

DISSERTATION

INSIGHTS INTO THE MOLECULAR MECHANISMS UNDERLYING PROPAGATION AND
ADAPTATION OF NOVEL EMERGENT CHRONIC WASTING DISEASE PRION STRAINS

Submitted by

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Graduate Degree Program in Cell and Molecular Biology

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Summer 2026

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ABSTRACT

INSIGHTS INTO THE MOLECULAR MECHANISMS UNDERLYING PROPAGATION AND ADAPTATION OF NOVEL EMERGENT CHRONIC WASTING DISEASE PRION STRAINS

Prion diseases are a group of invariably fatal neurodegenerative disorders caused by the conformational misfolding of the cellular prion protein (PrP^{C}), a membrane-bound glycoprotein expressed abundantly in neurons and cells of the immune system, into an infectious form, PrP^{Sc} . The term “prion” was coined by Stanley Prusiner in 1982 to describe a proteinaceous infectious agent (PrP^{Sc}) devoid of nucleic acids and associated with several neurodegenerative diseases in humans and animals, including cattle, sheep, goats, mink, dromedary camels, and cervids. While most human prion diseases arise sporadically, familial forms are associated with point mutations or insertions in the coding sequence for PrP^{C} . Others, such as human variant Creutzfeldt-Jakob disease (vCJD)—which has been linked to consumption of beef from cattle affected by bovine spongiform encephalopathy (BSE) or commonly known as “mad cow disease”—remain a compelling example of the zoonotic risk posed by prion diseases.

Chronic wasting disease (CWD) is a prion disease that affects species of the *Cervidae* family, including deer, moose, elk, red deer, and reindeer. In North America, CWD is endemic and has been reported in 36 US states and in five Canadian provinces. It is the only known prion disease affecting free-ranging cervids, largely due to its efficient horizontal transmission and the remarkable environmental stability of infectious prions. The most recent body of research on CWD has shown that North American CWD is characterized by low strain diversity, high tropism for lymphoid tissues, and high capacity for contagious transmission.

The emergence of CWD in Northern Europe in 2016, initially in reindeer and subsequently in moose and red deer, raised concerns about the possible introduction of North American CWD into Europe, as occurred in South Korea. However, studies by our group and others have demonstrated that Nordic CWD exhibits properties distinct from those of North American CWD, supporting a sporadic origin as the most plausible explanation for its emergence. Moreover, Nordic moose CWD in moose is non-lymphotropic, suggesting low risk of contagious transmission, though, this may not be the case for Norway reindeer CWD, which has been found to replicate in lymphoid tissues.

With the development of a gene-targeted (Gt) mouse model by the Telling lab, which expresses physiologically regulated levels of cervid PrP^C and recapitulates key features of CWD, including its lymphotropic properties, we are well-positioned to address, with greater precision, key questions regarding the strain diversity and properties of Nordic CWD. This model also facilitates investigation into the evolution and adaptation of prion strains and risks associated with the acquisition of properties that may enhance sympatric transmission, as well as the mechanisms by which unstable prion strains display unprecedented adaptation. This was demonstrated in a 2021 study, in which a non-lymphotropic Nordic moose strain unexpectedly evolved North American-like properties, including lymphotropism, following serial passage in the Gt mouse model.

This dissertation will discuss in detail our use of gene-targeted mouse models to better understand 1) the unprecedented strain heterogeneity in Nordic moose CWD, 2) its potential for the evolution of greater contagious transmission potential through the acquisition of lymphotropic properties and 3) The effects of polymorphic variants of the cervid prion protein that are found exclusively in Nordic moose and how they may impact CWD pathogenesis and risk of spread to other cervid species. Nordic moose CWD is most likely of sporadic origin, with a unique strain heterogeneity with diverse mechanisms of infectious adaptation that could also draw parallels and differences with human neurodegenerative diseases of sporadic etiology.

ACKNOWLEDGEMENTS

The present dissertation represents the end result of my combined contributions to the field of prion biology and chronic wasting disease. This work could not have been possible without the continuous support, hard work, and exceptional talent of key members of the Telling lab: Sehun Kim, Jenna Crowell, and my talented and hard-working undergraduate mentee, Madeline Judson. Thank you to Drs. Alyssa Block and Carlos Diaz for their mentorship, their guidance with difficult questions, and for reading my manuscript drafts. I am, as well, grateful to former members of the Telling lab who set the foundations of this work: Drs. Jifeng Bian, Julie Sun, and Sarah Kane, who have always been incredible mentors. To everyone else in the lab, thank you for the years of sharing science and laughs during conferences, symposiums, farewell lunches, and more.

I am deeply appreciative of the exceptional guidance and support of my academic committee, Drs. Candace Mathiason, Mark Zabel, and Eric Ross; their insight, expertise, and commitment to mentorship were fundamental for my progress as a PhD candidate and a scientist. I couldn't have been more fortunate than to be a PhD student in the Cell and Molecular Biology graduate program (CMB), a remarkable program under the leadership of Dr. Carol Wilusz, who always goes above and beyond for all of the students and strives for us to become our very best. Thank you to Mercedes Cooper, Ivy Hardy, Emma Eckley and Mitskie Kumagai for your hard work in supporting me in any way during my time in CMB. Thank you to CSU Writes, Kristina Quynn, and Kimi Conro for providing us grad students with great opportunities to become better writers and communicators. Your contributions to who I am today as a scientist are of immeasurable value.

I also want to acknowledge the immense privilege of having incredible collaborators who have enriched my training and have taught me the value of collaborative science. Dr. Sylvie Benestad at the Norwegian Veterinary Institute and Maria Nöremark and Dolores Gavier-Widen

at the Swedish National Veterinary Institute. Dr. Witold Surewicz, Dr. Xiangzhu Xiao, and Dr. Qiuye Li at Case Western Reserve University for your support, opening the doors of your laboratory, and allowing me to learn so much. Dr. Christopher Snow and Pegah Eizadkhah for your support and enthusiasm in collaborating on this project.

Thank you to all my sources of funding during my PhD journey, the qCMB training program, the OVPR Fellows program, the IDRRN training program, and the STRONG fellows program. Your support has been key in keeping focus on my research and reaching this milestone.

Now, I would like to thank my family. My mom for her unwavering love and support, and my dad for igniting in me the passion for books, never-ending learning, and perseverance. My dear sister and brother, sister-in-law, and beautiful nephew, and all of my family, for being there in my most challenging times. My greatest supporter, my wonderful husband, Gary. My friends, particularly those I have met during my time in the CMB program, have kept a smile on my face with their unconditional friendship. To mentors in the past, those who helped me find my way into a scientific career when I had no references for what being a scientist looked like.

Finally, I would like to personally acknowledge Dr. Glenn Telling for allowing me the privilege of being mentored in his laboratory. He has helped me develop a scientific mindset, to always question, to build resilience, and to become a more rigorous scientist. I am deeply grateful for his support, for believing in me, and for guiding me in achieving this lifelong dream.

DEDICATION

In memoriam:

Dr. Richard A. Bessen: a brilliant scientist and exceptional mentor.

Damelis Naranjo: my childhood friend and soul-sister.

Rojina Shrestha: the light of our PhD cohort, gone too soon.

The mice that gave their lives in the name of science.

TABLE OF CONTENTS

ABSTRACT	ii
ACKNOWLEDGEMENTS	iv
DEDICATION	vi
CHAPTER 1 – INTRODUCTION	1
REFERENCES	30
CHAPTER 2 – DETAILED INVESTIGATION OF THE STRAIN PROPERTIES OF MOOSE CWD PRIONS FROM SWEDEN IN GENE TARGETED MICE	48
Introduction	48
Materials and Methods	52
Results	61
Discussion	87
REFERENCES	91
CHAPTER 3 – ACQUISITION OF LYMPHOTROPIC PROPERTIES IN NON-LYMPHOTROPIC NORDIC MOOSE CWD STRAINS AFTER PASSAGES IN GENE TARGETED MICE	98
Introduction	98
Materials and Methods	102
Results	108
Discussion	120
REFERENCES	124
CHAPTER 4 – GENE-TARGETED MICE AS A MODEL TO INVESTIGATE THE EFFECT OF THE K109Q POLYMORPHISM IN THE PATHOGENESIS OF CWD	130
Introduction	130
Materials and Methods	134
Results	141
Discussion	167
REFERENCES	174
CONCLUDING REMARKS AND OVERALL SUMMARY	182

CHAPTER 1 - INTRODUCTION

A brief history of prion research: the origins of scrapie and scrapie research

In the eighteenth century, a premium commodity was one of the most important trading products of the British Empire. The demand for English wool fueled an exponential rise of England as a global trade superpower and the advent of the Industrial Revolution. It was in this significant and historic economic background that a fatal, contagious disease affecting sheep flocks was most seriously discussed by the British parliament, stressing the urgency to find a solution (1). Though the precise origin of the disease is not completely known, reports of what would eventually be named “scrapie” have been known in Europe since before the 1700s. The first formal descriptions of scrapie were made by clever observations of English sheep herders like Thomas Comber, who gave a first-person account in 1772 of “the shaking” as a collective of signs of a contagious disease that affected sheep on his farm for decades (2). It is possible that agriculturalist Edward Lisle described “the shaking” much earlier, as the precise date of his notes on the matter is not entirely known; but his work, compiled in the 1720s, emphasizes the pronounced weakness of the hindquarters. The term “scrapie,” which was later adopted to describe the disease, derives from the presence of intense pruritus that causes sheep to rub or “to scrape” their coat; descriptions of this behavior were not found in Lisle’s notes or earlier accounts of the scrapie epidemic. The presence of an early non-pruritic scrapie epidemic has been attributed to the predominance of atypical forms of scrapie (3), while the pruritic disease is now known as classical scrapie. The spread of scrapie among sheep in England and other European countries was attributed to the inbreeding practices that were imposed in efforts to improve the quality and yield of wool. This fact will be crucial to improve the breeding practices that led to the decline of scrapie among sheep in England in the twentieth century.

The studies by Besnoit and colleagues in the mid 1800s, established the neurological hallmarks of scrapie that eventually will characterize prion diseases, neuronal vacuolation. This was the first concrete evidence of scrapie as a neurological disease. Moreover, they attempted the first experimental transmission of scrapie, which failed to account for the remarkably prolonged incubation period characteristic of prion diseases, reporting negative results (2,4). After brilliant epidemiological observations by French scientists Cuillé and Chelle, they successfully achieved the first experimental transmission of scrapie in 1936, which established the long pre-clinical phase of the disease and its infectious nature (5–7). The transmissible nature of scrapie was first observed during the Moredun “louping-ill vaccine incident”. In 1935, the after the Moredun Research Institute developed a vaccine against a tick-borne disease of sheep caused by the louping-ill virus, based on preparations of neural tissues of sheep. One of the batches was prepared from tissues of young sheep that had acquired scrapie by vertical transmission. The vaccination effort resulted in a major scrapie outbreak (1,8). This vaccine was prepared with formalin-inactivated sheep brain, bringing attention to the resistance of scrapie to methods used to inactivate conventional viruses. Wilson also attempted to identify anti-scrapie antibodies but failed to find any (9). This was the start of a quest to establish the biological and chemical nature of the scrapie agent, which would result in one of the major scientific debates in modern biology.

Origins of the concept of prion strains

In order to standardize formal controlled laboratory studies on scrapie, a scrapie pool was established from three natural cases of scrapie and maintained by passages in Cheviot sheep. This pool was denominated sheep scrapie brain pool 1 (SSBP/1) (9,10). This isolate became one of the most widely known scrapie inoculum for laboratory use and the origin of

most rodent- adapted cloned prion strains that defined prion research for decades. Since scrapie had also been observed to occur in goats (11), in the 1950s, Pattison administered the SSBP/1 pool to goats, obtaining a complete disease attack rate, contrasting to the poor transmissibility observed in sheep. Pattison found a surprising phenomenon – after inoculation, the goats developed two distinctive phenotypes of disease, which were denominated “scratching” and “drowsy”; this finding represented the first experimental evidence of the existence of prion strains (12).

A major breakthrough came with the pioneering observations of Tikvah Alper, D.A. Haig, and M.C. Clarke that doses of ionizing radiation and UV-light radiation required for the inactivation of the scrapie agent were far superior to conventional viruses, ruling out the possibility of the agent relying on nucleic acids for replication, a subversive and revolutionary concept at the time (13,14). The work of Alper and colleagues was also accompanied by additional evidence derived from work demonstrating scrapie thermal resistance (12,15) and stability in low pH and alkylating agents (8,15). Resistance to the possibility of a nucleic-acid-free biological agent capable of infectivity and replication came with alternate hypotheses suggesting that scrapie activity was composed of a combination of scrapie-specific nucleic acid with normal membrane components that are inactive by themselves, to an animal viroid, to a slow virus, a concept pioneered by Sigurdsson in 1954 (16). Pattison and Jones had already proposed that scrapie could be a self-replicating protein (17). This hypothesis inspired J. S. Griffith, a British mathematician, to derive, using principles of thermodynamics, three potential theories for a nucleic-acid-free mechanism of replication. According to Griffith’s second theory, scrapie could be a host protein that existed in a “stable” conformation and a “reactive” form, where the reactive form can self-propagate its own conformation under favorable conditions, setting the foundations of ‘the protein-only hypothesis’ (18). In his words, “there is no reason to fear,” considering that polypeptide chains could replicate in a similar fashion as DNA, and to

completely dismantle the central dogma of biology; however, his theory remained unrevised for over a decade.

A revolution in prion research came with the first successful transmission of scrapie SSBP/1 pool into mice by Chandler in 1961 (19). This achievement represented a remarkable progress from the inconvenience of working with sheep and goats; however, it only amplified the debate on the perplexing nature of scrapie. Intracranial administration of scrapie to laboratory mice rendered “relentlessly progressive, and invariably fatal, disease of the central nervous system” (20). The continuous passage of brain extracts from these mice into new cohorts gave rise to stabilization of the incubation period and reproducibility of the phenotypes of disease. While the SSBP/1 pool isolate was transmitted to cohorts of mice and hamsters to generate some of the classic cloned prion strains we know today (i.e., 22L, 22A, 22CH), another isolate derived from the “drowsy” phenotype (due to the failure to achieve transmission with the “scratching” isolate) of scrapie transmission in goats was alternatively administered to cohorts of mice, rats and hamsters. The passage of “drowsy” into Swiss mice derived the 139A Chandler strain. An isolate from a mouse carrying this strain was then sent to Rocky Mountain Laboratories, where, after continuous and carefully controlled passage into CD-1 Swiss mice gave origin to the RML strain, one of the most studied mouse-adapted prion strains in the history of prion research. 139A Chandler passage through rats and then hamsters is the origin of the 263K strain of hamster prions (21–23), which became the first strain whose molecular structure was solved by cryo-electron microscopy many decades later (24). Another important strain found during the studies of transmissions in mice was derived from the transmission of natural scrapie isolates. After reproducing similar results with natural scrapie isolates from multiple sources across the United Kingdom (UK), the ME7 strain was determined to be the source of infection in all the scrapie cases. The successful transmission of ME7 infected mice

brains back into sheep and goats confirmed that it was indeed scrapie, the source of infectivity to mice (25). The modern concept of strains will be discussed later in the chapter.

One gene in control: discovery of the *sinc* gene

As mentioned previously, the finding of different phenotypes of disease in scrapie transmission in goats gave the first evidence of the existence of scrapie strains. After sequential passages of scrapie isolates to mice and other rodents, and reproducible, stable phenotypes of disease were established, strains were denominated due to the predictability of their pathogenesis. Those supporting the theory of a “slow virus” attributed strain differences to variations in the nucleic acid. In the classical, best documented full-scale experiment of the dynamics and evolution of the titers of the scrapie strain 139A Chandler in different organs, Eklund, Kennedy, and Hadlow attributed their results in virological terms, emphasizing potential explanations for the long incubations and lack of immunological response (20,26). The extensive studies of pioneers in prion pathogenesis, like A.G. Dickinson, R.H. Kimberlin, C. Walker, and colleagues, made groundbreaking observations that established the role of the lymphoreticular system, the effect of route of inoculation, inoculation dose, the strain of scrapie administered, and, importantly, the strain of mice used in the studies. Dickinson and colleagues observed consistent, reproducible differences in incubation period when identical doses of the strain ME7 were administered to different lines of inbred mice. Using restriction enzymes, they identified a gene responsible for controlling the incubation of scrapie. This gene, denominated *sinc* for scrapie-incubation, carried two alleles named *s7* and *p7* (s for short, p for prolonged, 7 for ME7 strain), acting as a single gene or two genes that were in proximity; the segregation of the short and prolonged phenotypes pointed to a single autosomal locus. This finding suggested that sheep and perhaps other scrapie-susceptible species must express the same gene. The identification of a gene that had such profound effects in scrapie pathogenesis provided a solid

preliminary explanation for the problem of scrapie strains. While Kimberlin and colleagues make their mark in the foundations of prion research, Hadlow, who had proposed a viral origin for scrapie, made a brilliant observation that brought prion research front and center in modern neuroscience.

A connection made on the other side of the world: human prion diseases

As mentioned, American veterinarian W. J. Hadlow, stationed as a scrapie researcher from the United States Department of Agriculture in Berkshire, England, heard about an exhibit at the Wellcome Medical Museum in London on kuru, a strange and newly discovered brain disease affecting the fore peoples of Papua New Guinea. Hadlow, highly trained in the neuropathology of scrapie, immediately made a connection with kuru after recognizing the neuronal vacuolation that had become a hallmark of the fatal disease in sheep (27). In his letter to Lancet in 1959, Hadlow suggested that if kuru and scrapie were caused by similar agents, kuru should be transmissible after a long incubation period, suggesting that any experimental transmission should be followed for a much longer period than the usual for other infectious agents. (1,28).

Only a few years earlier, Gadjusek and Zigas had introduced their observations of kuru. The name was derived from a native word describing the tremors and involuntary movements experienced by the affected individuals (29). Many potential causes were considered until the connection was made to the practice of ritualistic cannibalism of diseased relatives, which was a ceremony intended to pay respect to the dead. The definite connection was made in 1966 when Gadjusek's research group successfully transmitted kuru to chimpanzees, confirming Hadlow's early speculations, and further transmissions into other primates confirmed the initial results(30). Years before this crucial finding was made, neuropathologist I. Klatzo had extensively studied

several brains of people affected with kuru and made another unexpected connection. He knew of only one other human brain disease that resembled kuru in his experience –Creutzfeldt-Jakob disease (31).

While working independently, two German neurologists-neuropathologists, Hans Gerhard Creutzfeldt and Alfons Maria Jakob, described unusual cases of a brain neurodegeneration between 1920 and 1921. Creutzfeldt reported his finding as an unusual case in a young female of only 22 years (Bertha E.) who died from the disease only two and a half years later. Their neuropathological assessment described the classic spongiform changes that define neuronal vacuolation and gliosis, predominantly in the grey matter, which scrapie scientists knew well. The clinical manifestations of spasms and hyperalgesia also hinted at a new nosological entity. A clinical history of unusual signs and symptoms that preceded the terminal phase by years would also be a crucial observation in the recognition of Creutzfeldt-Jakob disease (CJD) as a prion disease many years later (32,33). Klatzo had again demonstrated the scientific impact of skilled neuropathology with the successful transmission of CJD to chimpanzees in 1968 by Gibbs and colleagues (34,35). With the inclusion of other neurological diseases in humans that resembled the slow course of pathogenesis observed for scrapie, the picture of what would be known as the group of transmissible spongiform encephalopathies (TSEs) started to take form.

The emergence of other animal prion diseases in the pre “protein only hypothesis” era

Transmissible mink encephalopathy.

By the time the scrapie-kuru-CJD connection started to form, a disease affecting farmed mink was first reported in Wisconsin in 1947 (36,37), and most outbreaks were observed during the 1960s (38). The early behavioral alterations are followed by ataxia and other motor compromise, which leads to death by inanition as the debilitated stage progresses. The

neuropathological findings are similar but by no means identical to Kuru, scrapie, and CJD. The definite link between this neurological disease of mink and these other diseases, came with the successful transmission from affected to healthy mink. The new denominated transmissible mink encephalopathy (TME) was, in fact, the first scrapie-like disease of a carnivore species. The origin of TME is hypothesized to be contaminated feed from cattle or ovine origin, possibly L-type BSE, as the farms affected used the same feed supplier (39). Most importantly and of great consequences for prion biology, R. F. Marsh and colleagues achieved the transmission of TME to hamsters, providing the second example of a TSE successful adaptation to a rodent model. (40).

Prion a “proteinaceous infectious particle.”

Though the early experimental work of Alper, Pattison, and Jones in the physicochemical properties of scrapie and the brilliant insights of Griffith remained unrevised for a while, with a few exceptions (41,42), it was the unconventional scientific mind of Stanley B. Prusiner that brought forward, based on his predecessors, the audacious idea of a protein-only pathogen and dared to produce experimental evidence of it. His road to the Nobel Prize in physiology and medicine in 1997 for his discovery of a “new principle of infection” was filled with controversy and resistance from those proposing the “slow virus” hypothesis (16,43–46). In the golden age of molecular biology, following the postulates of Watson and Crick and the central dogma of biology, in which biological information is transferred from nucleic acid to protein exclusively, the idea of a protein encoding heritable characteristics of pathogenesis was deemed impossible (47). Prusiner quickly realized that more refined techniques needed to be developed to isolate and study the scrapie agent; these efforts identified a 27-30 kDa protein that purified only with infectious fractions and was proteinase K resistant. PK digestion of the agent could decrease infectivity (48). In his reflection upon the amount of evidence pointing to the non-conventional

nature of scrapie, he suggested this agent to be a novel infectious entity and proposed the term “prion” for *proteinaceous infectious particle*, “which are resistant to inactivation by most procedures that modify nucleic acids” (43). The concept was clearly heretical.

If a protein were the infectious unit, the question was now whether that protein was foreign or host related. Following Prusiner’s prion hypothesis, remarkable progress was made in identifying the origin of the proteinaceous infectious particle. Since the purification of the 27-30 kDa protein (27-30PrP) from scrapie fractions, B. Chesebro and colleagues synthesized an oligonucleotide mRNA probe that hybridized to the complementary coding sequence to the amino acid sequence of 27-30PrP. Since they found this in both infected and uninfected mouse brains, they concluded that this mRNA must correspond to a normal host protein(49). This led to the discovery by Oesch and colleagues that a normal gene, the *Prnp/PRNP* gene in mice and humans, encoded the PrP27-30 protein (50).

The unambiguous role of the PrP27-30 protein (now denominated cellular prion protein or PrP^C) in the pathogenesis of prion diseases was conclusively demonstrated through the work of Büeler et. al. with the creation of the first PrP knockout mice. Mice devoid of PrP^C expression were resistant to infection by scrapie prions, with the absence of replication of the infectious agent and lack of clinical disease (51,52). Moreover, restoration of PrP expression in PrP knockout mice through the introduction of a hamster transgene resulted in not only further evidence of the absolute necessity of PrP^C for infection by scrapie prions but also introduced more clearly the issue of the species barrier and the role of the PrP^C primary sequence homology. These mice were now susceptible to hamster prions but remained resistant to mouse-adapted scrapie prions (16,51,52). These enormous steps in the new field of prion research were fueled by one of the gravest public health crises of the XX century, the emergence of bovine spongiform encephalopathy (BSE).

An unexpected crisis: the emergence of Bovine Spongiform Encephalopathy

G. A. H. Wells and colleagues reported in 1987 the first study of a new neurological entity in cattle with close resemblance in clinical presentation and neuropathology to other spongiform encephalopathies like scrapie and chronic wasting disease, with predominant vacuolation in the grey matter, which they denominated bovine spongiform encephalopathy (BSE) (53,54). The disease that had been reported since 1986 in the United Kingdom and spread quickly among thousands of cattle was commonly named “mad cow disease”. At the height of the BSE epidemic, it was estimated that around one million cattle had contracted the disease and nearly 200,000 died, with devastating economic consequences; around 4 million animals were culled with losses above 4 billion pounds in the UK alone (16,55–57). The origin of BSE was linked to the use of meat and bone meal (MBM) feed that was given primarily to dairy cows as a way to increase the protein content of cattle feed. Changes in the processing of MBM implemented in the late 1970s likely allowed any infectious prions contained in the feed to survive the process of rendering. The rapid spread and simultaneous emergence of BSE throughout England, also suggested that horizontal transmission was unlikely (16,56). Although the banning of MBM as cattle feed was implemented in the late 1980s, the crisis reached its peak in the early 1990s. The stricter regulations imposed during the following years throughout Europe have made BSE “of negligible risk status” (58).

The zoonotic risk of animal prion diseases: The case of variant CJD

The emergence of a new form of Creutzfeldt-Jakob disease was first reported by J. Collinge, T. C. Britton, D. Bateman and colleagues in two letters published in Lancet in 1995 describing two cases of CJD with onset of disease at an unusual young age (59,60). The landmark publication by J. Collinge and A. F. Hill in 1996 officially described it as “variant” CJD

(vCJD) (61,62). This new form of CJD had different clinical manifestations and age of onset compared to “sporadic” (sCJD), whose clinical manifestations are more common in individuals over 60 years old, with no known mutations or specific etiology (63) or “familial” CJD with linkage to autosomal dominant mutations of the *PRNP* gene (64). vCJD impacts younger individuals, with a median age of 28 years at the time of death, with over 200 cases reported in several countries (63). Collinge and Hill combined biochemical, neuropathological, and transmission studies to characterize this unusual rise in early-onset CJD cases. Not only did they find differences between these CJD cases and the sporadic form, but they also noticed similarities with the molecular signatures of BSE. Moreover, while sCJD was not transmissible to mice, vCJD, as well as BSE, rendered successful transmission (62,65). The role of polymorphism of methionine (M) or valine (V) at residue 129 is particularly striking, with virtually all affected individuals expressing both alleles for methionine at position 129. This suggests a protective role of the variant valine at this position (66)

The devastating consequences of BSE transmission to humans underscore the unpredictability of prion adaptation and challenge the robustness of the species barrier. While sCJD prions are not commonly found in tissues of the lymphoid system, vCJD acquired lymphotropic properties during its adaptation from BSE. It is estimated that, due to the widespread distribution of BSE-contaminated cattle, 1 in 2000 individuals could be vCJD silent carriers (67,68).

Other human prion diseases: inherited and genetic.

Gerstmann-Sträussler syndrome (GSS). First described in 1936 by neuropathologists Josef Gerstmann, Ernst Sträussler, and Ilya I. Scheinker in an Austrian family (69). GSS is a rare autosomal-dominant inherited prion disease (70). Typical disease manifestations are an

early-onset progressive cerebral ataxia and progressive cognitive decline. Its incidence is lower than sCJD, with less than 1 case per million per year (71,72). The main genetic factor is the presence of a causative mutation with wild type proline (P) substitution for leucine (L) at position 102, which can also manifest into four different phenotypes based on the molecular signature (73,74). Other mutations related to GSS have been identified at codons 105, 117, 129, 131, 145, 187, 198, 202, 212, 217, and 219 (75). P105L is related to GSS with spastic paraparesis. Residue 129 can influence the duration of the clinical phase (76). The experimental evidence of GSS transmissibility came with the successful infection of squirrel and spider monkeys (77).

Familial CJD (fCJD). First identified in 1924, another inheritable dominant autosomal prion disease is caused by mutations of the *PRNP* gene (76,78). fCJD accounts for 10-15% of the total cases of CJD, and the onset of clinical disease is slightly earlier than sCJD. The most common mutation impacting susceptibility to fCJD is located at position 200 with substitution of glutamic acid (E) for lysine (K) known as E200K (76). Interestingly, the equivalent mutation in the bovine *PRNP* gene encoding for E211K, is also a pathogenic mutation related to atypical BSE (79). Insertions in the octapeptide repeat domain are particularly related to fCJD age of onset and disease course, with more insertions correlating with earlier onset and more severe phenotypes (80,81).

Fatal Familial Insomnia (FFI). The earlier report of FFI was published in 1986 in a 53-year-old man with progressive insomnia and dysautonomia. The disease progressed in nine months, leading to coma and death. Neuropathological analysis found a degeneration targeted at the thalamic nuclei and a family history of a similar disease (82). FFI is another inheritable prion disease related to a germline mutation of D178N of the *PRNP* gene (83) with a compounded effect of methionine at codon 129 (84). The presence of the alternative allele valine at 129 with the D178N mutation results in a fCJD phenotype (84). The concept of strain diversity encoded in

the molecular structure of prions was in part derived from seminal transmission studies of FFI and fCJD into transgenic mice expressing human PrP protein (85).

Modern prion research: Structure of the cellular prion protein

The identification of similar levels of mRNA PrP²⁷⁻³⁰ in preparations from infected and non-infected Syrian hamster brains, which showed differential sensitivity to proteolysis and solubility, led to the conclusion that the infectious agent existed in two forms: the physiological cellular protein (PrP^C) and the disease-causing form that exhibited the unusual biochemical characteristics (PrP^{Sc}) (16,50,86). PrP^C (PrP) is encoded by the *PRNP* gene located on chromosome 20 in humans and encompasses 2 exons, while mouse *Prnp* is located on chromosome 2 and encompasses 3 exons. The open reading frame of the full-length human PrP protein is located in exon 2 and encodes a 253-amino acid (aa) protein. The expression of PrP starts during embryogenesis, mainly in the hippocampus, and is expressed in many tissues in adults, though the higher levels of expression are localized to neurons and cells of the immune system, like glia, astrocytes, and follicular dendritic cells in the spleen, but also B and T cells and macrophages. Although PrP is denominated as the mammalian prion protein, its expression has been found also in birds and zebrafish. PrP is likely a highly conserved protein across all vertebrates (87–90).

The initial studies into the structure of PrP^C were achieved using nuclear magnetic resonance (NMR). Initially, mouse PrP^C (91) followed by human PrP^C by Kurt Wüthrich and colleagues (91). Wüthrich was awarded the 2002 Nobel Prize in chemistry for his achievements in the development of NMR, particularly devoted to solving the structure of human PrP^C.

The cellular processing of the mature PrP encompasses the removal of the N-terminal and C-terminal signal peptide (aa 1-22 and 232-253, respectively) and the addition of a

glycosylphosphatidyl-inositol (GPI) anchor at the C-terminal end (92). This ensures the anchoring of PrP to the cell membrane. The transit of the prion protein through the Golgi apparatus comes with the addition of two N-linked glycans at positions 181 and 197. Additionally, a stabilizing disulfide bond linking cysteine 179 and 214 connects alpha-helices 2 and 3 (93) (Figure 1.1). Full-length anchored PrP can undergo additional proteolytic processing. The α -cleavage that occurs between amino acids 110 and 111 results in a misfolding-resistant C1 fragment, which remains bound to the membrane, and a soluble N1 fragment, described for its neuroprotective effects (94,95). An alternate proteolytic processing called β -cleavage, with a more upstream cleavage site, has been linked to the response of PrP to oxidative stress, is formed during infection - derived from proteolytic cleavage of PrP^{Sc} (96,97). A notable feature of the prion protein is a flexible, intrinsically disordered N-terminal domain containing an octapeptide repeat sequence with affinity for Cu²⁺ and Zn²⁺ ions which is believed to play a role in the cell protective role of PrP^C (98). Octapeptide repeat insertions are highly correlated with genetic and familial CJD (99) and GSS (100). Also located in the flexible N-terminal domain, two regions flanking the octapeptide repeat are known as the polybasic regions or charged clusters CC1 and CC2. Three mutations related to the post-octapeptide repeat domain (CC2), including P102L, P105L, and A117V, are associated with familial prion disease (101). Transgenic mice overexpressing P101L (the mouse equivalent of P102L in humans) develop transmissible spontaneous disease (102). Moreover, the specific residues that composed sequence 105 to 111 were found to be critical for prion propagation (103).

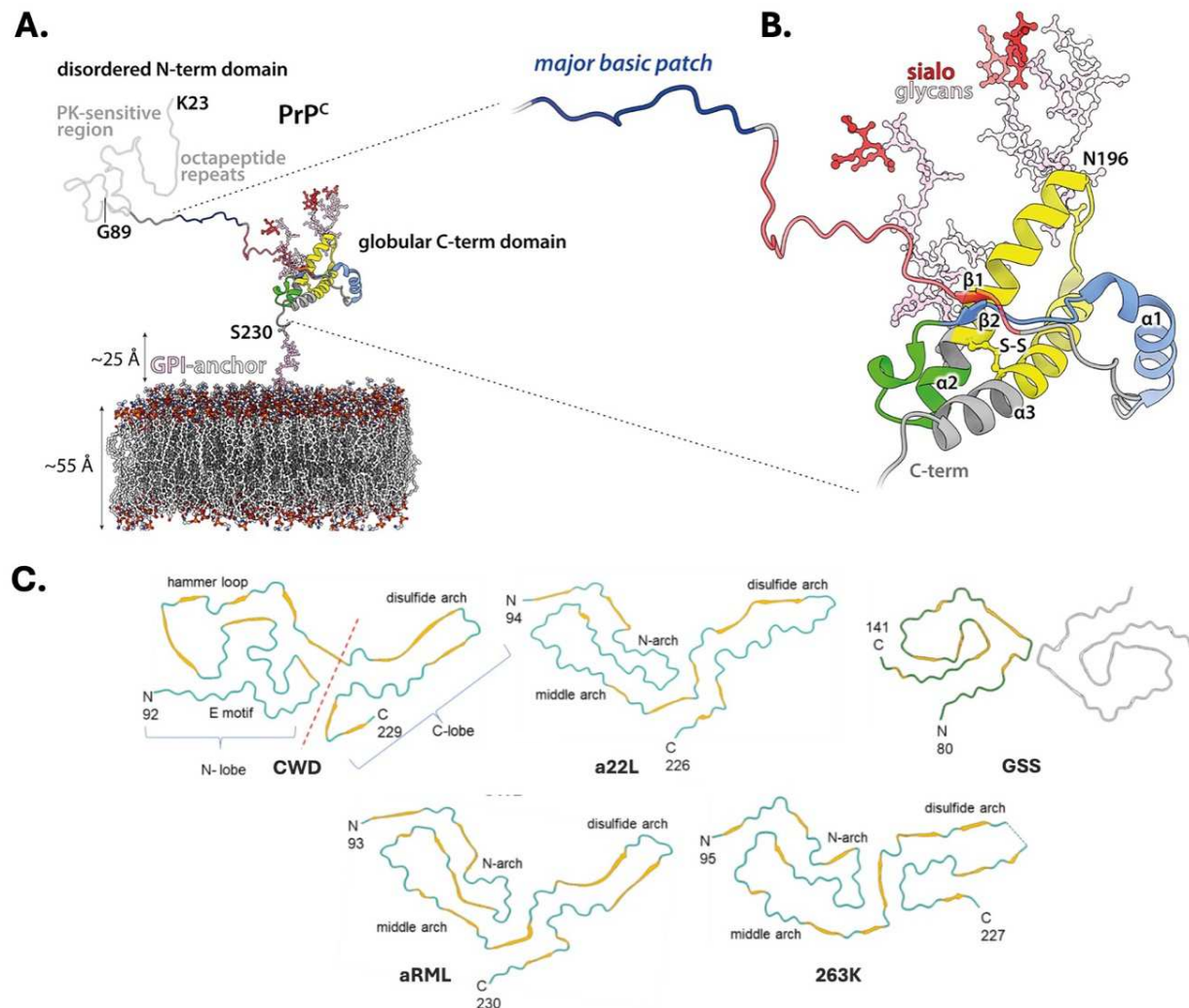


Figure 1.1. Structure of PrP^{Sc} bound to the cell membrane and comparison of the protofilament folds that define several prion strains **A.** Cellular prion protein amino acid 23-230 bound to the cell membrane by its GPI-anchor showing the globular domain secondary organization and the disordered, flexible N-terminal domain **B.** Location of the basic patch ~aa 100-110, the major alpha-helices and beta-sheets, the disulfide bridge and the post-translational N-linked glycosylation. From: Manka et al. 2023. **C.** Ribbon diagrams showing protofilaments for cervid CWD, anchorless 22L (a22L), Human GSS, anchorless RML (aRML) and hamster 263K. The individual cryo-EM resolved structures demonstrate that prion strains are defined by their unique structural polymorphism. From: Alam, et al. 2024.

The globular domain of the human prion protein comprises amino acids 122 to 231, and it is structurally formed by 3 alpha-helices spanning residues 144-154, 173-194, and 200-228 and two antiparallel beta-sheets at residues 128-131 and 161-164 (104). This region also influences susceptibility to familial prion diseases and species barriers (105,106), most importantly and well described, the susceptibility to CJD posed by polymorphism M129V located in the first beta-sheet strand (106).

Modern prion research: Prion strains and species barrier

The perplexing observations on the existence of multiple disease phenotypes caused by the misfolding of a single protein, were recognized early during the first transmissions of scrapie into rodent models and the identification of human prion diseases as a single group of disorders (12). How a protein-only pathogen could encode specific information leading to diverse disease outcomes was highly debated for years; indeed, the existence of strains was the main argument against the protein-only hypothesis and evidence that prion replication had to be mediated by an unidentified nucleic acid (107). While strains in conventional pathogens are traced to variations in the sequence of their nucleic acids, the definition of strain identity in prion biology has relied on an operational definition in which transmission renders heritable phenotypes of disease that are faithfully recapitulated under controlled experimental conditions (108,109).

Two key observations during the 1990s established an evidence-based hypothesis to explain strain information in a protein-only mechanism of disease. In 1992, R. A. Bessen and R. F. Marsh had reported two phenotypes of disease in studies of hamster-adapted TME. Due to the specific clinical signs, these strains were denominated hyper (HY), characterized by hyperexcitability, tremors, and ataxia, while drowsy (DY) presented with progressive lethargy (110). Two years later, an extensive biochemical characterization of HY and DY agents

demonstrated that they had distinct protein conformations, and this conformation accounts for the heritable strain information (111). These findings were also supported by G. C. Telling in 1996, who identified differences in the molecular size of PrP^{Sc} species between FFI (19 kDa) and sCJD (21 kDa), effectively proposing that differences in the conformational variants of various neurodegenerative disorders are the basis for their distinct phenotypes. In the last years of the twentieth century and the start of a new century, several low and medium-resolution techniques were used to demonstrate differences in the secondary structure of prion strains using more direct biophysical techniques (112), as well as immunoreactivity (113) and response of prions to chaotropic denaturants (114).

The transmission of a prion disease between members of the same species had been observed to be much more efficient than between members of different species. The differences between the primary structure of PrP^C and PrP^{Sc} of the prion between species, constitute the basis of the species barrier (115). Nonetheless, the picture was much more complicated. Transmission of scrapie in inbred lines of mice had resulted in different phenotypes of disease that were classified into specific prion strains; the role of the primary structure of PrP in these phenotypic differences could be excluded (116,117), which highlights the potential role of host factors. A similar case was observed in the transmission of BSE to humans as vCJD and the efficient transmission of vCJD prions to wild-type mice, while transmission of sCJD prions was only possible in transgenic mice expressing human PrP (61,118). To offer a hypothesis to the problem of prion strains, J. Collinge and A. Clark proposed the *conformational selection model*, which postulates that only a subset of the available cloud of molecular conformers or quasispecies that exist in a prion mixture is selected and propagates due to its conformational compatibility with the host PrP^C (119). Collinge adapted the term quasispecies from the concept first proposed in the 1970s by M. Eigen and P. Schuster originally used to explain the high mutation rate of RNA viruses that gives rise to a “mutant spectra or *cloud*” (120,121) but instead

of representing nucleotide variations, it is referred to conformational variants referring to multiple prion species. was adapted Additionally, strains have been observed to shift their molecular and phenotypic signature. According to the conformational selection model, this is due to the selection of a different or minor conformer in different conditions rather than mutations of the former strain (122,123). An alternative model developed in later years, denominated *deformed templating*, postulates that during interspecies transmissions, PrP^{Sc} intermediates that form from the inefficient templating of mismatching PrP sequences can facilitate the eventual adaptation and breach of a previously robust species barrier. This model originated with the advent and successful transmission of synthetic prion strains (124,125). Deformed templating considers the role of other cellular factors, echoing the original hypothesis of “Protein X,” a cellular co-factor that facilitates prion conversion (126). Alternate selection of conformers can arise as a consequence of drug resistance, allowing minor species to propagate with unpredictable outcomes (127,128)

The biophysical properties of prions, such as their high insolubility, posed a monumental challenge to the study of their structure at high molecular resolution. In the early 2000s the use of synthetically generated fibrils of human prions allowed the first structural studies of prion fibrils. Prions share an architecture based on the stacking of misfolded PrP monomers forming a fibrillar parallel-in-register intermolecular β -sheet (PIRIBS) structure (129). The development and refinement of cryo-electron microscopy and macromolecule atomic-resolution by Jacques Dubochet, Joachim Frank, and Richard Henderson, who were awarded the 2017 Nobel Prize in chemistry for their achievements, made the impossible, possible. In 2021, the groundbreaking report of the first high-resolution structure of a mammalian prion, the hamster-adapted strain 263K by A. Kraus and colleagues, opened a new era of prion research and demonstrated for the first time the accuracy of the PIRIBS model (24). The subsequent report of several cryo-em structures of rodent-adapted prion strains like RML (130), ME7 (131), and anchorless RML

(132), natural chronic wasting disease (CWD) from deer (133), human prion disease GSS (134) and synthetic fibrils containing the Y145Stop (tyrosine at position 145 is replaced by a premature stop codon) human PrP mutation associated to familial cerebrovascular amyloidosis (135) (Figure 1.1) . Have confirm that the PIRIBS molecular arrangement is common to all these prion strains, and that the individual protofilaments forming the filament stack adopt unique folds, these folds are now proposed to be the molecular signature of a prion informational identity (131,136–138).

Advances in high-molecular resolution through cryo-em and helical reconstruction algorithms have facilitated the resolution of other prion-like neurodegenerative diseases, finding that similar rules apply: high-resolution near atomic resolution of similar related pathologies like Alzheimer's disease (AD), Parkinson's disease (PD), multiple system atrophy (MSA), fronto-temporal dementia (FTD), dementia with Lewy bodies (DLB) and others, have revealed the same arrangement of protofilament with specific folds with specific packing arrangements (139,140). Although the wave of precise molecular resolution has a profound impact on our current definition of strain diversity, the real practical applications of the cryo-em resolution of prion structures in terms of disease diagnosis, progression assessment, and therapeutic interventions are yet to be fully understood. The biological relevance of these purified structures in terms of disease pathogenesis is still unknown.

Chronic Wasting Disease: the burgeoning prion disease

With the exception of the reported outbreaks of scrapie and BSE in the past two centuries, the finding of human or animals affected by prion disease is sporadic. This section will discuss the only prion disease affecting not only farmed but also free-ranging animals and has

established an endemic in North America: chronic wasting disease (CWD) of the members of the *Cervidae* family.

Chronic wasting disease was first discovered in 1967 in mule deer (*Odocoileus hemionus hemionus*) in a captive deer research facility in Fort Collins, Colorado. The clinical presentation of CWD suggested this was an entirely new disease, and its neuropathological features (neuronal vacuolation and astrocyte hypertrophy) resembled other transmissible spongiform encephalopathies (TSEs) or prion diseases. It was initially identified mostly in captive cervids; however, cases of the disease in several free-ranging cervid species were reported by the early 1990s (141,142). CWD was first recognized as a contagious wasting syndrome by biologists in Colorado before the first formal report by E. S. Williams and S. Young in 1980, recognizing CWD as a spongiform encephalopathy (141). The report described behavioral alterations, progressive weight loss with emaciation, polydipsia and polyuria, and excessive salivation. Death is caused by emaciation and secondary complications. Disease course can last up to 4 years, with a clinical phase from a few weeks to up to 1 year (143). After the initial studies in Northern Colorado, CWD had expanded to Wyoming and was identified in more species, including Rocky Mountain elk (*Cervus elaphus nelsoni*), captive black-tailed deer (*Odocoileus hemionus columbianus*), and white-tailed deer (*Odocoileus virginianus*) (144–146). The potential of CWD for remarkably facile transmission was recognized as a significant ecological threat, agricultural concern, and high risk for trade of captive cervids, both regionally and internationally (142).

Geographic and natural host range

The expansion of CWD in North America started slowly. In a period of ~30 years (from the first CWD case found in the late 1960s to the year 2000), CWD had only been reported in free-ranging cervids in Colorado and Wyoming, and in captive facilities in 4 US states and 1 Canadian province (147). The last report by the United States Geological Survey and the

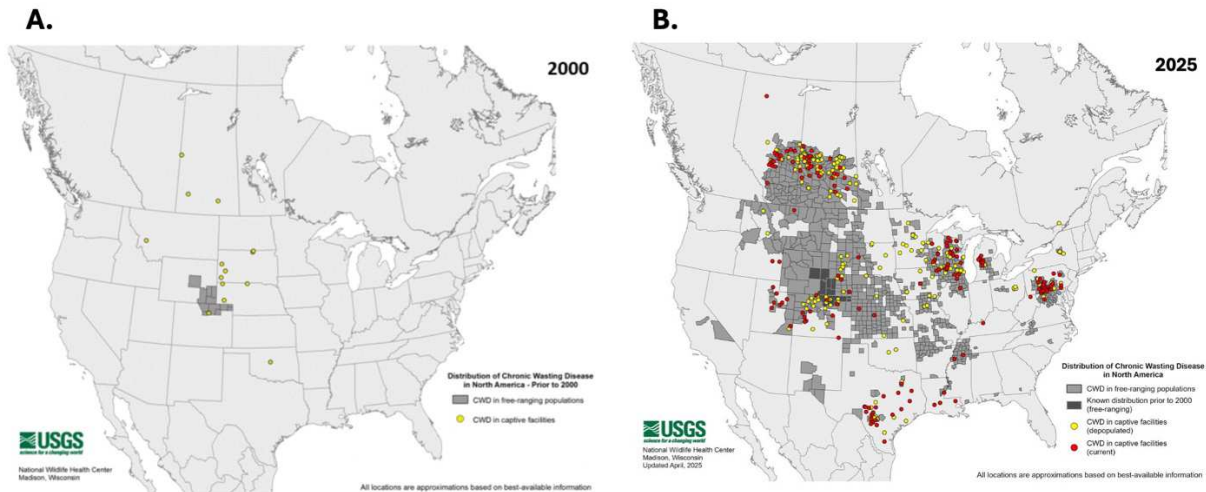


Figure 1.2 Geographic distribution of CWD in North America prior to the year 2000

compared to 2025. A. North American map displays the geographic range of chronic wasting disease (CWD) prior to the year 2000 B. Map reveals the exponential geographic spread of CWD throughout North America by April 2025. Source:

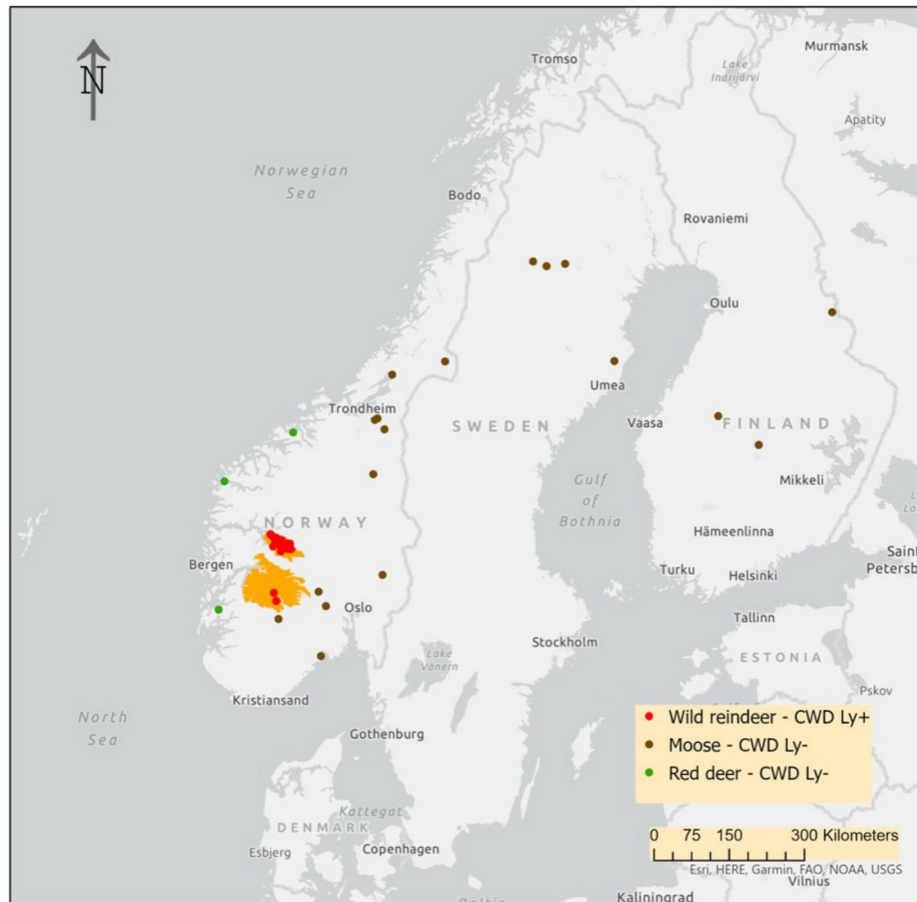
<https://www.usgs.gov/media/images/distribution-chronic-wasting-disease-north-america-0>.

National Wildlife Health Center in April 2025, CWD in free-ranging cervids has been reported in 36 US states and five Canadian provinces (Figure 1.2). The economic impact of CWD is enormous in terms of losses to game farming and hunting activities. Between the years 2000 and 2001, the US government spent nearly \$300 million in CWD-related efforts with very little success (148).

The range of CWD expanded beyond the North American Continent with the inadvertent export of a sub-clinically infected elk from Saskatchewan, Canada, into South Korea in 1997, causing the eventual spread of CWD among farmed cervids (149). Recent studies have concluded that the strain properties between North American and South Korean CWD are indistinguishable, even after two decades of transmission among red deer (*Cervus elaphus*) and

sika deer (*Cervus nippon*), demonstrating the stability of the North American CWD strain (150). In September 2005, the first case of CWD was detected in a hunted wild North American moose (*Alces alces shirasi*) in Colorado, and two additional cases were detected in 2006 (151). An additional case, the first one in Canada, was identified in 2013 (152).

In 2016, the first case of CWD in European cervids was identified in free-ranging Norwegian reindeer (*Rangifer tarandus tarandus*) (153). This unexpected finding was followed by the identification of additional cases in reindeer and wild Norwegian (Nordic) moose (*Alces alces alces*) (154,155), Norwegian red deer (156), Swedish moose (157), and Finnish moose (154) (Figure 1.3). The initial assessment of the clinical animals confirmed CWD positivity using ELISA-based rapid tests, TeSeE SAP ELISA CWD (Bio-Rad) and/or IDEXX HerdCheck ELISA. While reindeer were found to test positive for prion infectivity in lymph nodes, moose and red deer samples were found to render negative results in samples from those tissues (153–157). The reaction by Norwegian veterinarians and governmental authorities was swift to prevent the rapid expansion of CWD and prevent an outcome similar to that in North America. A difficult measure was taken to cull ~2000 reindeer from the original herd (154,158). The implementation of these approaches to control CWD has been controversial. The significant cultural and economic value of reindeer in Norway and other Nordic countries is no doubt an important factor to consider in approaching CWD surveillance and control (159,160). To date, the aggressive surveillance program in Northern Europe has reported ~21 positive reindeer, 15 moose, and 3 red deer in Norway; while Sweden and Finland have reported cases exclusively in moose with 4 and 3 cases, respectively (157,161).



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Figure 1.3 Geographic distribution of confirmed cases of CWD in Fennoscandia by the year 2023. Location of confirmed cases of CWD in Norway, Sweden and Finland. The orange shadowed areas represent the free-range reindeer populations affected with CWD, red dots represent individual cases. Brown dots represent individual moose cases found in each country. Red deer CWD cases are represented with green dots. Wild reindeer was the only species found with prion positivity in lymph nodes. Source: EFSA panel on biological biohazards. Scientific opinion on the monitoring of Chronic Wasting Disease (CWD) (IV). March 2023. Norwegian Institute for Nature Research: all rights reserved.

<https://doi.org/10.2903/j.efsa.2023.7936>

Pathogenesis and transmission of CWD

The fitness of CWD for horizontal transmission is unlike any other prion disease (162). Studies have estimated that the prevalence of CWD in free-ranging cervids in endemic areas of North America can be as high as 30% (163) and reach 90% in captive mule deer populations (141). Undoubtedly, CWD represents a serious ecological threat to the sustainability of cervid populations (164).

Like other prion diseases, CWD has a prolonged incubation period that can last several years, depending on the life span of the host. It is likely that the oral route through the consumption of water sources or vegetation contaminated with CWD, contact with contaminated carcasses, or interactions in which exchange of saliva, or contact with urine or feces can expose healthy animals, representing the main transmission route (142,162,165–167). Evidence shows that the likely cause of CWD's efficient horizontal transmission stems from its unparalleled tropism for the lymphoid system (168,169), constituting a site of early prion replication and long-term shedding of CWD prions into the environment through body fluids and excreta, long before neuroinvasion and clinical disease. This fact makes CWD an incredibly difficult wildlife disease to manage (145). CWD prions have also been detected in antler velvet, which is a highly demanded resource in Asian medicine (170), and blood (171). Vertical transmission is also possible, and CWD prions can be detected in fetal tissues in free-range sub-clinical white-tailed deer (172–174).

CWD prions gain access to the gastrointestinal tract by the oral route and travel along neuroanatomic tracts like the vagus and the splanchnic nerve after interaction with lymphoid-associated tissue in the gut (175,176), followed by early colonization and replication in the spleen and peripheral lymph nodes. Alternatively, accumulation of CWD prions in the retropharyngeal lymph nodes suggests a direct transport after oral exposure (177). Infection can also be contracted by the nasal cavity, allowing direct neuroinvasion via olfactory receptor

neurons (178,179). Neuroinvasion from secondary lymphoid organs is the most likely event. Accumulation of CWD prions in lymphoid organs seems to be more prevalent in deer compared to elk and moose (180,181). North American CWD is notable for its peripheralization, with multiple tissues involved besides the central nervous system (CNS) and the lymphatic system (152). CWD infectivity has been found in skeletal muscle (182) and adipose tissues (174), raising concerns about the level of exposure of humans to CWD-contaminated cervid meat. In the brain, CWD prions, as most prion strains, preferentially invade the grey matter. In horizontally acquired CWD, the most prominent accumulation of PrP^{Sc} is seen in the medulla oblongata and olfactory cortex, with large plaque-like aggregates.

The emergence of CWD in Northern Europe has increased the complexity of CWD pathogenesis as we knew it. The initial reports on the cases in reindeer show some similarities with North American CWD in terms of the presence of lymphotropism and plaque-like accumulation in the CNS; however, Norwegian reindeer CWD is biochemically distinct from North American CWD, confirming that they are not related strains (153). In moose, the distinctive properties of CWD are even more removed from North American CWD, and even from Nordic reindeer CWD, suggesting yet again, a distinct CWD strain (152,154). The origin and pathogenesis of Nordic CWD is still poorly understood. Horizontal transmission seems a plausible explanation for reindeer CWD in Norway, though its true origin is unknown. The most accepted hypothesis on Nordic moose CWD is a sporadic origin, due to the geographical distance between cases and their atypical prion properties (152).

Experimental transmission of CWD: natural host and mouse models

The first experimental transmissions on the natural host contributed to characterizing the pathogenesis of CWD (183) and reflected the natural prevalence of CWD in elk and deer

(143,180). In 2006, the first experimental transmission in Shira's moose (*Alces alces shirasi*) was achieved, demonstrating that CWD had a wide host range among cervids (181). Several research groups have also achieved CWD transmission in European red deer (184) muntjac deer (*Montiacus reevesi*) (185), and reindeer (176), while fallow deer seems to be partially resistant (186).

As ideal as experimental studies in the natural host may be, they are lengthy, difficult, and costly. Therefore, the development of a mouse model would bring major advantages for researchers. The development of transgenic mouse models during the 1990s solved the issue of the species barrier that precluded the use of mice in the study of human prion diseases; this same technology could be used to generate mice that expressed cervid PrP^C. In 2004, Browning and colleagues developed the first transgenic mouse (Tg) model, Tg(CerPrP)1536 mice, based on strategies developed by Stanley Prusiner's group. These mice overexpress cervid PrP (deer sequence) 5-6 times higher than physiological levels, due to the random integrations of the cloned cervid *PRNP* sequence (187). The Tg(CerPrP)1536 mice recapitulate key aspects of CWD in the CNS; however, they do not accumulate prion infection in the lymphoid system, a unique and highly important aspect of CWD pathogenesis. Years later, Angers and colleagues developed the Tg(ElkPrP) during their investigation of the role of amino acid 226 of the cervid protein. Deer and moose express glutamine (Q) and elk expresses glutamic acid (E) at this position in their PrP amino acid sequence (170,188)

A new era in CWD research came with the development of the gene-targeted (Gt) mouse model by the Telling lab (189). This model, in contrast to the generation of transgenic mice, was constructed following a homologous recombination strategy that comprises two key elements: first, the homologous recombination allows a precise replacement of the *Prnp* coding sequence of the mouse with the *PRNP* coding sequence of cervids, and second, it preserves the regulatory elements of the mouse sequence. In consequence, the Gt mouse model expresses

wild-type levels of PrP^C and recapitulates the lymphotropic properties of CWD, making them susceptible to peripheral challenge and natural routes of transmission (189). The syngeneic (identical genetic backgrounds between animals) Gt mice are also of great value to evaluate the effect of polymorphic variants under physiological expression of alleles of interest (189–191). With the emergence of Nordic CWD strains, R. Nonno and colleagues took advantage of the natural susceptibility of bank voles to a wide range of prion strains. These studies gave the first published experimental evidence that Nordic CWD strains were different from North American CWD (192). Using Gt mice models, Bian and colleagues found further evidence of the strain diversity of Nordic CWD from reindeer and Nordic moose and the unexpected adaptation of a moose strain towards a North American CWD phenotype (190). Yet again, the characterization of Finnish moose CWD showed distinct properties from North American CWD and even other Nordic CWD cases (161). The precise genetics of Gt mice will contribute to characterizing emergent strains of CWD and predict how their adaptation can represent risk for wildlife sustainability and for zoonosis.

CWD strains: from certainty to unpredictability

With the development of the Tg(CerPrP) lines, characterization of CWD strains was now possible in controlled conditions that facilitate the identification of specific phenotypes of disease. The results from almost 20 years of research suggest that North American CWD has low strain diversity and likely, a single, dominant strain is found in almost all cases of CWD studied from deer, elk, and even naturally infected moose (188–190). The first North American strains to be suggested as CWD1 and CWD2, were observed as outcomes during intracerebral transmissions of deer or elk-derived prions into PrP overexpressing Tg mice (188). Recent data from the Telling lab using the Gt mouse model suggest that these outcomes could be due to intracerebral inoculations in the overexpressing mouse, since transmission of different CWD

inoculum showed phenotypic differences when administered intracranially, but exhibited no differences during intraperitoneal transmission, which resembles more closely the natural route of transmission (193). These results strongly suggest that infectious CWD prions found in North American cervids are composed of a stabilized, dominant strain. One should not rule out that sub-strains could be present, but it seems likely that they are strongly suppressed by a dominant strain.

The emergence of Nordic CWD has completely changed the landscape of CWD strain diversity and evolution. The evidence points to Nordic CWD as an emergent form of the disease, unrelated to North American CWD. Strain characterization of Nordic reindeer and moose CWD shows not only striking differences in transmission, biochemistry, and neuropathological properties with North American CWD, but also these differences are observed between cases of the same species (161,190,192). Studies of Nordic moose CWD from Norway and Finland already suggest that Nordic CWD is composed of an unprecedented strain diversity, and iterative passages in mice models reveal unpredictable adaptation and evolution. Other projects in progress in the Telling lab demonstrate that this phenomenon is not exclusive to Nordic moose but is also found in Nordic red deer and reindeer. This dissertation will discuss new studies that expand and support this observation in Nordic moose, strongly supporting the current hypothesis of a sporadic origin for Nordic CWD.

Why this all matters: are humans at risk from CWD prions?

The zoonotic nature of BSE is a powerful reminder of the unpredictable nature of prion adaptation. Cervids are an important food source for millions, and reindeer herding is a fundamental way of life for Nordic indigenous populations. Antler velvet is a precious commodity in demand in Asian traditional medicine. All these circumstances make it evident that exposure

of humans to CWD is significant. Epidemiological data have found no evidence of humans developing CJD or prion disease as a consequence of consumption of CWD-contaminated cervids (194,195). The stronger evidence of potential zoonosis comes from CWD transmission studies in squirrel monkeys, which are susceptible to other human prions and scrapie (196,197); however, studies in cynomolgus macaques (*Macaca fascicularis*), which are evolutionarily closer to humans, failed to transmit CWD. Multiple studies using Tg mice expressing human PrP, have not shown conclusive results or are mostly negative, suggesting the species barrier between humans and cervids is robust (198,199). *In vitro* prion amplification assays like protein misfolding cyclic amplification (PMCA) offer a valuable tool to assess interspecies prion conversion. These studies have found that the potential of CWD prion to convert human PrP is very limited, requiring several passages to stabilize a strain before human PrP conversion could occur, or being dictated by polymorphic variations in both cervids and humans. (67,200,201).

In conclusion, the evidence suggests that the zoonotic risk of CWD prions is low, perhaps negligible, but not “nonexistent”. While the risk is uncertain, further research and surveillance efforts cannot be neglected. With the rise of a new group of unstable, unpredictable CWD strains emerging in Europe, these challenges are renewed.

Summary

The field of prion research has challenged the near-sacred status of the central dogma of biology and revolutionized our understanding of how pathogens encode heritable information of disease. Prion diseases have had far-reaching consequences including devastating economic losses, ecological disruption, and public health crises. The geographic expansion of CWD demands greater efforts to contain its potential consequences in terms of wildlife conservation and public health safety. In this dissertation, a detailed investigation of the strain properties and

adaptation of emergent Nordic moose CWD demonstrates its unprecedented strain diversity, unpredictable adaptation, the acquisition of lymphotropic properties in Nordic non-lymphotropic moose CWD, and the role of the unique Nordic moose *PRNP* polymorphism K109Q in CWD pathogenesis.

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CHAPTER 2 – DETAILED INVESTIGATION OF THE STRAIN PROPERTIES OF MOOSE CWD PRIONS FROM SWEDEN IN GENE-TARGETED MICE

Introduction

Prion diseases are transmissible and invariably fatal neurodegenerative diseases that afflict humans and animals caused by an entirely novel mechanism of pathogenesis (1). The host encoded prion protein (PrP^c) is a GPI-anchored glycoprotein expressed in the cellular surface, particularly abundant in central nervous system (CNS) with also notable expression in secondary lymphoid tissues and lymphoid structures in the gastrointestinal system (2). Prions are formed by the template-directed, conformational refolding of the physiological prion protein into the disease-causing form (PrP^{Sc}) (3). During this process, PrP^{Sc} imposes its infectious β -sheet rich structure onto the normal α -helical PrP^c with the transfer of its specific structural information followed by progressive accumulation of PrP^{Sc} , neurodegeneration and the inexorable death of the host.

Prions lack nucleic acids but similarly to conventional pathogens, have the capacity of transferring heritable strain information that defines disease outcomes in terms of incubation period, clinical signs, morphology and deposition patterns of PrP^{Sc} in the CNS and, neuronal vacuolation (4). Strains can be defined as well by biochemical properties of disease-associated PrP like electrophoretic mobility, resistance to chaotropic denaturation, replicative properties in vitro, among others (5). These properties are highly relevant to transmissibility among closely related species sharing geographical range as well as, events that lead to host range expansion. Strain heritable information appears to be encoded in the molecular structure of PrP^{Sc} and it is faithfully recapitulated during prion replication (6). Recent cryo-EM advances have allowed the resolution of several structures from rodent adapted prion strains (7–10) genetic human prion diseases (11,12) and naturally occurring chronic wasting disease in deer (13).

These groundbreaking studies have confirmed what low and medium resolution structural techniques had originally proposed –prions share a structural architecture based on fibrillar parallel-in-register intermolecular β -sheet (PIRIBS) (14) assemblies formed by stacks of PrP monomers that adopt unique folds, unique to each prion strain. Moreover, the near-atomic resolution of multiple amyloid structures related to other neurodegenerative diseases like tau and α -synuclein, shows that individual protofilaments within the stack form unique folds that are correlated to different diseases (15). These distinctive molecular motifs are now proposed to be the molecular basis of the operational definition of prion strains.

The infectious transmission of prion diseases is highly influenced by the amino acid sequence identity of PrP^c between species, the relative abundance of PrP^{Sc} conformations and selective pressures they encounter in the host. The adaptation of a prion conformer to a new host can be related to strain instability (prion strain instability and evolution to more pathogenic forms) (16–18). The devastating consequences following the emergence of bovine spongiform encephalopathy (BSE), including significant economic loss and the transmission of BSE to humans in the form of variant Creutzfeldt-Jacob disease (vCJD), emphasize the unpredictable adaptation of prion strains and the risk they pose on human health (19).

Chronic wasting disease (CWD) is a remarkably contagious prion disease found in captive as well as free-range members of the *Cervidae* family. CWD was reported for the first time in a captive deer facility in Fort Collins, Colorado in 1967 (20). In the last 20 years, the geographical spread of CWD in North America has reached endemic proportions with prevalence in 36 US States, five Canadian provinces and increasing; posing a threat to the sustainability of cervid populations (21). CWD is particularly frequent in white-tail deer (*Odocoileus virginianus*), mule deer (*Odocoileus hemionus*) and rocky mountain elk (*Cervus canadensis nelsoni*). CWD cases in North American moose (*Alces alces shirasi*) are less frequent (22). The inadvertent import of sub-clinical rocky mountain elk to South Korea in the late 1990s, has consequently established

the disease among the native red deer (*Cervus elaphus elaphus*) and farmed sika deer (*Cervus nippon*) populations, with all the outcomes described for North American CWD (23–25). CWD has a distinctive facility for horizontal transmission under natural circumstances, due to the elevated tropism of CWD for peripheral tissues which facilitates the shedding of prion infectivity to the environment through saliva, urine and feces in levels sufficient for effective transmission (26,27). Studies in captive mule deer, estimate that the prevalence of transmission can be as high as 90% (28).

In 2016, Norwegian veterinarian authorities reported the first case of CWD in Europe, found in free-ranging reindeer (*Rangifer tarandus tarandus*) in the locality of Nordjella (29). This remarkable and alarming discovery led to a monumental effort to contain the potential spread of CWD among the last population of wild tundra reindeer in Europe which led to the culling of approximately 2,000 animals (30). After the detection of CWD in additional reindeer within this population, intense surveillance efforts were mandated by the European Union (31). As result of these policies, CWD infected red deer (*Cervus elaphus*) in Norway and several Nordic moose (*Alces alces alces*) from Norway and Finland were reported by 2018 (32–35). The first reports of Nordic moose CWD described the unusual biochemical and morphological properties of these prions when compared to Norwegian reindeer and NA CWD, proposing the finding of a novel CWD strain of likely spontaneous origin. In March 2019, Sweden reported the first case of CWD in the country in a female moose that was euthanized due to clinical signs (36). Overall, three additional cases of CWD in moose have been reported in Sweden from 2019 to 2021, three of them geographically clustered around Norrbotten county. The initial assessment of PrP^{Sc} by western blot and IHC analysis revealed the similarities between Sweden moose CWD and all other cases found in Nordic moose, lower molecular migration, intraneuronal deposition and absence of PrP^{Sc} in lymph nodes.

The development of suitable mouse models capable of recapitulating the properties of diverse prion strains, have been pivotal in our understanding of prion strain biology and evolution. These models have allowed us to overcome the transmission barrier posed by differences in PrP primary structure between prion susceptible species and mice. In a more refined approach compared to classical transgenic models, we have shown that gene-targeted (Gt) mice allow, not only the precise replacement of the mouse PrP coding sequence with cervid PrP expressing glutamine (Q) or glutamate (E) at amino acid 226, but provides wild-type levels of PrP expression that facilitate true recapitulation of strain properties, including the peripheralization of prion infection, a cardinal feature of CWD infectious transmission (37). Our previous studies in characterization of Norwegian reindeer, moose and Finnish moose CWD using GtQ (Q226) and GtE (E226) mice as well as others using diverse transgenic (Tg) mice and bank voles (Bv) have clearly demonstrated that Nordic CWD is unrelated to North American strains (38–40). In the present chapter, we utilize gene-targeted GtQ and GtE mice to thoroughly investigate the properties of CWD prions found in four moose from Sweden, providing a closer look to the unprecedented diversity of newly emergent prion strains and their unpredictable evolution.

Materials and Methods:

Ethics Statement

All animal work was performed in an Association for Assessment and Accreditation of Laboratory Animal Care International accredited facility in accordance with the Guide for the Care and Use of Laboratory Animals. All procedures used in this study were performed in compliance with and were approved by the Colorado State University Institutional Animal Care and Use Committee.

CWD Field Isolates

The moose CWD field isolates from Sweden designated M-SW1, M-SW2, M-SW3 and M-SW4 (M: moose, SW: Sweden, number: case number) have been described previously (36,41). Information on official codes, age, sex, *PRNP* polymorphisms, clinical signs and geographical origin are detailed in Table 2.1. The North American CWD isolate used as positive control in multiple analysis represents the passaged brain material of diseased Rocky Mountain elk, 99w12389 (E-US1) in GtQ or GtE mice and has been described previously (42,43). The isolates from moose CWD from Sweden were generously provided as part of a collaboration with Dr. Sylvie Benestad at the Norwegian Veterinary Institute and Maria Nöremark and Dolores Gavier-Widen at the Swedish National Veterinary Institute.

Mouse models and transmission studies

The generation and characterization of gene-targeted GtQ and GtE mice has been described previously (37). Heterozygous GtEQ mice were generated by breeding GtQ and GtE mice, while GtQ/TgQ (TgT) hybrid mice expressing the Gt and Tg alleles for glutamine (Q) at

226 of cervid prion protein were generated as previously described (44). Ten percent brain homogenates (BH) were established by mechanical disruption (MP Biomedical, Irvine, CA) using sterile phosphate-buffered saline (PBS) lacking calcium and magnesium ions. The same number of male and female mice between four and six weeks were anaesthetized with halothane and intracerebrally inoculated with 30µL of 1 % BH into the right parietal lobe using a 26-gauge needle. One hundred microliters of 1 % BH were administered for intraperitoneal inoculations. Mice were assessed three times weekly for signs of prion disease, including truncal ataxia, loss of extensor reflex, hunched posture, slowed movements, plastic tail, wobbling/flattened gait, head bobbing/tilt, limb paralysis and rough coat. Incubation period is defined as the time elapsed between inoculation day and onset of clinical signs which was defined as the first day when clinical signs were identified. Brains collected from animals terminated due to intercurrent illness, were assessed for western blot and real-time quaking-induced conversion assay (RT-QuIC) for prion positivity when possible. Only animals clinically sick and positive by western blot were included in the statistics for attack rate and incubation period. Animals from studies where no clinical signs were detected, were terminated up to ~600 days post inoculation.

Analysis of PrP^{Sc} by western blotting

Protein concentration for all 10% brain homogenates was determined by bicinchoninic acid assay (BCA, ThermoFisher Scientific™). Samples were treated with 50 µg/mL of proteinase K (Roche) in the presence of 2% sarkosyl for 1 hour at 37°C in water bath. PK digestion was terminated with the addition of 2 mM of phenylmethylsulfonyl fluoride (PMSF) and incubated for 10min. XT-sample buffer (Bio-Rad Laboratories) was added to 1X and samples boiled at 100°C for 10 min prior to gel electrophoresis. Boiled samples were loaded into a

precast 12% discontinuous Bis-Tris gels (Bio-Rad Laboratories) at 140 W for 90 min. Proteins were transferred overnight into a PVDF-FL membranes (EMD Millipore) in a Trans-blot transfer system (Bio-Rad Laboratories). Membranes were blocked for 1 hour in 5% nonfat milk in freshly made TBST buffer (20 mM Tris in distilled water + 0.5% Tween 20) and probed with PRC5 and PRC1 monoclonal antibodies (mAb) at 1:5000 and 1:500 dilutions respectively for 1 hour, followed by horseradish peroxidase (HRP)-conjugated anti-mouse IgG secondary antibody (Cytiva) at a 1:5000 dilution for 1 hour. Membranes were developed using ECL 2 western blot substrate (Thermo Scientific™) in chemiluminescence Fujifilm imaging system.

Immunohistochemical analysis

Immunohistochemistry (IHC) was performed as described previously (45). Brains collected were fixed in 10% formalin and embedded in paraffin blocks. 5 µm coronal sections were obtained and placed onto positively charged glass slides and heated to 60°C for 30 minutes. Next, slides are treated with xylene and graduated ethanol treatment, followed by 88% formic acid for 30 minutes. Antigen retrieval was performed in the 2100 Retriever (Proteo-Genix, Schiltigheim, France) using citrate buffer followed by endoperoxidase quenching in 3 % hydrogen peroxide. Slides are blocked in 5% nonfat milk for 30 minutes at room temperature before overnight incubation with primary antibody D18 at 1:2,500 followed by exposure to biotin labelled goat Fab anti-human IgG secondary antibody (Southern biotech, Birmingham, AL) at 1:2,500 dilution for 1 hour at room temperature. Slides are developed for 30 minutes at room temperature using avidin-conjugated horseradish peroxidase (HRP) with diaminobenzidine (DAB) as substrate (Vector Laboratories, Burlingame, CA). Images were captured using an Olympus microscope at 1 mM (4x) or under oil immersion at 10 µM (100x).

Lesion Profiling

Scoring of spongiform neurodegeneration was performed as previously described (46). Coronal sections of paraffin-embedded mouse brains were stained with hematoxylin and eosin. Sections of frontal, mid and hindbrain were randomly selected and evaluated independently by at least two investigators. Scores for severity of vacuolar degeneration were assigned to nine cerebral gray matter areas and four white matter tracts. Lesions were evaluated at a magnification of 100 μ M (10x).

Conformational stability assay

Conformational stability assay was performed as previously described (37,38,47). Protein concentration of 10% brain homogenates (BH) was determined by BCA assay (Thermo Fisher Scientific™). Dilutions of 10% BH containing from 5 to 30 μ g of protein were incubated with increasing concentrations of guanidinium hydrochloride (GdnHCl) in 96 well plates for 1 hour at room temperature with constant agitation. Treated samples were transferred to a nitrocellulose membrane (Whatman GmbH, Dassel, Germany) through a dot blot apparatus. After two PBS washes, membranes were air dried for a minimum of 1 hour before rehydration with PBS followed by digestion with 5 μ g/mL PK in cell lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, and 0.5% Igepal CA-630) for one hour and PK digestion was halted with PMSF at 2 mM in PBS for 20 minutes. To expose PRC epitope, membranes were denatured with 3M guanidinium isothiocyanate for 10 minutes at room temperature and blocked for 1 hour with 5% nonfat milk in TBST. Membranes were incubated overnight at 4°C with mAb PRC5 antibody 1:5000 followed by IRDye® 800CW goat anti-mouse IgG (H+L) (LiCOR Biosciences, Lincoln, NE, USA) at 1: 25000 for 1 hour. After washes with TBST and

TBS 1X, membranes were imaged in LiCOR Odyssey DLx imaging system. Signal quantification was performed in Li-COR image studio™ acquisition software.

Protein misfolding cyclic amplification

PMCA assay was performed as previously described (48). PMCA brain homogenate substrates were prepared by manual homogenization of uninfected, perfused GtQ and GtE mice brains in PMCA conversion buffer (1X phosphate-buffer saline, 150mM sodium chloride, 1% Triton X-100) with addition of protease inhibitors (cOmplete™ Protease Inhibitor Mini, Roche). Previous to PMCA reactions, substrates are supplemented with 12mM EDTA and 0.05% digitonin (Invitrogen). Ten percent brain homogenates from mice clinically diagnosed and confirmed as CWD infected were used to initiate PMCA seeding reactions. The initial seeding reaction is considered the 10^{-2} dilution in the series and subsequently diluted in 10-fold increments to 10^{-9} or 10^{-11} in uninfected substrates of the homologous genotype (GtQ derived seeds in GtQ substrates and similarly with GtE seeds and substrates). PMCA was performed using a QSonica700 sonicator at an amplitude of 12-15 A for a target of 120-130 W per sonication pulse for 10 seconds and incubation of 14 minutes and 50 seconds (144 sonication/incubation cycles). All PMCA conditions were held constant across all PMCA experiments. three independent PMCA experiments were run for each study.

Real-Time Quaking-Induced Conversion Assay (RT-QuIC)

Hamster recombinant PrP production. The expression plasmid containing the hamster prion protein coding sequence (90-231) was kindly provided by Dr. Byron Caughey (Rocky Mountain Laboratories, Hamilton, MT). The truncated Syrian hamster PrP^C coding sequence was expressed and consequently purified from BL Rosetta (Novagen) *Escherichia coli*. After the

generation of glycerol stock, cells were cultured in lysogeny broth (LB) media with selection antibiotic kanamycin and growth agent chloramphenicol. Inclusion bodies were harvested after bacterial cell lysis with Bugbuster™ (Sigma-Aldrich) supplemented with Lysonase™ (EMD Biosciences). Recombinant rHaPrP90-231 was purified by incorporating inclusion bodies in NiNTA superflow resin (Qiagen) and loading to a XK chromatograph column (Cytiva) on AKTA Fast Protein Liquid Chromatograph (GE). Proteins were denatured and refolded on the column. Following protein elution from the column, the fractions from the elution peak were combined and dialyzed overnight at 4 °C. Purified recombinant HaPrP concentration was measured and adjusted to 0.5 mg/mL, aliquoted in Eppendorf tubes and frozen at -80°C.

RT-QuIC assays. RT-QuIC was performed as follows. Ten percent brain homogenates or 10% spleen homogenates from GtQ and GtE diseased mice, as well as North American CWD (GtQ and GtE mice passaged – E-US1) as positive controls and uninfected GtQ and GtE brains and spleens as negative controls were diluted to 10^{-3} in PBS and then to 10^{-4} working dilutions in 0.1% sodium dodecyl sulfate (SDS) in PBS 1X. Spleens were seeded in RT-QuIC reactions at 10^{-4} to assess positive amyloid formation. Brains dilutions were used to generate serial $10_{\log}$ dilutions. RT-QuIC reaction mixture contains 1X phosphate buffer saline, 1mM EDTA, 170mM NaCl, 0.01mM Thioflavin T and recombinant hamster PrP (rHaPrP90-231) 0.1 mg/mL. Two microliters of brain homogenate dilutions are used to seed 98 μ L of reaction mixture loaded in 96-well optical black bottom plates, covered with plate sealing tape and loaded in FLUOstar® Omega plate reader (BMG Labtech). Reaction plate was run for 48 hours at 42C with readings recorded every 15 minutes.

Determination of threshold, lag times and seeding dose for RT-QuIC positives and analysis.

Threshold of positive reaction for RT-QuIC was determined by the sum of the mean plus five times the standard deviation for the first 4 readings (after excluding the first read) to avoid variability due to temperature differences and spontaneous aggregation of the recombinant PrP. Lag times were calculated as the time in hours for seeding reactions to cross the estimated fluorescence threshold. All RT-QuIC-generated data were plotted as quadruplicated technical replicates from 3 biological replicates. Seeding dose (SD_{50} /g tissue) was calculated using the Spearman–Kärber method (49).

Prion purification by NaPTA precipitation (non-protease treated).

Aliquots (200 μ L) of 10% (w/v) brain homogenate in D-PBS were followed by sarkosyl to 2% (w/v). NaPTA (pH 7.4) was added to a final concentration of 0.3% (w/v). Iodixanol was then added to 35% (w/v) and the sample was centrifuged at $16,100 \times g$ for 90 minutes to separate into an insoluble pellet (P1), a clarified supernatant (S1), and a buoyant surface layer. S1 was carefully collected and mixed with an equal volume of 2% (w/v) sarkosyl in D-PBS containing 0.3% (w/v) NaPTA (pH 7.4). After 10 minutes at 37 °C, the sample was centrifuged for 90 minutes at $16,100 \times g$ to yield pellet P2, which contained partially purified prions. At this point, P2 can be used for observation by AFM (atomic force microscopy).

To obtain P4, highly purified prions for EM-negative staining, P2 is sequentially washed, resuspended in 200 μ L wash buffer with 0.3% (w/v) NaPTA and benzonase, followed by centrifugation at $16,100 \times g$ for 30 minutes, producing pellets P3 and then P4. The final P4 pellet was resuspended in D-PBS containing 0.1% (w/v) sarkosyl at one-tenth the volume of the starting brain homogenate and stored at -70 °C. (50)

Atomic Force Microscopy.

A 10 μ L aliquot of fibril suspension (P2) was deposited onto a mica substrate and incubated for 5 min. The surface was washed three times with Milli-Q water and dried. Images were acquired with NanoScope 9.1 using ScanAssist mode and a silicon probe (spring constant 40 N/m) on a Bruker multimode atomic force microscope equipped with a Nanoscope V controller (v2.55).

Electron Microscopy:

Immunogold labelling. The primary antibody SAF-32 (epitope Octarepeat region) was used to probe PrP^{Sc}. Briefly, 10 μ l of the purified sample was resuspended in 500 μ l TBS containing 0.1% (w/v) sarkosyl and 2 μ g/ml mouse monoclonal antibody SAF-32 (Cayman Chemical Company) and incubated overnight at 25 °C on a rotor. After incubation, 40.5 μ l of 4% NaPTA was added to the solution to a final concentration of 0.3% NaPTA. The sample was centrifuged at 21,000 *g* for 90 minutes, and the collected pellet was resuspended in 9.5 μ l of TBS containing 5% (v/v) glycerol and 0.5 μ l of 12 nm Colloidal Gold AffiniPure® Goat Anti-Mouse IgG (H+L) (Jackson Immuno Research Inc. Cat no. 115-205-166). The sample mixture was incubated at 25 °C for 4 hours on a rotor. Finally, the sample mixture was short spun for 5 seconds ready for negative staining. (50)

Negative staining. For negative staining, the purified prion (PrP^{Sc}) in P4 was used. Briefly, 4 μ l of the sample was loaded on carbon-coated 300-mesh copper grids (CF300-Cu-50; Electron Microscopy Sciences), which were pre-glow-discharged for 40 seconds using a PELCO easiGlow™ Glow Discharge (TED PELLA Inc.). After 2 minutes of incubation, the grids were blotted with filter paper, briefly washed three times with a 50 μ l droplet of water, and finally, stained twice with 2% (w/v) uranyl acetate (50 μ l each) for 40 seconds. All the steps of grid preparation

were done within a biosafety cabinet in a biosafety level 2 (BSL-2) room. The grids were loaded onto a dedicated sample holder for the prion sample and screened on an FEI Tecnai G2 TF20 electron microscope (FEI) at an acceleration voltage of 200 kV. The images were recorded on an Eagle 4k × 4k multiport CCD camera (FEI company) with a 4-port readout at various magnifications. After the EM session, the sample holder was cleaned, disinfected, and properly stored in a dedicated cabinet. (50)

Both protocols of negative staining and immunogold labelling TEM with prion samples were approved by Environmental Health and Safety (EHS) at Case Western Reserve University (CWRU).

Statistical analyses.

Statistical analyses were conducted using GraphPad Prism software (San Diego, CA).

Results

Sweden moose CWD isolates exhibit widely different transmission properties after passages into GtQ and GtE mice.

We previously demonstrated that gene-targeted (Gt) mice expressing cervid prion protein (CerPrP) are susceptible to infection with Nordic moose CWD prions (38,40), establishing a more precise model for investigating this emerging prion disease. Here, we used Gt mice expressing CerPrP with either glutamine (Q) or glutamic acid (E) at residue 226 to characterize the transmission properties of CWD prions present in brain homogenates from the four confirmed Swedish moose cases (Table 2.1). The most notable finding was the rapid adaptation of the second Swedish isolate (M-SW2) in GtQ mice, which reached clinical onset at ~90 days post-inoculation (dpi) following an initially prolonged and inefficient primary transmission (Fig. 2.1B). This represents the fastest CWD transmission observed in our cervid PrP Gt mouse with wild-type expression levels of cervid PrP. Although GtE mice were initially resistant to M-SW2, secondary passage resulted in a complete attack rate with a longer incubation period of ~300 dpi (Fig. 2.1B). In contrast to the other Swedish isolates, the first isolate (M-SW1) was the only one capable of transmitting to GtE mice during primary passage, although infections were predominantly subclinical. This subclinical phenotype persisted across all passages involving GtE mice (Fig. 2.1A). As expected, M-SW1 transmitted efficiently in both primary and secondary passages in GtQ mice, but incubation periods remained stable (Fig. 2.1A). This pattern differed markedly from M-SW2, M-SW3, and M-SW4, all of which exhibited substantial reductions in incubation time after serial passage in GtQ mice. M-SW2 decreased from >500 dpi to ~90 dpi (Fig. 2.1B), Table S4). M-SW3 showed a ~100-day reduction, from ~380 dpi to ~280 dpi, with complete attack rates in both passages (Fig. 2.1C). M-SW4 resembled M-SW2 in showing a low primary-passage attack rate (~10%) followed by complete transmission in secondary passage, though without the extreme

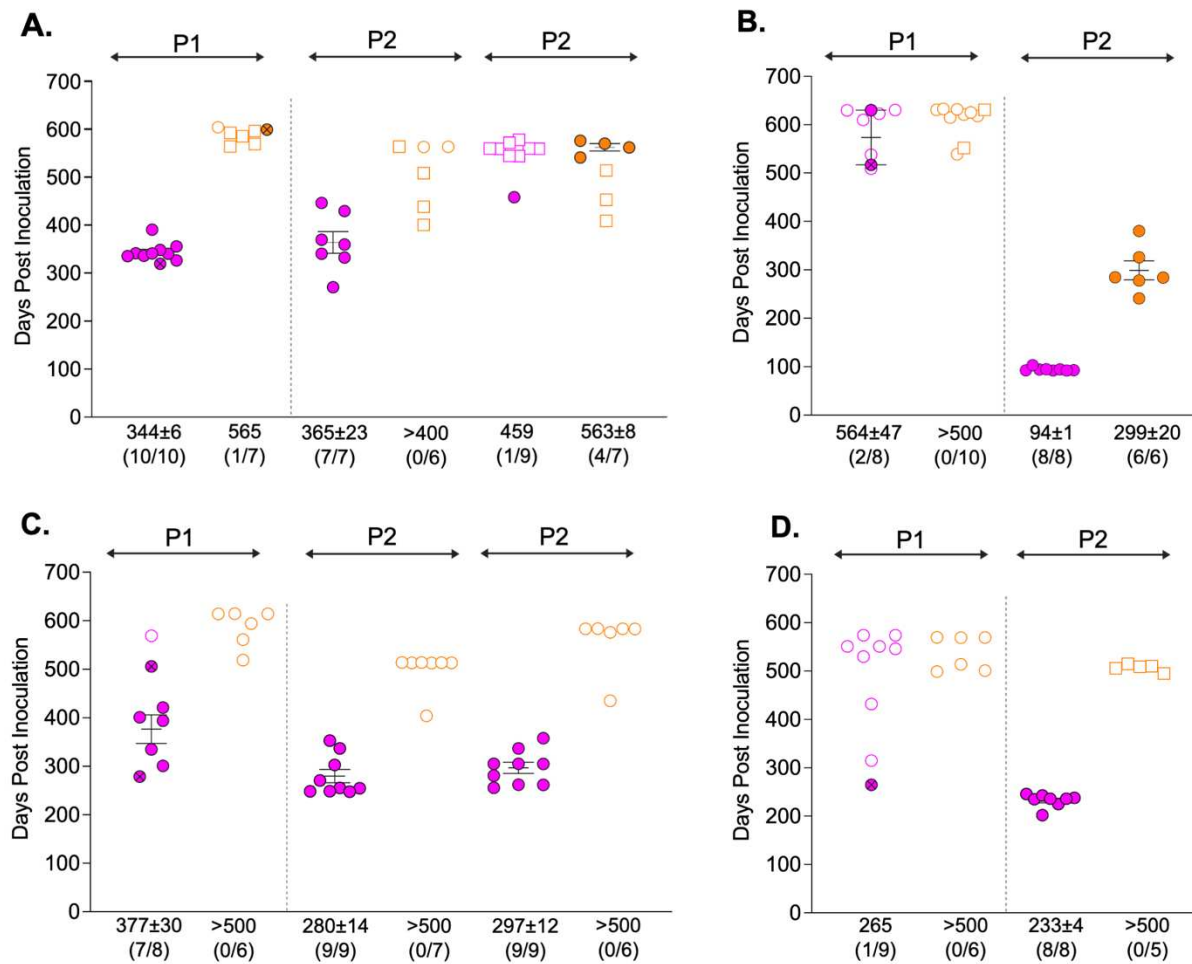


Figure 2.1. Primary and secondary transmissions of Sweden moose CWD isolates in gene-targeted (Gt) mice expressing glutamine (GtQ) or glutamic acid (GtE) at amino acid 226 of the cervid prion protein. Scattergrams showing the transmission properties of Sweden moose CWD isolates after intracerebral challenges in GtQ and GtE mice. Gt mice positive for clinical signs and PrP^{Sc} in the brain are represented (GtQ: full magenta circles, GtE: full orange circles). Sub-clinical cases: absence of clinical signs but presence of PrP^{Sc} in the brain are noted (GtQ: magenta empty squares, GtE: orange empty squares), mice without clinical signs and absence of PrP^{Sc} in the brain (GtQ: empty magenta circles, GtE: empty orange circles). Animals whose brains were selected for serial transmissions are labeled (a) and (b) (crossed circles). **A.** Primary and secondary transmissions of the first Sweden moose CWD isolate (M-SW1) in GtQ and GtE mice. **B.** Primary and secondary passages of isolate M-SW2 into GtQ and GtE mice. **C.** Primary and secondary transmission of the M-SW3 isolate in GtQ and GtE mice. **D.** Primary and secondary transmission of the fourth Sweden moose CWD isolate (M-SW4) in GtQ and GtE mice. P2(a): secondary passage of the brain labeled (a); P2(b): secondary passage of the brain labeled (b). Incubation period in days ($\pm$ SEM) and attack rate are shown and were calculated based on mice confirmed CWD positive by related clinical signs and presence of PrP^{Sc} in the brain by western blot. GtE and GtQ: CWD-susceptible gene-targeted mice in which the prion protein coding sequence was replaced with one encoding glutamate (E) at codon 226 or with glutamine (Q).

shortening of the incubation period observed for M-SW2 (Fig. 2.1D). While M-SW4 did not transmit to GtE mice in primary passage, secondary passage of GtQ-adapted M-SW4 into GtE mice resulted in mostly subclinical disease at >500 dpi, indicating ongoing adaptation to the GtE genotype (Fig. 2.1D). Notably, GtE mice remained completely resistant to M-SW3 infection in both primary and secondary passages after 500 days (Fig. 2.1C). Serial dilution analysis and estimation of prion titers by RT-QulC (SD_{50}/g tissue) is consistent with the kinetics of transmission in primary passages (Table 2.2). Collectively, these pronounced differences in transmission kinetics among Swedish moose CWD isolates indicate: 1) substantial strain diversity among Nordic moose CWD cases, 2) the adaptation and evolution of the transmissions properties of these CWD strains during serial transmission are influenced by *PRNP* polymorphism Q226E and 3) naturally emergent, unstable CWD strains, such as those found in Nordic moose, can undergo unpredictable adaptation during passage, including the emergence of a fast transmitting strain.

Table 2.1. Demographic data of Sweden moose CWD isolates.

Case number	Code	Age (y)	Clinical signs	Location
M-SW1	19-VLT000541	16	Emaciated, circling, presumably blind	Arjeplog
M-SW2	19-VLT000876	16	Emaciated, Behavioral changes	Arvidsjaur
M-SW3	19-VLT002232	10	Hunted, apparently healthy with altered behavior	Arjeplog
M-SW4	20-VLT002459	14	Walking on three legs	Västerbotten

Tertiary and quaternary transmissions of M-SW2 into GtQ and GtE mice, as well as transmissions into additional PRNP genotypes, further demonstrate the remarkable and progressive adaptation of M-SW2 toward a rapidly transmitting strain.

Following the unusually rapid adaptation of the second Swedish isolate (M-SW2) to a ~90-day incubation period in GtQ mice during secondary passage, we examined whether this accelerated disease kinetics—and the comparatively delayed onset observed in GtE mice—would undergo further changes during tertiary and quaternary passages. Transmission outcomes for brains derived from GtQ- and GtE-infected mice into both genotypes are shown in Fig. 2.2A. Tertiary passage of either GtQ- or GtE-derived inoculum into GtQ mice consistently produced rapid disease onset (GtQ-derived: 95 ± 1 dpi; GtE-derived: 107 ± 3 dpi). In contrast, transmission into GtE mice resulted in a further reduction of incubation periods relative to secondary passage, decreasing from 299 ± 20 dpi to 225 ± 2 dpi (GtQ-derived) and 251 ± 15 dpi (GtE-derived inoculum). Quaternary passage again revealed stable, rapid disease progression in GtQ mice, independent of the genotype of the inoculum (GtQ-derived: 93 ± 2 dpi; GtE-derived: 100 ± 2 and 99 ± 2 dpi). GtE mice exhibited slightly shorter incubation periods than in tertiary passage, though still exceeding 200 dpi (208 ± 0 and 223 ± 7 dpi). These findings indicate that once M-SW2 adapts to the GtQ genotype—matching the natural host *PRNP* sequence—the rapid 90–100 day disease onset becomes stable, whereas GtE mice undergo a more gradual adaptation process (Fig 2.2A).

To further investigate the stability of this adaptation, we transmitted a second M-SW2-infected brain from the primary passage, which was PrP^{Sc}-positive by western blot but collected at 610 dpi, approximately 100 days after the first clinical mouse used for earlier secondary transmissions. Secondary transmission of this ~610 dpi brain into GtQ

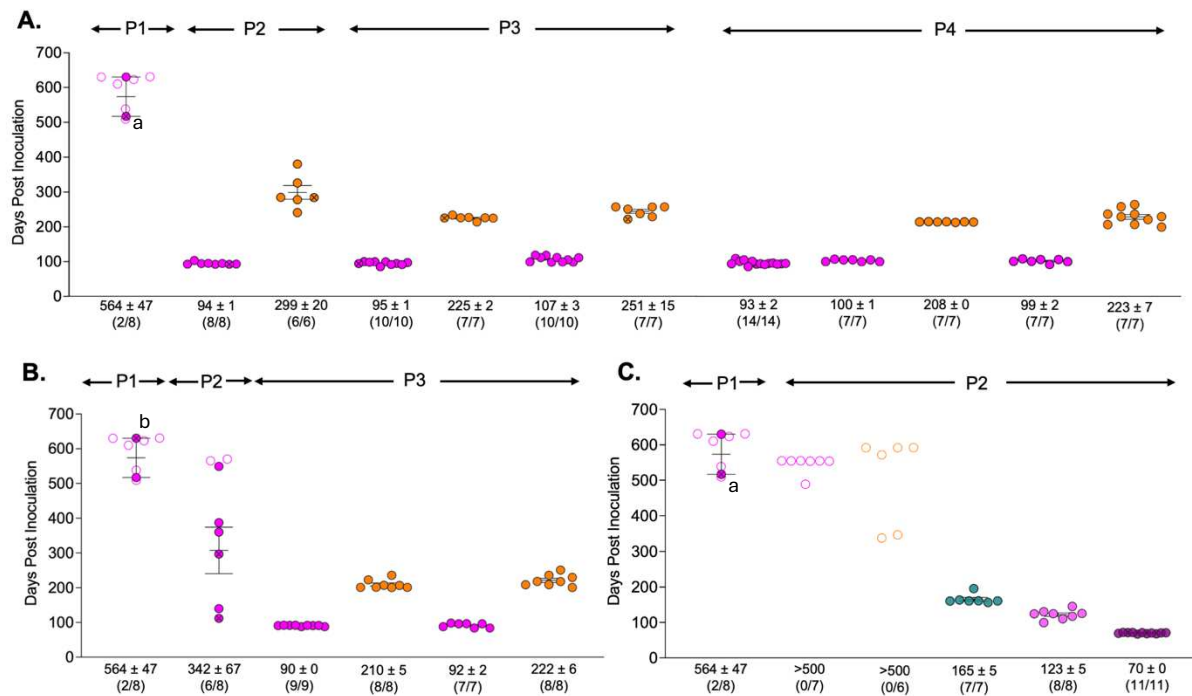


Figure 2.2. Tertiary and Quaternary transmissions of M-SW2 in GtQ and GtE mice and secondary transmissions of M-SW2 in GtEQ, TgQ and TgTQ mice and by intraperitoneal route in GtQ and GtE mice. Scattergrams showing the transmission properties of the second Sweden moose isolate after iterative passages in GtQ, GtE, GtEQ, TgQ and TgTQ mice. Mice positive for clinical signs and PrP^{Sc} in the brain are represented (GtQ: full magenta circles, GtE: full orange circles, GtEQ: full teal circles, TgQ: full pink circles, TgTQ: full plum circles). Sub-clinical cases: absence of clinical signs but presence of PrP^{Sc} in the brain are noted (GtQ: magenta empty squares, GtE: orange empty squares), mice without clinical signs and absence of PrP^{Sc} in the brain (GtQ: empty magenta circles, GtE: empty orange circles). Animals whose brain was selected for serial transmissions are labeled (a) and (b) in representation of primary passages or for iterative passages (crossed circles). **A.** Primary to quaternary transmissions from one brain selected from primary passage of M-SW2 isolate collected at 517 days in GtQ and GtE mice. **B.** Primary to tertiary transmission of a second brain selected from M-SW2 primary passage that was collected at 610 days into GtQ and GtE mice. **C.** Secondary transmission of the M-SW2 primary passage brain collected at 517 days into GtEQ, TgQ and TgTQ mice and by intraperitoneal route in GtQ and GtE mice. P2: secondary passage of brain labeled (a) or (b); P3: tertiary passage of brain labeled (crossed circles); P4: quaternary passage of brain labeled (crossed circles); Incubation period in days (± SEM) and attack rate were calculated based on mice confirmed CWD positive by related clinical signs and presence of PrP^{Sc} in the brain by western blot. GtE and GtQ: CWD-susceptible gene-targeted mice in which the prion protein coding sequence was replaced with one encoding glutamate (E) at codon 226 or with glutamine (Q); GtEQ: CWD-susceptible gene-targeted mice heterozygous EQ for codon 226; TgQ: PrP overexpressing transgenic mice expressing glutamine (Q) at codon 226; TgTQ: hybrid TgQ/GtQ mice.

mice produced a wide range of incubation periods (112–550 dpi) (Fig. 2.2B). We therefore performed tertiary passages using two brains representing early (112 dpi) and intermediate (297 dpi) disease onset. In both cases, transmission kinetics closely reflected those observed for tertiary passages of the first primary-passage brain, with GtQ mice developing disease at ~90 dpi and GtE mice approximately 120–130 days later (Fig. 2.2B). These results reinforce that M-SW2 consistently converges toward a fast-transmission phenotype during serial passage in GtQ mice, stabilizing at <100 dpi.

To evaluate whether the rapidly transmitting GtQ-adapted M-SW2 strain could transmit via peripheral routes, we performed a secondary passage of the primary-passage brain homogenate by intraperitoneal inoculation that adapted to the ~90 dpi transmission. Despite its accelerated intracerebral transmission, this inoculum failed to infect either GtQ or GtE mice when administered intraperitoneally (Fig. 2.2C). To further characterize the transmission properties of the GtQ-adapted M-SW2 strain, we performed secondary transmissions of primary-passage brain into three additional genotypes: GtEQ mice (heterozygous for Q226E), TgQ mice with a 6x expression of PrP-Q226 (27,37,51), and TgTQ mice (GtQ × TgQ), a model previously shown to accelerate disease onset for multiple prion strains (44). Transmission into GtEQ mice resulted in disease at 165 ± 5 dpi (Fig. 2.2C), intermediate between GtQ and GtE secondary passages. Surprisingly, TgQ mice exhibited a longer incubation period (123 ± 5 dpi) than GtQ mice (94 ± 1 dpi), indicating that PrP overexpression alone, does not enhance the rapid adaptation of this strain. In contrast, TgTQ mice displayed the fastest disease onset observed for M-SW2, with an average incubation period of 70 days, showing that PrP overexpression can influence the shortening of the incubation period but only when acting within the genetic and physiological context of the Gt mouse system. Collectively, these findings highlight the exceptional adaptability of M-SW2 which consistently

evolves toward a fast-transmission phenotype of ~90 dpi in GtQ mice and an even more accelerated course in TgTQ mice. Although this strain is capable of establishing efficient and fast disease when inoculated intracranially, it was unable to infection by the intraperitoneal route.

Analysis of electrophoretic properties of Swedish moose CWD prions in field isolates and serial transmissions in GtQ and GtE mice.

We assessed the presence of proteinase K (PK)–resistant CWD PrP in Swedish moose CWD field isolates and in the brains of GtQ and GtE mice diagnosed with prion disease by Western blot analysis. With the exception of M-SW1, detectable levels of PK-resistant PrP in the Swedish moose CWD field isolates were generally very low; however, these levels increased markedly during iterative passages. All Swedish CWD serial transmissions exhibited the characteristic electrophoretic migration pattern previously reported for other Nordic CWD cases, including the occasional presence of a second unglycosylated band of lower molecular weight than the dominant counterpart (30,38,40) (Fig. 2.3A-D). Differences in PrP^{Sc} glycoform distribution were observed during passages of M-SW1 in the GtE background, which were characterized by dominance of the diglycosylated band, whereas passages in the GtQ background showed predominance of the unglycosylated band (Fig. 2.3A). This effect of the Q226E polymorphism on PrP^{Sc} glycoform distribution was not observed in passages of M-SW2, M-SW3, or M-SW4 CWD isolates (Fig. 2.3B–D).

Western blot analyses were further performed to characterize PrP^{Sc} in CNS tissues from GtQ and GtE mice inoculated with Swedish CWD prions using monoclonal antibodies targeting distinct regions of the cervid PrP protein. The PRC5 monoclonal antibody recognizes an epitope in the globular domain between residues 135 and 162, whereas PRC1 targets an epitope N-terminal to the C1 cleavage site (52). All brain samples derived from Swedish moose CWD

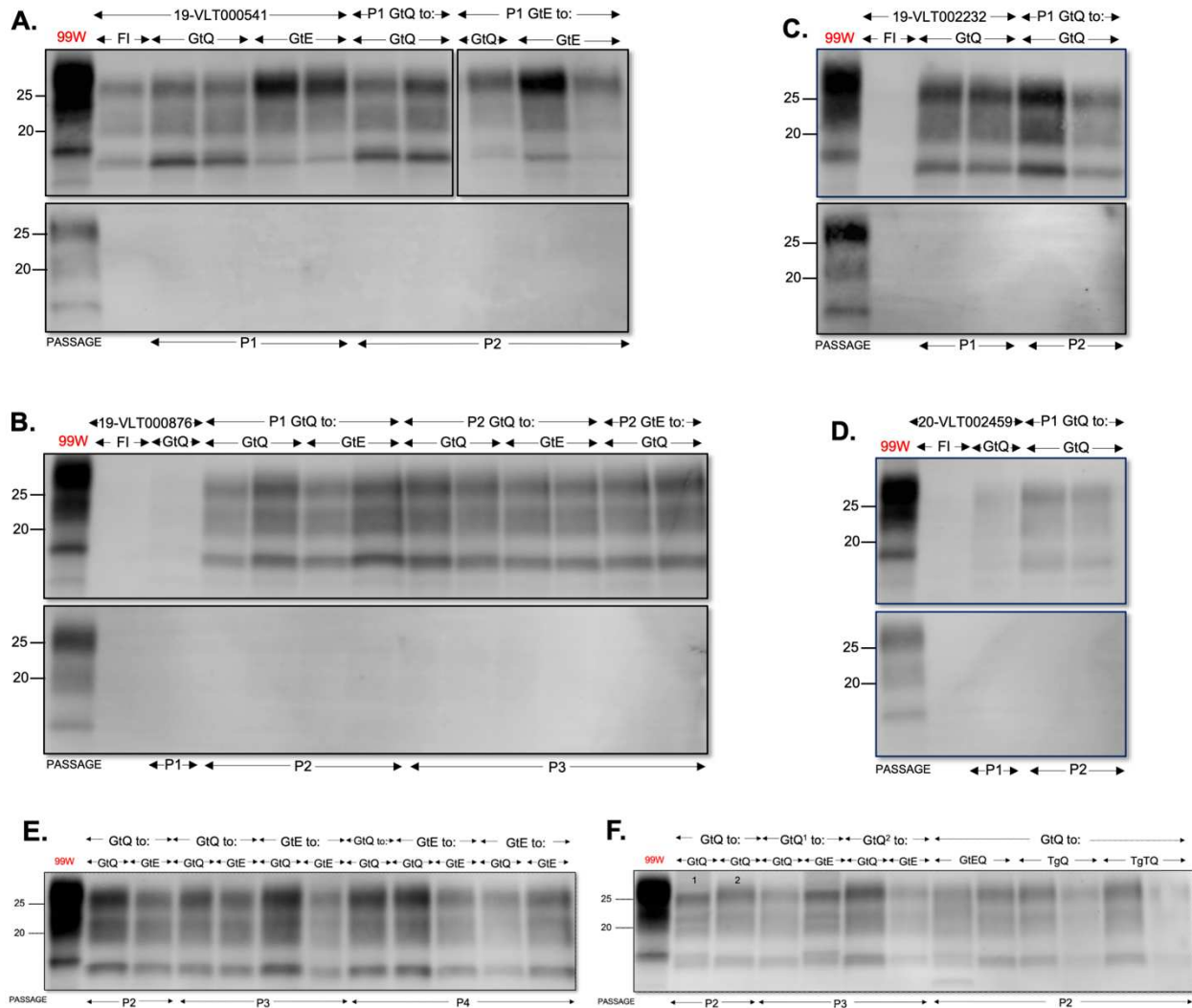


Figure 2.3. Immunoblot analysis of PrP^{Sc} in Sweden Moose CWD-infected gene-targeted mice. Western blot analysis of PrP^{Sc} from CNS tissue from GtQ and GtE mice infected with Sweden moose CWD isolates after iterative passages. **A.** First Sweden moose isolate (M-SW1) first and secondary passages in GtQ and GtE mice. **B.** Second Sweden moose isolate (M-SW2) first to tertiary passages in GtQ and GtE mice. **C.** Third Sweden moose isolate (M-SW3) first and secondary passages in GtQ mice. **D.** Fourth Sweden moose isolate (M-SW4) first and secondary passages in GtQ mice. **E.** Tertiary and Quaternary transmissions of M-SW2 into GtQ and GtE mice. **F.** Tertiary passages of the second 630d brain from M-SW2 transmission into GtQ and GtE mice, 1: 112 days; 2: 297 days brains used from secondary transmission. Immunoblots were probed with mAb PRC5 (top blot) and PRC1 (bottom blot). The position of molecular weight markers approximating 25, 20 kDa is shown; 99W: North American elk CWD isolate ; P1: primary transmission; P2: secondary transmission.

isolates and their passages in Gt mice were reactive to mAb PRC5, exhibiting faster electrophoretic mobility relative to North American (99W) CWD (Fig. 2.3A–D). In contrast, the absence of mAb PRC1 reactivity further distinguished Swedish CWD from North American CWD, indicating that N-terminal cleavage of Swedish CWD prions occurs at a more C-terminal site. For the M-SW2 isolate, these electrophoretic properties remained unchanged through tertiary and quaternary passages and upon transmission into GtEQ, TgQ, and TgTQ mouse genotypes (Fig. 2.3E-F). These findings demonstrate that the electrophoretic properties of Swedish moose CWD prions are consistent with those described for the majority of Nordic moose CWD cases and are clearly distinct from those of North American CWD.

Diverse conformational properties among the moose CWD isolates from Sweden demonstrate the unprecedented strain diversity in Nordic CWD

The marked differences in transmission profiles observed among the moose CWD isolates suggest that these cases are caused by distinct CWD strains. To support this hypothesis, we characterized the conformational properties of moose CWD prions in the brains of diseased GtQ and GtE mice. Previous studies examining naturally occurring and synthetic prion strains have reported a correlation between conformational stability and incubation period, such that less stable prions replicate more efficiently and are associated with shorter incubation times (53,54). To explore if prions in Sweden CWD cases conform to this model, we first assessed the response of M-SW1, M-SW2, M-SW3, and M-SW4 isolates, passaged in the homologous moose genotype (GtQ mice), to increasing concentrations of the denaturant guanidinium hydrochloride (GdnHCl) (55).

Analysis of the half-maximal denaturation concentration values ($\text{GdnHCl}_{1/2}$) for PK-resistant PrP at secondary passage revealed substantial differences in conformational stability

among the GtQ-passaged moose cases ($p < 0.0001$). M-SW2 exhibited the lowest GdnHCl_{1/2} value (2.2 M), indicating the least conformational stability, followed by M-SW4 and M-SW3, which displayed intermediate values of 3.0 M and 3.2 M, respectively. In contrast, M-SW1 demonstrated the highest GdnHCl_{1/2} value (4.1 M), consistent with increased conformational stability (Fig. 2.4). These values also differed significantly from those obtained for the North American CWD strain 99W following passage in GtQ226 mice (approximately 2.5 M; $p < 0.0001$) (Fig. 2.5A-F). These data indicate that the four moose CWD cases represent distinct prion strains.

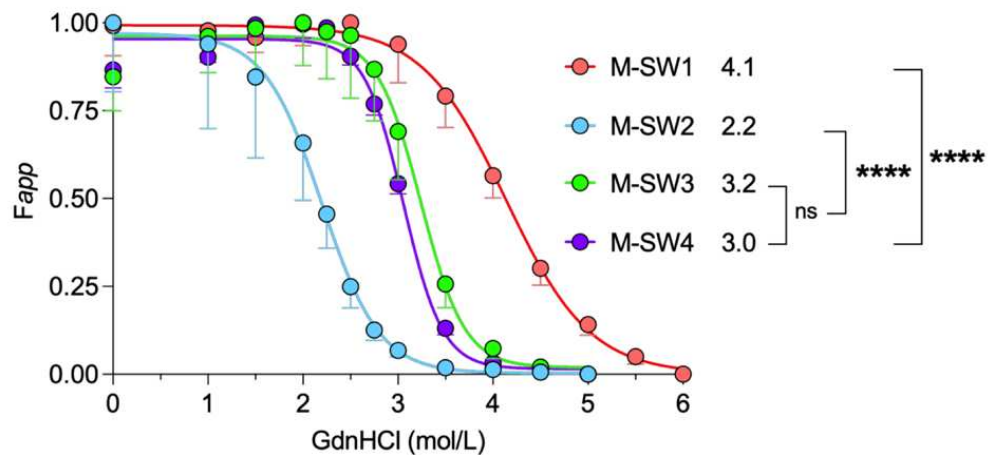


Figure 2.4. Conformational properties of Sweden moose CWD prions passaged in GtQ mice.

Response of Sweden moose CWD prions to denaturation with increasing concentrations of GdnHCl. The average fractional apparent signal (F_{app}) of PK resistant PrP^{Sc} was obtained by densitometry of probed dot blots plotted as a function of GdnHCl concentration (M) **A**. Comparison of denaturation response of Sweden moose CWD prions passaged in GtQ mice at secondary passage: M-SW1 (salmon circles) M-SW2 (light blue circles) M-SW3 CWD (green circles) M-SW4 (purple circles). GdnHCl_{1/2} values (Mol/L) for each Sweden moose CWD dose-response curve is shown. Sigmoidal dose-response curves were plotted using a four-parameter algorithm and nonlinear least-square fit. Error bars indicate $\pm$ SD of data from ≥ 3 animals per study. Statistical significances were calculated by pairwise analysis of GdnHCl_{1/2} values from best fit curves (**** $p \leq 0.0001$). ($F_{app} = (\text{maximum signal} - \text{individual signal}) / (\text{maximum signal} - \text{minimum signal})$). GdnHCl, guanidine hydrochloride; GtQ, CWD-susceptible gene-targeted mice expressing glutamine at codon 226; PK, proteinase K; R^2 (goodness of fit) for each curve: > 0.9 .

To determine whether the conformational properties of Swedish moose CWD prions remain stable or undergo adaptation during serial transmission, and to assess the potential influence of the Q226E PRNP polymorphism, we compared denaturation profiles across iterative passages in both GtQ and GtE genotypes. The conformational properties of all four isolates remained stable during serial passage. Specifically, comparisons of M-SW2 at secondary and quaternary passages (Fig. 2.5A-B) and of M-SW1, M-SW3, and M-SW4 at primary and secondary passages revealed no significant differences in $\text{GdnHCl}_{1/2}$ values ($p > 0.05$) (Fig. 2.5C-F). Moreover, no significant differences in conformational stability were observed during transmission of M-SW1 and M-SW2 in GtE mice compared with GtQ mice ($p > 0.05$) (Fig. 2.5A-D).

These findings indicate that each Swedish moose CWD isolate possesses distinct conformational properties that remain stable during serial passage and are not influenced by the Q226E polymorphism. Furthermore, we observed a clear correlation between conformational stability and incubation period among these moose CWD strains. The least stable strain, M-SW2, exhibited the shortest incubation period, whereas the most stable strain, M-SW1, was associated with the longest time to disease onset. M-SW3 and M-SW4, which displayed intermediate $\text{GdnHCl}_{1/2}$ values, were associated with correspondingly intermediate incubation periods.

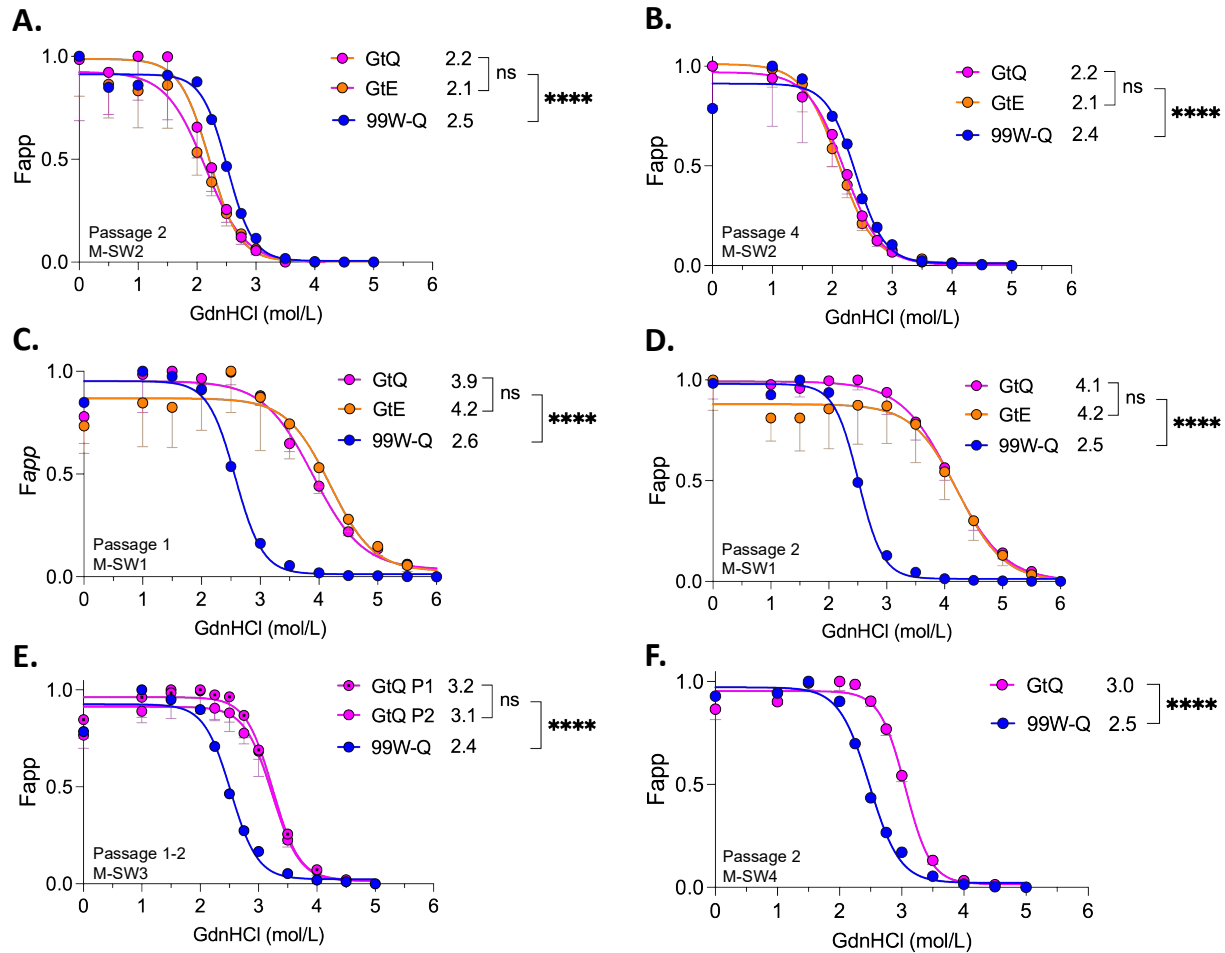


Figure 2.5. Conformational properties of Sweden moose CWD prions during iterative passages in GtQ and GtE mice. Response of Sweden moose CWD prions to denaturation with increasing concentrations of guanidinium hydrochloride (GdnHCl). The average fractional apparent signal (F_{app}) of PK resistant PrP^{Sc} was obtained by densitometry of probed dot blots plotted as a function of GdnHCl concentration (M) **A.** Comparison of denaturation response of Sweden moose 2 (M-SW2) CWD prions passed in GtQ and GtE mice at secondary passage **B.** Responses of M-SW2 CWD prions passed in GtQ and GtE mice to GdnHCl denaturation at quaternary passage **C.** Denaturation profiles of Sweden moose 1 (M-SW1) CWD prions after primary passage in GtQ and GtE mice **D.** Denaturation profiles of GtQ and GtE passed M-SW1 CWD at secondary passage **E.** Responses of Sweden moose 3 (M-SW3) CWD prions to GdnHCl denaturation after primary and secondary passages in GtQ mice **F.** Responses of Sweden moose 4 (M-SW4) to chaotropic denaturation at secondary passage in GtQ mice. All brain samples have been compared to North American CWD isolate 99W passed in GtQ mice. $GdnHCl_{1/2}$ values (Mol/L) for each Sweden moose CWD dose-response curve and the corresponding R^2 values. Sigmoidal dose-response curves were plotted using a four-parameter algorithm and nonlinear least-square fit. Error bars indicate mean $\pm$ SD of data from ≥ 3 animals per study. Statistical significances were calculated by pairwise analysis of $GdnHCl_{1/2}$ values from best fit curves. (F_{app} = (maximum signal - individual signal)/(maximum signal – minimum signal)) . GdnHCl, guanidine hydrochloride; GtQ, CWD-susceptible gene-targeted mice expressing

glutamine at codon 226; GtE, CWD-susceptible gene-targeted mice expressing glutamate at codon 226; PK, proteinase K. *** $p \leq 0.001$.

CWD isolates from moose from Sweden exhibit distinct patterns of PrP^{Sc} deposition and neuropathology in the central nervous system (CNS) after passage in Gt mice.

We analyzed brains from CWD-affected Gt mice inoculated with isolates from moose from Sweden to assess the morphological properties and regional distribution of PrP^{Sc} accumulation by immunohistochemical (IHC) analysis. Distinct morphological patterns and region-specific deposition of PrP^{Sc} were observed throughout the central nervous system (CNS). M-SW2 and M-SW4 were characterized by fine, diffuse PrP^{Sc} staining detectable only at high magnification (100 $\times$; scale bar, 10 μ m), which was present throughout the brain but most prominent in the thalamus and frontal cortex, respectively. No additional PrP^{Sc} deposition patterns were observed in the brains of Gt mice infected with these isolates (Fig. 2.6A, D). Although the intensity of PrP^{Sc} accumulation following tertiary and quaternary passages of M-SW2 in GtQ and GtE mice increased, the underlying morphology remained unchanged (Fig. 2.8A-B). Similarly, IHC analyses of PrP^{Sc} deposition in GtEQ and TgTQ mice revealed morphologies comparable to those observed in GtQ and GtE mice, whereas TgQ mice displayed a mixed pattern consisting of diffuse staining and dense punctate accumulations (Fig. 2.8C-E).

In contrast, the M-SW1 isolate exhibited a unique combination of PrP^{Sc} morphologies, including intense perineuronal staining localized to the substantia nigra in the midbrain (Fig. 2.6B), fine diffuse and granular staining in the hindbrain, and denser punctate deposits in both frontal and hindbrain regions. These region-specific patterns were observed in both GtQ226 and GtE226 mice (Fig. 2.7A-B). M-SW3 was distinct from the other isolates, displaying a pronounced coarse granular PrP^{Sc} pattern primarily localized to the neuropil of the thalamus (Fig. 2.6C).

The diversity of PrP^{Sc} deposition patterns across the CNS of Gt mice highlights the heterogeneity of CWD prion strains present in moose from Sweden. Notably, unlike North American CWD, the *PRNP* Q226E polymorphism had little to no effect on the morphology or regional distribution of PrP^{Sc} after intracerebral challenges of these moose isolates. Furthermore, the PrP^{Sc} deposition patterns observed in Gt mice closely recapitulate those previously reported in the natural host (41), underscoring the accuracy of the Gt mouse model in reproducing strain-specific pathological features.

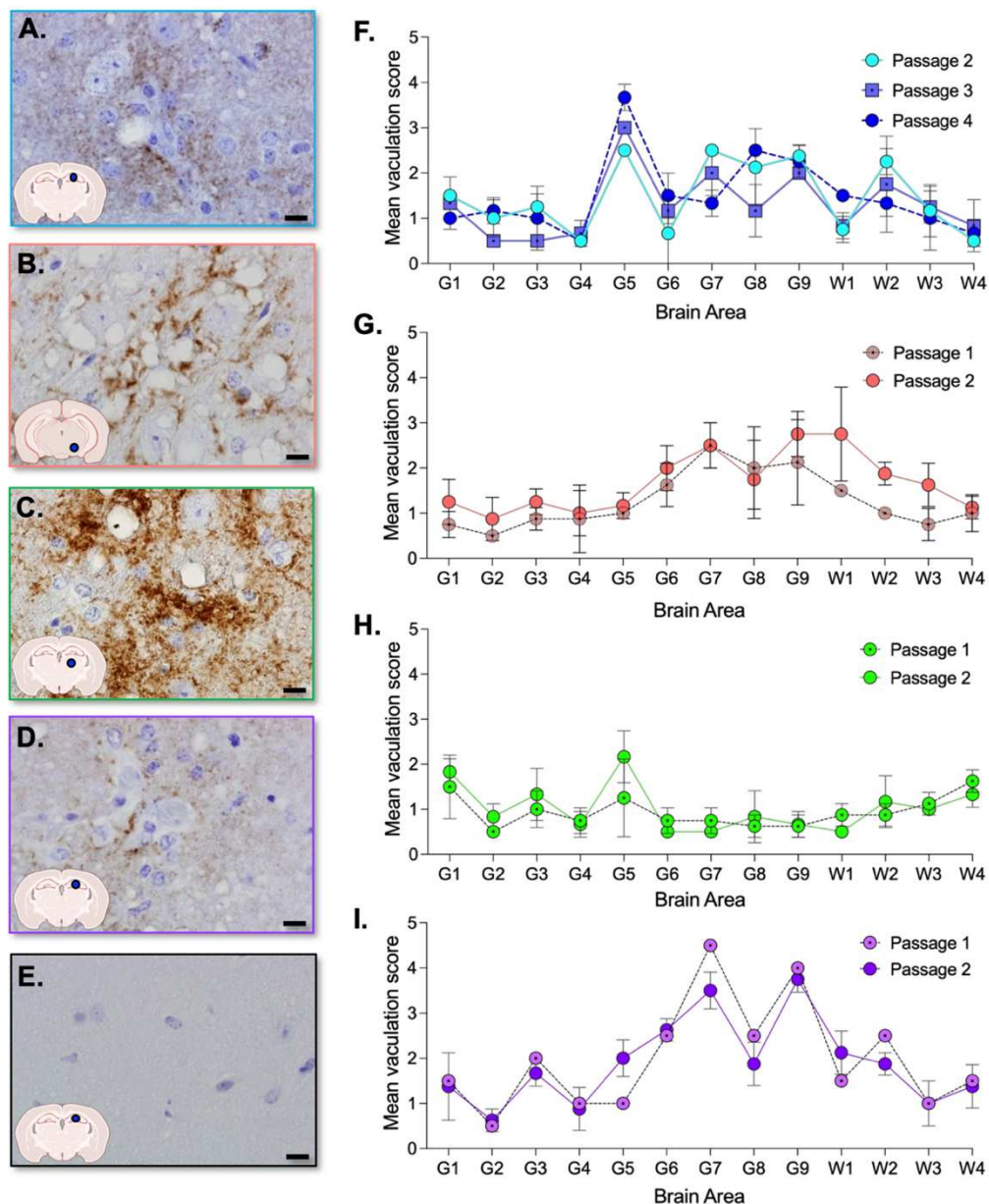


Figure 2.6. Immunohistochemical analysis of PrP^{Sc} and lesion profiling of coronal brain sections of Gt mice infected with moose CWD isolates from Sweden. Deposition and morphology of disease-related PrP^{Sc} in the CNS of diseased Gt mice and analysis of vacuolar lesion scores. **A.** PrP^{Sc} deposition in GtQ mice infected with Sweden moose 2 (M-SW2) in the region of the hippocampus **B.** PrP^{Sc} deposition in GtQ mice infected with Sweden moose 1 (M-SW1) isolate in the substantia nigra region of the midbrain **C.** PrP^{Sc} deposition in GtQ mice infected with Sweden moose 3 (M-SW3) isolate in the thalamus **D.** PrP^{Sc} deposition in GtQ mice infected with the Sweden moose 4 (M-SW4) isolate in the hippocampus. **E.** Stained hippocampus section of uninfected age-matched GtQ mouse. IHC sections were proven with Fab D18 antibody. Bar scale in A-E = 10µm **F.** Lesion profiles of secondary to quaternary passages of Sweden moose 2 (M-SW2) in GtQ mice (blue circles and squares) **G.** Lesion profiles of primary and secondary passages of Sweden moose 1 (M-SW1) in GtQ mice (salmon circles) **H.** Lesion profiles of primary and secondary passages of Sweden moose 3 (M-SW3) in GtQ mice (green circles) **I.** Vacuolation profiles of primary and secondary passages of Sweden moose 4 (M-SW4) in GtQ mice (purple circles). Assessment of vacuolar lesions was performed in the following regions: G1) medulla oblongata (Mobl); G2) cerebellum cortex (Cb ctx); G3) superior colliculus (Sup Col); G4) hypothalamus (Hy); G5) thalamus (Th); G6) hippocampus (Hp); G7) septum (Sep); G8) cerebral cortex at the level of the thalamus (Th ctx); G9) frontal cerebral cortex (Fr ctx); W1) cerebral peduncle (Cb pd dec); W2) corpus callosum (Cb WM); W3) caudoputamen (Cp); W4) cerebellar white matter (Crb WM). Data points represent the mean ± SD of data from ≥ 3 animals per study except primary passage of M-SW4 which represents the only animal positive from the study. CNS: central nervous system.

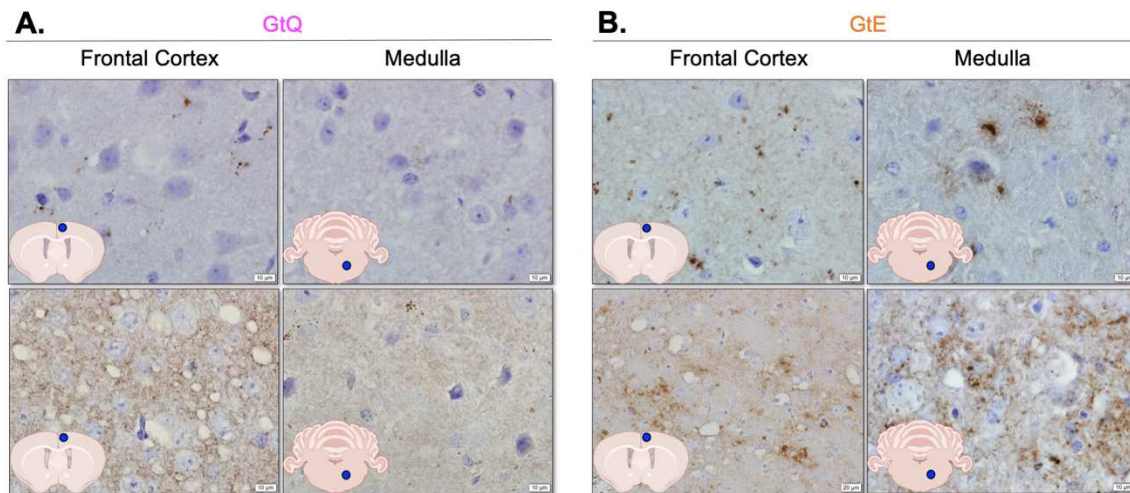


Figure 2.7. Immunohistochemical analysis of Sweden moose 1 (M-SW1) CWD prions in GtQ and GtE mice at secondary passage. Deposition and morphology of CWD prions from M-SW1 in the CNS of diseased GtQ and GtE mice at secondary passage. Coronal sections from septum and cerebellum are shown. IHC sections were proven with Fab D18. Bar scales = 10µm. GtE and GtQ: CWD-susceptible gene-targeted mice in which the prion protein coding sequence was replaced with one encoding glutamate (E) at codon 226 or with glutamine (Q).

To further substantiate the extensive strain diversity among CWD cases from moose from Sweden, we performed a semiquantitative assessment of spongiform degeneration by lesion profiling following the criteria described by Fraser et al. (46). The analysis revealed that both the severity and regional distribution of spongiform lesions varied among Gt mice infected with these CWD isolates, and these profiles were maintained during iterative passages in GtQ mice. M-SW4 and M-SW2 were associated with more severe neuropathology, predominantly affecting the frontal cortex and thalamus, respectively, consistent with their corresponding patterns of PrP^{Sc} deposition (Fig. 2.6F, I; Fig. 2.9B-D). M-SW1-associated pathology was also most prominent in frontal brain regions, though it was less severe than that observed for M-SW4. Moderate pathology in white matter tracts was observed for M-SW1, M-SW2, and M-SW4; however, while M-SW1 primarily affected the cerebral peduncle in the midbrain (Fig. 2.6G), M-SW2 and M-SW4 exhibited higher spongiosis scores in the corpus callosum (Fig. 2.6F, I). In contrast, M-SW3 displayed a distinctive lesion profile with minimal neuropathology across most brain regions that was preserved upon serial passage, with only moderate lesions observed in the thalamus (Fig. 2.6H). This finding contrasts with the intense PrP^{Sc} accumulation detected in M-SW3-infected GtQ mice (Fig. 2.6C).

Because quaternary passages were conducted for M-SW2, we additionally evaluated whether the Q226E polymorphism influenced spongiform lesion profiles. Although spongiosis scores increased with successive passages, no differences in lesion severity or regional distribution were observed between GtQ and GtE mice (Fig. 2.9B-D). These analyses of PrP^{Sc} deposition patterns and lesion profiles demonstrate that: 1) CWD isolates from moose from Sweden exhibit distinct and strain-specific patterns of PrP^{Sc} accumulation that are preserved across serial passages in Gt mice; 2) even when similar PrP^{Sc} morphologies are observed among isolates, their CNS target regions differ, as exemplified by M-SW2 and M-SW4; 3) the PrP^{Sc} deposition patterns described here closely mirror those reported in the natural host; and

4) each isolate displays a unique lesion profile with variable severity and regional involvement. Collectively, these findings provide strong evidence for remarkable strain diversity within Nordic CWD.

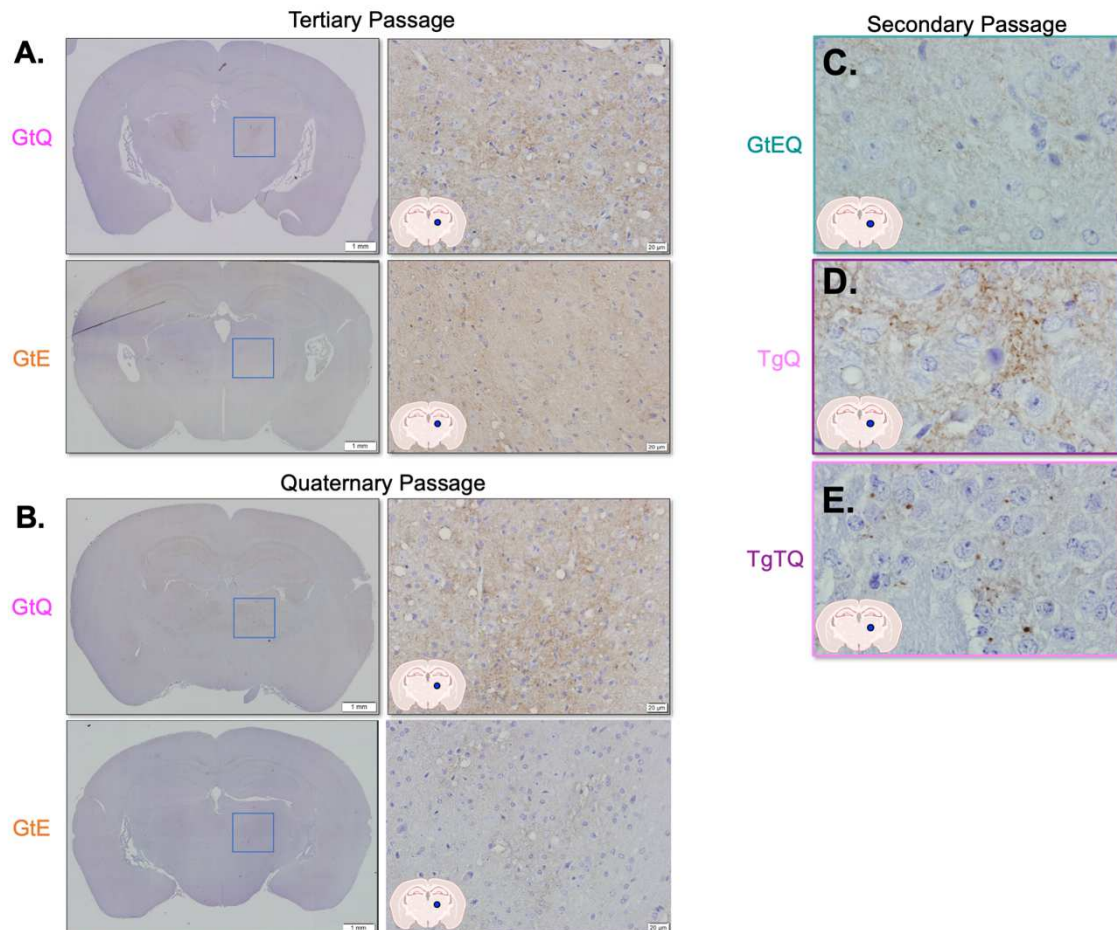


Figure 2.8. Immunohistochemical analysis of PrP^{Sc} after iterative passages of Sweden moose 2 (M-SW2) CWD in GtQ, GtE, GtEQ, TgQ and TgTQ mice. **A.** Deposition and morphology of CWD prions from M-SW2 in the CNS of diseased GtQ and GtE mice at tertiary and **B.** Quaternary passages and **C-E.** GtQ-Adapted CWD from M-SW2 into GtEQ, TgQ and TgTQ mice respectively. Coronal hippocampal sections are shown. IHC sections were proven with Fab D18. Bar scales = 1mm, 10μm. GtE and GtQ: CWD-susceptible gene-targeted mice in which the prion protein coding sequence was replaced with one encoding glutamate (E) at codon 226 or with glutamine (Q). GtEQ: CWD-susceptible gene-targeted mice in which the prion protein coding sequence expresses heterozygous glutamate (E) and glutamine (Q) at codon 226. TgQ: transgenic mouse expressing transgene glutamine (Q) at codon 226. TgT: hybrid GtQ/TgQ mouse expressing glutamine (Q) at codon 226 from both transgenic and gene-targeted backgrounds.

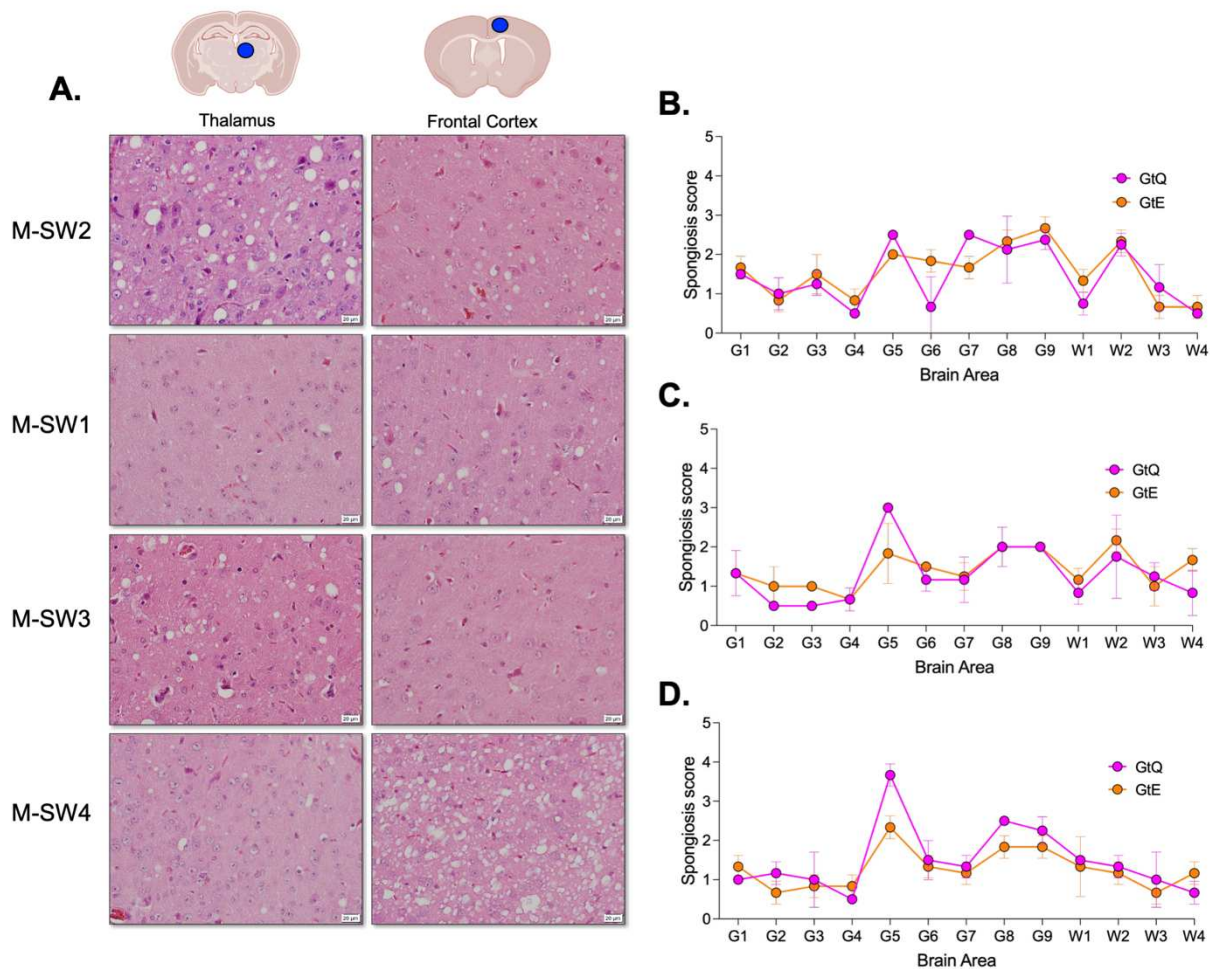


Figure 2.9. Hematoxylin and eosin-stained brain sections of Gt mice passed Sweden moose CWD and assessment of vacuolar degeneration during iterative passages of Sweden moose 2 (M-SW2). **A.** Representative images of hematoxylin-eosin stained sections of the thalamus and the frontal cortex at the level of the hippocampus and septum layers respectively from GtQ mice infected with Sweden moose CWD isolates at secondary passage. Bar scale = 100µm. **B.** Lesion profiles of secondary passages of Sweden moose 2 (M-SW2) in GtQ (magenta circles) and GtE mice (orange circles) **C.** Vacuolar lesion scores of tertiary passages of M-SW2 in GtQ (magenta circles) and GtE mice (orange circles) **D.** Lesion profiles M-SW2 in GtQ (magenta circles) and GtE mice (orange circles) at quaternary passage. Assessment of vacuolar lesions was performed in the following regions: 1) medulla oblongata (Mobl); 2) cerebellum cortex (Cb ctx); 3) superior colliculus (Sup Col); 4) hypothalamus (Hy); 5) thalamus (Th); 6) hippocampus (Hp); 7) septum (Sep); 8) cerebral cortex at the level of the thalamus (Th ctx); 9) frontal cerebral cortex (Fr ctx); 10) cerebral peduncle (Cb pd dec); 11) cerebral white matter at the septum (Cb WM); 12) caudoputamen (Cp); 13) cerebellar white matter (Crb WM). Data points represent the mean $\pm$ SD of data from ≥ 3 animals per study.

In vitro amplification reveals diverse conversion efficiencies and seeding properties among natural isolates and Gt mice infected with CWD from moose from Sweden assessed by PMCA and RT-QuIC assays.

Previous studies have shown that protein misfolding cyclic amplification (PMCA) can broadly recapitulate strain properties (56,57), while real-time quaking-induced conversion (RT-QuIC) assay has been used to discriminate prion strains based on seeding kinetics (58–60) as well as seeds from other neurodegenerative diseases (61–63). We therefore used PMCA and RT-QuIC assays to assess the replicative properties and seeding activity of Sweden moose CWD prions and passages in GtQ and GtE mice. For PMCA, seed and substrate genotypes were matched (i.e., GtQ-derived seeds amplified in GtQ substrate and GtE-derived seeds in GtE substrate). RT-QuIC reactions were seeded with GtQ- or GtE-diseased brains using a master reaction mixture containing recombinant hamster PrP. To initially evaluate replicative properties of all isolates by both PMCA and RT-QuIC assays, we selected brain seeds from the secondary passages, once moose isolate transmission properties had stabilized.

PMCA amplification profiles varied among the different isolates. GtQ and GtE seeds from isolate 19-VLT000541 amplified efficiently in their respective substrates, with higher endpoint for the GtQ seeds (Fig. 2.10B). GtQ seeds from 19-VLT000876, derived from the rapid ~90 d disease onset, failed to amplify in its matching substrate, while GtE substrate sustained amplification of GtE seeds, derived from an onset of disease at ~300 d (Fig. 2.10A). Isolates 19-VLT002232 and 20-VLT002459 only caused disease in GtQ mice, we only evaluated their amplification efficiency in GtQ substrate. While GtQ substrate failed to sustain amplification of 19-VLT002232 infected GtQ brains (Fig. 2.10C), it facilitated moderate amplification of GtQ for brain seeds from 20-VLT002459 transmissions (Fig. 2.10D). When we evaluated the seeding kinetics of the same prion seeds from GtQ and GtE mice by the RT-QuIC assay, we found that GtQ seeds from 19-VLT000876 and 19-VLT002232, which failed to sustain PMCA amplification

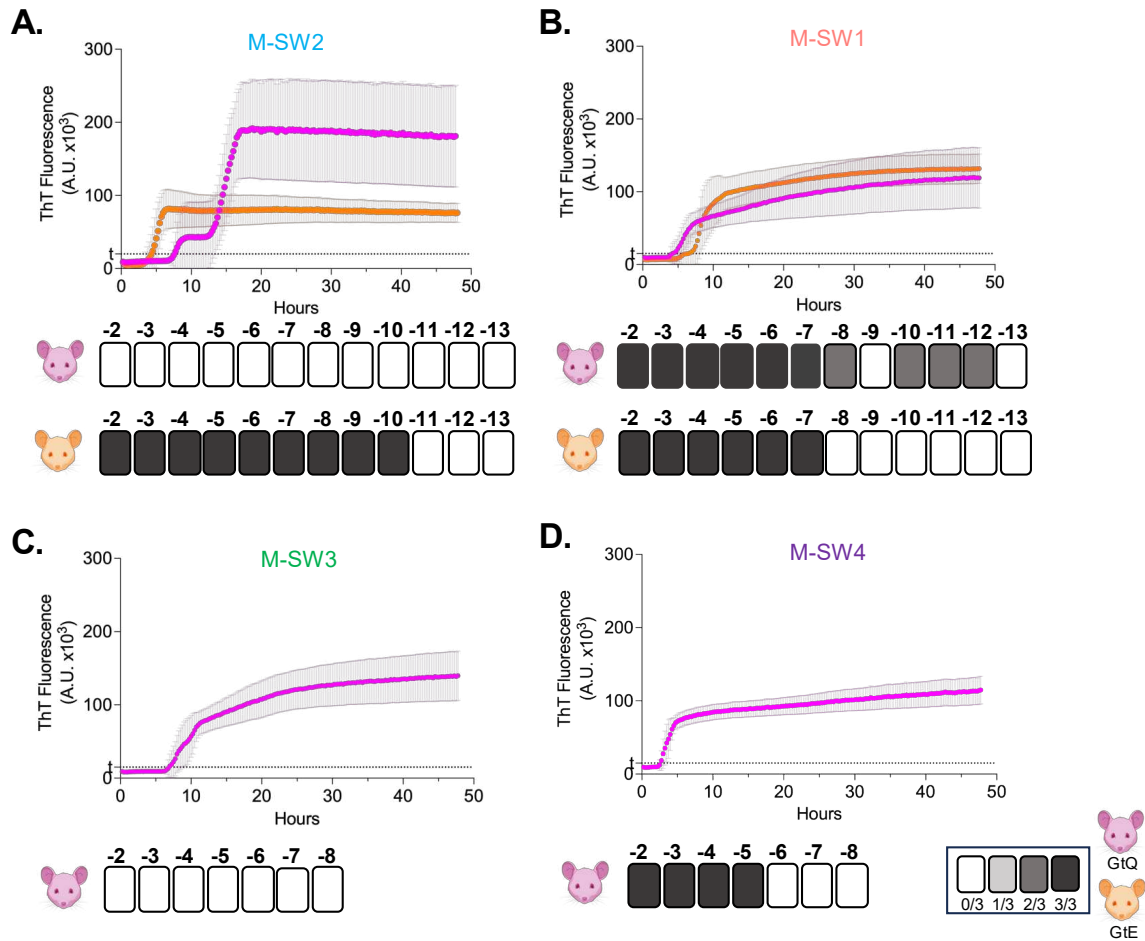


Figure 2.10. In vitro replicative and seeding properties of Sweden moose CWD after passages in GtQ and GtE mice by protein misfolding cyclic amplification (PMCA) and real-time quaking-inducing conversion assay (RT-QuIC). 10% brain homogenates derived from diseased GtQ and GtE mice from secondary passages of Sweden CWD isolates were used to initiate PMCA reactions at 10^{-2} dilution. This starting reaction is serially diluted in 10-fold increments to 10^{-8} or 10^{-13} in uninfected brain homogenate substrates from GtQ and GtE mice homologous to the seed source. RT-QuIC seeding reactions were spiked with 2 μ L of 10^{-4} dilution of brain homogenate from the same mice selected for PMCA assays and run for 48h at 42°C. **A.** PMCA endpoint amplification and RT-QuIC fluorescence readouts for GtQ and GtE passaged Sweden Moose 2 (M-SW2) at secondary passage. **B.** PMCA endpoint amplification and RT-QuIC fluorescence readouts for GtQ and GtE passaged Sweden Moose 1 (M-SW1) at secondary passage. **C.** PMCA endpoint amplification and RT-QuIC fluorescence readouts for GtQ passaged Sweden Moose 3 (M-SW3) at secondary passage. **D.** PMCA endpoint amplification and RT-QuIC fluorescence readouts for GtQ passaged Sweden Moose 4 (M-SW4). PMCA data representative of at least three independent experiments. RT-QuIC readout kinetic curves represent the mean $\pm$ SD of technical quadruplicates from $\geq$ three biological replicates per study. Legend indicates number of positive PMCA reactions out of 3 independent experiments.

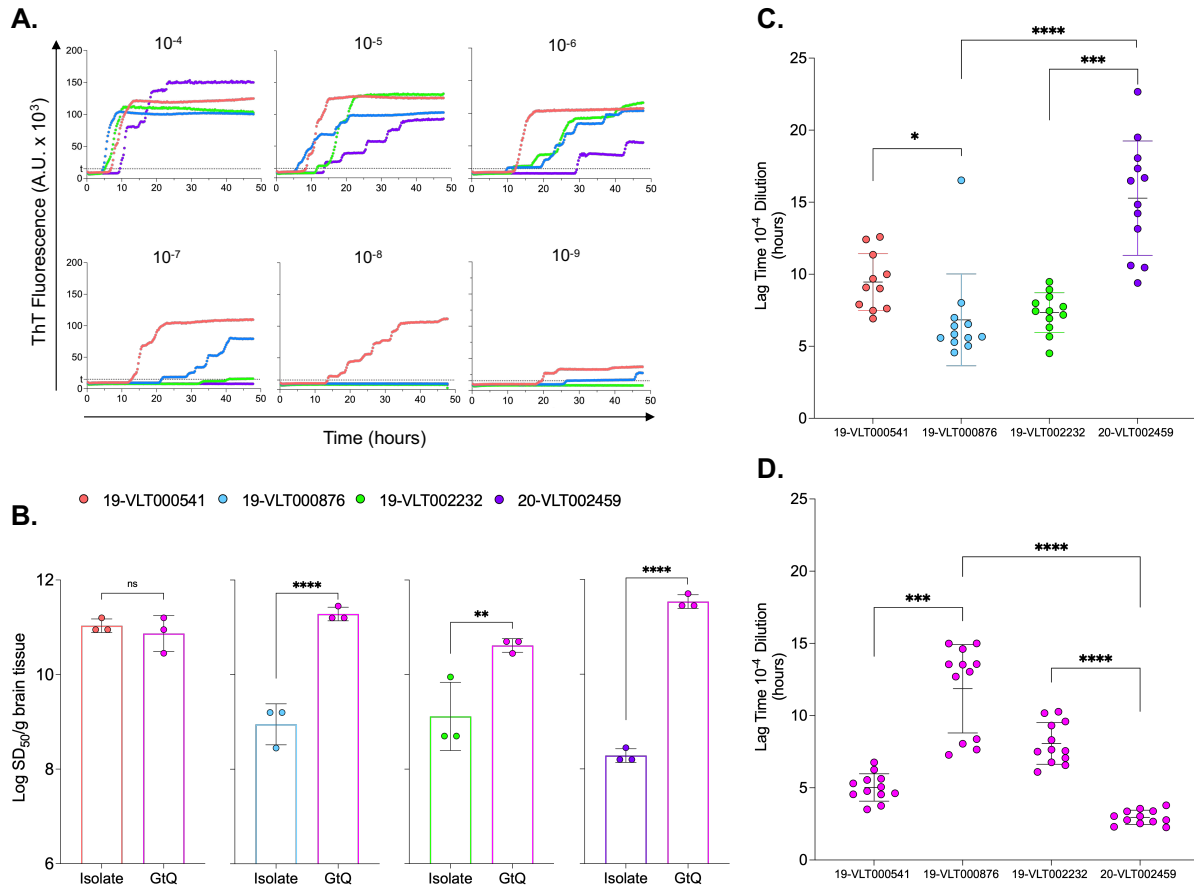


Figure 2.11. In vitro replicative and seeding properties of Sweden moose CWD after passages in GtQ and GtE mice by real-time quaking-inducing conversion assay (RT-QulC). RT-QulC seeding reactions were spiked with 2 μ L of 10^{-4} dilution of brain homogenate from the Sweden moose CWD isolates and their passages in GtQ and GtE mice and run for 48h at 42°C. **A.** PMCA endpoint amplification and RT-QulC fluorescence readouts for GtQ and GtE passaged Sweden Moose 2 (M-SW2) at secondary passage. **B.** PMCA endpoint amplification and RT-QulC fluorescence readouts for GtQ and GtE passaged Sweden Moose 1 (M-SW1) at secondary passage. **C.** PMCA endpoint amplification and RT-QulC fluorescence readouts for GtQ passaged Sweden Moose 3 (M-SW3) at secondary passage. **D.** PMCA endpoint amplification and RT-QulC fluorescence readouts for GtQ passaged Sweden Moose 4 (M-SW4). PMCA data representative of at least three independent experiments. RT-QulC readout kinetic curves represent the mean $\pm$ SD of technical quadruplicates from $\geq$ three biological replicates per study. Legend indicates number of positive PMCA reactions out of 3 independent experiments.

In the GtQ substrate, robust seeding curves by RT-QuIC (Fig. 2.10A, C), similar to those that promoted efficient amplification by PMCA (Fig. 2.10A-D). Discrepancies in PMCA conversion efficiencies were not attributable to titer levels, since 19-VLT000876 in GtQ mice displayed the second highest SD_{50}/g levels among the moose isolates, even higher than isolate 19-VLT000541, which efficiently sustained PMCA conversion (Table 2.2, Fig. 2.10B). The failure of GtQ mice brains from isolate 19-VLT000876 to sustain PMCA amplification contrasts with the high endpoint achieved by GtE seeds, indicating that rapid disease progression does not necessarily correspond to efficient in vitro amplification by PMCA. Moreover, both GtQ and GtE 19-VLT000876 prions were highly competent at seeding RT-QuIC, potentially reflecting subtle conformational differences between prion assemblies generated in GtQ and GtE hosts that explain the differences in PMCA conversion. These divergent amplification properties of CWD prions from GtQ and GtE mice support the notion of strain differences in these isolates.

Given the discordant amplification properties observed between PMCA and RT-QuIC assays, we used the RT-QuIC assay to evaluate in more detail the seeding properties of prions in the moose field isolates and diseased GtQ and GtE mice. Serial dilutions of each moose CWD brain tissue homogenate were prepared down to 10^{-10} to analyze the kinetics of fluorescence response by RT-QuIC. All isolates showed strong RT-QuIC seeding responses at lower dilutions (Fig. 2.11A). 20-VLT002459 was the first isolate to reach an endpoint. At 10^{-7} , only isolate 19-VLT000541 had at least 3 out of 4 replicate wells with positive responses, and all isolates reached an endpoint at 10^{-9} dilution (Fig 2.11A). Since we performed the serial dilution analysis and estimation of seeding dose ($\log SD_{50}/g$ tissue) for all moose CWD field isolates, we therefore estimated the SD_{50}/g in brain tissues of GtQ mice infected with the moose isolates at secondary passage to determine how changes in titer levels can indicate differences in seeding properties of the moose CWD isolates. Although 19-VLT000541 isolate had the highest titer (SD_{50}/g) values among the isolates, prion titers did not differ significantly after iterative

transmissions, whereas the isolate with the lower prion titers, 20-VLT002459 ($P < 0.0001$) experienced the highest increase in titer levels after passage. Similarly, but slightly less pronounced, was the increase in titer for 19-VLT000876 ($P < 0.0001$) and 19-VLT002232 ($P < 0.001$) (Fig. 2.11B). The estimation of lag times (time to estimated fluorescence threshold) for both CWD moose isolates and iterative passage in GtQ mice offered insights into the seeding potency of prion seeds in these isolates. We found no correlation between prion titers and lag times, as seeds from 19-VLT000876 that showed the second lowest titer among the isolates showed the shortest lag phase among the isolates, while the lag phase for the isolate with the highest titer, 19-VLT000541, was not significantly different than 20-VLT002459, which carried the lowest titer of all isolates (Fig. 2.11C). When we analyzed the lag times of prion seeds in the brains of GtQ mice, we found that isolates 20-VLT002459 and 19-VLT000541 experienced a substantial reduction in lag time after iterative transmission, while 19-VLT000876 showed a delay with two mice clustered at a longer lag time compared to a third mouse with a faster lag time (Fig. 2.11D). Seeds from GtQ mice infected with 19-VLT002232 did not experience a reduction of the lag time. The distinct profiles observed in log SD_{50}/g variation in seeding titers and lag phase duration provide evidence that 1) replication efficiency is an intrinsic strain property rather than a function of prion titer in the inoculum and 2) prion seeds in the moose CWD isolates have distinct seeding properties, consistent with conformationally distinct strains.

Table 2.2. Estimation of SD_{50/g} prion titers in Sweden moose CWD isolates and passages in gene targeted mice by RT-QulC assay

	Field Isolate	Primary passage		Secondary passage	
		GtQ	GtE	GtQ	GtE
M-SW1	11.0 ± 0.14	11.53 ± 0.38	10.20 ± 0*	10.87 ± 0.38	11.12 ± 0.38
M-SW2	8.9 ± 0.43	9.82 ± 0.18**		11.28 ± 0.14	11.86 ± 0.14
M-SW3	9.1 ± 0.72	10.70 ± 0.5		10.62 ± 0.14	
M-SW4	8.3 ± 0.14	11.19 ± 0*		11.53 ± 0.14	10.45 ± 0.18*

Initial assessments of Sweden moose 2 CWD prion morphology by EM-nanogold negative staining.

Due to the unprecedented fast transmission kinetics of the (M-SW2) isolate after stabilization at secondary passage (~90 days) and the paradoxical results obtained by PMCA in contrast to RT-QulC (Figure 2.10-A), it was hypothesized that this isolate could be composed of a strain with unusual structural features, potentially a more oligomeric form. A collaboration was established with the laboratory of Dr. Witold Surewicz to address these questions. A laboratory visit was agreed to train on atomic force microscopy (AFM) and electron microscopy (EM) and obtain images of these purified CWD prions to determine if they are of a fibrillar or non-fibrillar morphology.

The first part of this project was the initial imaging and optimization of the EM-nanogold negative staining protocol, initially on non-PK-treated samples. After testing numerous antibodies, the mAb SAF-32, which targets an epitope on the octarepeat region of PrP, was

determined to be the best alternative; this restricted the possibility of using proteinase-K for eliminating PrP^C. The results shown in this chapter are the initial test of the nanogold labeling technique. Ongoing progress of the project has established an adequate mild-protease treatment that preserves the SAF-32 epitope and successfully eliminates PrP^C. The next step is the inoculation of these purified fractions into Gt-mice.

The preliminary results showed that in multiple fields during AFM and EM imaging, we have found no evidence of fibrillar structures in the purified fractions of M-SW2, when compared to a North American CWD strain purified side by side with M-SW2. Instead, we only observed amorphous particles, covered by nanogold staining that tend to aggregate, as seen in EM (Fig. 2.12E-F). The positive controls show that the fibrillar prions are covered by nanogold particles, which indicates that the labeling was successful (Fig. 2.12A-D). If M-SW2 is composed mainly of non-fibrillar structures, the bioassay will be of great importance in the debate around the true biological relevance of cryo-EM solved structures of fibrillar prions and other amyloid structures.

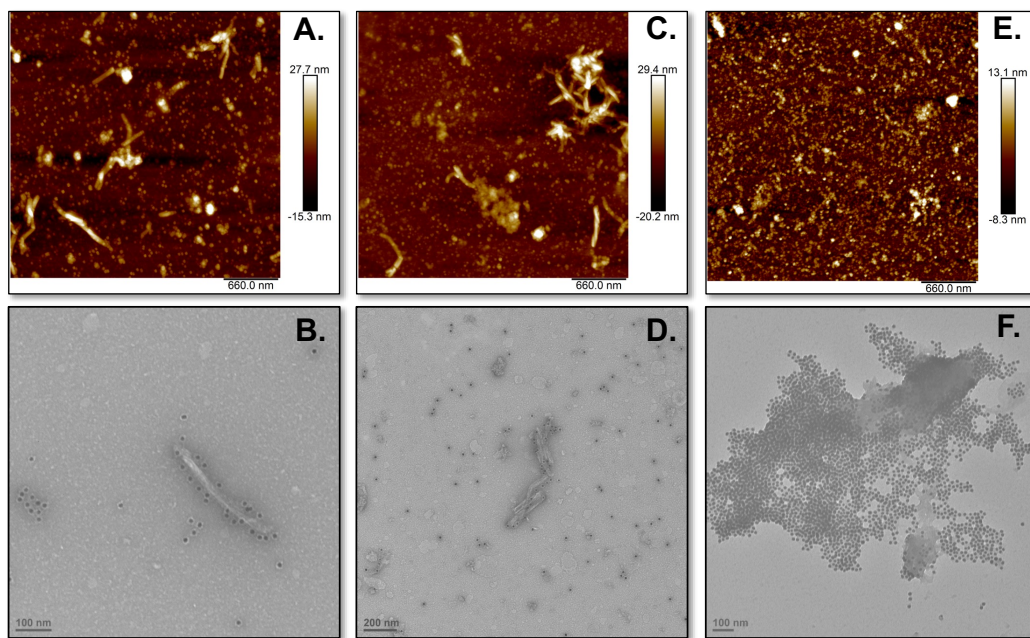


Figure 2.12. Morphology of North American CWD US-1E (Elk) from GtQ and GtE infected mice and Sweden moose 2 (M-SW2) infected GtQ mice at quaternary passage. Images from AFM and EM-nanogold negative staining from non-protease-treated purified prions. **A.** AFM

image of GtE-derived purified prions from US-1E inoculations. **B.** EM image obtained from purified prion rods from (A) coated with nanogold particles against SAF-32. **C.** AFM image of GtQ-derived purified prions from US-1E inoculations. **D.** EM image obtained from purified prion rods from (C) coated with nanogold particles against SAF-32. **E.** AFM image of GtQ-derived purified prions from M-SW2 inoculations (fourth passage in GtQ mice). **F.** EM image obtained from purified prion from (E) coated with nanogold particles against SAF-32 shows not fibrils but amorphous particles.

Discussion

The expanding geographic range and prevalence of chronic wasting disease (CWD) in North America, and its inadvertent expansion to South Korea, illustrate the unique efficiency of CWD prions in infectious transmission. The strain properties of North American (NA) CWD have long been recognized for their highly consistent biochemical and neuropathological properties, as well as clinical disease manifestations—clear indications that CWD in North America is composed of a dominant strain with stable properties that are reliably reproduced in experimental transmissions in the native host and engineered mouse models (64,65). In contrast, characterization studies performed by our group and others have found that newly emergent CWD prions from reindeer, moose, and red deer from Norway and Finland exhibit pronounced heterogeneity in their properties, excluding the possibility of accidental introduction of classic NA CWD into Europe(30,39,40,66). Nordic moose CWD also shows unconventional properties, including the absence of detectable prion infection in the lymphoreticular system. In this study, we sought to investigate the strain properties of the four cases of CWD in moose identified in Sweden. We found: 1) remarkable strain diversity, where each moose CWD case exhibited distinct properties upon serial transmission in gene-targeted (Gt) mice expressing cervid *PRNP*; and 2) the unexpected adaptation of the second moose case, M-SW2, to a fast-transmission phenotype, unprecedented in our studies of CWD strains in Gt mice. These findings add to the growing body of evidence that Nordic moose CWD is likely of sporadic origin and composed of unstable PrP^{Sc} conformers with unpredictable adaptation.

Similar to our previous reports on transmission of Nordic moose CWD cases from Norway and Finland (38,40), moose CWD cases from Sweden also exhibited a preference for transmission in Gt mice expressing the host genotype Q226 (GtQ), while partial to complete resistance was observed when passaged in the elk variant E226 (GtE). We have also reported the effect of the natural variant Q226E on CWD susceptibility for North American and additional

Nordic CWD strains (37,38,40). This pattern is consistent with the influence of polymorphic variants in other prion diseases of humans and animals (43,67). We observed that resistance of some moose CWD strains to the GtE genotype can be overcome when inoculum from primary passage in GtQ mice is administered to GtE mice in secondary transmission. This supports the view that different PrP^{Sc} conformers present in the original isolate are selected during serial transmission in the same host genotype, consistent with the conformational selection model (38,58,68). Such strain diversity is expected in naturally occurring prion disease. Alternatively, one could propose that low prion titers in field isolates explain these findings. However, estimated prion titers of M-SW2 in primary passage in GtQ mice were lower than those of M-SW3. Yet efficient transmission in GtE mice was observed for M-SW2 in secondary passage, while complete resistance persisted for M-SW3. This favors pre-existing conformers as the likely mechanism of adaptation to a new cervid *PRNP* genotype.

The evolution of M-SW2 in GtQ mice began with inefficient transmission in primary passage and a prolonged incubation period, despite identical primary structure. This was followed by accelerated adaptation to a fast-transmission phenotype of ~90 days—unique among our studies of CWD in Gt mice. The clinical phase was extremely fast, sometimes not exceeding one to two days from diagnosis, and was characterized by limb paralysis, ataxia, hunched posture, and inability to reach water or food. The morphological properties of PrP^{Sc} generally similar to those described for moose CWD in Norway and Finland; however, they differed from those in M-SW1 and M-SW3, reflecting strain heterogeneity among Nordic moose CWD. M-SW2 prions were highly diffuse and widely distributed throughout the brain, causing moderate neuropathology. Among the passaged isolates, M-SW2 prions were the least stable, while M-SW1, which was the most stable, exhibited the longest incubation periods. Studies of natural and synthetic prions have found an inverse correlation between incubation period and conformational stability, while cloned hamster strains are an exception (54,69,70). We found no

conformational differences between GtQ and GtE CWD prions, even after iterative passages for any of the isolates, suggesting that conformational properties are preserved across passages. For rapidly adapting strains like M-SW2, low prion titer in the field isolate limited our assessment of native conformational properties; therefore, we cannot determine whether changes in denaturation profile occurred during adaptation.

The accelerated adaptation of M-SW2 is consistent with a highly toxic PrP^{Sc} species. RT-QuIC traces from Gt mice infected with this isolate provide a potential clue: compared with other moose field isolates, M-SW2 showed the fastest time to the seeding threshold despite having the second-lowest estimated titer. This suggests high seeding potency that may facilitate efficient replication. M-SW4 showed a similar pattern: a marked reduction in seeding lag time from field isolate to primary and secondary passages, indicating that, under favorable conditions, this strain may possess structural advantages that outweigh low infectivity titer. Beyond lower stability and seeding potency, the shorter incubation periods of these highly toxic prion species might also reflect proteolytic processing and clearance requirements that make these species more difficult to eliminate from neurons and glia, particularly in critical brain regions (71)

Despite high infectivity, M-SW2 seeds from GtQ mice consistently failed to amplify in PMCA, whereas GtE seeds reached high amplification endpoints. These unexpected PMCA differences between GtQ- and GtE-passaged M-SW2 suggest subtle structural differences, beyond the resolution of current biochemical assays, that could contribute to differences in disease kinetics between genotypes. Similarly, GtQ-derived seeds from M-SW3 did not amplify in PMCA, while M-SW4 seeds amplified only to lower dilutions (10^{-5}), despite strong RT-QuIC seeding and notable neurotoxicity. The opposite effect was observed for M-SW1, whose GtQ seeds amplified more efficiently than GtE seeds. Notably, M-SW1 was the only moose isolate

causing infection in GtE mice during primary passage and showed mixed morphology, including granular, punctate, and diffuse deposition patterns.

Three of the four CWD cases in moose reported in Sweden were found sequentially from 2019 to 2020 in a defined geographic cluster. This context is important in light of our findings. After iterative passages of these isolates in a precise Gt mouse model—syngeneic except at residue 226 of the cervid prion protein—each CWD case exhibited a unique profile of strain properties. This supports the hypothesis that Nordic CWD is a newly emergent form of prion disease with high strain diversity and likely sporadic origin. Characterization of emergent natural CWD strains in models that recapitulate strain properties challenges long-held assumptions from cloned mouse and hamster strains, and rodent-adapted scrapie. We found that Nordic moose CWD does not follow the convention that infectivity correlates with inoculum titer, resulting in unpredictable transmission properties. In human sporadic CJD, multiple strains have been identified and are influenced by *PRNP* codon 129 (72–74). By contrast, the four CWD cases in Sweden were identified in moose with identical primary structure and wild-type variants of known polymorphisms in moose subspecies, excluding host genetic variability as the driver of strain diversity in these cases.

Although direct evidence supporting zoonotic transmission of CWD remains lacking, it has long been proposed that emergent and structurally unstable prion strains pose an increased risk of cross-species adaptation. Such adaptation may arise either through conformational selection of minor pre-existing strains or through structural features that confer enhanced compatibility with novel PrP sequences (64,75,76).

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CHAPTER 3 – ACQUISITION OF LYMPHOTROPIC PROPERTIES IN NON-LYMPHOTROPIC NORDIC MOOSE CWD STRAINS AFTER PASSAGES IN GENE-TARGETED MICE

Introduction

The importance of the lymphoreticular system in prion infection was first described in the 1970s by Clarke and Haig from studies of peripheral challenges of scrapie in mice after splenectomy significantly increased the post-infection survival time (1,2), though earlier reports of scrapie infectivity in multiple peripheral compartments, including lymphoid tissues, had been published (3). Later, Kimberlin and Walker established that the role of the spleen was less critical once prion infection had reached the spinal cord, and that other lymphoid structures could facilitate neuroinvasion in the absence of the spleen (4,5). The role of the quiescent cells in the stroma was determined after fractionation of radiated lymphoid cells; these cells were later identified as the follicular dendritic cells (FDCs) of the splenic germinal centers, which express important levels of physiological prion protein (PrP) (6,7). Moreover, Additional studies observed that in immunodeficient engineered mice, like the SCID and RAG-/- lines, intraperitoneal prion transmission was impaired, suggesting the crucial role of the immune system for neuroinvasion after peripheral challenge (8,9). Prinz and Aguzzi complemented these studies with the development of mouse models that lacked relevant components of the lymphoid system, mainly mature FDCs, which prevented prion replication in the spleen (10). More recently, using PrP knock-out (PrPKO) mouse lines in which PrP^c (cellular prion protein) expression has been restricted to FDCs using *cre*-recombinase system, was demonstrated to be enough to sustain prion replication in the spleen, establishing FDCs and the germinal centers of the spleen as the most important site for non-central nervous system (CNS) prion replication (11).

The role of FDCs in prion disease is not limited to a cell line with high levels of PrP expression that facilitates prion conversion and replication. Zabel and Aguzzi demonstrated that

mice deficient in the complement receptor CD21/35 experienced a delay in disease and lower attack rates; it was also determined that this protection was related exclusively to the lack of expression of this receptor in FDCs, suggesting this molecule plays a role in the targeting of prions to the FDCs (12,13). In recent years, studies have looked at the involvement of tertiary lymphoid structures as supporters of prion replication and have suggested that involvement of FDCs in lymphoid structures is strain dependent, with additional factors like expression levels and sialylation playing an important role in PrP^c trafficking to secondary lymphoid organs (14–16), however, studies comparing HY-TME (lymphotropic) and DY-TME (non-lymphotropic) found that both strains reach the same lymphoid structures at similar rate, suggesting that degradation rate rather than sialylation or other modifications that regulate FDC targeting, are determinant of the establishment of prion replication in lymphoid organs (17). Interestingly, not all prion diseases are known to involve the spleen, though secondary lymphoid organs in the digestive tract could play a role in spleen-independent neuroinvasion. Examples of non-lymphotropic prion strains are the DY-TME hamster prion strain (17,18), bovine spongiform encephalopathy (BSE) (19,20), Nor98 atypical scrapie (21,22), sporadic Creutzfeld-Jacob disease (sCJD) (23), and newly emergent chronic wasting disease (CWD) strains in Nordic moose and red deer (24–26). It remains to be elucidated if the role of specific cellular factors in the rate of replication and clearance determines prion colonization and replication in a strain-independent manner, or if lymphotropism is encoded as part of the structural information in the misfolded PrP^{Sc} structure and pre-existing lymphotropic sub-strains, contained within the conformational cloud, are favored by specific host compartments.

Among animal prion diseases, chronic wasting disease (CWD) is notable for its extraordinary fitness for horizontal transmission, which is attributed mostly to the high tropism of CWD strains for the spleen and other lymphoid tissues and its environmental robustness (27). These characteristics make CWD prevalent not only among captive but also among free ranging

cervids. The most common route of infection in wildlife is the oral route. Following the consumption of CWD-contaminated material, CWD prions can be found in several lymphoid structures, including draining retropharyngeal lymphoid nodes, tonsils, and gut-associated lymphoid tissue (GALT) like the Peyer patches (28–31). The uptake of prions by dendritic cells and macrophages targets them to the spleen and other lymphoid organs, starting the process of neuroinvasion via the mesenteric and autonomic nervous system (32). Moreover, peripheralization of prion infection is most effective in CWD. Besides involvement of the lymphoid system, prion infection in CWD-affected cervids and animal models has been found in tissues of the skeletal system, heart, pancreas, kidney, adipose tissue, and reproductive organs (33–37). The cycle of infectivity is completed with the shedding of CWD prions to the environment from the lymphoid tissues via body fluids and excreta (urine, saliva, and fecal matter) or from other compartments like antler velvet and blood, which can be exposed by injury or carcasses (38,39).

Effective peripheralization is a defining property of stable strains of North American CWD. This fact is illustrated in the exponential growth of the geographical range of CWD in North America in the last 20 years (40) and the efficient spread of accidentally imported CWD to South Korea among the native and farmed cervid population (41,42). Lymphotropism is also present in naturally occurring cases of CWD among semi-domesticated reindeer in Norway (*Rangifer tarandus tarandus*), though the properties of these CWD prions differ from North American CWD (43,44). The extreme and difficult decisions in containment measures taken by the Norwegian government were informed by the possibility of CWD establishing an endemic situation, similar to North America. Due to the economic and cultural value centered around reindeer herding in the Nordic countries, the spread of CWD would have a significant impact in these activities (26,45–48). The only cases of non-lymphotropic CWD reported to date have been found exclusively among Nordic moose and red deer (26,43,49). These findings provided

the hope that CWD among these species would not represent a risk of horizontal transmission and reinforced the hypothesis of a sporadic origin for non-lymphotropic Nordic CWD. All reports of CWD among Nordic moose could not find any evidence of prion infection in lymphoid tissues by immunohistochemistry (IHC) or rapid ELISA-based tests (26,49–51).

For nearly 20 years, CWD research was dependent on transgenic mouse models that overexpress cervid PrP. These mouse lines have been indispensable in the current understanding of CWD pathogenesis (52); however, these transgenic mice are not susceptible to CWD prion peripheralization, and the potential to study this crucial aspect of CWD pathogenesis was not possible. The development of the gene-targeted (Gt) cervidized mice by the Telling laboratory demonstrated that by precisely using a homologous recombination approach to replace the coding sequence of mouse *prnp* with the sequence of cervid *PRNP*, while preserving the regulatory elements of the mouse *prnp* coding sequence, this new line was permissive to lymphoid replication of CWD prions, faithfully recapitulating this key aspect of the disease (53). The key importance of this advance in CWD research is now evident in the ongoing efforts to study the properties of the newly emergent CWD strains in Northern Europe. During the investigation of the strain properties of CWD in moose from Sweden, evidence was found that Nordic moose CWD can evolve lymphotropic properties during iterative passages in Gt mice. This evolution differs from the report by the Telling lab in 2021(43), in which the evolution of lymphotropic properties in Nordic moose isolates was correlated to the acquisition of North American CWD properties. This chapter will detail two cases in which Nordic moose CWD evolved lymphotropic properties without adaptation to a North American CWD phenotype and discuss how lymphotropic strains can converge between different cervid species.

Materials and Methods

Ethics Statement

All animal work was performed in an Association for Assessment and Accreditation of Laboratory Animal Care International accredited facility in accordance with the Guide for the Care and Use of Laboratory Animals. All procedures used in this study were performed in compliance with and were approved by the Colorado State University Institutional Animal Care and Use Committee.

CWD Field Isolates and controls

The moose CWD field isolates from Sweden, designated M-SW1, M-SW2, M-SW3, and M-SW4 (M: moose, SW: Sweden, number: case number) have been described previously (50,54). Information on official codes, age, sex, *PRNP* polymorphisms, clinical signs, and geographical origin is detailed in Table 2.1. The North American CWD isolate used as positive control in multiple analyses represents the passaged brain or spleen material of diseased Rocky Mountain elk, 99w12389 (E-US1) in GtQ mice, and has been described previously (29,55). The isolates from moose CWD from Sweden were generously provided as part of a collaboration with Dr. Sylvie Benestad at the Norwegian Veterinary Institute and Maria Nöremark and Dolores Gavier-Widen at the Swedish National Veterinary Institute.

Mouse models and transmission studies

The generation and characterization of gene-targeted GtQ and GtE mice has been described previously (53). 10% brain homogenates (BH) were established by mechanical disruption (MP Biomedical, Irvine, CA) using sterile phosphate-buffered saline (PBS) lacking calcium and magnesium ions. The same number of male and female mice between four and six weeks were anaesthetized with halothane and intracerebrally inoculated with 30 μ L of 1% BH into the right parietal lobe using a 26-gauge needle. Mice were assessed three times weekly for

signs of prion disease, including truncal ataxia, loss of extensor reflex, hunched posture, slowed movements, plastic tail, wobbling/flattened gait, head bobbing/tilt, limb paralysis, and rough coat. Incubation period is defined as the time elapsed between inoculation day and the onset of clinical signs, which was defined as the first day when clinical signs were identified. Brains were dissected down the midline, and one hemisphere was used for preparing 10% brain homogenates in PBS for biochemical analysis, while the opposite hemisphere was immersed in formalin for neuropathological analysis. Spleens were collected at necropsy and homogenized to 10% in PBS by mechanical disruption using zirconium-silicate spheres (lysis matrix D, MP Biomedicals). All available mouse brains and spleens collected from animals terminated due to intercurrent illness were assessed for Western blot and real-time quaking-induced conversion assay (RT-QuIC) for prion positivity when possible. Only animals clinically sick and positive by Western blot were included in the statistics for attack rate and incubation period. Animals from studies where no clinical signs were detected were terminated up to ~600 days post inoculation.

Analysis of PrP^{Sc} by western blotting

Protein concentration for all 10% brain homogenates or 20% spleen homogenates was determined by bicinchoninic acid assay (BCA, ThermoFisher Scientific™). Brain samples were treated with 50 µg/mL of proteinase K (PK, Roche) in the presence of 2% sarkosyl for 1 hour at 37°C in water bath. PK digestion was terminated with the addition of 2mM of phenylmethylsulphonyl fluoride (PMSF) and incubated for 10min. 500 µg of protein from spleen homogenates were treated with 1 mg/mL DNase in the presence of 5 mM MgCl₂ for 90 minutes at 37 °C with constant agitation before PK digestion with 50 µg/ml PK and 2% sarkosyl for 1 hour at 37 °C. Digestion was terminated with 2 mM phenylmethylsulfonyl fluoride (PMSF) for 10 min at room temperature, followed by ultracentrifugation at 100,000 x g for 1 hour at 4 °C (43). The resulting pellet was resuspended in XT-sample buffer (Bio-Rad Laboratories) at 1.5X in the

absence of reducing agents, and the digested brain samples at 1X. Samples were boiled at 100°C for 10min prior to gel electrophoresis. Boiled samples were loaded into a precast 12% discontinuous Bis-Tris gels (Bio-Rad Laboratories) at 140W for 90min. Proteins were transferred overnight into a PVDF-FL membranes (EMD Millipore) in a Trans-blot transfer system (Bio-Rad Laboratories). Membranes were blocked for 1h in 5% nonfat milk in freshly made TBST buffer (20mM Tris in distilled water + 0.5% Tween 20) and probed overnight with PRC5 and PRC1 monoclonal antibodies (mAb) at 1:5000 and 1:500 dilutions, respectively, followed by horseradish peroxidase (HRP)-conjugated anti-mouse IgG secondary antibody (Cytiva) at a 1:5000 dilution for 1 hour. Membranes were developed using ECL 2 western blot substrate (Thermo Scientific™) in chemiluminescence Fujifilm imaging system.

Immunohistochemical analysis

Immunohistochemistry (IHC) was performed as described previously (56). Brains collected were fixed in 10% formalin and embedded in paraffin blocks. 5µm coronal sections were obtained and placed onto positively charged glass slides and heated to 60°C for 30 min. Next, slides are treated with xylene and graduated ethanol treatment, followed by 88% formic acid for 30 minutes. Antigen retrieval was performed in the 2100 Retriever (Proteo-Genix, Schiltigheim, France) using citrate buffer followed by endoperoxidase quenching in 3% hydrogen peroxide. Slides are blocked in 5% nonfat milk for 30 min at room temperature before overnight incubation with primary antibody D18 at 1:2,500, followed by exposure to biotin-labelled goat Fab anti-human IgG secondary antibody (Southern Biotech, Birmingham, AL) at 1:2,500 dilution for 1 h at room temperature. Slides are developed for 30 min at room temperature using avidin-conjugated horseradish peroxidase (HRP) with diaminobenzidine (DAB) as substrate (Vector Laboratories, Burlingame, CA). Images were captured using an Olympus microscope at 1mM (4x) or under oil immersion at 10µM (100x).

Lesion Profiling

Scoring of spongiform neurodegeneration was performed as previously described (57). Coronal sections of paraffin-embedded mouse brains were stained with hematoxylin and eosin. Sections of the frontal, mid, and hindbrain were randomly selected and evaluated independently by two investigators. Scores for severity of vacuolar degeneration were assigned to nine cerebral gray matter areas and four white matter tracts.

Conformational stability assay

Conformational stability assay was performed as previously described (43,53,58). Protein concentration of 10% brain homogenates (BH) was determined by BCA assay (Thermo Fisher Scientific™). Brain homogenate dilutions containing from 1-3 µg/ul of protein were incubated with increasing concentrations of guanidinium hydrochloride (GdnHCl) in 96 well plates for 1 hour at room temperature with constant agitation. Treated samples were transferred to a nitrocellulose membrane (Whatman GmbH, Dassel, Germany) through a dot blot apparatus. After two PBS washes, membranes were air dried for a minimum of 1 hour before rehydration with PBS, followed by digestion with 5 µg/mL PK in cell lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, and 0.5% Igepal CA-630) for one hour. PK digestion was halted with PMSF at 2 mM in PBS for 20 minutes. To expose the PRC epitope, membranes were denatured with 3M guanidinium isothiocyanate for 10 minutes at room temperature and blocked for 1 hour with 5% nonfat milk in TBST. Membranes were incubated overnight at 4°C with mAb PRC5 antibody 1:5000 followed by IRDye® 800CW donkey anti-mouse IgG (H+L) (LiCOR Biosciences, Lincoln, NE, USA) at 1: 25000 for 1 hour. After washing with TBST and TBS 1X, membranes were imaged in LiCOR Odyssey DLx imaging system. Signal quantification was performed in Li-COR image studio™ acquisition software.

Real-Time Quaking-Induced Conversion Assay (RT-QuIC)

Hamster recombinant PrP production. The expression plasmid containing the hamster prion protein coding sequence (90-231) was kindly provided by Dr. Byron Caughey (Rocky Mountain Laboratories, Hamilton, MT). The truncated Syrian hamster PrP^C coding sequence was expressed and consequently purified from BL Rosetta (Novagen) *Escherichia coli*. After the generation of glycerol stock, cells were cultured in lysogeny broth (LB) media with selection antibiotic kanamycin and growth agent chloramphenicol. Inclusion bodies were harvested after bacterial cell lysis with Bugbuster™ (Sigma-Aldrich) supplemented with Lysonase™ (EMD Biosciences). Recombinant rHaPrP90-231 was purified by incorporating inclusion bodies in NiNTA superflow resin (Qiagen) and loading to an XK chromatograph column (Cytiva) on AKTA Fast Protein Liquid Chromatograph (GE). Proteins were denatured and refolded on the column. Following protein elution from the column, the fractions from the elution peak were combined and dialyzed overnight at 4 °C. Purified recombinant HaPrP concentration was measured and adjusted to 0.5mg/mL, aliquoted in Eppendorf tubes, and frozen at -80°C.

RT-QuIC assay. It was performed as follows. 10% brain homogenates or 20% spleen homogenates from GtQ and GtE diseased mice, as well as North American CWD (GtQ and GtE mice passaged – E-US1) as positive controls, and uninfected GtQ and GtE brains and spleens as negative controls were diluted to 10⁻³ in PBS and then to 10⁻⁴ working dilutions in 0.1% sodium dodecyl sulfate (SDS) in PBS 1X. Spleens were seeded in RT-QuIC reactions at 10⁻⁴ to assess positive amyloid formation. Brain dilutions were used to generate serial 10_{log} dilutions. RT-QuIC reaction mixture contains 1X phosphate buffer saline, 1mM EDTA, 170mM NaCl, 0.01mM Thioflavin T and recombinant hamster PrP (rHaPrP90-231) 0.1mg/mL. 2 µL of brain homogenate dilutions are used to seed 98 µL of reaction mixture loaded in 96-well optical black

bottom plates, covered with plate sealing tape, and loaded in FLUOstar® Omega plate reader (BMG Labtech). The reaction plate was run for 48h at 42C with readings recorded every 15 minutes.

Determination of threshold and lag times for RT-QulC positives and analysis.

Threshold of positive reaction for RT-QulC was determined by the sum of the mean plus five times the standard deviation for the first 4 readings (after excluding the first read) to avoid the effect of variability due to temperature differences and spontaneous aggregation of the recombinant PrP. Lag times were calculated as the time in hours for seeding reactions to cross the estimated fluorescence threshold. All RT-QulC-generated data were plotted as quadruplicated technical replicates from at least three biological replicates, unless the number of diseased mouse samples available in a study is less than three.

Statistical analyses.

Statistical analyses were conducted using GraphPad Prism software (San Diego, CA).

Results

Non-lymphotropic moose CWD strain from Sweden acquires lymphotropic properties influenced by the polymorphism 226E in gene-targeted mice

Intracerebral primary and secondary transmissions of the four Sweden moose isolates were described in Chapter 2 (Figure 2.1). Seminal reports on the finding of the Sweden moose CWD isolates describe the absence of prion infection in retropharyngeal lymph nodes (RPLN) or other lymphoid tissue when available. These determinations were made using an ELISA-based rapid assay (TeSeE SAP ELISA CWD (Bio-Rad) and/or IDEXX HerdCheck ELISA (26). RPLN from moose studied in this chapter were analyzed by Nordic scientists. These tissues were not available for analysis by our group.

To investigate the potential acquisition of lymphotropism during iterative passages of the moose CWD isolates from Sweden, spleen tissues harvested from all mice collected from transmissions (clinical mice and mice euthanized at the end of a study in the absence of clinical signs) were analyzed for seeding in real-time quaking-induced conversion assay (RT-QuIC) reactions. Spleens analyzed from all passages of M-SW2 and M-SW3 isolates show no evidence of amyloid formation (prion positivity), despite the high levels of PrP^{Sc} in the brains of these mice (Figure 3.1-B, 3.1-C). The absence of spleen prion seeding persisted for M-SW2 after secondary passage but was also observed for M-SW3 infected mice that showed a longer incubation period compared to M-SW2 (>300 days). These observations suggest the absence of a lymphotropic strain in these isolates, or if present, this sub-strain is at very low levels. Likewise, no evidence of prion spleen replication was found in primary passages of the M-SW1 isolate in GtQ or GtE mice. Unexpectedly, we found that spleens from secondary transmissions of the M-SW1 isolate were positive for prion infection. Interestingly, spleen positivity was seen

exclusively when iterative transmission involved the GtE (glutamic acid at PrP amino acid 226) genotype (Figure 3.1-A).

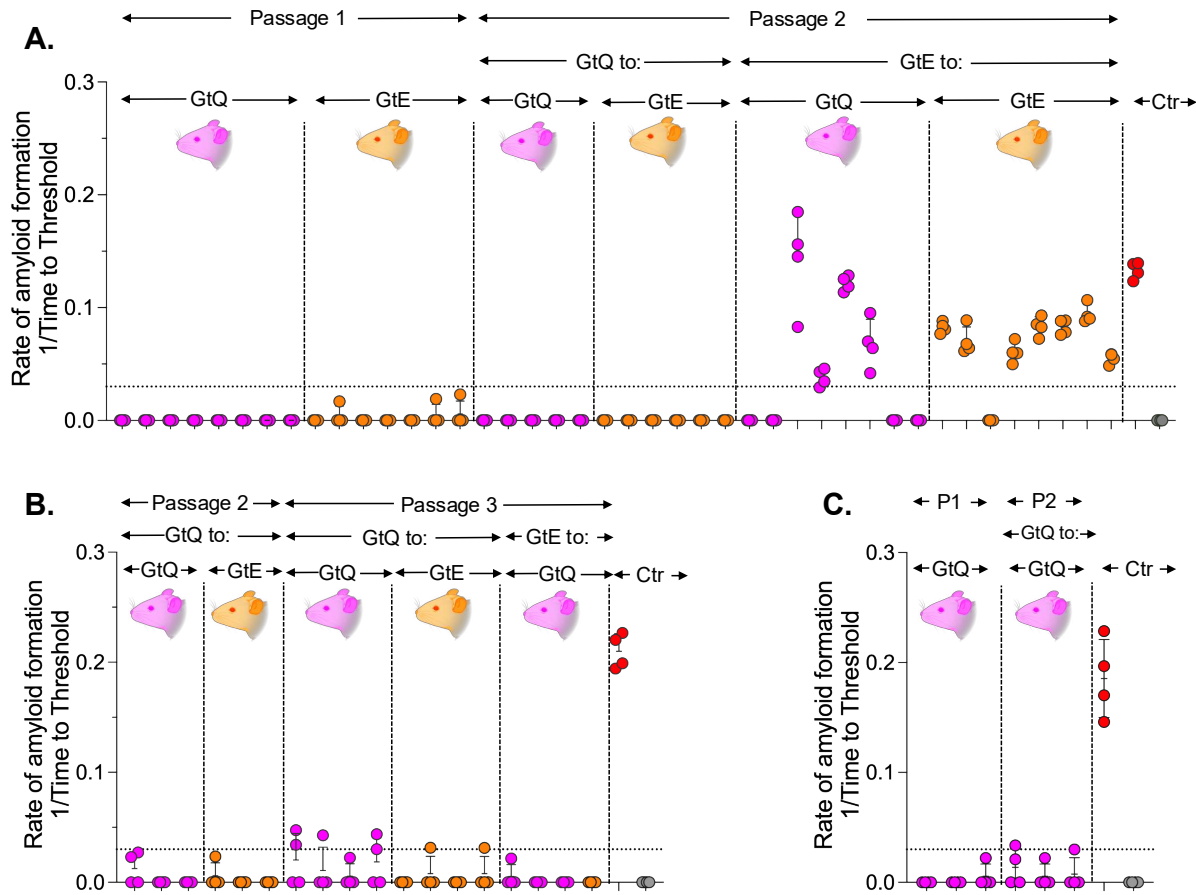


Figure 3.1. Prion positivity in spleens from iterative passages of Sweden moose isolates into GtQ and GtE mice. RT-QuIC positive seeding responses of spleen homogenates obtained from Sweden CWD diseased GtQ and GtE mice. Individual circles represent four technical replicates of individual mice spleen homogenates (GtQ: magenta circles, GtE: orange circles). **A.** Spleens from primary and secondary transmissions of the first Sweden moose CWD isolate (M-SW1) in GtQ and GtE mice. **B.** Spleens from secondary and tertiary transmissions of the second Sweden moose CWD isolate (M-SW2) in GtQ and GtE mice. **C.** Spleens from primary and secondary transmissions of the third Sweden moose CWD isolate (M-SW3) in GtQ mice. P1: primary passage; P2: secondary passage. Ctr: control (positive control: red circles, negative control: grey circles). Dashed line: threshold of positive seeding response, positive response is considered 3 technical replicates above the dashed line. GtE and GtQ: CWD-susceptible gene-targeted mice in which the prion protein coding sequence was replaced with one encoding glutamate (E) at codon 226 or with glutamine (Q).

It was also observed that spleen prion levels were higher, although the positivity rate was stochastic when the GtQ genotype (glutamine at PrP amino acid 226) was involved in transmission (GtE to GtQ). In contrast, complete rate of spleen prion positivity was only achieved when transmission occurred exclusively between GtE genotypes (GtE to GtE) (Figure 3.1A). Transmission kinetics of passages of M-SW1 in GtQ and GtE mice, as discussed in Chapter 2, revealed that M-SW1 was the only Sweden moose isolate capable of infecting GtE mice upon primary passage. However, most of these transmissions were sub-clinical and had a much longer incubation time compared to GtQ mice (560 days versus 350 days; Figure 2.1A). At secondary passage, all transmissions that resulted in spleen involvement, irrespective of the recipient genotype, displayed significantly prolonged incubation periods compared to those that resulted in no spleen infection.

These observations support the conclusion that Nordic moose strains have the potential to acquire lymphotropic properties when transmission occurs in a genotype that permits the selection and replication of strains with tropism for the lymphoid system. The marked differences in disease kinetics between passages resulting in spleen involvement or absence thereof, suggest that polymorphism Q226E dictates the replication of different strains contained in M-SW1 isolate.

Western blot of M-SW1 transmissions reveals distinct glycosylation profiles between M-SW1-infected GtQ and GtE mice.

The presence of proteinase K (PK)–resistant CWD PrP in Sweden moose CWD field isolates, as well as in the brains of GtQ and GtE mice diagnosed with prion disease by Western blot analysis, was discussed in Chapter 2. All Swedish CWD serial transmissions exhibited the characteristic electrophoretic migration pattern previously reported for other Nordic CWD cases

(26,43,49). Likewise, Chapter 2 discussed differences in PrP^{Sc} glycoform distribution observed during passages of M-SW1.

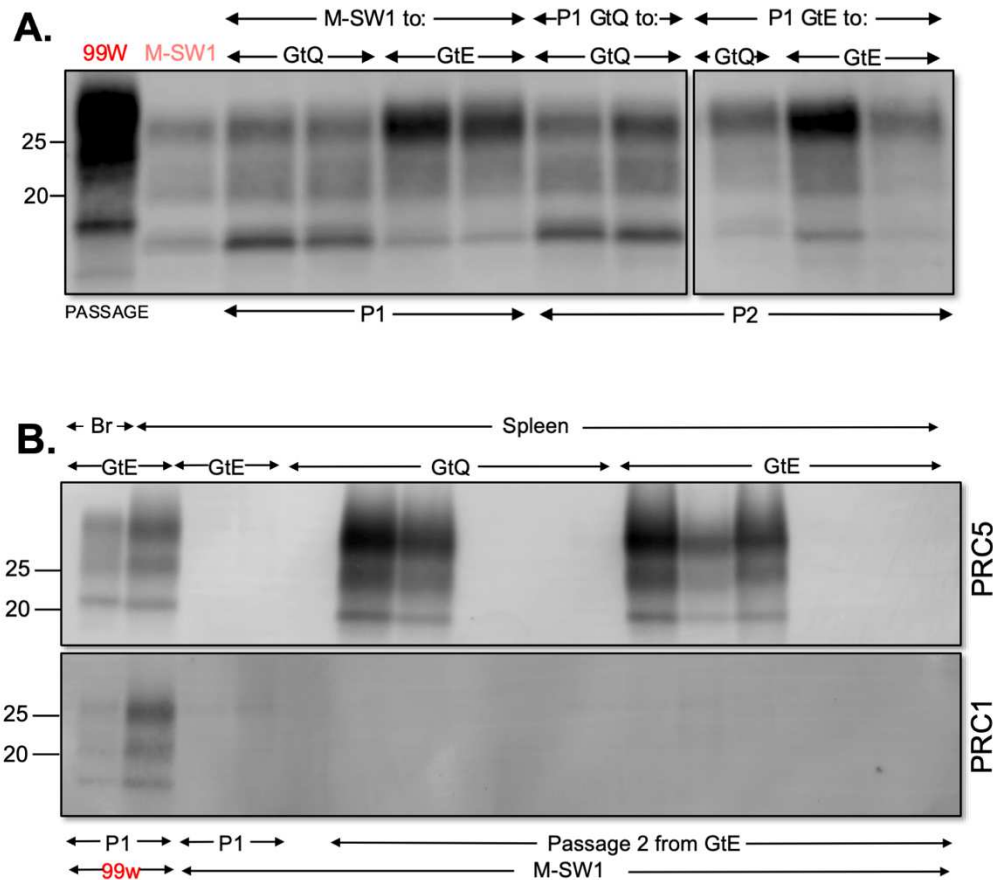


Figure 3.2 Immunoblot analysis of PrP^{Sc} in Sweden Moose 1 (M-SW1) brains and spleens from CWD-infected gene-targeted mice. Western blot analysis of PrP^{Sc} from CNS and spleen tissue from GtQ and GtE mice infected with Sweden moose 1 (M-SW1) CWD isolate after iterative passages. **A.** Brain PK-resistant PrP from M-SW1 after primary and secondary passages. **B.** Spleen PK-resistant PrP from M-SW1 after primary and secondary passages. Immunoblots were probed with mAb PRC5 and PRC1 (PRC5 if not indicated). The position of molecular weight markers approximating 25, 20 kDa is shown; 99W: North American elk CWD isolate; P1: primary transmission; P2: secondary transmission. Br: brain. GtE and GtQ: CWD-susceptible gene-targeted mice in which the prion protein coding sequence was replaced with one encoding glutamate (E) at codon 226 or with glutamine (Q).

We observed that the presence of a higher level of glycosylation in M-SW1 is observed only in passages with involvement of the GtE genotype, which also presented positivity for spleen

infection (Figure 3.2-A). Western blot analysis of spleens from GtE primary passage and corresponding secondary passages showed that only a small number of mice exhibited levels of spleen prion accumulation sufficient for detection by Western blot. Spleen prions displayed the same increased glycosylation pattern observed in brain-derived prions (Figure 3.2-B).

This increased glycosylation is not observed in passages through GtQ mice-only, which are negative for spleen infection. Based on this data, it can be derived that the strain that causes disease in GtQ-only passages is biochemically different than the strain acquiring lymphotropic properties through the GtE genotype, and that glycosylation status could mediate the acquisition of lymphotropic properties in non-lymphotropic strains.

Occurrence of lymphotropism in M-SW1 coincides with the presence of a distinct morphology of PrP^{Sc} deposition in the dentate gyrus of the hippocampus.

Immunohistochemistry (IHC) was performed to investigate the morphologies of PrP^{Sc} in the brains of M-SW1 and to assess if additional evidence could be gathered to support the presence of a distinct CWD prion strain within the M-SW1 isolate that is associated with the acquisition of lymphotropism during passages in Gt mice. As discussed in Chapter 2, a mixture of morphologies of PrP^{Sc} was common across all passages of both GtQ and GtE mice (Figure 2). Further IHC analysis revealed that a distinctive stellate pattern of PrP^{Sc} deposition in the dentate gyrus of the hippocampus was present only in mice positive for prion infection in the spleen at secondary passage (Figure 3.3-C, 3.3-F).

Notably, the presence of this pattern of deposition preceded the occurrence of lymphotropism, as it was observed at primary passage in GtE mice that were negative for prions

in the spleen but developed lymphotropism in secondary passage (Figure 3.3-D). Taken together, from the analysis of prion infection in the spleen and the presence of dentate gyrus staining led to the definition of a new phenotype named DGL (dentate gyrus + lymphotropism). The DGL designation refers to the concurrent presence of dentate gyrus staining and spleen prion infection, whereas DG denotes dentate gyrus staining only (as observed in GtE mice in primary passage), and L indicates spleen infection in the absence of dentate gyrus staining. The most common phenotype observed in GtE passages was the DGL phenotype. The L phenotype was only found in one mouse that was collected early in the study (~450 days), exhibiting low levels of spleen positivity, suggesting that the presence of DG staining may not be strictly required for lymphotropism but could facilitate this adaptation.

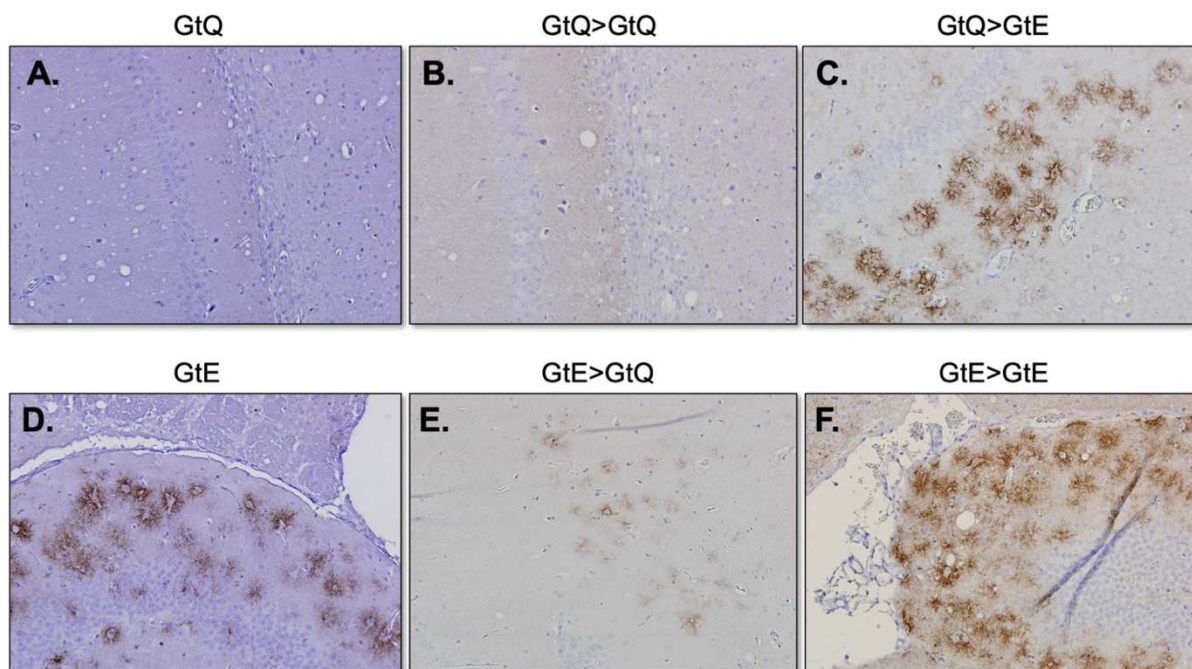


Figure 3.3. Dentate gyrus stellate pattern (DG) linked to lymphotropic adaptation analyzed by immunohistochemistry of PrP^{Sc} of brain coronal hippocampus sections in Gt mice diseased with M-SW1 isolate. Deposition and morphology of disease-related PrP^{Sc} in the CNS of diseased Gt mice. **A.** PrP^{Sc} deposition in GtQ mice infected with Sweden moose 1 (M-SW1) in primary passage **B.** PrP^{Sc} deposition in GtQ mice infected GtQ-infected brain from primary passage. **C.** PrP^{Sc} deposition in GtE mice infected GtQ-infected brain from primary passage. **D.** PrP^{Sc} deposition in GtE mice infected with Sweden moose 1 (M-SW1) in primary

passage. **E.** PrP^{Sc} deposition in GtQ mice infected GtE-infected brain from primary passage. **C.** PrP^{Sc} deposition in GtE mice infected GtE-infected brain from primary passage. GtE and GtQ: CWD-susceptible gene-targeted mice in which the prion protein coding sequence was replaced with one encoding glutamate (E) at codon 226 or with glutamine (Q). Slides taken at 10x (100 μ M) magnification

Differences in the conformational properties of M-SW1 CWD during iterative passage that involves GtQ or GtE exclusively suggest a strain divergence event.

Chapter 2 discussed how M-SW1 primary passages in GtQ and GtE mice, as well as secondary passages through GtE mice (GtE to GtQ and GtE to GtE) showed no significant differences in half maximum denaturation values (GdnHCl_{1/2}). However, comparison of secondary transmissions exclusively through GtQ (GtQ to GtQ) or GtE (GtE to GtE) mice revealed that GtQ prions were significantly less stable than GtE prions (Figure 3.4-A). This indicates that after iterative passages within the same genotype, a strain divergence event may occur, whereby passage through GtQ selects a different strain than passage through GtE. This interpretation is supported by the observation that inoculation of GtQ mice with GtE-infected brain tissue from primary passage resulted in undistinguishable kinetics of disease from those observed in GtE to GtE mice (Figure 2.1).

No differences in conformational properties are found between DGL, DG, and L phenotypes.

The presence of three phenotypes associated with dentate gyrus stellate staining and lymphotropism suggested the possibility that distinct strains dictate the occurrence of these phenotypes. Analysis of chaotropic denaturation by incubation with increasing concentrations of GdnHCl of brain homogenates derived from mice presenting DGL, DG, and L phenotypes, revealed no significant differences in their (GdnHCl_{1/2}) of PK-resistant PrP; however, the

highest value was observed when both DG and L were present. These results show that the observed phenotypes are associated with the same strain (Figure 3.4-B).

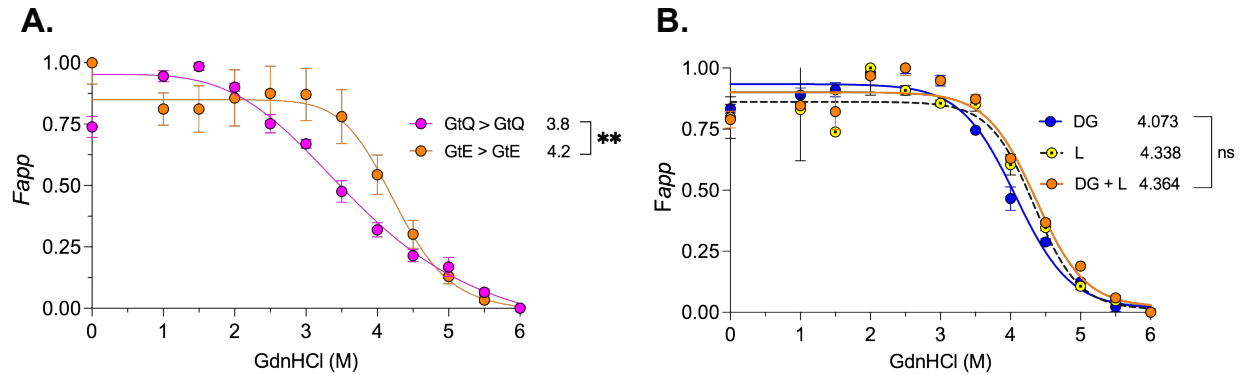


Figure 3.4. Conformational properties of Sweden moose 1 (M-SW1) CWD prions passed exclusively in GtQ and GtE mice and comparison of DGL phenotypes.

Response of Sweden moose 1 (M-SW1) CWD prions to denaturation with increasing concentrations of GdnHCl. The average fractional apparent signal (F_{app}) of PK resistant PrP^{Sc} was obtained by densitometry of probed dot blots plotted as a function of GdnHCl concentration (M) **A.** Comparison of denaturation response of M-SW1 prions passed exclusively through GtQ and GtE mice at secondary passage. **B.** Comparison of denaturation response of prions from DGL phenotypes (secondary transmissions of M-SW1 prions in the GtE genotype). GtQ: magenta circles, GtE: orange circles. DG: dentate gyrus staining only (dark blue circles), L: lymphotropism only (yellow dotted circle), DGL: dentate gyrus staining + lymphotropism (orange circles). $GdnHCl_{1/2}$ values (Mol/L) for each dose-response curve is shown. Sigmoidal dose-response curves were plotted using a four-parameter algorithm and nonlinear least-square fit. Error bars indicate $\pm$ SEM of data from ≥ 3 animals per study if available. Statistical significances were calculated by pairwise analysis of $GdnHCl_{1/2}$ values from best fit curves ($** p \leq 0.001$). ($F_{app} = (\text{maximum signal} - \text{individual signal}) / (\text{maximum signal} - \text{minimum signal})$). GdnHCl, guanidine hydrochloride; GtQ or GtE, CWD-susceptible gene-targeted mice expressing glutamine or glutamic acid at codon 226; R^2 (goodness of fit) for each curve: > 0.9 .

Lesion profile analysis supports the notion that M-SW1 passage through GtQ and GtE leads to a strain divergence event.

To identify additional evidence of strain differences between lymphotropic and non-lymphotropic M-SW1 strains and to assess the potential occurrence of a strain divergence event, a semiquantitative assessment of spongiform degeneration was performed by lesion

profiling, following the criteria described by Fraser et. al. (57) with the addition of four white-matter tracts. This analysis showed that spongiform neurodegeneration profiles between primary passages of M-SW1 in GtQ and GtE mice differed mostly in lesion severity within the frontal brain areas (septum and frontal cerebral cortex), which were more pronounced in GtQ mice (Figure 3.5-A).

Interestingly, upon serial passage through the GtE genotype, neuropathological profiles between GtQ and GtE mice become indistinguishable, supporting the observation that once M-SW1 is transmitted in the GtE mice, it acquires properties reflective of this genotype independently of the recipient genotype (GtQ or GtE) (Figure 3.5-B). In contrast, secondary passage exclusively through the GtQ genotype preserved the neuropathology profile observed in primary passage. Collectively, these findings indicate that M-SW1 causes more severe neuropathology in GtQ mice and discrete lesions in GtE mice. The profile of neuropathology observed in GtE mice is maintained upon serial passage, regardless of G226E polymorphism, and it is associated with mice that developed lymphotropism. This also supports that GtQ and GtE select for distinct prion strains through divergence dictated by Q226E.

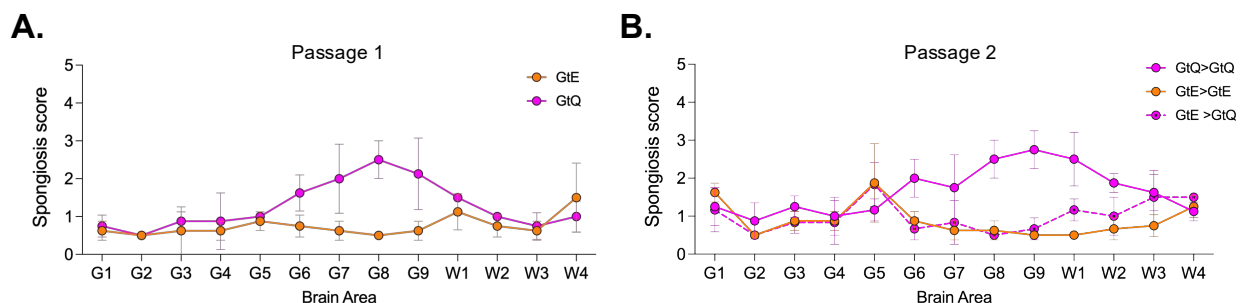


Figure 3.5. Lesion profiling of coronal brain sections of Gt mice infected with Sweden moose 1 (M-SW1) CWD isolate after iterative passages. Analysis of vacuolar lesion scores. **A.** Lesion profiles of primary and secondary passages of M-SW1 in GtQ and GtE mice. GtQ: magenta circles, GtE: orange circles **B.** Lesion profiles of secondary passages of M-SW1 in GtQ mice (GtQ to GtQ): magenta circles, GtQ mice (GtE to GtQ): dotted magenta circles, GtE mice (GtE to GtE): orange circles. Assessment of vacuolar lesions was performed in the following

regions: G1) medulla oblongata (Mobl); G2) cerebellum cortex (Cb ctx); G3) superior colliculus (Sup Col); G4) hypothalamus (Hy); G5) thalamus (Th); G6) hippocampus (Hp); G7) septum (Sep); G8) cerebral cortex at the level of the thalamus (Th ctx); G9) frontal cerebral cortex (Fr ctx); W1) cerebral peduncle (Cb pd dec); W2) corpus callosum (Cb WM); W3) caudoputamen (Cp); W4) cerebellar white matter (Crb WM). Data points represent the mean $\pm$ SD of data from ≥ 3 animals per study.

Intraperitoneal transmission of M-SW1 field isolates, fails to establish infection in the spleen of GtQ or GtE mice.

To address the question if a pre-existing lymphotropic strain exists in the natural isolate, the M-SW1 1% BH inocula was administered by the intraperitoneal (IP) route into GtQ and GtE mice. GtQ and GtE mice were collected after 550 days in the absence of clinical signs (Figure 3.6-A). Spleens were collected from all mice and homogenized to 10% and 10^{-3} dilutions were established for their analysis by the RT-QuIC assay. RT-QuIC reactions resulted in no evidence of prion seeding activity in the spleens of GtQ or GtE mice (Figure 3.6-B). These results suggest that the M-SW1 isolate is composed of pre-existing lymphotropic strains that are in a titer too low to sustain IP infection to the spleen or the lymphotropic-adaptation occurs without a pre-existing lymphotropic strain in the inoculum.

Sweden moose 4 (M-SW4) acquires lymphotropic properties through a GtE-independent mechanism.

Previous studies describing the development of lymphotropism through adaptation of Nordic moose CWD to a North-American (NA) CWD phenotype (43), as well as the case of Sweden moose 1 (M-SW1), demonstrated that *de novo* spleen involvement is contingent upon the transmission of these isolates through the GtE genotype. This suggests that polymorphism

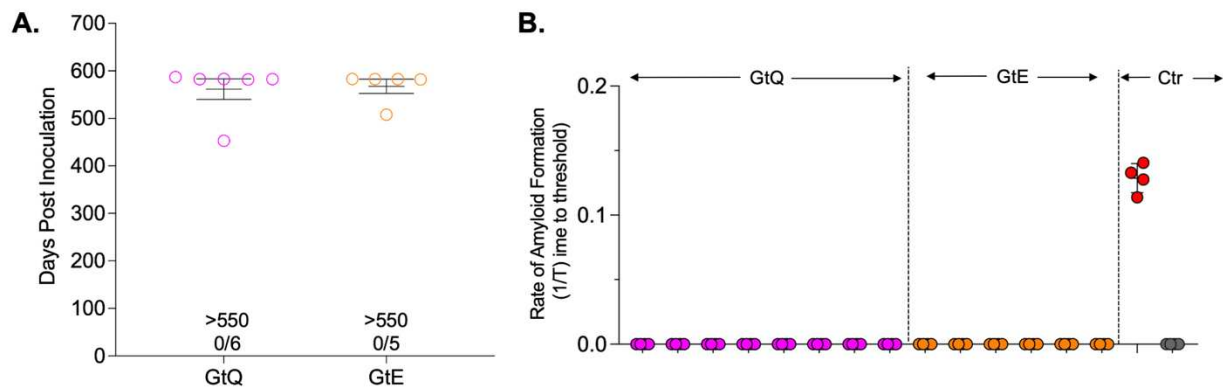


Figure 3.6. M-SW1 fails to cause disease or spleen positive seeding activity after inoculation by the intraperitoneal route. **A.** Scattergram represents intraperitoneal transmissions of M-SW1 in GtQ and GtE mice. Time to collection and attack rate are shown. absence of clinical signs or PrP^{Sc} in the brain: GtQ, magenta empty circles; GtE orange empty circles. **B.** Seeding activity by the RT-QuIC assay from spleen homogenates from GtQ and GtE mice intraperitoneally inoculated with M-SW1 isolate. Ctr: positive and negative controls.

226E either facilitates the selection of a lymphotropic strain present within the natural isolate or promotes the acquisition of novel lymphotropic properties.

RT-QuIC analysis of spleens obtained from the iterative passages of Sweden moose CWD isolates, revealed that the fourth isolate (M-SW4), which exhibited very inefficient transmission in primary passage but achieved a complete attack rate in GtQ mice and represented the second fastest transmission among the four Swedish isolates (Figure 2.1), results in the acquisition of lymphotropic properties in GtQ mice at secondary passage (GtQ to GtQ) (Figure 3.7-E). It was described in Chapter 2 that M-SW4 PrP^{Sc} morphology in the CNS is characterized by a diffuse, widespread deposition that is visible only under high magnification (100X). There is no evidence of a DG-like staining or changes in the glycosylation profile of PK-resistant PrP (Figure 2.3, Figure 3.7-A, 3.7-B, 3.7-C, 3.7-D). These observations demonstrate that the acquisition of lymphotropic properties in Nordic moose CWD strains can occur

independently of the GtE genotype. These findings suggest that multiple, strain-dependent mechanisms of lymphotropic adaptation may operate in Nordic moose.

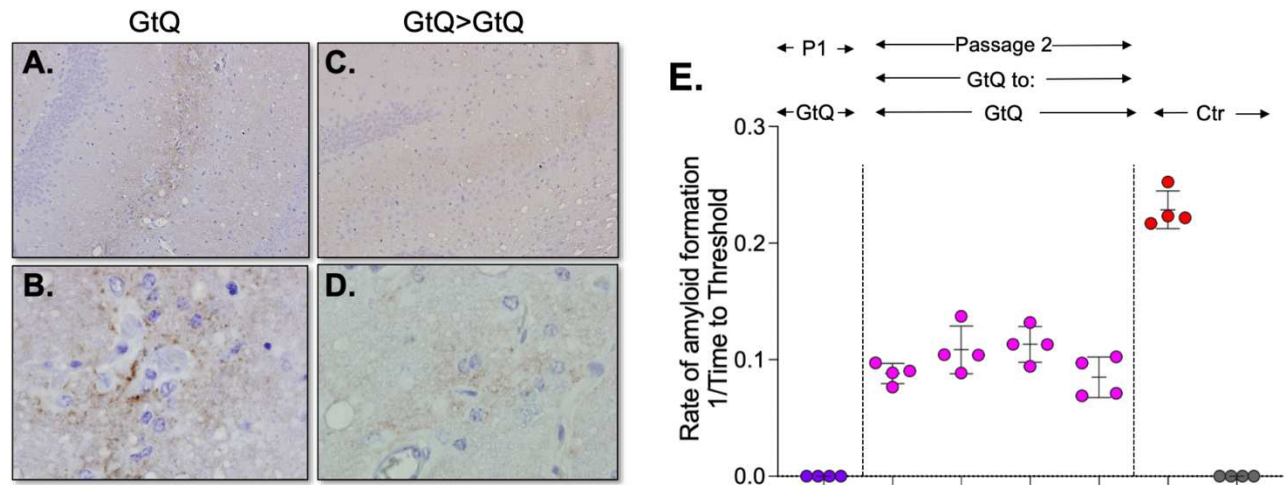


Figure 3.7. Development of lymphotropism in GtQ mice infected with M-SW4 isolate without GtE genotype involvement and absence of DG staining. All images represent the area of the hippocampus and the dentate gyrus region. **A.** Immunohistochemistry (IHC) analysis of D18-stained coronal sections from the primary passage of the M-SW4 isolate in GtQ mice. **B.** Magnification of image in (A). **C.** IHC analysis of secondary passage of M-SW4 in GtQ mice. **D.** Magnification of image in (C). **E.** RT-QuIC seeding activity of spleen homogenates from primary and secondary passages of M-SW4 in GtQ mice. Individual circles represent four technical replicates of individual mice spleen homogenates. P1: passage 1, Ctr: controls.

Discussion

The exceptional tropism of chronic wasting disease (CWD) prions for replication in secondary lymphoid organs is considered a major contributor to the facile horizontal transmission that has driven the exponential expansion of its geographic distribution in North America over the last 20 years. Following initial infection, CWD prions are detected in the lymphoreticular system at early stages of the asymptomatic period (28,31,59,60). Consequently, infectious prions can be shed to the environment in the absence of clinical signs for months or years, and while free-ranging cervids migrate across wide geographic areas. Although the strain properties of Nordic CWD and North American CWD differ substantially, it is expected that emergent, unstable strains can evolve and adapt in unpredictable ways. Nordic moose CWD has been described as non-lymphotropic due to the absence of detectable levels of PrP^{Sc} in lymphoid tissues (26,45,50,51). However, our recent studies have shown that Nordic moose CWD can acquire North American-like properties, including the development of a lymphotropic phenotype(43).

In this chapter, we report a detailed biochemical characterization of a lymphotropic strain that emerges during iterative passages of a Nordic moose isolate from Sweden. This adaptation occurs under the influence of a polymorphism in the wild-type cervid prion protein primary sequence, specifically the expression of glutamic acid at residue 226. After initial transmission in both GtQ and GtE mice, RT-QuIC analysis showed no evidence of prion seeding activity in spleens. However, following secondary passage of brain inoculum from a GtE mouse positively diagnosed with prion disease, both GtQ and GtE mice exhibited substantial prion seeding activity in the spleen. In contrast, secondary passage from GtQ mice into both GtQ and GtE recipients resulted in negative RT-QuIC detection in spleen samples. These results indicate that, after the initial absence of detectable lymphotropism in both the natural host and primary

passage in Gt mice, iterative passage through the GtE genotype favors selection of a lymphotropic strain and/or facilitates *de novo* acquisition of lymphotropic properties under the influence of the GtE genotype.

We determined that CWD prions from Sweden moose 1 (M-SW1) undergo a potential strain divergence event, in which the biochemical properties of prions passaged in GtQ and GtE mice do not differ significantly until secondary passages, when transmission occurs within the same genotype. CWD prions from mice that develop lymphotropism differ from those that do not in glycosylation profile, conformational stability, and the severity of vacuolation in the CNS. Lymphotropic strains exhibit increased glycosylation, higher conformational stability as reflected by resistance to denaturation, and reduced neurotoxicity. Notably, CWD prions from GtQ mice that develop lymphotropism upon secondary passage adopt properties similar to those observed in GtE spleen-positive mice. These findings suggest that glutamic acid at residue 226 has a strong influence on the adaptation of M-SW1 prions.

Wagner et al. observed that biochemical properties of CWD prions can be dictated by the tissue environment, and lymphotropic prions display differences with neurotropic prions (61). While sialylation may not determine efficient replication of prions in lymphoid organs (17), it has been described as an important factor for their trafficking within those tissue compartments (16). We also observe that the M-SW1 lymphotropic strain displays the highest stability and is associated with lower neurotoxicity, longer incubation periods, and a higher prevalence of subclinical disease (Figure 2.1-A). Previous studies have shown that increased sialylation can slow prion replication, potentially due to electrostatic repulsion acting as a constraint on replication (62,63), and may play a determinant role in strain selection (64). Lymphotropic adaptation may therefore represent a favorable adaptation in terms of delayed clinical onset and reduced neuropathology; however, it comes with a cost, a CWD strain with increased infectivity.

This adaptation may suggest two potential scenarios: 1) there is no pre-existing lymphotropic strain in the natural inoculum, and spleen tropism arises *de novo* within the CNS environment, potentially involving cellular factors of the neuroimmune system; in this case, deformed templating may play a central role, whereby a strain with a primary sequence mismatch—here, glutamine versus glutamic acid—favors the emergence of intermediate conformations that ultimately adapt to a new phenotype (65,66). The observation of strain divergence in this study may represent plausible evidence. Immune cells and cellular factors may play a role. It has been shown that astrocytes in the CNS and follicular dendritic cells (FDCs) in the spleen can sustain prion replication in mouse models in which PrP^C expression is restricted to these cell populations (11,67), and that components of the complement system play a key role in prion trafficking (12,13).

2) Alternatively, a preexisting lymphotropic strain may be present within the natural isolate. This scenario is consistent with the conformational selection model, in which a cloud of preexisting conformers co-exists within a population of prion strains and is selected under favorable conditions (68). The failure of intraperitoneal transmission to produce CWD infection in GtQ or GtE mice may suggest that no pre-existing lymphotropic strain is present in M-SW1. However, we cannot exclude the possibility that a lymphotropic strain, which emerges with involvement of the GtE genotype, exists at a very low titer relative to the dominant strain that transmits efficiently in the GtQ genotype, as in the natural host, thereby imposing a barrier to efficient replication in the spleen.

Remarkably, the same phenotype observed during lymphotropic adaptation of the Sweden moose 1 isolate was also identified in additional isolates currently under investigation in our laboratory. We found that Norwegian moose 3 and Norwegian red deer undergo a similar lymphotropic adaptation involving the GtE genotype, including development of the DGL phenotype, and exhibit indistinguishable biochemical properties compared with M-SW1. This

observation suggests that one mechanism of lymphotropic adaptation in non-lymphotropic Nordic CWD strains may involve convergent evolution of lymphotropic variants under the influence of the GtE genotype.

This investigation also established that the DGL convergent evolution is not the exclusive mechanism of lymphotropic adaptation of non-lymphotropic Nordic CWD strains, as a mechanism, not influenced by the GtE genotype is observed for the fourth Swedish moose case (M-SW4). This adaptation occurs without detectable changes in the strain properties and a complete absence of DG staining. However, substantial seeding activity was found at secondary passage in spleens within the GtQ genotype. This adaptation strongly suggests that pre-existing conformers with the properties favorable for the spleen replication may occur. Contrasting with the DGL phenotype, the development of lymphotropism does not correlate with a delay in time to disease or reduced severity of neuropathology.

In summary, the collective results from this study, together with findings from additional non-lymphotropic Nordic CWD cases, emphasize the precision and value of the Gt mouse model in the context of the emergence of new CWD strains in Europe. The initial results from surveillance studies established that Nordic moose and red deer CWD were non-lymphotropic; however, our findings suggest that alternative outcomes may arise as these strains evolve and adapt in unpredictable ways, underscoring the importance of continued surveillance, management, and assessment of the zoonotic risk of unstable, evolving CWD strains.

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CHAPTER 4 – GENE-TARGETED MICE AS A MODEL TO INVESTIGATE THE EFFECT OF THE K109Q POLYMORPHISM IN THE PATHOGENESIS OF CHRONIC WASTING DISEASE

Introduction

Prion diseases are a group of inexorably fatal neurodegenerative conditions that afflict humans and animals, caused by the aberrant misfolding of the alpha-helical-rich, membrane-bound glycoprotein, the cellular prion protein (PrP^c), especially abundant in neurons and cells of the immune system. The defining paradigm of the prion hypothesis is the capacity of misfolded PrP^c (PrP^{Sc}) to propagate its beta-sheet-rich infectious conformation in the absence of nucleic acids. Prions are therefore infectious agents of solely proteinaceous nature (1). The exact mechanism of prion propagation has remained elusive, and its exact role in the outcome of disease is still unclear; however, the progressive accumulation of infectious prions in the central nervous system (CNS) is a related event (2).

Most remarkably, a protein-only pathogen is responsible for diseases of various etiologies, since prion diseases can be spontaneous, genetic, or acquired (3). Prion diseases in animals that include scrapie in sheep and goats, transmissible mink encephalopathy (TME) in captive mink, classic bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) of farmed and free-range cervids and more recently, camel prion disease (CPD) in dromedary camels, are mostly of acquired origin; although spontaneous forms of scrapie (Nor98) and BSE (H-BSE, L-BSE) (4–8) and genetically linked BSE have been identified (9,10). Emergent CWD in Norwegian reindeer (11) could have an infectious origin, while cases in Nordic red deer (12) and moose (13–15) are likely sporadic (16–19). Creutzfeldt-Jacob disease (CJD) in humans can arise spontaneously (sCJD), due to genetically linked factors (fCJD), or acquired in the form of variant CJD (vCJD) in consequence of the consumption of bovine

spongiform encephalopathy (BSE) contaminated cattle, as the most devastating example of the zoonotic risk of animal prion diseases (20). While sporadic human prion diseases account for ~80-85% of cases, genetic factors represent 10-15% of their annual incidence (3).

The pathogenic heterogeneity of prions is explained by the existence of distinct conformers or strains, which are operationally defined as a transferable phenotype of disease that remains stable during controlled conditions. The phenotype is defined by the length of the incubation period, clinical signs, biochemical properties, cellular tropism, and neuropathology (21–23). Breakthrough advances in cryo-electron microscopy techniques have provided high-resolution structural information from multiple rodent-adapted strains (24–27), human prion diseases (28), and, recently, naturally occurring CWD in deer (29), as evidence that prion strains encode heritable information through conformational differences reflected in their structural polymorphism.

Chronic wasting disease (CWD) of the Cervidae family is the only prion disease known to occur naturally in free-ranging wildlife and captive animals. CWD is endemic in North America among wild and farmed deer, elk, and red deer, with isolated cases in moose. Since its first identification in a research facility in Fort Collins, Colorado, in 1967 (30), the prevalence of CWD has increased markedly, particularly in the last decade, and has now been identified in 36 US states and four Canadian provinces. CWD was inadvertently introduced to South Korea from Canada in the late 1990s and has become endemic among farmed cervids (31). Recent studies have confirmed that South Korean and North American CWD have indistinguishable properties. In 2016, CWD was reported for the first time in Northern Europe. The first cases were identified in free-ranging reindeer in Norway (11), followed by cases in red deer (12) and moose (13). Additional moose CWD cases were also found in Sweden and Finland (14,15). Studies using bank voles as well as transgenic and gene-targeted mouse models have demonstrated that

North American and Nordic CWD are not connected, with striking differences observed in their strain properties (15,17,32). The zoonotic potential of CWD remains uncertain, as studies using both in vivo and in vitro systems have yielded mixed or inconclusive results (33–37). Although the majority of evidence suggests the species barrier between cervids and humans is robust, the emergence of novel and unstable strains warrants renewed assessment of the public health risk posed by CWD.

Variations in a strain's ability to cause disease in a host are dictated by the species barrier, which is largely determined by differences in the primary structure of PrP^C (38). Polymorphisms of the *PRNP* gene have been demonstrated to influence susceptibility to genetic, acquired, and sporadic forms of human prion disease. Methionine at codon 129 is overrepresented among affected individuals, illustrating the profound impact of single amino acid substitutions on prion disease susceptibility and pathogenesis (39–41).

In animal prion diseases, classical scrapie in sheep represents the most well-characterized example of a species with a highly polymorphic variation in *PRNP* that dictates variability in susceptibility. Polymorphisms at codons A136V, R154H, and Q171R are especially influential, with the VRQ haplotype conferring the greatest risk of disease and ARR complete resistance (3,42). The best-described examples of polymorphic variations in cervid *PRNP* include the G96S variant in deer (43,44) and the M132L variant in elk, both of which confer reduced susceptibility to CWD in their minor allele forms (S and L, respectively) (45,46). Notably, *PRNP* codon 132 in elk is equivalent to 129 in humans. Multiple polymorphic variants are notable in the signal peptide region (47), as well as codons for positions 225 and 116 (48). In addition, Nordic reindeer affected by CWD have an increased prevalence of the serine allele at position 225, and multiple polymorphisms have been identified (49). Despite these variations, the wild type cervid *PRNP* sequence is nearly identical in most cervid species, except for

polymorphism at residue 226. At this position deer, moose and reindeer express glutamine, moose, and reindeer, whereas glutamic acid is found in elk and red deer, although red deer may also express glutamine (50).

A unique polymorphic variant has been identified exclusively in the Nordic moose subspecies (*Alces alces alces*). This variant is located at codon 109, where the wild-type lysine is substituted with glutamine (51). Genetic surveys in Nordic cervid populations indicate that approximately 20-25% of moose in Norway are homozygous for glutamine at codon 109 (52,53). Codon 109 lies within the second polybasic domain of PrP, a region previously associated with pathogenic mutations that increase the risk of inherited prion diseases in humans (P101L, P105L) (39,54), spontaneous disease in bank voles (M109I) (55,56), and mice (L108I) (57), and has been found to play a key role in prion conversion for several strains (58).

Among the CWD cases in Nordic moose reported to date, three individuals were found homozygous for glutamine at position 109 (16,52). In the present study, we utilize the advantages of the gene-targeted mouse model to investigate the effects of this unique polymorphic variant on CWD susceptibility and pathogenesis. Given both the high prevalence of this polymorphism in Nordic moose populations and its critical location within the PrP sequence, defining its role in CWD pathogenesis is of utmost importance, as it may inform intervention strategies to protect wild moose and other sympatric species from CWD.

Materials and Methods:

Ethics Statement

All animal work was performed in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility in accordance with the Guide for the Care and Use of Laboratory Animals. All procedures used in this study were performed in compliance with and were approved by the Colorado State University Institutional Animal Care and Use Committee.

CWD Field Isolates

The CWD field isolates from moose expressing the 109Q polymorphism from Norway are designated 18-CD24724 (M-NO4), moose 19-CD24854 (M-NO6), and moose 22-CD29 (M-NO10), (M: moose, NO: Norway, number: case number) have been described previously (53,59,60). Demographic information is described in Table S1. The Canada moose isolate CA-1, the American moose isolate 06057822F derived from lymphoid tissue, and the mule deer isolate 001-39697 have been described (17,61). Norway moose 1 (P138). GtQ-Ad-V876 refers to the Sweden moose 2 isolate 19-VLT000876 that has been transmitted and adapted to GtQ mice with an incubation period of ~90 days. The isolate 19-VLT000876 has been previously described (13,59,62). North American CWD isolate 99w12389 (E-US1) was used as a control for PMCA and spleen RT-QulC reactions.

Mouse models and transmission studies

The generation and characterization of gene-targeted GtQ and GtE mice expressing glutamine (Q) and glutamic acid (E) at amino acid 226, respectively, and wild-type lysine (K) at amino acid 109 have been described previously (63). The GtQ109 mouse line was generated from GtQ homozygous mice by a CRISPR knock-in strategy to introduce the 109Q point mutation. Homozygosity for the 109Q allele was confirmed by PCR genotyping, and GtQ, GtE, and

GtQ109 lines were maintained on identical FVB inbred backgrounds. Ten percent brain homogenates (BH) from 109Q Nordic moose CWD and North American CWD were established by mechanical disruption (MP Biomedicals, Irvine, CA) using sterile phosphate-buffered saline (PBS) lacking calcium and magnesium ions. The same number of male and female mice between four and six weeks were intracerebrally inoculated with 30 μ L of 1% BH into the right parietal lobe using a 26-gauge needle. One hundred microliters of 1% BH was administered for intraperitoneal inoculations. Thirty microliters of 1% homogenates was administered for serial passage of spleen tissue. Mice were assessed three times weekly for signs of prion disease, including truncal ataxia, loss of extensor reflex, hunched posture, slowed movements, plastic tail, wobbling/flattened gait, head bobbing/tilt, limb paralysis, and rough coat. Incubation period is defined as the time elapsed between inoculation day and onset of clinical signs, which was defined as the first day when clinical signs were identified. Brains were dissected down the midline and one hemisphere used for preparing 10% brain homogenates in PBS for biochemical analysis while the opposite hemisphere was immersed in formalin for neuropathological analysis. Spleens were collected at necropsy and homogenized to 10% in PBS by mechanical disruption using zirconium-silicate spheres (lysis matrix D, MP Biomedicals). All available mice brains and spleens collected from animals terminated due to intercurrent illness, were assessed for western blot and/or real-time quaking-induced conversion assay (RT-QuIC) for prion positivity when possible. Only animals clinically sick and positive by western blot were included in the statistics for attack rate and incubation period. Animals from studies where no clinical signs were detected, were terminated between 350 days to ~600 days post inoculation.

Analysis of PrP^{Sc} by western blotting

Protein concentration for all 10% brain homogenates was determined by bicinchoninic acid assay (BCA, ThermoFisher Scientific™). Samples were treated with 50ug/mL of proteinase K

(PK, Roche) in the presence of 2% sarkosyl for 1 hour at 37°C in water bath. PK digestion was terminated with the addition of 2mM of phenylmethanesulfonyl fluoride (PMSF) and incubated for 10 minutes. For non-PK-treated samples, sarkosyl was added to 2% with no further treatment. XT-sample buffer (Bio-Rad Laboratories) was added to 1X to all samples before boiling at 100°C for 10 minutes prior to gel electrophoresis. Boiled samples were loaded into a precast 12% discontinuous Bis-Tris gel (Bio-Rad Laboratories) at 140W for 90 minutes. Proteins were transferred overnight into a PVDF-FL membrane (EMD Millipore) in a Trans-blot transfer system (Bio-Rad Laboratories). Membranes were blocked for 1 hour in 5% nonfat milk in freshly made TBST buffer (20mM Tris in distilled water + 0.5% Tween 20) and probed overnight with PRC5 and PRC1 monoclonal antibodies (mAb) at 1:5000 and 1:500 dilutions, followed by 1 hour incubation with IRDye® 800CW Goat anti-Mouse IgG (H + L) (LI-COR Biosciences, Lincoln, NE, USA). membranes were imaged in LiCOR Odyssey DLx imaging system. Signals were analyzed in Fiji open source software (64). Western blots from PMCA reactions were treated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG secondary antibody (Cytiva) at a 1:5000 dilution for 1 hour and developed using ECL 2 western blot substrate (Thermo Scientific™) in a chemiluminescent Fujifilm imaging system.

Immunohistochemical analysis

Immunohistochemistry (IHC) was performed as described previously (65). Brains collected were fixed in 10% formalin and embedded in paraffin blocks. 5µm coronal sections were obtained and placed onto positively charged glass slides and heated to 60°C for 30 min. Next, slides are treated with xylene and graduated ethanol treatment, followed by 88% formic acid for 30 minutes. Antigen retrieval was performed in the 2100 Retriever (Proteo-Genix, Schiltigheim, France) using citrate buffer followed by endoperoxidase quenching in 3% hydrogen peroxide. .

Slides are blocked in 5% nonfat milk for 30 minutes at room temperature before overnight incubation with Fab D18 at 1:2,500, followed by exposure to biotin-labelled goat Fab anti-human IgG secondary antibody (Southern Biotech, Birmingham, AL) at 1:2,500 dilution for 1 hour at room temperature. Slides are developed for 30 minutes at room temperature using avidin-conjugated horseradish peroxidase (HRP) with diaminobenzidine (DAB) as substrate (Vector Laboratories, Burlingame, CA). Images were captured using an Olympus microscope at 1mm, 50 μ M (4x, 40X) or under oil immersion at 10 μ M (100x).

Lesion Profiling

Scoring of spongiform neurodegeneration was performed as previously described(66). Coronal sections of paraffin-embedded mouse brains were stained with hematoxylin and eosin. Sections of frontal, mid, and hindbrain were randomly selected and evaluated independently by two investigators. Scores for severity of vacuolar degeneration were assigned to nine cerebral gray matter areas and four white matter tracts. Lesions were evaluated at a magnification of 100 μ M (10x).

Conformational stability assay

Conformational stability assay was performed as previously described (17,63,67). Protein concentration of 10% brain homogenates (BH) was determined by BCA assay (Thermo Fisher ScientificTM). Dilutions of 10% BH containing from 5 to 30 ug of protein were incubated with increasing concentrations of guanidinium hydrochloride (GdnHCl) in 96-well plates for 1 hour at room temperature with constant agitation. Treated samples were transferred to a nitrocellulose membrane (Whatman GmbH, Dassel, Germany) through a dot blot apparatus. After two PBS washes, membranes were air dried for a minimum of 1 hour before rehydration with PBS followed by digestion with 5 ug/mL PK in cell lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM

NaCl, 0.5% sodium deoxycholate, and 0.5% Igepal CA-630), and PK digestion was halted with PMSF at 2 mM in PBS for 20 minutes. To expose PRC epitope, membranes were incubated with 3M guanidinium isothiocyanate for 10 minutes at room temperature and blocked for 1 hour with 5% nonfat milk in TBST. Membranes were incubated overnight at 4°C with mAb PRC5 antibody 1:5000 followed by IRDye® 800CW goat anti-mouse IgG (H+L) (LiCOR Biosciences, Lincoln, NE, USA) at 1: 25000 for 1 hour. After washing with TBST and TBS 1X, membranes were imaged in LiCOR Odyssey DLx imaging system. Signal quantification was performed in LiCOR image studio™ acquisition software.

Protein misfolding cyclic amplification

PMCA assay was performed as previously described (68). PMCA brain homogenate substrates were prepared by manual homogenization of uninfected, perfused GtQ and GtQ109 mice brains in PMCA conversion buffer (1X phosphate-buffered saline, 150mM sodium chloride, 1% Triton X-100) with the addition of protease inhibitors (cOmplete™ Protease Inhibitor Mini, Roche). Previous to PMCA reactions, substrates are supplemented with 12mM EDTA and 0.05% digitonin. 10% brain homogenates from mice clinically diagnosed and confirmed as CWD-infected were used to initiate PMCA seeding reactions. The initial seeding reaction is considered the 10^{-2} dilution in the series and subsequently diluted in 10-fold increments up to 10^{-13} in uninfected substrates. PMCA was performed using a QSonica700 sonicator at an amplitude of 15A for a target of 120-130W per sonication pulse for 10 seconds and incubation of 15 minutes (144 sonication/incubation cycles). All PMCA conditions were held constant across all PMCA experiments.

Real-Time Quaking-Induced Conversion Assay (RT-QuIC)

Hamster recombinant PrP production. The expression plasmid containing the hamster prion protein coding sequence (90-231) was kindly provided by Dr. Byron Caughey (Rocky Mountain

Laboratories, Hamilton, MT). The truncated Syrian hamster PrP^C coding sequence was expressed and consequently purified from BL Rosetta (Novagen) *Escherichia coli*. After the generation of glycerol stock, cells were cultured in lysogeny broth (LB) media with selection antibiotic kanamycin and growth agent chloramphenicol. Inclusion bodies were harvested after bacterial cell lysis with Bugbuster™ (Sigma-Aldrich) supplemented with Lysonase™ (EMD Biosciences). Recombinant rHaPrP90-231 was purified by incorporating inclusion bodies in NiNTA superflow resin (Qiagen) and loading to a XK chromatograph column (Cytiva) on AKTA Fast Protein Liquid Chromatograph (GE). Proteins were denatured and refolded on the column. Following protein elution from the column, the fractions from the elution peak were combined and dialyzed overnight at 4 °C. Purified recombinant HaPrP concentration was measured and adjusted to 0.5mg/mL, aliquoted in Eppendorf tubes and frozen at -80°C.

RT-QuIC assays. RT-QuIC was performed as follows. Ten percent brain homogenates or 10% spleen homogenates from GtQ and GtE diseased mice, as well as North American CWD (GtQ and GtE mice passaged – E-US1) as positive controls and uninfected GtQ and GtE brains as negative controls were diluted to 10⁻³ in PBS and then to 10⁻⁴ working dilutions in 0.1% sodium dodecyl sulfate (SDS) in PBS 1X. Spleens samples and controls were seeded in RT-QuIC reactions at 10⁻³ in SDS to assess positive amyloid formation. Brains dilutions were used to generate serial 10_{log} dilutions. RT-QuIC reaction mixture contains 1X phosphate buffer saline, 1mM EDTA, 170mM NaCl, 0.01mM Thioflavin T and recombinant hamster PrP (rHaPrP90-231) 0.1mg/mL. 2 µL of brain homogenate dilutions are used to seed 98 µL of reaction mixture loaded in 96-well optical black bottom plates, covered with plate sealing tape and loaded in FLUOstar® Omega plate reader (BMG Labtech). Reaction plate was run for 48 hours at 42C with readings recorded every 15 minutes.

Determination of threshold and lag times for RT-QuIC positives and analysis.

Threshold of positive reaction for RT-QuIC was determined by the sum of the mean plus five times the standard deviation for the first 4 readings (after excluding the first read) to avoid variability due to temperature differences and spontaneous aggregation of the recombinant PrP.

Lag times were calculated as the time in hours for seeding reactions to cross the estimated fluorescence threshold. All RT-QuIC-generated data were plotted as quadruplicated technical replicates from at least 3 biological replicates when possible.

Longitudinal analysis of moose-6 19-CD24854 infection in GtQ109 mice

The same number of male and female mice between four and six weeks were intracerebrally inoculated with 30 μ L of 1% 19-CD24854 isolate homogenate into the right parietal lobe using a 26-gauge needle. Assessment of clinical signs was performed as described above. Groups of 5 mice were collected every 10 days after inoculation day until terminal disease, approximately 100 days after inoculation (dpi). Per group, three mouse brains were collected for biochemical analysis, and two were collected whole and fixed for IHC analysis. IHC slides were obtained and stained as described above, as well as western blots of brain homogenate samples. Western blots were imaged in LiCOR Odyssey DLx imaging system. Semi-quantification analysis of PrP^{Sc} was performed in Fiji open source software (64)

Statistical analyses.

Statistical analyses were conducted using GradPad Prism software (San Diego, CA).

Results

Development of Gene-Targeted Mice Expressing the Cervid *PRNP* 109Q Variant

The development of gene-targeted (GtQ and GtE) mice encoding cervid *PRNP* glutamine (Q) or glutamic acid (E) at amino acid 226, respectively, has been described previously (69). The 109Q point mutation was generated from GtQ mice expressing the wild-type lysine (K) allele at amino acid 109 using a CRISPR-mediated knock-in strategy to obtain GtQ109 founder mice (Figure 4.1-D). Verified N1 mice were bred, and homozygosity for the 109Q allele was confirmed by PCR genotyping. GtQ, GtE, and GtQ109 lines were maintained on identical FVB inbred backgrounds. Quantification of PrP found comparable relative expression of GtQ109 mice to the GtQ line previously determined to express physiological levels of cervid PrP (70) (Figure 4.1-A, 4.1-B). GtQ109 mice were aged up to 664 days and euthanized due to non-specific clinical signs associated with aging. Brains from aged GtQ109 mice were collected and bisected, with one half used for biochemical analysis and the other for immunohistochemical (IHC) analysis. Ten percent brain homogenates were used to seed real-time quaking-induced conversion (RT-QuIC) assays, which revealed no evidence of amyloid formation activity (Figure 4.1-C). IHC and lesion profile analyses showed no evidence of PrP^{Sc} accumulation or neuropathology in aged 109Q mice

GtQ mice expressing wild-type K109 are resistant to infection from 109Q moose-derived isolates, while GtQ109 mice develop rapid disease

We established 10% homogenates from brain tissues collected from moose CWD cases, which will be denominated from now on as 18-CD24724 (M-NO4), moose 19-CD24854 (M-NO6), and moose 22-CD29 (M-NO10). These cases of chronic wasting disease were

identified in Nordic moose (*Alces alces alces*) in Norway and were found to carry homozygosity for the glutamine (Q) allele at residue 109 in the *PRNP* sequence (2–4). We performed intracerebral inoculations of the three 109Q moose-derived field isolates into GtQ and GtE mice

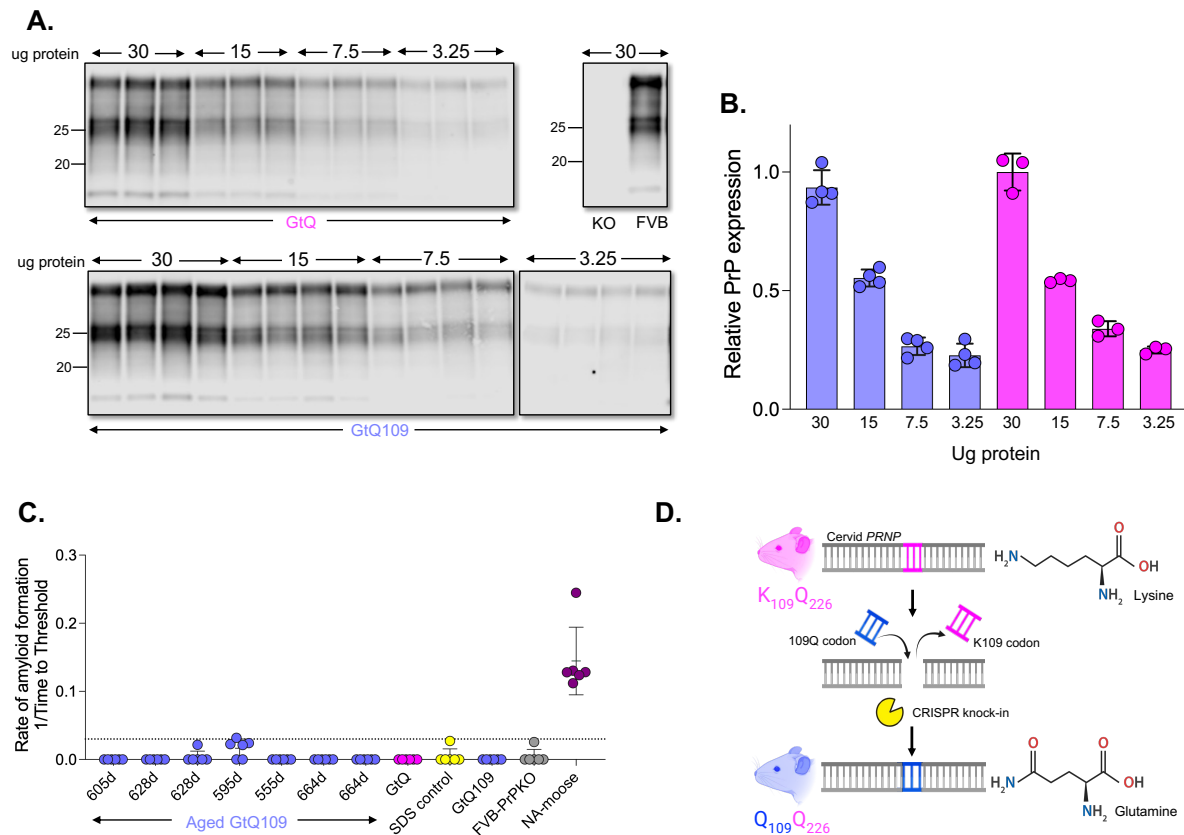


Figure 4.1. Generation and characterization of the GtQ109 mice line. **A.** Immunoblot analysis of relative PrP expression in brain tissue from CNS tissue from the GtQ109 mice line compared to the characterized GtQ (wild-type K109) mice, PrP knockout FVB mice (KO) and wild-type FVB/nj mice (FVB). **B.** Quantification of relative signal for PrP expression for GtQ109 and GtQ mice from immunoblots (A). **C.** RT-QuIC seeding analysis of aged GtQ109 mice at 555 days to 664 days compared to uninfected GtQ and GtQ109 mice < 300 days old, PrP knockout FVB and North American CWD control. Immunoblot was probed with mAb PRC5 **D.** Schematic shows the generation of GtQ109 mice by CRISPR knock-in point mutation insertion from wild-type GtQ mice to replace lysine (K) for glutamine (Q) at amino acid 109 of cervid *PRNP*.

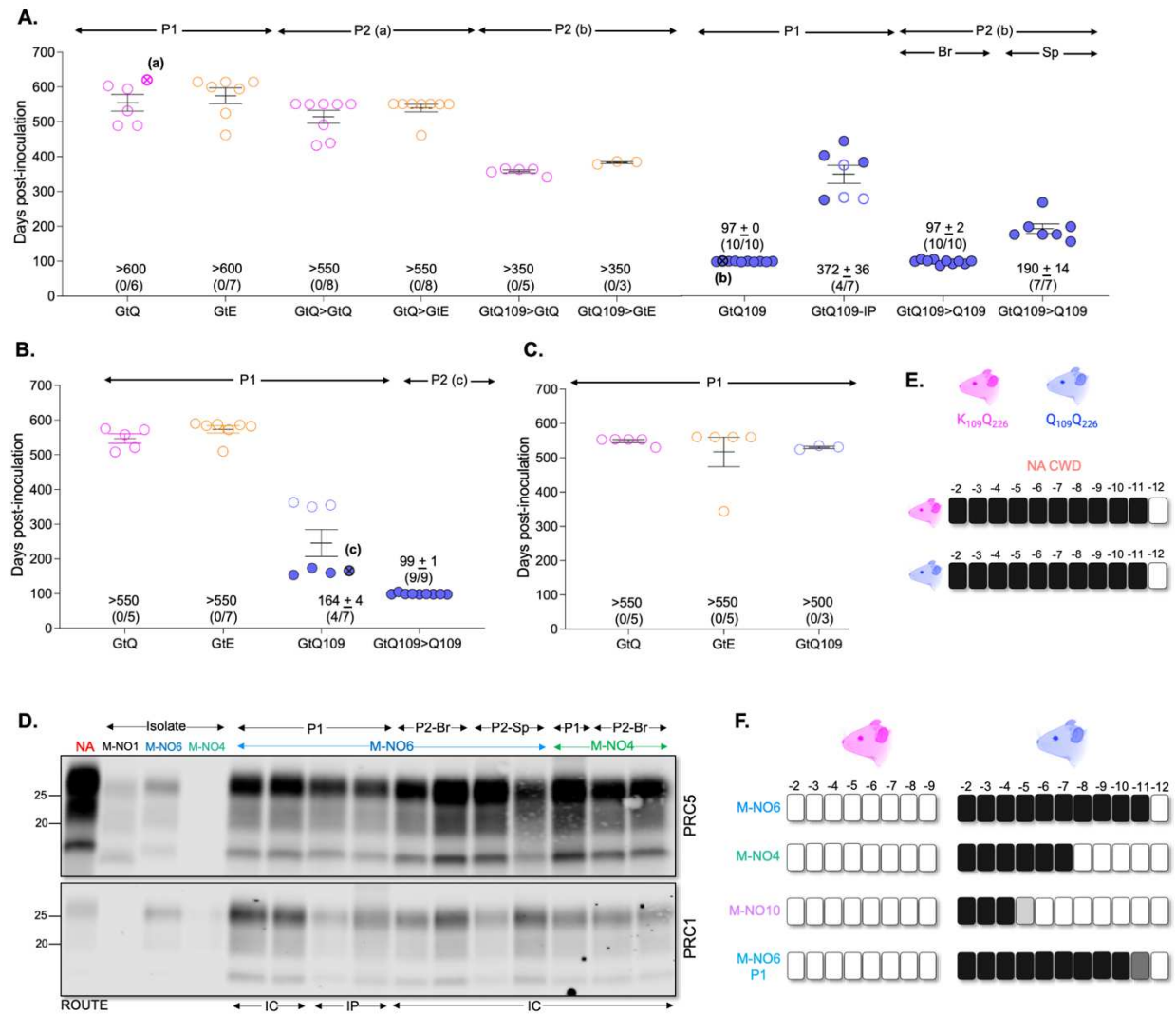


Figure 4.2. Primary and secondary transmissions of 109Q Norway moose CWD isolates in GtQ, GtE and GtQ109 mice. Scattergrams showing the transmission profiles of 109Q Norway moose CWD isolates after challenges in GtQ, GtE and GtQ109 mice. Gt mice positive for clinical signs and PrP^{Sc} in the brain are represented (GtQ: full magenta circles, GtE: full orange circles, GtQ109: full orchid circles). Sub-clinical cases: absence of clinical signs but presence of PrP^{Sc} in the brain are noted (GtQ: magenta empty squares, GtE: orange empty squares, GtQ109: empty orchid squares), mice without clinical signs and absence of PrP^{Sc} in the brain (GtQ: empty magenta circles, GtE: empty orange circles, GtQ109: empty orchid circles). Animals whose brain was selected for serial transmissions are labeled (a) and (b) (crossed circles). **A.** Primary and secondary transmissions of 109Q moose isolate 19-CD24854 (M-NO6) in GtQ, GtE and GtQ109 mice. **B.** Primary and secondary passages of isolate 18-CD24724 (M-NO4) into GtQ, GtE, GtQ109 mice. **C.** Primary and secondary transmission of isolate 22-CD29 (M-NO10) in GtQ, GtE, GtQ109 mice. **D.** Immunoblot analysis of PrP^{Sc} from CNS tissue from GtQ109 inoculated with isolate 19-CD24854 and 18-CD24724 at primary and secondary passages. Immunoblots were probed with mAb PRC5 and PRC1. The position of

molecular weight markers approximating 25, 20 kDa is shown. **E.** PMCA controls: North American CWD isolate 99w12389 (E-US1) seeded in GtQ and GtQ109 mice substrate. **F.** PMCA amplification profiles of the three 109Q moose isolates in GtQ and GtQ109 mice substrate, and from the primary transmission of 19-CD24854 isolate into GtQ109 mice, in GtQ and GtQ109 mice brain substrate. P2(a): secondary passage of brain labeled (a); P2(b): secondary passage of brain labeled (b); P2(c): secondary passage of brain labeled (c). Incubation period in days ($\pm$ SEM) and attack rate are shown and were calculated based on mice confirmed CWD positive by related clinical signs and presence of PrP^{Sc} in the brain by western blot. P1: primary passage, P2: secondary passage IP: intraperitoneal, Br: brain inoculum, Sp: spleen inoculum, NA: North American CWD control. GtE and GtQ: CWD-susceptible gene-targeted mice in which the prion protein coding sequence was replaced with one encoding glutamate (E) at codon 226 or with glutamine (Q). GtQ109: CWD-susceptible gene-targeted mice expressing codon 226 with glutamine (Q) in which the codon 109 expressing lysine (K) has been replaced with glutamine (Q).

both expressing the wild-type homozygous lysine (K) allele at position 109 and glutamine (Q) or glutamic acid (E) at position 226 and assessed them weekly until the onset of clinical signs or until termination after 550 days in the absence of signs of prion disease.

We found that wild-type K109 GtQ and GtE mice were resistant to prion infection by all three 109Q moose CWD isolates (Fig. 1A–C; Table S2), which was confirmed by real-time quaking-induced conversion assay (RT-QuIC) (Figure 4.3-A). These results showed that the presence of the PrP 109Q variant in the CWD isolates posed a significant barrier to sympatric transmission. Therefore, we proceeded to perform intracerebral inoculations of the three 109Q moose CWD isolates into our developed GtQ109 mouse line and evaluated their progress weekly.

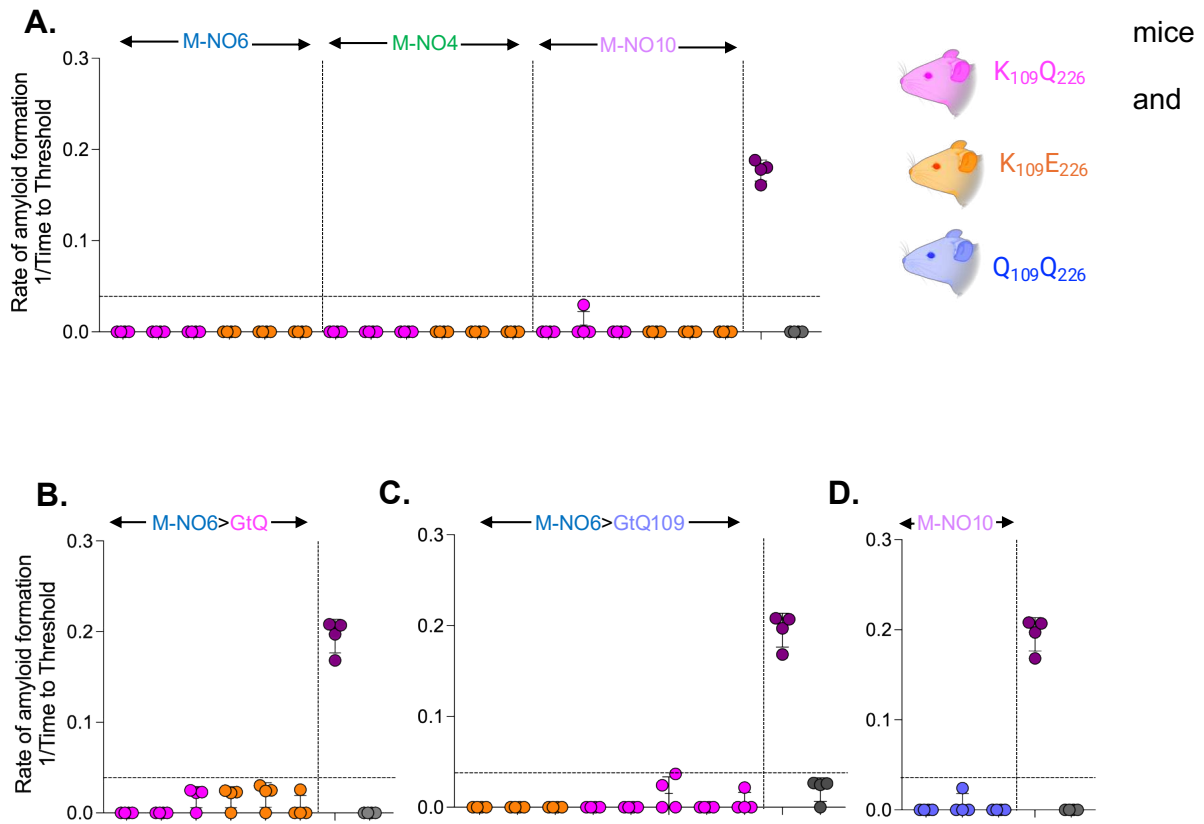
Remarkably, at primary passage, GtQ109 mice inoculated with the M-NO6 isolate manifested the onset of clinical signs at approximately 90–100 days with a 100% attack rate, while M-NO4 inoculations produced disease at approximately 160 days with an attack rate of approximately 60% (Figure 4.2-A, 4.1-B). GtQ109 mice inoculated with M-NO10 survived beyond 500 days post-inoculation without clinical signs and were terminated (Figure 4.2-C). We

rationalized that, due to the high attack rate and short incubation period observed for the M-NO6 isolate, 1) differences in attack rate and incubation period in the transmission of M-NO4 in GtQ109 mice compared to M-NO6 could be due to a titer effect, and 2) secondary passage of brains from M-NO6 transmission in GtQ109 mice back into wild-type GtQ and GtE mice could result in subclinical or clinical disease.

Consequently, we performed secondary transmissions from clinically affected M-NO6- and M-NO4-infected GtQ109 mice into GtQ109 mice and from M-NO6-infected GtQ109 mice back into GtQ and GtE mice. We additionally performed secondary transmissions from GtQ mice originally inoculated with the M-NO6 isolate that were terminated at the end of the study without clinical signs of prion disease, to rule out any possibility of residual prion infection present below the limit of detection of our assays. As anticipated, at secondary passage, M-NO6 and M-NO4 were indistinguishable in their kinetics (approximately 90–100 days) with 100% attack rates for both transmissions (Figure 4.2-A, 4.2-B) suggesting these isolates may be composed of the same CWD strain.

Secondary transmission of M-NO6-positive GtQ109 material back into wild-type GtQ and GtE mice resulted in no disease at >300 days post-inoculation and no evidence of prion infection by RT-QuIC analysis of brain homogenates (Figure 4.3-B). Secondary passage of brains from GtQ mice originally inoculated with M-NO6 resulted in no transmission, thereby confirming that no residual infection was present in the brains of GtQ mice at primary passage (Figure 4.2-A) (Figure 4.3-C). Analysis of brain tissues from GtQ109 mice inoculated with the M-NO10 isolate found no evidence of CWD infection by RT-QuIC assay (Figure 4.3-D).

The transmission properties displayed by the three 109Q moose isolates were mirrored by their replicative profiles observed by protein misfolding cyclic amplification (PMCA). We established 10% brain PMCA substrates, as described previously (5), from GtQ and GtQ109



performed 10-fold serial dilutions of each 109Q field isolate. We found that none of the 109Q isolates was able to amplify in GtQ substrate, whereas GtQ109 substrate was permissive to amplification by all three isolates, with M-NO6 having the highest endpoint amplification

Figure 4.3. RT-QulC seeding analysis of brain homogenates from asymptomatic GtQ, GtE and GtQ109 mice after inoculations with M-NO6, M-NO4 and M-NO10 isolates. A. Brain homogenates from primary transmissions of M-NO6, M-NO4 and M-NO10 isolates in GtQ and GtE mice. **B.** Brain homogenates from secondary transmissions of GtQ mice inoculated with M-NO6 inoculum into GtQ and GtE mice. **C.** Brain homogenates from transmissions of M-NO6 into GtQ109 mice back into GtQ and GtE mice. **D.** Brain homogenates from primary transmissions of the M-NO1 isolate into GtQ109 mice. Plum circles: North American CWD, grey circles: uninfected GtQ109 brain homogenate.

(10^{-11}), M-NO10 showing the lowest (10^{-4}), and M-NO4 exhibiting an intermediate endpoint (10^{-7}) (Figure 4.2-F). In contrast, North American CWD sustained a high endpoint amplification in both GtQ and GtQ109 substrates. (Figure 4.2-E).

In line with the inability of M-NO6-infected GtQ109 mice to cause disease when transmitted back into wild-type GtQ mice, GtQ substrate was unable to sustain amplification of these prion seeds, whereas efficient amplification was observed in the GtQ109 substrate. These results also demonstrate that negative transmissions of the M-NO10 isolate in GtQ109 mice could be due to a titer effect.

A notable finding of primary and secondary transmissions of M-NO6 and M-NO4 in GtQ109 mice was the presence of prion infection in spleens, with both M-NO4 and M-NO6-infected mice showing incomplete spleen involvement at primary passage, consistent with a stochastic mechanism (Figure 4.4-B, 4.4-D) while all M-NO6 and M-NO4-infected mice were positive for prions in the spleen at secondary passage (Figure 4.4-B, 4.4-D). Lymphotropism is a prevalent feature of CWD that accounts for its highly contagious nature; however, it has not been observed naturally in tissues of Nordic moose afflicted with CWD, likewise, we did not observe any amyloid forming activity in lymphoid tissue homogenate analyzed from M-NO6 and M-NO10 (Figure 4.4-A). A recent study identified prion positivity in M-NO6 lymphoid tissues by serial PMCA (6). Therefore, we investigated the potential of M-NO6 for intraperitoneal (IP) transmission. Intraperitoneal inoculations of M-NO6 into GtQ109 mice resulted in disease as early as approximately 280 days in one mouse, while the remaining mice exhibited signs of disease nearly 100 days later (Figure 4.2-A). Despite an incomplete attack rate, all mice intraperitoneally inoculated with the M-NO6 isolate were found positive for PrP^{Sc} in the spleen (Figure 4.4-B). These results indicate the pre-existence of a lymphotropic strain in the brain tissue derived from the natural host.

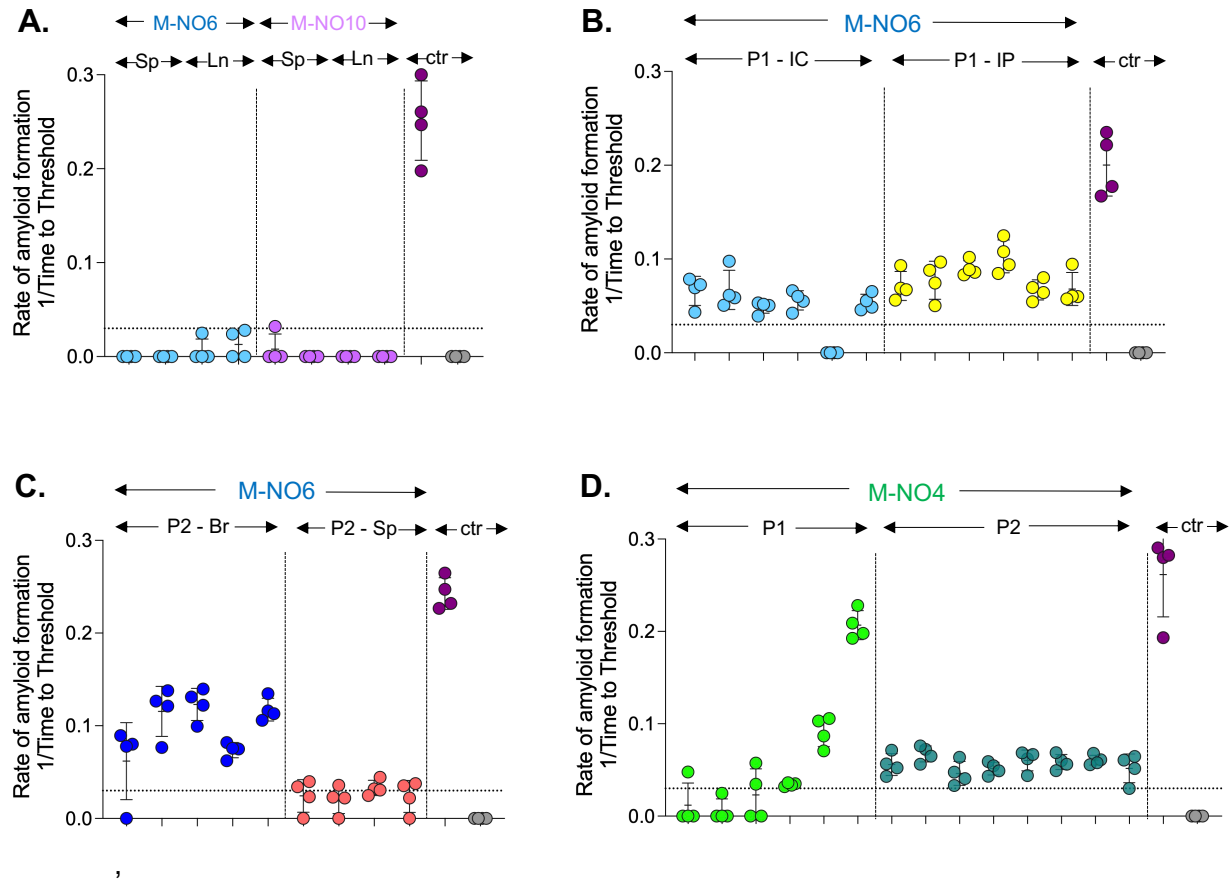


Figure 4.4. RT-QuIC seeding analysis of spleen and lymph node homogenates from M-NO6 and M-NO10 field isolates and spleen homogenates from primary and secondary passages of M-NO6, M-NO4 isolates into GtQ109. **A.** Analysis of spleen and lymph node-derived homogenates from M-NO6 and M-NO10 field isolate tissues. **B.** Analysis of spleen homogenates from primary transmissions of M-NO6 inoculum into GtQ109 mice. **C.** Analysis of spleen homogenates from secondary transmissions of M-NO6 brain and spleen inoculum into GtQ109 mice. **D.** Analysis of spleen homogenates from primary and secondary transmissions of M-NO4 inoculum into GtQ109 mice. Plum circles: North American CWD spleen homogenate, grey circles: uninfected GtQ109 spleen homogenate. P1: primary transmission, P2: secondary transmission, IC: intracerebral, IP: intraperitoneal, Sp: spleen, Ln: lymph node, Br: brain, ctr: controls.

Since prion infectivity was identified in the spleens of M-NO6-infected mice at primary passage, we assessed the transmission properties of spleen-derived inoculum and compared its transmission kinetics to those of GtQ109-passaged M-NO6 brain inoculum. Intracerebral inoculations of spleen inoculum derived from intracerebrally transmitted M-NO6 at primary

passage resulted in an incubation period of nearly 200 days with a 100% attack rate, approximately double the time required for intracerebral transmission of brain inoculum from the same mouse (Figure 4.2-A). Surprisingly, inoculation of spleen inoculum did not result in prion accumulation in the spleen, as was observed for the transmission of brain inoculum (Figure 4.4-C)

The transmission profiles of different isolates from CWD-positive Nordic moose expressing the 109Q variant demonstrate: 1) the pronounced effect of this polymorphism on CWD transmission, with wild-type K109 mice showing complete resistance to infection by these isolates and markedly efficient transmission observed in the presence of the homologous 109Q genotype; 2) that 109Q moose-derived isolates contain CWD prion strains with tropism for the lymphoid system; 3) that PMCA amplification faithfully recapitulates the transmission properties of these isolates; and 4) that the indistinguishable disease kinetics exhibited by M-NO6 and M-NO4 after iterative passages strongly suggest these isolates contain the same CWD strain.

The electrophoretic, conformational, and histopathological properties of 109Q Nordic moose CWD isolates M-NOR6 and M-NOR4 are indistinguishable and remain stable during iterative passages in GtQ109 mice.

In contrast to our previous studies (Chapter 2) of Nordic K109 moose CWD, which demonstrated remarkable strain diversity among individual moose CWD cases in Sweden (5,7), secondary transmissions of M-NO6 and M-NO4 isolates in 109Q mice exhibited indistinguishable transmission kinetics. This finding suggests that expression of the 109Q polymorphism in Nordic moose may pose a constraint to strain diversity, allowing replication of a dominant strain common across 109Q moose CWD cases. Accordingly, we characterized the

biochemical and histopathological properties of CWD prions derived from passages of M-NO6 and M-NO4 isolates in GtQ109 mice.

Proteinase K (PK)-resistant CWD PrP^{Sc} was assessed in 109Q moose CWD field isolates and in the brains of GtQ109 mice diagnosed with prion disease by Western blotting. We observed that the natural M-NO6 isolate contained higher levels of PrP^{Sc} than M-NO4, correlating with differences in primary-passage transmission kinetics. A marked increase in PK-resistant PrP^{Sc} was observed after iterative passages for both isolates (Figure 4.2-D). Analysis of 109Q moose isolates and GtQ109-derived prions using the PRC5 monoclonal antibody demonstrated electrophoretic properties resembling North American CWD (3), with slower electrophoretic mobility than Nordic K109 moose (M-NO1) PrP^{Sc} and reactivity with the PRC1 monoclonal antibody (equivalent to 12B2), which targets an N-terminal epitope (Figure 4.2-D). These findings suggest that 109Q-derived prions are distinct from K109 moose prions in their PK cleavage site and exhibit high replication efficiency, as evidenced by increased PrP^{Sc} levels despite short incubation periods in GtQ109 mice.

Resistance of PrP^{Sc} to chaotropic denaturation by guanidinium hydrochloride (GdnHCl) is one of the most precise biochemical methods for assessing strain conformational stability. The indistinguishable transmission properties of M-NO6 and M-NO4 at secondary passage suggested that these two 109Q moose CWD cases harbor the same strain. The half-maximum denaturation value (GdnHCl_{1/2}) for the M-NO6 field isolate was approximately 2.8 M. This value remained unchanged following primary and secondary passages in GtQ109 mice, indicating replication of an unaltered M-NO6 strain during iterative passage (Figure 4.5-A, 4.5-B). No differences were observed between secondary intracerebral transmissions of M-NOR6-infected GtQ109 mice brain versus spleen inoculum (Figure 4.5-D). Significance was observed when M-NO6 prions were transmitted intracerebrally versus intraperitoneally (Figure 4.5-C).

Secondary passages of M-NO6 and M-NO4 displayed similar GdnHCl_{1/2} values (~2.6–2.8 M), supporting the conclusion that they are composed of the same strain (Figure 4.5-F).

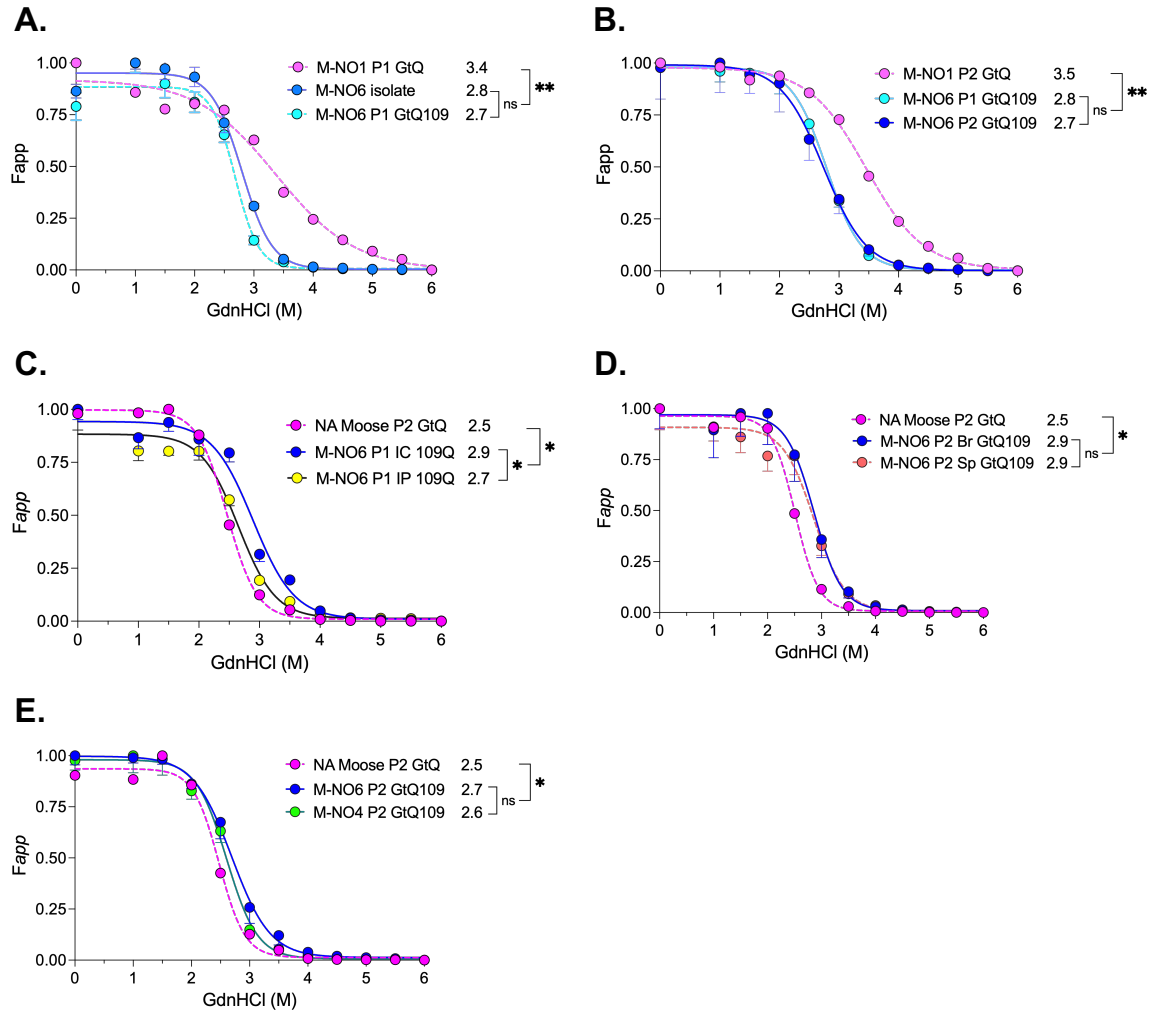


Figure 4.5. Conformational properties of 109Q Norway moose CWD prions during iterative passages in GtQ109 mice. Response of 109Q moose CWD prions to denaturation with increasing concentrations of guanidinium hydrochloride (GdnHCl). The average fractional apparent signal (F_{app}) of PK resistant PrP^{Sc} was obtained by densitometry of probed dot blots plotted as a function of GdnHCl concentration (M) **A.** Comparison of denaturation response of M-NO6 native CWD prions and passage into GtQ109 mice. **B.** Responses to GdnHCl denaturation of M-NO6 primary versus secondary passages in GtQ109 mice. **C.** Denaturation profiles of intracerebral versus intraperitoneal primary passage of M-NO6 in GtQ109 mice. **D.** Denaturation profiles of brain versus spleen inoculum at M-NO6 secondary passage **E.** Responses to GdnHCl denaturation of M-NO6 versus M-NO4 at secondary passages in GtQ109 mice. All brain samples have been compared to Norway moose 1 (M-NO1) or North American

moose CWD isolate CA-1 passaged in GtQ mice. GdnHCl_{1/2} values (Mol/L) for each CWD dose-response curve values are shown. Sigmoidal dose-response curves were plotted using a four-parameter algorithm and nonlinear least-square fit. Error bars indicate mean $\pm$ SD of data from ≥ 3 animals per study. Statistical significances were calculated by pairwise analysis of GdnHCl_{1/2} values from best fit curves. (F_{app} = (maximum signal - individual signal)/(maximum signal - minimum signal) . GdnHCl, guanidine hydrochloride. ** $p \leq 0.01$, * $p < 0.05$.

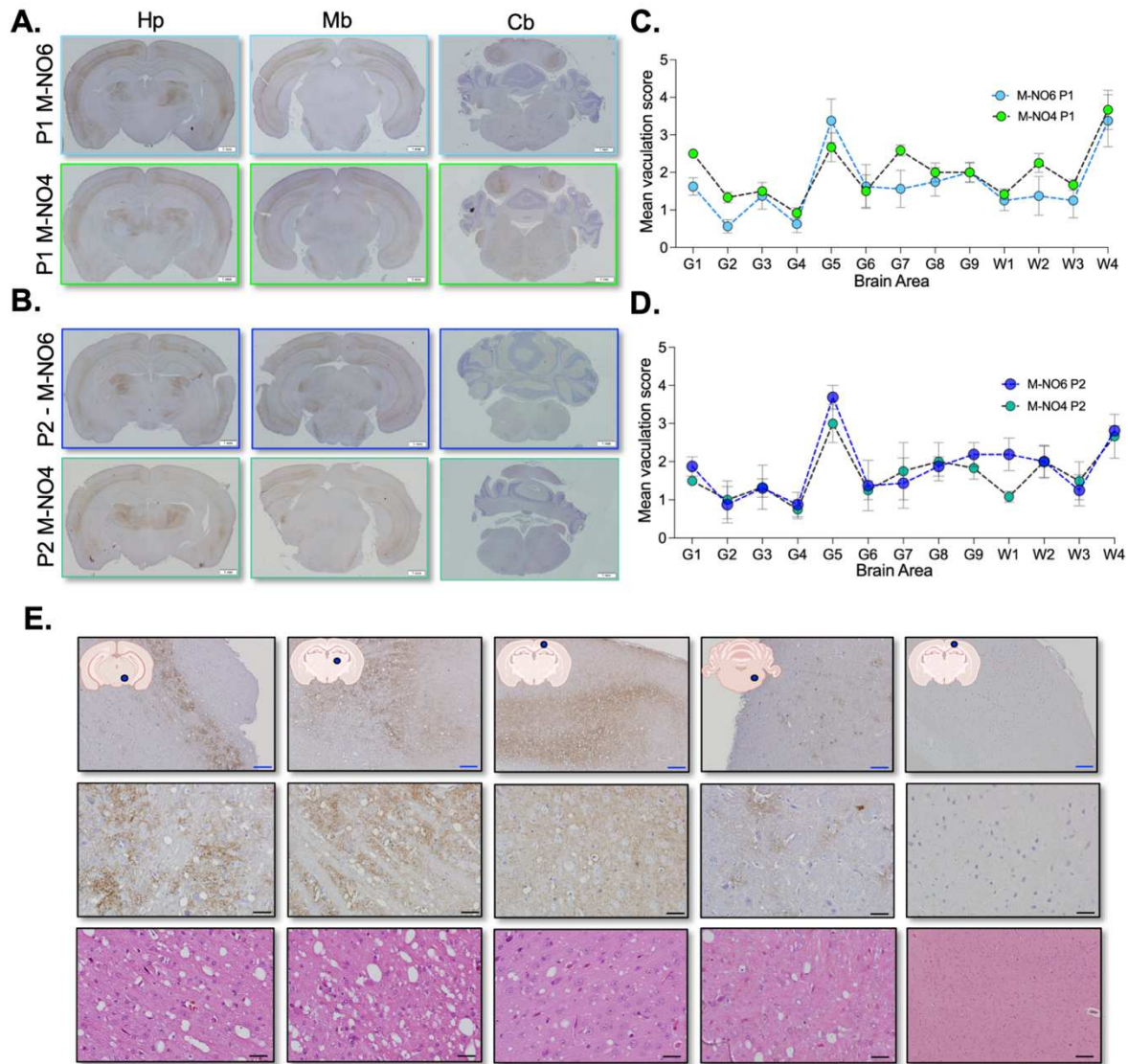


Figure 4.6. Immunohistochemical analysis of PrP^{Sc} and lesion profiling of coronal brain sections of GtQ109 mice infected with 109Q Norway moose CWD isolates. Deposition and morphology of disease-related PrP^{Sc} in the CNS of diseased Gt mice and analysis of vacuolar lesion scores. **A.** PrP^{Sc} distribution in coronal sections of GtQ109 mice infected with M-NO6 and M-NO4 isolate. **B.** PrP^{Sc} distribution in coronal sections of GtQ109 mice infected with M-NO6 and M-NO4 isolate at secondary passage. **C.** Lesion profiles of primary passages of M-NO6 and M-NO4 isolate. **D.** Lesion profiles of secondary passages of M-NO6 and M-NO4 isolate. **E.** Representative IHC and H&E stained coronal sections of 109Q moose CWD-infected GtQ109

mice and aged GtQ109 mouse (~600 days). Sections depicting from left to right: substantia nigra, thalamus, frontal cortex, cerebellar medulla, frontal cortex of control aged GtQ109 mouse. IHC sections were proven with Fab D18 antibody. Bar scale in blue = 100 μ m (10x), Bar scale in black = 20 μ m (40x). Assessment of vacuolar lesions was performed in the following regions: G1) medulla oblongata (Mobl); G2) cerebellum cortex (Cb ctx); G3) superior colliculus (Sup Col); G4) hypothalamus (Hy); G5) thalamus (Th); G6) hippocampus (Hp); G7) septum (Sep); G8) cerebral cortex at the level of the thalamus (Th ctx); G9) frontal cerebral cortex (Fr ctx); W1) cerebral peduncle (Cb pd dec); W2) corpus callosum (Cb WM); W3) caudoputamen (Cp); W4) cerebellar white matter (Crb WM). Data points represent the mean $\pm$ SD of data from ≥ 3 animals per study.

Brain sections from CWD-affected GtQ109 mice were examined by immunohistochemical (IHC) analysis to assess the morphological properties of PrP^{Sc} accumulation. PrP^{Sc} deposition in M-NO6- and M-NO4-infected GtQ109 mice was indistinguishable with respect to morphology and regional distribution in D18 anti-PrP-stained sections, even after iterative passages (Figure 4.6-A, 4.6-B). The predominant PrP^{Sc} pattern consisted of intense coarse granular deposition localized primarily to the somatosensory cortex, thalamic regions of the hippocampus, substantia nigra, and medial geniculate complex of the midbrain (Figure 4.6-E). Additional PrP^{Sc} patterns were observed at lower frequency in the dentate gyrus of the hippocampus (Figure 4.7-B) and cerebellar medulla (Figure 4.7-C) as a stellate pattern, the corpus callosum (Figure 4.7-D) plaque like accumulation is found at secondary passage, and ring-like morphologies at the substantia nigra (Figure 4.7-E).

Neuropathological assessment revealed no differences in spongiform change between M-NO6 and M-NO4 isolates or across their iterative passages (Figure 4.6-C, 4.6-D). No differences were observed between intracerebral and intraperitoneal passages of M-NO6 inoculum (Figure 4.7-F) or between secondary passages derived from brain versus spleen inoculum in M-NO6-infected GtQ109 mice (Figure 4.7-G). the main target areas of spongiform lesions across all transmissions of M-NO6 and M-NO4 isolates were observed in the substantia nigra, the thalamus and the white matter of the cerebellum (Figure 4.6-C, 4.6-D, 4.6-E).

Collectively, these findings support the conclusions that 1) M-NO6 and M-NO4 harbor the same CWD strain, and 2) the biochemical, conformational, and histopathological properties of these isolates are faithfully preserved during passage in the GtQ109 model.

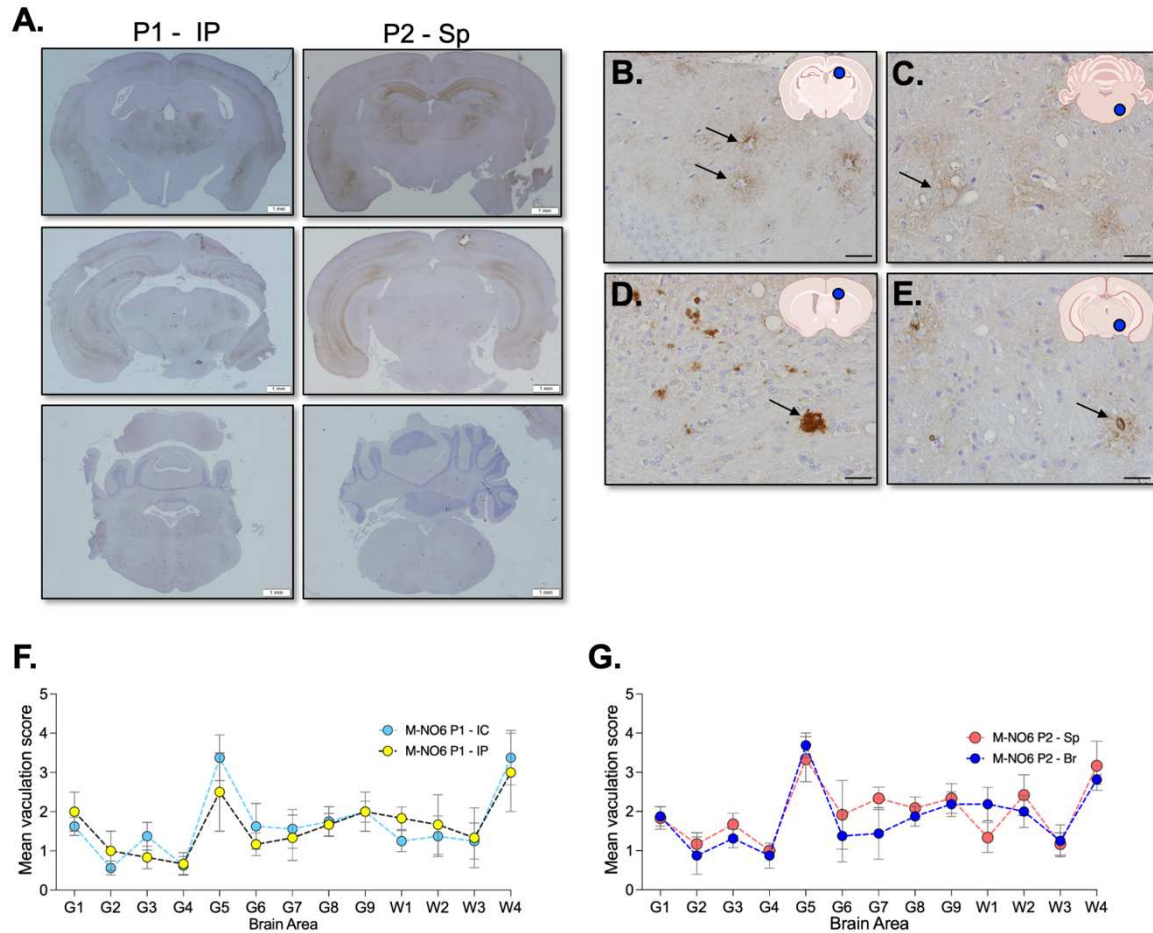


Figure 4.7. Immunohistochemical analysis of PrP^{Sc} and lesion profiling of coronal brain sections of GtQ109 mice infected with 109Q Norway moose CWD isolates by different routes of inoculation or iterative passages of different tissue inoculum. Additional morphologies of PrP^{Sc} found in coronal sections of GtQ109 mice infected with M-NO6 and M-NO4 isolates and analysis of vacuolar lesion scores in intraperitoneal and spleen inoculum transmissions of M-NO6. **A.** PrP^{Sc} distribution in coronal sections of GtQ109 mice infected with M-NO6 isolate by the intraperitoneal route or iterative transmission of spleen inoculum. **B.** Stellate PrP^{Sc} deposition in the dentate gyrus of the hippocampus **C.** Stellate PrP^{Sc} deposition in the cerebellar medulla. **D.** Plaque-like accumulation is found only in the corpus callosum after iterative passages of both M-NO6 and M-NO4. **D.** Ring-like morphology is observed in the substantia nigra after passages of M-NO6 and M-NO4 isolates. **E.** Lesion profiles found after primary passage of M-NO6 isolate by the intracerebral versus the intraperitoneal route. **F.**

Lesion profiles found after primary passage of M-NO6 isolate by the intracerebral versus the intraperitoneal route. **G.** Lesion profiles found after secondary passage of M-NO6 infected GtQ109 mice brain inoculum versus spleen inoculum.

Seeding dose of M-NO6 and M-NO4 isolates becomes indistinguishable after passage in GtQ109 mice, regardless of initial inoculum titer and route of inoculation.

Real-time quaking-induced conversion assay (RT-QuIC) has been used extensively to assess the replicative properties of prion seeds, and numerous studies have demonstrated its utility as an *in vitro* tool to distinguish prion strains. Therefore, we used RT-QuIC assays to 1) obtain additional evidence that M-NO6 and M-NO4 isolates comprise the same CWD strain and 2) interrogate the replicative properties of these isolates and monitor their adaptation during iterative passage in GtQ109 mice.

We analyzed seeding dose (SD_{50}/g) by serial dilution using RT-QuIC for all 109Q moose isolates and their iterative passages. We found that the M-NO6 isolate demonstrated an increase of less than one $\log_{10}$ unit after transmission in GtQ109 mice (Figure 4.8-A, 4.8-B, 4.8-E), regardless of inoculation route (intracerebral vs. intraperitoneal) or inoculum type (brain vs. spleen) (Figure 4.8-E). No differences in estimated brain titers were observed between primary and secondary passages (Figure 4.8-C, 4.8-E), suggesting that M-NO6 prion seeds reached maximal seeding capacity by primary passage. Strikingly, although the SD_{50}/g of the M-NO4 isolate was approximately two $\log_{10}$ units lower than that of M-NO6, prion titers in primary and secondary passages reached levels comparable to those of M-NO6 (Figure 4.8-A, 4.8-D, 4.8-E). The M-NO10 isolate exhibited SD_{50}/g values approximately one $\log_{10}$ lower than M-NO4, suggesting that the failure of M-NO10 to cause disease in GtQ109 mice is likely due to a titer effect (Figure 4.8-A, 4.8-B, 4.8-E).

Collectively, these results demonstrate that 1) M-NO6 and M-NO4 isolates have comparable RT-QuIC seeding properties, further supporting that they represent the same strain, and 2) the high seeding potency of this strain enables rapid adaptation, with prion titers peaking at primary passage for both isolates.

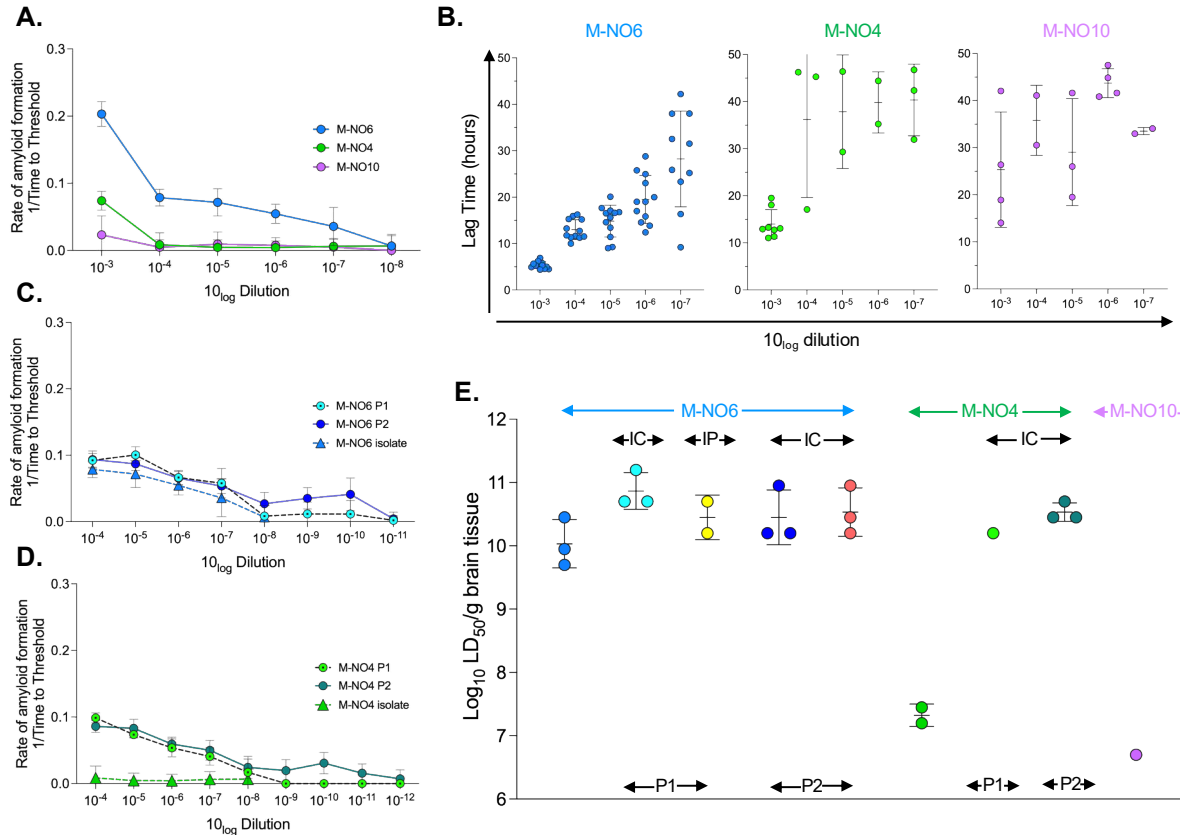


Figure 4.8. Seeding properties and end-point dilution titration analysis of 109Q Norway mouse CWD field isolates and iterative passages in GtQ109 mice by real-time quaking-inducing conversion assay (RT-QuIC). **A.** End-point dilution of 109Q moose CWD field isolates M-NO4, M-NO6 and M-NO10. **B.** Lag-time analysis of serial dilutions from field isolates M-NO4, M-NO6 and M-NO10. **C.** End-point dilution of M-NO6 isolate and iterative passages. **D.** End-point dilution of M-NO4 isolate and iterative passages. **E.** Estimation of seeding dose SD_{50} per gram of brain tissue from 109Q moose CWD field isolates M-NO4, M-NO6 and M-NO10 and iterative passages of M-NO6 and M-NO4. Lag time was determined as the time in hours for seeding reactions to cross a threshold, estimated as the average + five times the standard deviation (SD) of a baseline fluorescence. Symbols (circles) in endpoint and lag time analyses represent quadruplicate reaction wells from at least 3 biological replicates, except when less

than 3 biological samples were available. Bars show mean $\pm$ SD. SD₅₀ values were obtained by the Spearman-Kärber method. RT-QuIC assay was run for 48 hours.

The presence of the 109Q variant accelerates disease kinetics of wild-type K109 moose-derived CWD strains.

We demonstrated that the presence of the 109Q variant in natural Nordic moose CWD isolates poses a barrier to transmission into wild-type GtQ and GtE mice. Therefore, we examined the susceptibility of GtQ109 mice to CWD prions derived from K109 cervids. We selected three North American (NA) CWD inoculum: two derived from naturally infected moose brain (CA-1) and lymphoid tissue (06057822F), and one from mule deer (001-39697). In addition, we selected three Nordic K109 moose inoculum from Norway moose-1 (M-NO1, also known as P138), Sweden moose-2 (M-SW2, also known as 19-VLT000876), and a CWD isolate from M-SW2 adapted to GtQ mice (GtQAd-M-SW2), which displays rapid transmission kinetics, identical to those of GtQ109-adapted M-NO6 and M-NO4 (~90–100 days). Following intracerebral inoculation, GtQ109 mice were monitored until the onset of clinical signs, and disease kinetics were compared with those observed in wild-type GtQ mice. All groups of GtQ109 mice inoculated with K109 NA or Nordic CWD prions exhibited accelerated time to disease compared with transmission of the same isolates in GtQ mice. We observed a reduction in mean time to clinical onset of approximately 50 days (11% and 13%, respectively) for NA moose CA-1 (~350 vs. 400 days) and 06057822F (~325 vs. 375 days), while the mule deer NA isolate showed a more pronounced difference of ~150 days or ~39% (246 vs. 403 days) (Fig. 4.9A). This effect was more pronounced for Nordic moose isolates if compared to the North American moose, with a reduction of nearly 150 days (32%) to clinical onset for Nordic moose M-NO1, P138 (~300 vs. 450 days), a reduction for the isolate 19-VLT000876, M-SW2 moose of ~200 days or ~35% (367 vs. 564 days), and an approximately 30-day reduction (31%) for

GtQ-adapted M-SW2 (~65 vs. 95 days) (Fig. 4.9B). Overall, these results demonstrate that GtQ109 mice are not only susceptible to wild-type K109-derived CWD isolates, but that the presence of the 109Q variant markedly accelerates CWD disease kinetics, most notably for transmissions of Nordic moose K109 isolates and North American deer CWD.

To evaluate whether PMCA amplification profiles can provide insight into the accelerated disease progression observed in 109Q mice inoculated with North American and Nordic CWD isolates, PMCA reactions using GtQ and GtQ109 substrates were seeded with two North American and two Nordic K109 isolates. All wild-type CWD isolates generated amplification products in both GtQ and GtQ109 substrates, although with distinct amplification efficiencies. North American deer isolate exhibited higher amplification efficiency in the GtQ109 substrate, whereas no difference was observed for the 06057822F North American moose isolate. These findings are consistent with the greater reduction in time to clinical disease observed for the deer isolate compared to the 06057822F moose isolate. For the moose isolate P138, a modest increase in amplification efficiency was observed in the GtQ109 substrate relative to GtQ. Similarly, for moose 19 VLT000876, amplification was detected at higher dilutions in the GtQ109 PMCA reactions (Fig 4.9H). Overall, the relationship between PMCA amplification efficiency and reduction in time to disease remains inconclusive; however, these results suggest that the GtQ109 substrate may support more efficient amplification, irrespective of the CWD inoculum source.

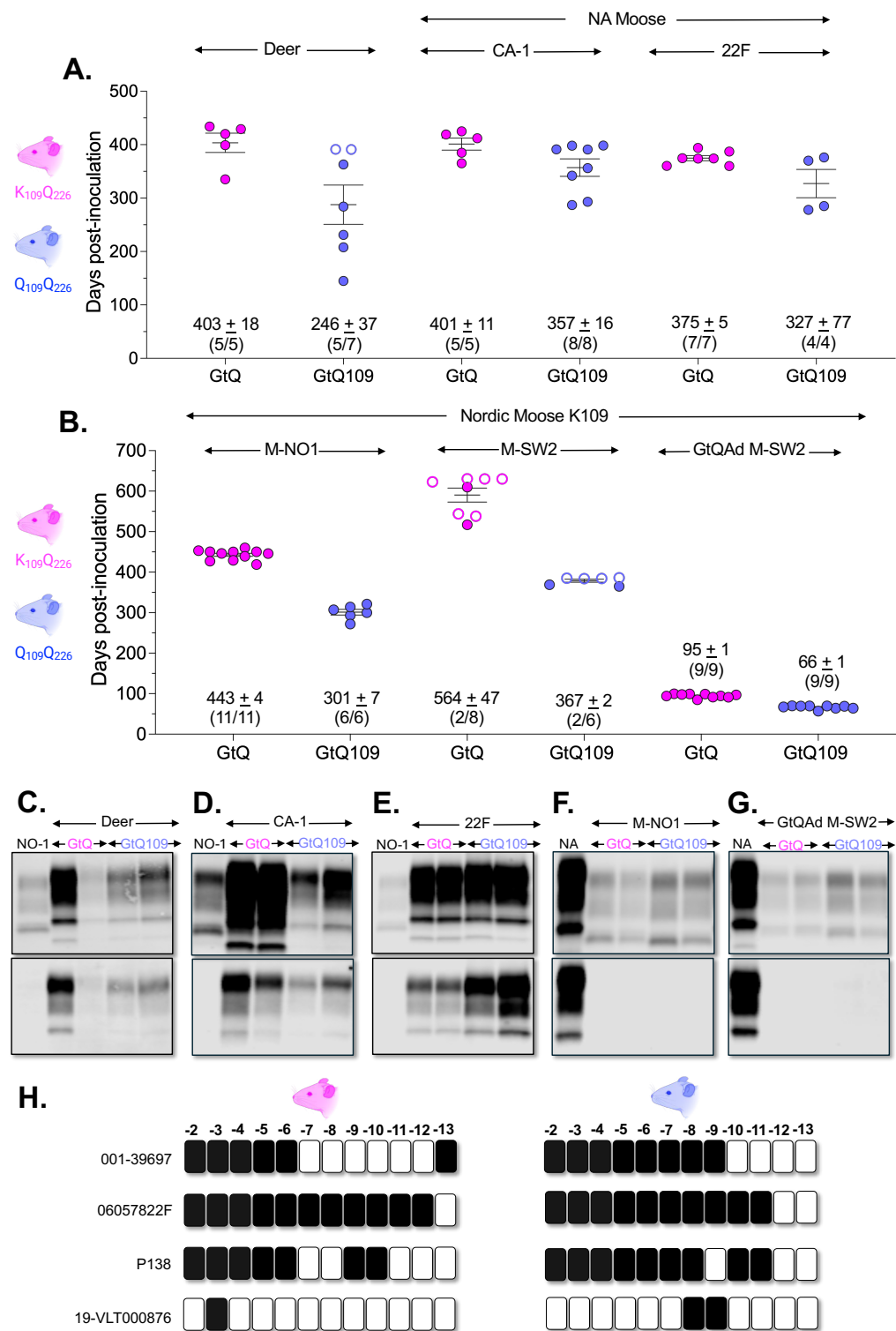


Figure 4.9. Primary and transmissions of wild-type K109/226Q North American and Nordic CWD isolates in GtQ and GtQ109 mice. Scattergrams showing the transmission profiles of North American and Nordic CWD isolates after challenges in GtQ and GtQ109 mice.

Gt mice positive for clinical signs and PrP^{Sc} in the brain are represented (GtQ: full magenta circles, GtQ109: full orchid circles). Sub-clinical cases: absence of clinical signs but presence of PrP^{Sc} in the brain are noted (GtQ: magenta empty squares, GtQ109: empty orchid squares), mice without clinical signs and absence of PrP^{Sc} in the brain (GtQ: empty magenta circles, GtQ109: empty orchid circles). **A.** Primary transmissions of North American CWD isolates: mule deer 001-39697, North American moose brain isolate CA-1, and lymph node derived isolate 06057822F in GtQ and GtQ109 mice. **B.** Primary transmissions of Nordic moose K109 isolates: Norway moose 1 (P138) M-NO1, Sweden moose 2 (19-VLT000876) M-SW2 and GtQ-Adapted M-SW2 into GtQ and GtQ109 mice. **C.** Immunoblot analysis of PrP^{Sc} from CNS tissue from GtQ and GtQ109 inoculated with North American and Nordic CWD K109 isolates. Immunoblots were probed with mAb PRC5 and PRC1. The position of molecular weight markers approximating 25, 20 kDa is shown. **F.** PMCA amplification profiles of two North American CWD isolates: moose lymph node derived isolate 06057822F and mule deer 001-39697, and the Nordic CWD moose isolates: Norway moose 1 (P138) M-NO1, Sweden moose 2 (19-VLT000876) M-SW2 in GtQ and GtQ109 brain homogenate PMCA substrate. Incubation period in days ($\pm$ SEM) and attack rate are shown and were calculated based on mice confirmed CWD positive by related clinical signs and presence of PrP^{Sc} in the brain by western blot. NA: North American CWD control. GtQ: CWD-susceptible gene-targeted mice in which the prion protein coding sequence was replaced with one encoding glutamate (E) at codon 226 or with glutamine (Q). GtQ109: CWD-susceptible gene-targeted mice expressing codon 226 with glutamine (Q) in which the codon 109 expressing lysine (K) has been replaced with glutamine (Q).

The morphology of patterns of PrP^{Sc} accumulation in the CNS and conformational properties of North American CWD and Nordic CWD prions remain invariant after passages in GtQ mice and GtQ109 mice.

We analyzed the morphologies of PrP^{Sc} accumulation in the CNS of GtQ and GtQ109 mice clinically affected with North American and Nordic K109 CWD isolates by immunohistochemistry (IHC). Overall, we found that the morphology of CWD prions in the CNS was unchanged when comparing GtQ and GtQ109 mice, regardless of the inoculum source. North American CWD maintained its classical plaque-like pattern, although the intensity of accumulation was lower in GtQ109 mice (Figure 4.10). A similar observation was made for Nordic CWD isolates. Both P138 and 19-VLT000876 Nordic moose isolates showed no changes in the morphology of PrP^{Sc} when passaged in 109Q mice, and no differences in the intensity of PrP^{Sc} accumulation were observed between the two genotypes (Figure 4.10). These

observations suggest that the 109Q variant may facilitate the accumulation of sufficient levels of PrP^{Sc} in CNS target areas within a shorter incubation period to cause clinical disease.

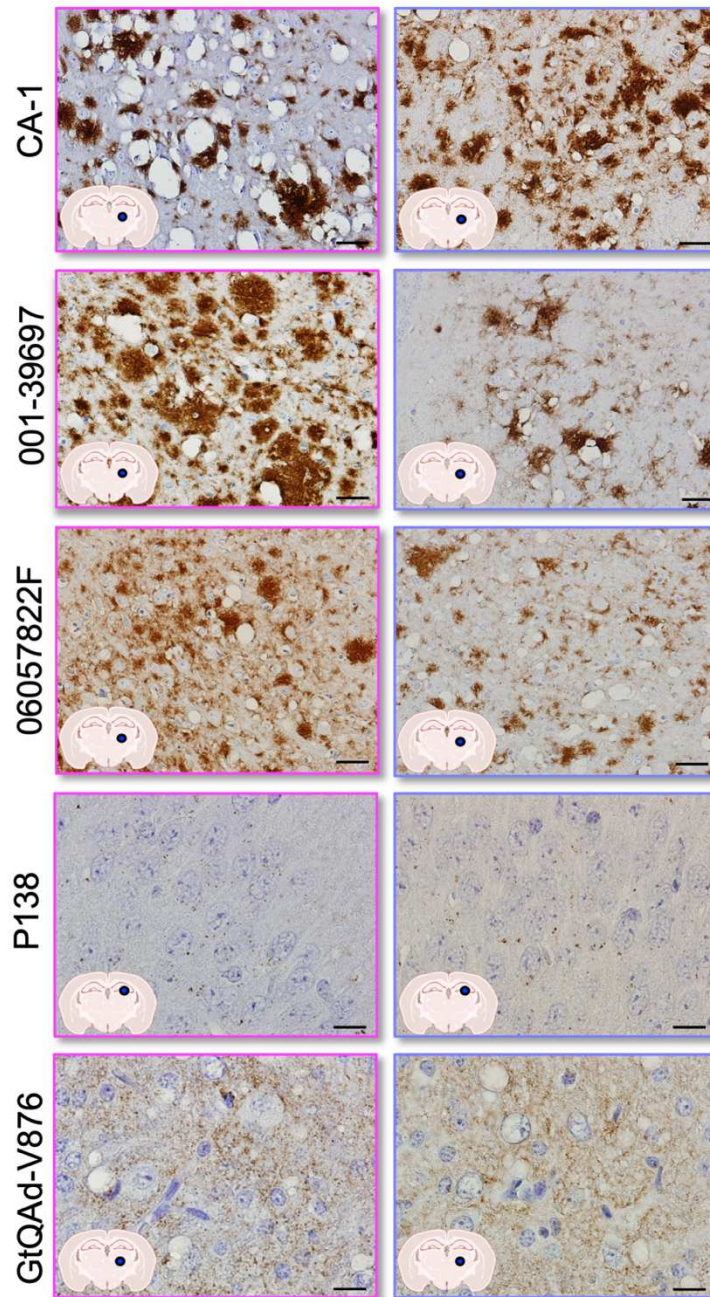


Figure 4.10. Immunohistochemical analysis of PrP^{Sc} morphologies in coronal brain sections of GtQ and GtQ109 mice infected with North American CWD isolates and Nordic moose isolates with lysine at amino acid 109 (K109). Deposition and morphology of disease-

related PrP^{Sc} in the CNS of diseased GtQ and GtQ109 mice. Coronal sections of GtQ109 mice infected with M-NO6 and M-NO4 isolates at secondary passage. IHC images from GtQ (magenta border) and GtQ109 (orchid border). Sections depicting from top to bottom: North American moose CA-1, 06057822F, and mule deer 001-39697 IHC images from thalamus. Nordic moose 1 (P138) M-NO1 IHC images from the granular layer of the hippocampus, GtQ-Adapted Sweden moose 2 (GtQ-Ad V876), IHC images from thalamus. IHC sections were stained with Fab D18 antibody. Bar scale = 10 μ m (100x) for P138 and GtQ-Ad SW2 (V876) , Bar scale = 20 μ m (40x) for North American CWD.

We analyzed the conformational properties of the North American moose CA-1 and The Nordic moose 19-VLT000876, which exhibited faster times to disease in GtQ109 mice, to assess whether conformational changes could be associated with this phenomenon. We found no differences in conformational stability, as reflected by the half-maximum denaturation values (GdnHCl_{1/2}), between GtQ and GtQ109 mice infected with either CWD strain (Figure 4.11A, 4.11-B). These results suggest that the acceleration in time to clinical disease is unlikely to be mediated by changes in PrP^{Sc} conformation during passage in Gt mice expressing the 109Q variant.

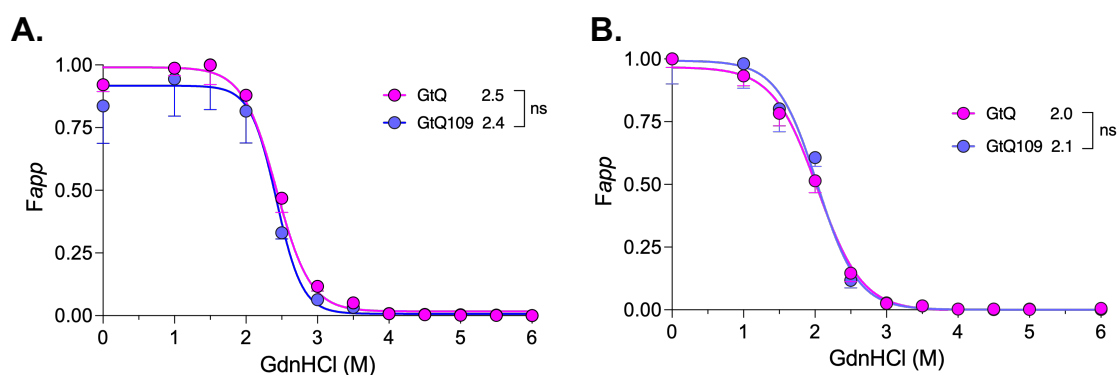


Figure 4.11. Conformational properties of North American and Nordic moose CWD prions after transmissions into GtQ and GtQ109 mice. Response of North American and Nordic CWD prions to denaturation with increasing concentrations of guanidinium hydrochloride (GdnHCl). The average fractional apparent signal (F_{app}) of PK-resistant PrP^{Sc} was obtained by densitometry of probed dot blots plotted as a function of GdnHCl concentration (M) **A**. Comparison of denaturation response of North American moose isolate CA-1 after passage into

GtQ and GtQ109 mice. **B.** Responses to GdnHCl denaturation of GtQ-Adapted Sweden moose 2 (19-VLT000876) CWD prions from GtQ and GtQ109 mice. GdnHCl_{1/2} values (Mol/L) for each CWD dose-response curve are shown. Sigmoidal dose-response curves were plotted using a four-parameter algorithm and nonlinear least-squares fit. Error bars indicate mean $\pm$ SD of data from ≥ 3 animals per study. Statistical significances were calculated by pairwise analysis of GdnHCl_{1/2} values from best fit curves. ($F_{app} = (\text{maximum signal} - \text{individual signal})/(\text{maximum signal} - \text{minimum signal})$). GdnHCl, guanidine hydrochloride; ns = non-significant

Longitudinal analysis of Norwegian 109Q moose-6 infection reveals a biphasic accumulation of PrP^{Sc} during the asymptomatic phase.

A longitudinal analysis was conducted to study the disease kinetics of 109Q moose 6 (19-CD24854) using IHC and Western blot semiquantitative assessment of PrP^{Sc} accumulation, in light of the very short time to clinical disease exhibited by this CWD strain (90–100 days). IHC staining revealed that the first clear accumulation of CWD prions in hippocampal sections is observed at 50 days post-inoculation (dpi) on the right hemisphere (the hemisphere where inoculation is performed) at the thalamus area (Figure 4.12-A), with less involvement of the amygdala nucleus in the cerebral cortex. The accumulation of PrP^{Sc} remains mostly limited to the right thalamus area until day 80 dpi. At day 90 dpi, bilateral accumulation of prions is observed in the thalamus and the first noticeable accumulation in the right somatosensory cortex that intensifies in terminally diseased mice (Figure 4.12-A).

Semi-quantitative analysis of PrP^{Sc} time-wise accumulation during the incubation phase was performed by running protein-normalized western blots of PK-resistant PrP of three mice per time point from day 30 to terminal disease at >90 days (Figure 4.12-B). Interestingly, the curve of CWD prion accumulation displays a biphasic pattern, where accumulation starts at 50 days with a plateau and decline at around 70 days, to then exponentially increase, reaching peak values at the end of the clinical phase at ~100 days (Figure 4.12-C).

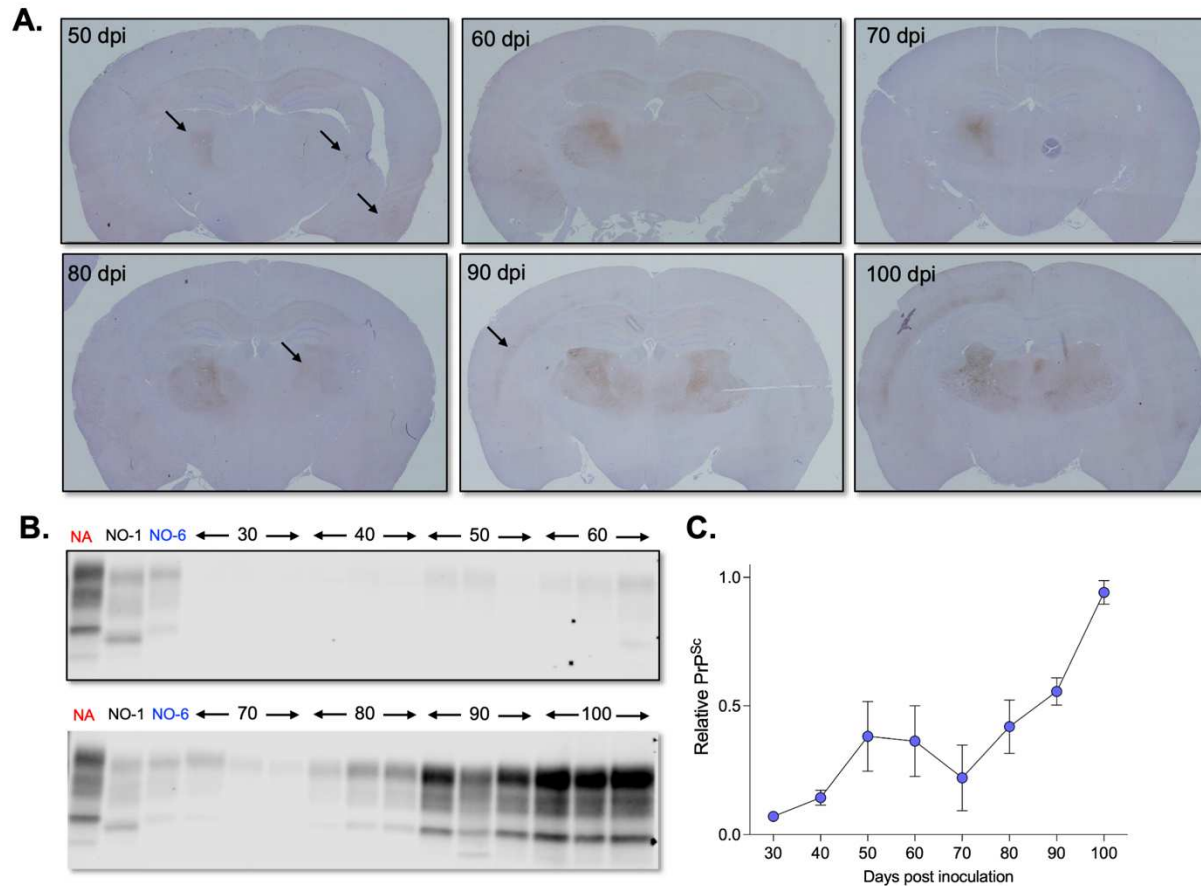


Figure 4.12. Longitudinal Analysis of Norwegian 109Q moose isolate 19-CD24854 in GtQ109 mice. **A.** Sequential analysis of IHC-stained coronal sections of GtQ109 mice infected with 19-CD24854 prions from day 50 to terminal disease at ~100 days. Arrows indicate main areas of PrP^{Sc} accumulation by time point. Day 50: Thalamus, amygdala nucleus at the cerebral cortex; Day 80: opposite hemisphere accumulation starts at the thalamus area; day 90: noticeable accumulation begins at the somatosensory cortex. **B.** Immunoblots show the progressive accumulation of PK-resistant PrP in the brains of GtQ109 mice. Each time point (from day 30 to day 100) is represented by 3 biological replicates. **C.** Semi-quantification of relative PrP^{Sc} signal from immunoblots across time points in the longitudinal study. NA: North American control, NO-1: Norway moose 1, NO-6: Norway moose 6 19-CD24854 isolate,

Additional materials:

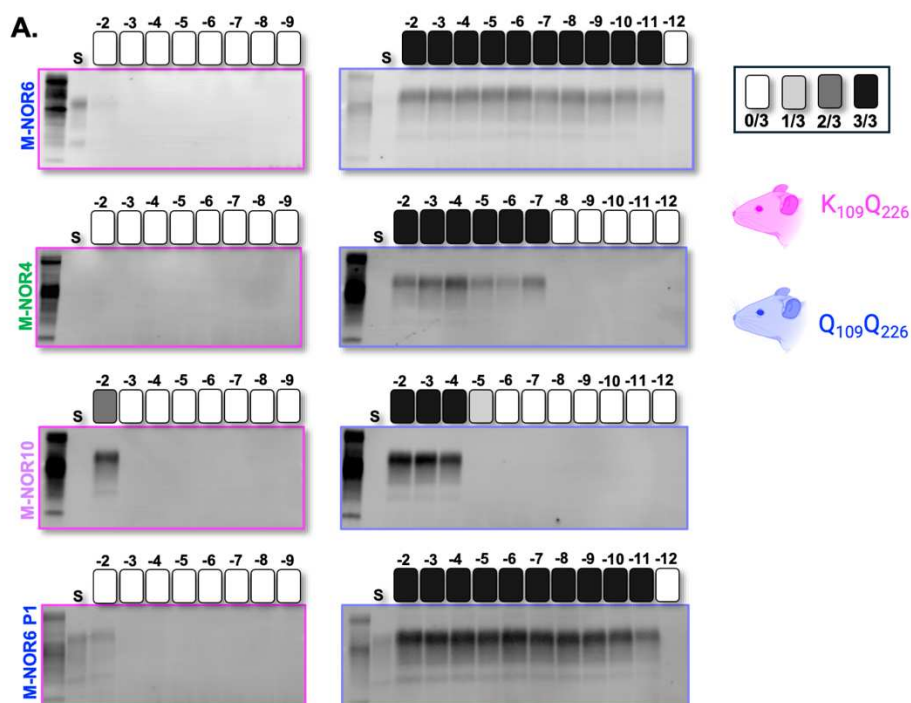


Figure 4.13. Immunoblots of PMCA reactions seeded with the three 109Q moose CWD isolates: M-NOR6, M-NOR4 and M-NOR10 and primary passage of M-NOR6 into GtQ109 mice. Blots are representative of 3 independent experiments. GtQ: wild type K109/Q226 gene-targeted mice (magenta borders), GtQ109: Q109/Q226 gene-targeted mice (orchid borders), S: non-amplified frozen controls of 10^{-2} reaction.

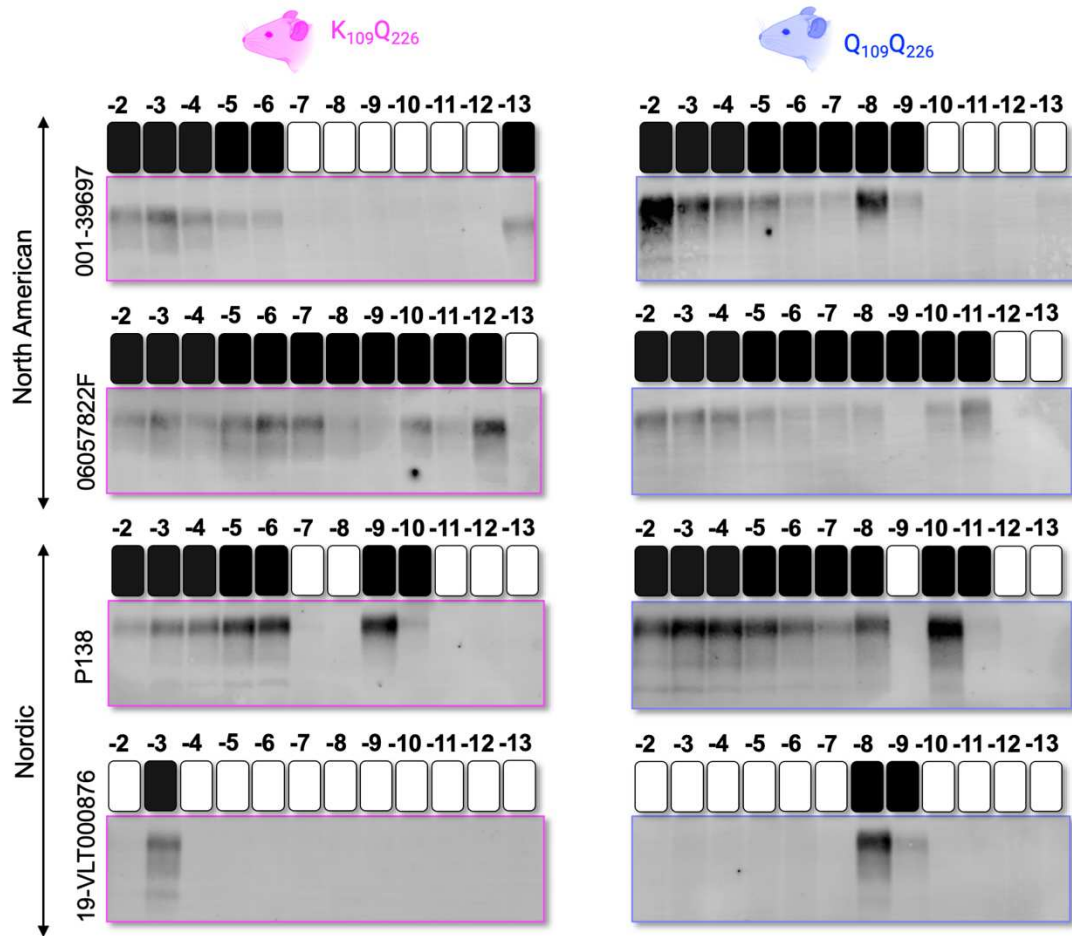


Figure 4.14. Immunoblots of PMCA reactions seeded with field isolate North American CWD and Nordic moose CWD from wild-type K109 cervids. North American CWD isolates and Nordic isolates were seeded into GtQ and GtQ109 substrates. GtQ: wild type K109/Q226 gene-targeted mice (magenta borders), GtQ109: Q109/Q226 gene-targeted mice (orchid borders).

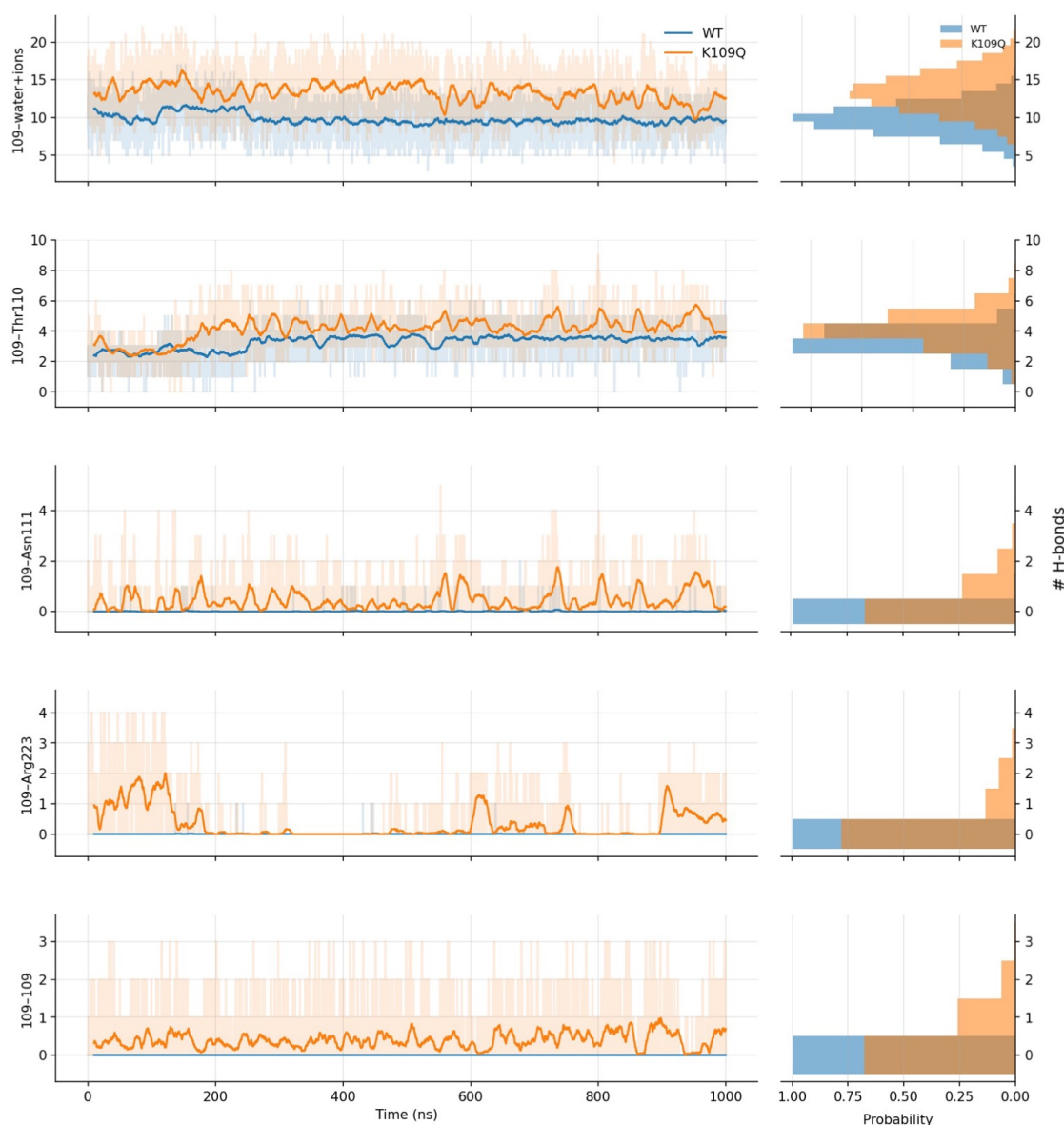


Figure 4.15. Molecular dynamics (MD) simulation of the deer CWD fibril cryo-EM structure (Alam et. al. 2024) with the 109 residue substitution lysine (K) for glutamine (Q). the 109Q fibril exhibited notably elevated hydrogen bond counts with solvent (109-solvent) and neighboring residues (Thr110, Asn111, and Arg223). While many of these interactions were transient, the trend to higher mean H-bond occupancy for the mutant was visible throughout the 1 μ s trajectory and is particularly clear in the probability histograms on the right. Whereas Lys109 is relatively lacking in favorable interaction partners (and must content with electrostatic repulsion from neighboring Lys107 and Arg223), the Gln109 can access a variety of hydrogen bonds with neighbors. Furthermore, the mutation allows a greater per-residue number of hydrogen bonds with water. Simulations were run at 1 μ s using the GROMACS MD software package.

Discussion

Naturally occurring polymorphic variants in the primary sequence of the prion protein (PrP^c) can significantly impact disease susceptibility. This phenomenon is most clearly illustrated by polymorphic variation at residue 129 of the human prion protein, which influences susceptibility to sporadic, inherited, and acquired forms of Creutzfeldt-Jakob disease (CJD) (39–41), as well as the strain properties observed in other inherited human prion diseases like GSS and FFI. The effect of single polymorphic variants can vary in penetrance levels, with some exerting profound effects on the phenotype of prion diseases, ranging from incubation period, clinical manifestations, biochemical properties, and patterns of deposition in the central nervous system (CNS) (71–73). Some single-amino-acid substitutions are known to cause spontaneous disease that can be transmitted in several models of transgenic mice overexpressing PrP (56,57,74–79). This is in striking contrast to polymorphisms known for conferring strong or complete resistance (80).

While wild-type PrP of several cervid species shares a consensus primary sequence that differs only at amino acid 226—with glutamine (Q) in deer, moose, and reindeer and glutamic acid (E) in elk and red deer—additional *PRNP* non-synonymous variants have been associated with increased or reduced susceptibility to chronic wasting disease (CWD). Homozygosity for lysine (K) at PrP residue 109 appears to be highly conserved among mammals, as the only known variant at this position, glutamine (Q), has been described exclusively in Nordic moose (*Alces alces alces*) (51,52,81). Polymorphic variants within the second polybasic region which includes residue 109 in the cervid PrP sequence, as well as their equivalents in other species within this domain, including human, mouse, and bank voles, are associated with inherited and

spontaneous disease (3,55,57,58,74). This suggests that primary sequence variations at PrP 109 could be of substantial consequence in CWD susceptibility and pathogenesis.

Among the first 13 positive CWD cases in Norwegian moose that were homozygous for lysine, around 23% of the cases (three moose) were homozygous for glutamine at amino acid 109. This frequency of 109Q CWD-positive animals is not higher than the relative frequencies observed in genetics surveys of the Norwegian moose population(51,52), suggesting that this polymorphism may not be associated with an increased risk of CWD. However, the low number of CWD cases does not allow for any definite conclusions. Initial assessments of 109Q moose CWD properties revealed pronounced differences compared to CWD prions from homozygous K109 moose, not only from Norway, but also those found in Sweden and Finland in terms of their electrophoretic migration properties and PrP^{Sc} deposition and morphology in the CNS (59,82,83). These findings indicate that the presence of glutamine at 109 in the PrP sequence results in PrP^{Sc} with distinct properties from those observed in K109 moose.

The use of gene-targeted mice provides a precise model to characterize the strain properties of these isolates and to follow the adaptation of these properties during iterative passages. Published and ongoing projects in the Telling lab have successfully transmitted CWD isolates from all reported cases of Nordic moose CWD (15,17). We found that isolates from 109Q moose do not transmit in our gene-targeted mice that express wild-type K109. No evidence of residual or subclinical disease was found by sensitive RT-QuIC testing of brain tissues from these mice. These surprising results led to the development of the GtQ109 mouse line, which expresses PrP at levels comparable to those of the previously established and characterized GtQ mouse line and demonstrated to be highly susceptible to 109Q moose prions. This finding indicates that the presence of glutamine at residue 109 in the inoculum imposes a significant barrier for PrP^{Sc} conversion of PrP^C in a host expressing wild-type K109.

The lack of conversion of wild-type GtQ mice (K109) substrate by 109Q isolates, in contrast to their efficient conversion of homologous 109Q substrate in PMCA, confirms the robustness of this sympatric transmission barrier.

The initial differences in transmission kinetics of the moose 6 and moose 4 isolates were likely due to titer effects. Iterative passages of these isolates in GtQ109 mice showed that their strain properties were indistinguishable, strongly suggesting that both animals harbored the same CWD strain. As predicted based on our previous results with the Gt model, studies in GtQ109 mice revealed that, despite the initial lack of evidence of lymphotropism in 109Q moose, aside from PMCA amplification data (83), CWD prions were present in the spleens of these mice following transmission of both moose 6 and moose 4 field isolates.

Conformational properties remained unchanged in native CWD prions and throughout iterative passages, demonstrating that GtQ109 mice recapitulate an unaltered CWD strain. Overall, the morphology and regional distributions of PrP^{Sc} were indistinguishable between the two 109Q moose isolates. Strain properties of 109Q CWD prions were discreetly different between intracranial and intraperitoneal transmission, while the time to disease onset could be attributed to the route of inoculation. This finding supports our previous observations that different host compartments have a role in strain selection (84). Similarly, 109Q prions derived from brain or spleen CWD-infected GtQ109 mice displayed indistinguishable properties.

The failure of moose 10 to transmission in GtQ109 mice is attributable to titer effects, as demonstrated by its low PMCA conversion efficiency and low RT-QuIC SD₅₀ values. Future studies will involve inoculations of PMCA products of moose 10 to evaluate whether its strain properties resemble those observed for the other two 109Q cases. However, it cannot be ruled out that CWD prions present in this isolate possess distinct properties compared to those

observed for the other two 109Q moose. A preliminary analysis of the RT-QuIC fluorescence traces of PMCA products from the three 109Q moose indicated that while those from moose 4 and moose 6 had similar RT-QuIC traces, moose 10 presented a distinct pattern that more closely resembled traces from PMCA products derived from aged GtQ109 mice. This observation may suggest that moose 10 CWD strains represent an earlier stage of prion adaptation from a sporadic origin. Although positive amplification was observed when seeding PMCA reactions with samples from aged 109Q mice, more controlled and reproducible data are required to conclude that expression of the 109Q variant leads to sporadic prion formation.

While the 109Q variant represents a strong barrier to CWD transmission, this polymorphism confers increased host susceptibility. Transmission of wild-type K109-derived CWD from North American and Nordic cervids into GtQ109 mice was substantially accelerated compared to their homologous GtQ mice expressing lysine at K109. Intriguingly, we found no evidence of notable alterations in the strain properties of these CWD prions between GtQ and GtQ109 mice including conformational changes, aside from a shift in the electrophoretic migration in the K109 Nordic CWD moose isolates transmitted in GtQ109 compared to GtQ mice.

Differences in incubation period may be attributable to events beyond the resolution of current prion-typing techniques. One possibility is that the presence of glutamine at residue 109 confers early structural advantages during fibril formation, shortening the lag (asymptomatic) phase without requiring strain adaptation. Future studies comparing infectivity versus total PrP^{Sc} accumulation over the course of disease progression could provide insight into the feasibility of this mechanism facilitated by this polymorphic variant. To explore this question, a collaboration with Dr. Chris Snow and Pegah Eizadkhah at the School of Biomedical and Chemical

Engineering at Colorado State University was formed to apply molecular dynamic (MD) simulations incorporating the 109Q point mutation into the recently published cryo-EM structure of deer CWD prions (29). The MD data shows that the presence of glutamine at 109 yields an increased flexibility in the number of potential hydrogen bond interactions with its neighboring residues while wild type lysine shows a more restricted in the number of favorable interaction partners within the structure of the deer CWD filament (Fig. 4.15). This increase in favorable hydrogen bonds with water conferred by the presence of variant 109Q, could play a role in favoring the early events of PrP^C to PrP^{Sc} conversion or rate of fibril formation by conferring greater conformational plasticity and could explain the acceleration in disease kinetics we observed in 109Q mice inoculated with the wild-type K109 isolates.

The faster kinetics of transmission observed in GtQ109 mice could be due the 109Q prions being more conformationally flexible, less susceptible to conformational mutations, or that sub-strains within 109Q isolates are less able to emerge since 109Q prions are more conversion efficient. We previously reported the effects of the cervid F225 polymorphic variant and its effects on CWD pathogenesis, which contrast sharply with the observations for 109Q. Molecular dynamics simulations noted that F225 increases the stability of the interactions between the β 2- α 2 loop and the α -helix 3 through a hydrogen bond. The overall effect is less solvent accessibility and higher stability/less plasticity; this effect is supported by *in vivo* data, with higher resistance to CWD infection and lower levels of PrP^{Sc} in the brain (48).

Examples of variants that can have important structural effects for pathogenicity are drawn by the occurrence of E211K in cattle, associated with a higher risk of sporadic H-Type bovine spongiform encephalopathy (BSE). E211K is equivalent to the human PrP mutation E200K, which is associated with the most common form of familial CJD. Biophysical studies have shown that these mutations have minimal effects on the native state but can alter

intermolecular interactions within folded PrP. Some studies suggest that substitution of glutamic acid with lysine at this position may not produce significant structural changes but may influence the conformational landscape of PrP misfolding (85–88). We do not exclude the possibility that the 109Q variant may also have pathogenic effects by promoting spontaneous conversion. Further experiments using PMCA and overexpressing transgenic mouse models will be necessary to evaluate these hypotheses in greater detail.

Overall, this investigation has shown that the 109Q variant in Nordic moose CWD poses a robust barrier to CWD transmission, whereby infectious prions from these cervids are unable to convert in wild-type PrP^C hosts and substrates. Future studies will evaluate whether, across iterative passages, strain adaptation can lead to breaching of this barrier. Based on the data presented, we infer that 109Q Nordic moose are highly susceptible to both 109Q- and K109-derived CWD prions, exhibiting an accelerated time to disease when exposed to wild-type CWD isolates and potentially harboring a variant that favors spontaneous PrP misfolding. These findings resemble those described for atypical scrapie and spontaneous forms of BSE. Given that approximately one quarter of the Norwegian moose population expresses a homozygous genotype for the 109Q variant, these results underscore the increasing need for enhanced surveillance and management strategies, particularly for this population.

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OVERALL SUMMARY AND CONCLUDING REMARKS

The discovery of a “new principle of infection” and the introduction of the term “prion” by Stanley Prusiner in 1982 was one of the most subversive and thought-provoking events in modern biology. The paradox of prions represented a challenge to the sacrosanct central dogma of molecular biology in the era following the discovery of the double helix by Franklin, Watson, and Crick. The visionary minds of Alper, Haig, Clarke, and Griffith proposed an improbable theory: a pathogen composed solely of protein, with a self-contained replication mechanism independent of nucleic acids. Further complicating this concept, Pattison observed the emergence of distinct phenotypes of disease, indicating the existence of strains that were later attributed to conformational changes in the molecular structure of prions. The surge in the field’s relevance came with the catastrophic events caused by the bovine spongiform encephalopathy outbreak of the late 1980s and its breach of the species barrier to humans, resulting in hundreds of documented cases of variant Creutzfeldt–Jakob disease. Suddenly, prions were no longer a mere biological curiosity, but pathogens causing incurable neurodegenerative disease with an unpredictable zoonotic potential.

A fundamental question in the field has been the molecular basis of prion strain diversity. Bessen and Telling established the first molecular evidence that structural conformation encodes the heritable properties of prion strains. For the next two decades, only limited understanding of this principle was possible, aided by the advent of nuclear magnetic resonance (NMR); however, the biophysical properties of prions made it difficult to apply higher near-atomic resolution techniques such as X-ray crystallography. The development of cryo-electron microscopy (cryo-EM) and computational reconstruction algorithms for amyloid-like structures enabled a breakthrough, culminating in the study by Kraus in 2021 with the publication of the first high-resolution structure of the hamster strain 263K. Several prion

strains have been resolved in the last 5 years, including structures for Gerstmann–Sträussler–Scheinker disease in humans and chronic wasting disease of deer.

Chronic wasting disease (CWD) is the only prion disease in animals that affects wildlife, specifically members of the Cervidae family, and is characterized by highly efficient contagious transmission. Since its discovery in the late 1960s in Colorado, it has spread extensively across North America and reached endemic status. Most of the current understanding of CWD is based on decades of research into the strain properties of North American CWD. The question of how many strains circulate in endemic regions has produced conflicting results. While some propose that a limited number of strains circulate among North American cervids, a substantial body of evidence suggests that a single dominant strain prevails. The Telling laboratory has pioneered the use of transgenic (Tg) mice as a model for the study of CWD and, in 2019, established a new generation of gene-targeted (Gt) mice. This more precise model offers the advantage of physiologically regulated PrP^C expression in terms of protein levels and tissue distribution, rendering these mice susceptible to prion infection in lymphoid and peripheral tissues and overcoming limitations associated with Tg models.

This body of work encompasses a comprehensive examination of the emergent form of CWD reported in Norway, Sweden, and Finland since 2016. The emergence of Nordic CWD has challenged current understanding of CWD prion biology. In particular, CWD in Nordic moose represents an atypical form of the disease that is hypothesized to have a sporadic origin. In Chapter 2, we selected four moose cases reported in Northern Sweden and demonstrated, through detailed strain characterization, that strain diversity in Nordic moose CWD is unprecedented. Moreover, these strains display an unpredictable capacity for adaptation, exemplified by the emergence of a rapidly transmitting strain—the fastest transmission observed in the Gt mouse model. This strain exhibited discordant amplification properties in RT-QuIC versus PMCA analyses, potentially explained by its structural features. Morphological

observations suggest that this strain may be largely composed of non-fibrillar prions, raising important questions regarding the biological relevance of highly fibrillar structures characterized by cryo-EM. Ongoing studies aim to confirm the non-fibrillar nature of this strain and to assess whether purified fractions reproduce the rapid transmission phenotype in Gt mice.

In Chapter 3, we present evidence that Nordic moose CWD can acquire lymphotropic properties independent of a North American–like phenotype. Early reports indicated no detectable prion infection in lymphatic tissues of natural cases in moose, leading to the classification of Nordic moose CWD as non-lymphotropic and suggesting a lower risk of contagious transmission. However, our studies of Swedish moose CWD show that two isolates develop lymphotropic properties through distinct mechanisms that are either influenced or not by the polymorphism encoding glutamic acid at position 226 of cervid PrP. The 226E-dependent pathway involves the development of a phenotype termed DGL, characterized by dentate gyrus–specific staining that precedes prion detection in the spleen. This phenotype is not restricted to a single Swedish case; similar observations have been reported in Norwegian moose and red deer, suggesting convergent evolution of a lymphotropic strain under the influence of residue 226E, regardless of host genotype. This strain diverges from that observed when isolates are passaged exclusively within the moose-homologous genotype containing glutamine at position 226 (Q226).

A second mechanism facilitates lymphotropism without involvement of the 226E polymorphism and is observed in another Swedish moose case that does not display the DGL phenotype. Additional Norwegian moose cases appear to share this phenotype. These findings suggest that multiple mechanistic pathways underlie lymphotropic adaptation in previously non-lymphotropic strains and that the risk of infectious transmission may emerge during disease evolution and sympatric spread. This underscores the importance of continued surveillance, management, and investigation into the zoonotic potential of evolving CWD strains.

In Chapter 4, we present an investigation of the effects of the 109Q polymorphism on the pathogenesis of CWD. The K109Q polymorphism of the cervid PrP sequence is a variant in which the wild type lysine is replaced by glutamine (Q) and is found exclusively in Nordic moose. The failure of natural isolates from 109Q Norwegian moose to cause disease in GtQ or GtE mice was puzzling; however, we reasoned that the presence of this polymorphic variant could represent a strong transmission barrier. The development of the GtQ109 mouse line enabled the successful transmission of 109Q moose CWD prions, which exhibited rapid transmission kinetics, even upon primary passage. Strain characterization demonstrated that the two successfully transmitted 109Q isolates harbored the same CWD strain.

We further observed that transmission of wild-type (K109) isolates from North American and Nordic cervids into GtQ109 mice resulted in accelerated disease kinetics, although this effect was more pronounced for Nordic moose isolates. This phenotype did not correlate with noticeable changes in strain properties in GtQ109 mice. Therefore, we performed molecular dynamics simulations using the backbone of the deer CWD cryo-EM structure to evaluate the effect of the 109Q substitution. These analyses revealed that the presence of glutamine at residue 109 significantly increases solvent accessibility and expands the range of intermolecular interactions with neighboring residues compared with the more limited interactions permitted by lysine at this position.

Overall, the results of this investigation will be reported in three peer-reviewed publications and are expected to contribute to a broader understanding of the prion biology underlying emergent Nordic CWD. Nordic moose CWD exhibits properties that resemble those of sporadic human neurodegenerative diseases, including high strain heterogeneity. As such, studies of Nordic moose CWD may provide valuable insights as an example of sporadic prion disease.