposition patterning in the brain. Histopathology of meadow vole passaged scrapie was found to be distinct from that of bank vole-passaged scrapie (Di Bari et al., 2008), ovinized transgenic mouse-passaged sheep scrapie (Beck et al., 2013), and scrapie in sheep (Ligios et al., 2002). No evidence for strain

selection was observed upon passage of sheep scrapie in meadow voles, although additional serial subpassages of agent in this host may be needed before this is conclusive.

In Chapter 4, I presented evidence demonstrating prion protein uptake by the model plant *Arabidopsis thaliana* and several crop species. Additionally, I established that prions taken up by plants maintain their ability to infect mammalian hosts, which is demonstrated by both intracerebral and peroral challenge experiments in mice. This work extends previous research implicating soil as an environmental reservoir for prions (Cooke et al., 2007; Johnson et al., 2007; Johnson et al., 2006; Leita et al., 2006; Ma et al., 2007) and highlights plants as a currently unrecognized, yet plausible, environmental exposure or transmission route for prions. Confocal miscroscopy experiments of plants hydroponically grown on fluorescently tagged prion containing media revealed internalization of prion protein in the roots of *A. thaliana,* alfalfa (*Medicago sativa*), barley (*Hordeum vulgare*), and tomato (*Solanum lycopersicum*). To confirm these observations, and determine whether prion protein remained in root tissues, or was translocated within the plant to aerial tissues, PMCAb amplification of prion protein from stem and leaf tissues was carried out on *A. thaliana,* corn, and alfalfa hydroponically grown on prion-containing media. Prion protein was detected in all three plant species using this approach. For plants to serve as an environmental source of prion infectivity, prions in plants must remain in an infectious conformation and be bioavailable to animals via the oral route of exposure. We found that TSE agent taken up by *A. thaliana* grown on either agar or liquid media containing prions is infectious when injected into the brains of laboratory mice. More importantly, TSE agent taken up by *A. thaliana* and corn (*Zea mays*) remains orally bioavailable to laboratory mice and able to

cause clinical TSE in this host. These data support the concept that plants may be capable of internalizing environmentally shed prions and serve as a source of environmental TSE exposure.

Discussion and Future Directions

Meadow voles as useful CWD strain typing tools

As demonstrated in Chapter 2 of this thesis, the passage of white-tailed deer CWD in meadow voles has provided the most conclusive evidence presented to date to support the existence of multiple CWD strains. Meadow vole passaged CWD strains are differentiable by both host-specific characteristics as well as biochemical characteristics intrinsic to the prion agent. This work highlights meadow voles as extremely valuable strain typing "tools" with which we may now begin to ask many unanswered questions that remain about both fundamental CWD biology as well as CWD management.

Wildlife management and health risk analysis decisions have both been based on the concept that CWD is one, identical disease agent (Canadian Cooperative Wildlife Health Center, 2005; Gillette et al., 2004; Nebraska Game and Parks Commission, 2003; Parmley et al., 2008; Petersen et al., 2002; Williams et al., 2002; Wisconsin Department of Natural Resources, 2010). Our findings suggest that CWD may actually exist as a mixture of strains, and that isolates of CWD from different genotypes of white-tailed deer (either 96GG or 96 GS in *Prnp*) may harbor different CWD strain profiles that are detectable upon transmission to meadow voles. These findings have important implications for CWD management in that a "one-size-fits-all" management strategy is likely not appropriate to mitigate CWD in captive and free-ranging cervids and may, in part, explain why current management efforts have failed to eradicate or even effectively control the disease on the landscape (Canadian Cooperative Wildlife Health Center, 2005; Gillette et al., 2004; Gross and Miller, 2001; Nebraska Game and Parks Commission, 2003; Parmley et al., 2008; Petersen et al., 2002; Wasserberg et al., 2009; Williams

et al., 2002; Wisconsin Department of Natural Resources, 2010). A question of priority that stems from this work is: what is the role, if any, of cervid genetics in CWD selection, transmission, and evolution among free-ranging cervids? To address this, strain diversity of circulating CWD agent from a number of geographically-disparate CWD isolates could be assessed by meadow vole bioassay and subsequent strain characterization, as carried out in Chapter 2 of this thesis. CWD isolates would include those from various genotypes of naturally infected cervids, including mule deer and elk to determine whether a true correlation exists between cervid genotype and CWD strain selection. We have yet to establish the susceptibility of meadow voles to mule deer CWD, but i.c. challenge experiments currently underway in our laboratory indicate that meadow voles are just as susceptible to elk CWD as they are to white-tailed deer CWD (unpublished data). Also, strain characterization between both recent and established outbreaks of CWD would provide insight into CWD adaptation and evolution over time. Given that many U.S. states with CWD endemicity in their cervid populations also have active CWD surveillance programs in place, archived CWD isolates from various locations are likely available and could be compared with present-day samples. Especially of interest are CWD isolates from regions where the ratios of 96GG to 96GS are different (i.e. eastern versus western U.S. herds), because these populations would give us an ideal opportunity to test our hypothesis that cervid host genotype determines strain selection. Determination of the strain profile of 96SS white-tailed deer CWD isolates may shed light on the relative CWD "resistance" these animals display; perhaps 96SS animals don't efficiently propagate currently prevalent CWD strains and are only susceptible to a minority strain circulating among white-tailed deer.

Alternatively, perhaps disease progression is slower in 96SS white-tailed deer as compared with the wild type 96GG counterpart.

While animal bioassay remains the gold standard by which prion strain typing is carried out, this method is time intensive, costly and laborious, even with rodents. Thus, it would be of great interest to incorporate the high conversion promiscuity of meadow vole PrP^C, as well as its strain distinguishing capability, into a rapid, *in vitro* assay. PMCA provides an ideal platform with which to pursue this because prions amplified by this method have been shown to maintain their original strain characteristics. Healthy meadow vole brain tissue could be used as "substrate" to amplify CWD isolates of interest via multiple rounds of sonication and incubation, and, ultimately strain differences could be detected by differences in electrophoretic migration patterns of the resulting amplified prion protein via immunoblotting. The advantages of a PMCA-based assay include short experimental timeframe (results are obtained within 48 – 72 hours, as compared with animal bioassay, which can take hundreds of days to years to complete), replacement of living animals with tissue samples, exceptionally high assay sensitivity, and the ability to maintain prion strain properties as they would be *in vivo*. Such an assay could be used not only for rapid strain differentiation, but also as a possible *ante mortem* CWD detection assay, which would improve current surveillance and diagnostic efforts since detection of infected animals is central to managing CWD and understanding its epidemiology and efficient techniques for *ante mortem* detection of CWD are lacking. Most testing is performed *post mortem*, typically hunter harvested or road kill, and the availability of specimens can be limiting Current *ante mortem* methods are clumsy, invasive, expensive and may detect <42% of cases for some deer (Thomsen et al., 2012). A PMCA based test would also provide the opportunity to

detect CWD prions from easily accessible tissues and bodily fluids (i.e. saliva, blood, urine) that don't require invasive techniques to acquire and usually exhibit low levels of prions. Given the high promiscuity of vole PrP^C for conversion of various TSEs, a vole-based PMCA assay may be applicable to other TSEs beyond CWD. Recently, we found that PMCA substrate derived from red-backed vole, another North American vole species, competently amplified human vCJD prions (Nemecek et al., 2013). We are currently working with the U.S. Food and Drug Administration in determining the efficacy of this assay in detecting human prions from blood toward development of a prototypic prion blood screening assay. We are also currently working to develop the meadow vole-based PMCA strain typing assay for CWD in our laboratory and plan to test it against additional animal TSEs, including scrapie and BSE.

Characterization of novel vole-passaged CWD strains

In Chapter 2 of this thesis, I successfully isolated two unique vole-passaged CWD strains. In addition to their differential disease presentation in meadow voles (with regard to incubation period, and histopathology) and differential electrophoretic migration patterns of the protein agent, much remains to be characterized about these two strains. Additional biochemical characterization of the agent associated with each strain is needed. Traditionally, this includes determinations of PK and guanidine hydrochloride sensitivities, glycoform analysis, aggregation state, and three-dimensional structural differences. Transmission characteristics of each strain should also be investigated. A common test of prion strain change is to try to inoculate strains isolated from cross-species challenges back into the original host species; a differential ability of each newly isolated strain to re-infect the original host can serve as another strain differentiation

parameter. In our case of vole-passaged white-tailed deer CWD isolates, this could be accomplished using transgenic cervidized mice. It would also be of interest to determine whether strain selection has changed the host range of the original agent. CWD does not readily infect mice and hamsters (Bartz et al., 1998; Raymond et al., 2007), so efficient infection of mice or hamsters with vole-passaged CWD would demonstrate an expansion of host range. Of greater relevance would be determination of host range of vole-passaged CWD strains in other wildlife, domestic animals, and humans. Toward this end, we are currently collaborating with the U.S. Department of Agriculture in testing the susceptibility of raccoons to vole-passaged CWD. We are also just beginning i.c. challenges of vole-passaged CWD into humanized transgenic mice to test the zoonotic potential of the CWD strains we isolated; this work is being carried out in collaboration with the U.S. FDA.

Plant uptake of prions

In Chapter 4, I present the initial observational work demonstrating that plants are capable of taking up prions and that those plant-internalized prions remain infectious to mammalian hosts. Although a wealth of previous research has been carried out on internalization of bacterial and viral pathogens by plants, this is the first work demonstrating uptake of prion agents. Thus, there is much that remains to be done. Determining the mechanism of prion uptake by plants follows naturally from the research presented in Chapter 4. Our studies indicate that prion uptake is occurring in the roots and endocytotic uptake mechanisms are hypothesized means by which plants are able to internalize whole proteins (Nishizawa and Mori, 1977; Nishizawa and Mori, 1980b; Paungfoo-Lonhienne et al., 2008). Since we've had success

detecting fluorescently-tagged prion proteins, extended microscopy studies investigating co-localization of prions and endocyctotic vesicles would provide a viable starting point from which to investigate endocytotic uptake mechanisms. Alternatively, endocytosis inhibitors, such as brefeldin A and latriculin B, would provide another experimental route to test the endocytosis uptake hypothesis. Root growth, including the development of lateral roots, results in tissue breakages and fissures to accommodate new structures; these openings provide another potential route by which prions may enter plant root tissues. The fate of infectious prion protein once inside the plant is also an important question. From our studies, it appears that prion infectivity is maintained in plant tissues for a timeframe ranging between 96 hours (for *A. thaliana)* to 50 days (for maize). A detailed kinetics study examining the turnover of prion protein and infectivity in plants would help determine whether plant contamination by prions presents an extended exposure threat, or whether plants are able to effectively catabolize prion protein. If plants sequester infectious prion protein, they may prove beneficial in improving CWD or scrapie contaminated environments via phytoremediation. Should plants accumulate prion protein, they could potentially be grown in contaminated areas, such as depopulated cervid farms, and then harvested to remove prions from the soil, effectively serving as a prion sink. Of course, it would first need to be determined whether plants are able to take up prion protein from soil environments. In Chapter 3 we investigated prion uptake from hydroponic and solid state agar media; soil uptake experiments are currently in progress in our laboratory.

Another mechanism of bioremediation is breakdown of contaminants by either plants (phytodegradation) or root or soil-associated bacteria or fungi (rhizodegradation). In the case of phytodegradation, contaminants are transformed through plant metabolic processes and are

thereby degraded or inactivated. The secretion of degradative enzymes from roots can lead to phytodegradation of contaminants external to the plant. Similarly, rhizodegradation of contaminants occurs through the action of root-associated microbes metabolizing pollutants. Rhizodegradation of contaminants occurs within the rhizosphere (zone of soil surrounding roots) of certain plants as microbial communities can be highly plant species-specific. Vegetation with the capability of inactivating prions could be cultivated in sites of suspected contamination to reduce environmental CWD agent levels.

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