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REVIEW

The Evolution of Experimental Rodent Models for Prion Diseases

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ABSTRACT

Prion diseases are a group of fatal, neurodegenerative diseases that affect animals and humans. These diseases are characterized by the conformational conversion of normal, host-encoded PrP^{C} into a disease-causing prion isoform, PrP^{Sc}. Significant advancements in biological, genetic, and prion research have led to the capability of studying this pathogenetic process using recombinant proteins, ex vivo systems, in vitro models, and mammalian hosts, the latter being the gold standard for assaying prion infectivity, transmission, and strain evolution. While devoid of nucleic acid, prions encipher strain information by the conformation of their constituent infectious proteins, with diversity altering pathogenesis, host-range dynamics, and the efficacy of therapeutics. To properly study the strain properties of natural prions and develop appropriate therapeutic strategies, it is essential to utilize models that authentically recapitulate these infectious agents in experimental mammalian hosts. In this review, we examine the evolution of research on prion diseases using non-transgenic and transgenic animals, primarily focusing on rodent models. We discuss the successes and limitations of each experimental system and provide insights based on recent findings in novel gene-targeted mice.

1 | Prion Biology Introduction

The prion protein (PrP^{C}) is a glycosylphosphatidylinositol (GPI)-anchored glycoprotein encoded by *Prnp*, a single-copy housekeeping gene on chromosome 2 in mice (Figure 1A) (Chesebro et al. 1985; Oesch et al. 1985; Westaway et al. 1994a). The open reading frame (ORF) of PrP^{C} is entirely contained within a single exon of mammalian prion protein genes (e.g., exon 3 of murine *Prnp*; exon 2 of hamster *Prnp*; exon 3 of cervid, bovine, and ovine *PRNP*; exon 2 of human *PRNP*). The *Prnp* coding sequence consists of an N-terminal signal peptide and a C-terminal GPI signal peptide regions that are cleaved off during protein translocation through the secretory pathway. The mature prion protein is comprised of a non-structured N-terminal domain containing two polybasic charged clusters, octapeptide repeats, and a hydrophobic region. The predominantly alpha-helical C-terminal domain of mouse PrP^{C} (MoPrP) consists of a disulfide bridge spanning from amino acids 176–203, two N-linked glycosylation sites at

Abbreviations: APP, amyloid precursor protein; BAC, bacterial artificial chromosome; Bov, bovine; bp, base pairs; BSE, bovine spongiform encephalopathy; BvPrP, bank vole cellular prion protein; CerPrP-E, cervid cellular prion protein encoding glutamic acid at residue 226; CerPrP-Q, cervid cellular prion protein encoding glutamine at residue 226; Chr, chromosome; CJD, Creutzfeldt-Jakob disease; CNS, central nervous system; Cryo-EM, cryogenic electron microscopy; CWD, chronic wasting disease; DY, Drowsy transmissible mink encephalopathy; f1, first filial generation; fCJD, familial Creutzfeldt-Jakob disease; FFI, fatal familial insomnia; GPI, glycosylphosphatidylinositol; GSS, Gerstmann-Sträussler-Scheinker; Gt, gene-targeted; HuPrP, human cellular prion protein; HY, Hyper transmissible mink encephalopathy; ic, intracerebral; IHC, immunohistochemistry; kb, kilobases; kDa, kilodaltons; LRS, lymphoreticular system; MoPrP, mouse cellular prion protein; ORF, open reading frame; phgPrP, half-genomic *Prnp* plasmid; PIRIBS, parallel in-register intermolecular β-sheet; PMCA, Protein misfolding cyclic amplification; *Prnp* or *PRNP*, mammalian prion protein gene; PrP^{C}, cellular prion protein; PrP^{Sc}, disease-causing prion protein (scrapie isoform); RML, Rocky Mountain Laboratories; RT-QuIC, real-time quaking-induced conversion; sCJD, sporadic Creutzfeldt-Jakob disease; SHaPrP, Syrian hamster cellular prion protein; SSBP/1, Sheep Scrapie Brain Pool 1; Tg, transgenic; TME, transmissible mink encephalopathy; vCJD, variant Creutzfeldt-Jakob disease.

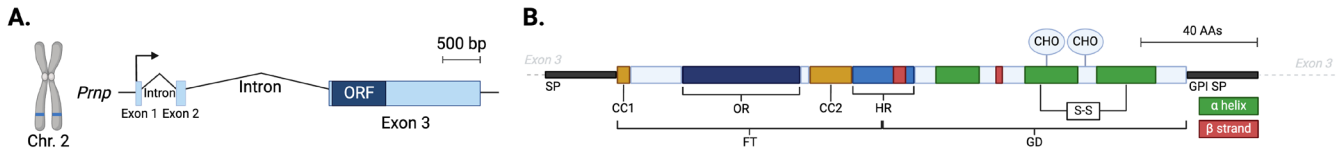

FIGURE 1 | Molecular composition of mouse *Prnp* and PrP^{C}. (A) Murine prion protein gene (*Prnp*) expressed on chromosome (Chr.) 2. The gene comprises three exons, with the entire ORF contained in exon 3. Scale bar = 500 base pairs (bp). The genomic promoter is identified as a black arrow. (B) The ORF of *Prnp* and structural components of PrP^{C}. Dotted lines represent the untranslated regions of exon 3 flanking the ORF. Thin black lines represent the N-terminal signal peptide (SP) and the C-terminal glycosylphosphatidylinositol (GPI) SP, which are cleaved off during secretory translocation to the cell membrane. The translated protein is separated into the flexible tail, FT (amino acids 23–125), and globular domain, GD (amino acids 126–231). Yellow regions are charged clusters (CC), the dark blue region is the octapeptide repeats (OR), the royal blue region is the hydrophobic region (HR), and structurally defined domains are α helical (green) and β strand (red). N-linked glycosylation sites (CHO) are present at amino acids 180 and 196, and a disulfide bridge (S-S) spans from amino acids 178 to 213. Scale bar = 40 amino acids (AAs) (Riesner 2003).

codons 180 and 196, and a GPI anchor (Figure 1B) (Naslavsky et al. 1997; Stahl et al. 1992; Stahl et al. 1987).

Prions are misfolded self-templating counterparts of host-encoded proteins (Prusiner 1982). During the pathogenesis of prion diseases, PrP^{C} undergoes global conformational rearrangement into a pathogenic isoform, PrP^{Sc}, consisting of a partially protease-resistant core primarily composed of beta-sheets. According to the "protein-only hypotheses", PrP^{Sc} is the only disease-causing agent (Griffith 1967; Prusiner 1998), with prion propagation occurring by recruiting other PrP molecules to form fibrillar assemblies. High-resolution structural analyses by cryogenic electron microscopy (cryo-EM) of ex vivo prion fibrils have recently shown a conserved parallel in-register intermolecular β-sheet (PIRIBS) architecture between rodent and higher mammalian prions (Alam et al. 2024; Hallinan et al. 2022; Hoyt et al. 2022a; Hoyt et al. 2022b; Kraus et al. 2021; Manka et al. 2023; Manka et al. 2022b).

Although the propagation and spread of PrP^{Sc} is a conserved feature of prion diseases, they can be categorized into three distinct etiologies: sporadic, genetic, and acquired. Genetic prion diseases are caused by pathogenic mutations within the prion protein coding sequence, resulting in a PrP misfolding event within the brain (Hsiao et al. 1989; Hsiao et al. 1990; Mead 2006). In contrast, acquired prion diseases result from transmission from an external source, either iatrogenic contamination or infectious spread, and generally require initial prion replication in an extraneural compartment before neuroinvasion (reviewed in Mabbott and MacPherson 2006). Despite these disparate etiologies, prion diseases generally have similar clinical and neuropathological presentations, such as PrP^{Sc} deposition, glial activation, and spongiform degeneration within the central nervous system (CNS) (Prusiner 1998).

While lacking informational nucleic acids, prion strain diversity is enciphered by the conformation and PIRIBS structure of the constituent infectious proteins (Bessen and Marsh 1992; Manka et al. 2022a; Telling et al. 1996b). The characteristics of the prion agent, host, and resulting disease phenotype define strain properties influencing pathogenesis, host-range dynamics, and the efficacy of therapeutics (Bartz 2016). Essential for the accurate evaluation of natural prion diseases is recapitulating *bona fide* prion strain properties in appropriate experimental models. In this review, we discuss the major progress of studying prion diseases in mammalian models (Figure 2A), focusing primarily on experimental systems that seek to recapitulate natural infection scenarios. We address the successes and limitations of each model and examine how novel genetic approaches have improved the veracity of current and future prion investigations.

2 | Brief Remarks on the Seminal Observations and Studies of Prion Diseases in the Natural Host

Early observations and investigations played a crucial role in identifying several distinctive features of prion diseases. Scrapie was first described around the beginning of the 18th century (Ness et al. 2023) and initially noted for its contagious nature, consistent symptoms, and the significant damage it could inflict on domesticated sheep and goats (Comber 1772; M'Gowan 1918; Thaer 1819). Neuronal vacuolation and notably prolonged incubation periods were documented in diseased sheep (Besnoit 1898; Stockman 1913) and established as pathological hallmarks of prion diseases (Brownlee 1940; Holman and Pattison 1943; Palmer 1957). However, in-depth examination of the disease was delayed due to several unsuccessful attempts to experimentally transmit scrapie during the late 19th and early 20th centuries (Dammann 1869; M'Fadyean 1918; Plummer 1946), which could be due to the low number of inoculated animals and short duration of observation periods post inoculation (Greig 1940).

Cuillé and Chelle first demonstrated scrapie transmission in sheep and goats following intraocular, epidural, subcutaneous, and intracerebral (ic) inoculations (Cuillé and Chelle 1936; Cuillé and Chelle 1938a, 1938b, 1939). They found that the disease could be transmitted by injecting several different tissues and noted that the incubation period was significantly longer than that of other known viral or bacterial pathogens. D.R. Wilson and colleagues generated Sheep Scrapie Brain Pool 1 (SSBP/1) following nine ic inoculations, primarily in Cheviot sheep, to facilitate and standardize scrapie experiments (Wilson et al. 1950). At the end of serial passaging, there were no noticeable differences in clinical signs or neuropathology, a process known as biological cloning (Dickinson 1976). Subsequent passages in goats led to two distinct phenotypes, "scratching" and "drowsy" which suggested the presence of different strains within SSBP/1 (Pattison and Millson 1961). Numerous subsequent studies on sheep and goats supported these early findings and contributed to the foundational understanding of prion diseases (Gordon 1946; Mackay et al. 1960; Pattison et al. 1959;

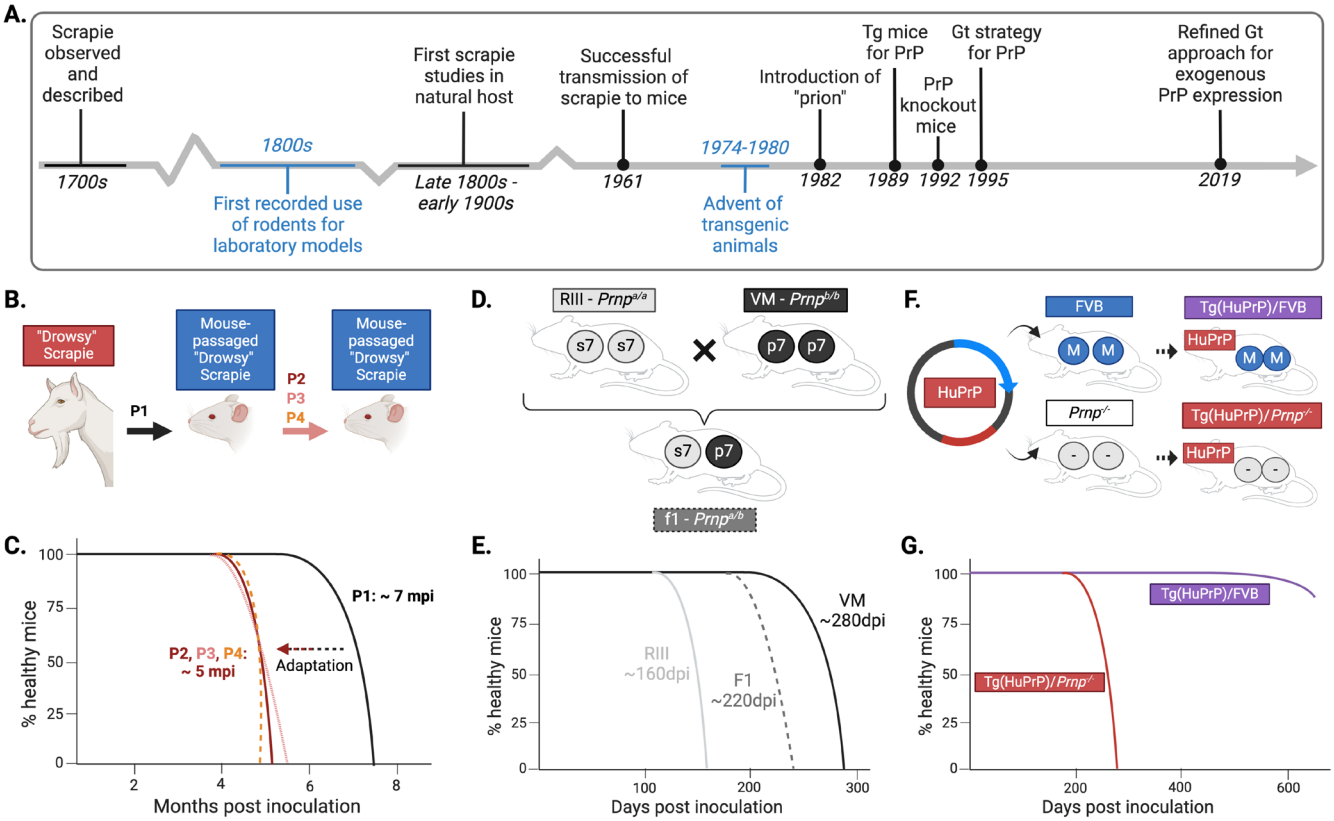

FIGURE 2 | Seminal discoveries and findings contributing to the evolution of experimental prion models. (A) Timeline of significant events of prion biology (black) and general science (blue) fostering the development of mammalian experimental models for prion diseases. (B) Illustration of the transmission of the "drowsy" scrapie strain to mice, resulting in the generation of mouse-passaged "drowsy" scrapie. (C) Graphical estimation of the transmissions reported in (Chandler 1961), which described a decreased time to disease onset between the first passage (P1) and subsequent second, third, and fourth passages (P2, P3, P4). (D) Illustration of the breeding of RIII/$Prnp^{a}$ mice harboring the s7 allele and VM/$Prnp^{b}$ mice harboring the p7 allele, resulting in the generation of f1 ($Prnp^{a/b}$) mice harboring both s7 and p7 alleles. (E) Graphical estimation of the transmissions reported in (Dickinson et al. 1968), which described intermediate disease kinetics for f1/$Prnp^{a/b}$ inoculated with ME7 prions compared to its progenitors RIII/$Prnp^{a}$ mice and VM/$Prnp^{b}$ mice. (F) Illustration of the production of Tg mice expressing HuPrP either on a wild-type (FVB) or $Prnp^{-/-}$ mouse background. (G) Graphical estimation of the transmissions reported in Telling et al. (1995), which described rapid disease kinetics following transmission of human prions in Tg(HuPrP)/$Prnp^{-/-}$ mice, while Tg(HuPrP)/FVB mice remained largely resistant to infection.

Pattison and Millson 1960; Stamp et al. 1959). Collectively, at the time, these findings suggested that the scrapie infectious agent was unique: (1) the infectious agent was self-replicating and exceptionally stable, requiring a prolonged incubation period compared to conventional pathogens; (2) scrapie was transmissible by several different routes of inoculation, and several tissues harbored infectivity (including the brain, spine, and spleen); and (3) scrapie possessed strain properties that influenced pathogenesis and host-range potential.

Following the discovery of prion diseases in various mammalian species, research in other natural hosts has elucidated central pathogenic features of prion diseases and informed risk analysis measures (e.g., Bartz et al. 1994; Hadlow et al. 1974; Hanson et al. 1971; Lambert et al. 2024; Mitchell et al. 2012; Moore et al. 2017; Robinson et al. 1994). For instance, pathogenesis studies following experimental infection of cattle revealed the critical involvement of the Peyer's patches in the transportation of classical bovine spongiform encephalopathy (BSE) prions to the CNS (Hoffmann et al. 2007; Kaatz et al. 2012; Terry et al. 2003). These findings support the suspected etiology of BSE, which is horizontal transmission through oral consumption. Transmission studies involving cervid hosts have underscored the crucial role of the lymphoreticular system (LRS) during the infection of chronic wasting disease (CWD) prions and the preclinical shedding in bodily fluids (e.g., Denkers et al. 2024; Henderson et al. 2015; Sigurdson et al. 1999). These insights not only inform the public health community but also highlight the highly transmissible nature of CWD prions in comparison to other naturally spreading prion diseases. Nevertheless, while these studies are inimitable and provide irreplaceable information to the prion field, conducting experiments with large models has several limitations: (1) it is difficult to establish and maintain the personnel/facilities required for these experiments; (2) some host animals have long lifespans and extended prion disease incubation periods; and (3) relatively little is understood about certain host species compared to others, complicating comparative pathological studies.

Furthermore, prion transmission experiments with human hosts are not feasible. Prion diseases in humans have a wide range of etiologies and are associated with a spectrum of clinical presentations. The most prevalent human prion disease is sporadic Creutzfeldt-Jakob disease (sCJD), accounting for

approximately 85% of human prion disease cases, with its exact cause remaining unknown (Uttley et al. 2020). Approximately 15% of human prion diseases are associated with over 60 autosomal dominant mutations in *PRNP* (Minikel et al. 2016). For example, familial CJD (fCJD) is the most common genetic prion disease and is primarily linked with the E200K missense mutation (Prusiner 1991). Gerstmann-Sträussler-Scheinker disease (GSS) is associated with a number of pathogenic mutations (e.g., P102L and A117V) (Prusiner and Hsiao 1994), while the clinicopathologic phenotype of fatal familial insomnia (FFI) is caused by the D178N mutation when amino acid residue 129 encodes methionine (Goldfarb et al. 1992; Monari et al. 1994). Fewer than 1% of human prion diseases are acquired via iatrogenic transmissions (such as during surgical procedures, corneal transplants, or human growth hormone prepared from human pituitary extracts) or through oral consumption of contaminated tissues/food, which can lead to kuru and variant CJD (vCJD) (Brown et al. 1992; Bruce et al. 1997; Gajdusek 1977; Hill et al. 1997). While non-human primates can recapitulate numerous physiological features of human aging and neurological diseases (Colman 2018; Emborg 2017), the eleven amino acid differences between human and primate PrP^C primary structures can alter prion strain properties and host-range dynamics. For instance, while there has been no correlation between human prion disease and consumption of scrapie-infected lamb, non-human primates are susceptible to disease following transmission of classical scrapie (Comoy et al. 2015). Collectively, these drawbacks have led to the development of experimental model systems that recapitulate the native strain properties of human and animal prions.

3 | Prion Biology Advancements Using Non-Transgenic Laboratory Rodents

The earliest documented use of vertebrate animal species to model diseases dates back to the 6th century BCE, credited to the Greek medical writer and philosopher–scientist Alcmaeon of Croton (Franco 2013). The first documentation of rodent animal models was domesticated rats in 1828 (Lindsey and Baker 2006), and experimentation on laboratory mice shortly followed (Henig 2000). Rodents have become a prominent model for scientific research due to their ease of handling, cheap housing and care, relative resistance to inbreeding, short lifespan, and rapid reproduction rate (Baumans 2007). Additionally, the use of rodents is often regarded as less ethically contentious than larger animal models (Rudacille 2000). PrP^C is highly conserved among mammals, making all members of the order Rodentia potential candidates for experimental studies (Erana et al. 2024); however, a few species have emerged as the preferred models.

3.1 | Transmission of Prion Diseases to Laboratory Rodents

In the 1940s and 1950s, Wilson and colleagues recognized the value of studying scrapie in laboratory animals; however, several attempts to transmit prions to rodents yielded negative results (Pattison 1972). Richard Chandler attempted to transmit "scratching" and "drowsy" scrapie into several different inbred and outbred murine strains (Chandler 1961). While "drowsy" scrapie was able to transmit to laboratory mice, "scratching" scrapie failed to cause disease, further supporting the idea of distinct prion strains within SSBP/1 (Figure 2B,C) (Chandler 1963). Notably, upon serial passaging infected murine brains into other mice, there was decreased disease kinetics, bolstering the notion of a "species barrier" for prion transmissions between different mammalian hosts (Pattison 1965). Importantly, the mouse-passaged scrapie strain ME7 was later shown to be infectious to goats and sheep (as well as hamsters and rats) (Zlotnik and Rennie 1962, 1963, 1965), confirming that it was, in fact, the scrapie infectious agent causing the disease in these experimental animals.

Prions from other mammalian hosts have also been successfully transmitted to wild-type rodents. Injections of human brain extracts from patients with sCJD (Brownell et al. 1975; Lawson et al. 2008; Tateishi et al. 1981), iatrogenic CJD (Tateishi 1996), GSS (Tateishi et al. 1979), FFI (Tateishi et al. 1995), and vCJD (Bruce et al. 1997; Hill et al. 1997) have successfully caused prion disease in mice and other rodents, such as rats and guinea pigs (Manuelidis et al. 1976). Furthermore, guinea pig-passaged sCJD has been shown to successfully transmit to hamsters and mice (Manuelidis et al. 1978). Native BSE prions and feline spongiform encephalopathy prions have also been shown to be transmissible to wild-type mice (Bruce et al. 1997; Hill et al. 1997). Supporting Chandler's findings, serial passage within the same species typically results in a shorter time to disease onset (e.g., Manuelidis et al. 1976; Muramoto et al. 1992; Tateishi et al. 1979). Taken together, unlike other neurodegenerative diseases—where experimental models simulate human or animal conditions (reviewed in Duyckaerts et al. 2008; Potashkin et al. 2011)—these studies demonstrate that rodents effectively recapitulate authentic prion disease in laboratory settings.

3.2 | Key Findings Using Non-Transgenic Mice

Examining prion diseases in laboratory murine models has proven to be highly useful for answering a multitude of questions in the field of prion biology. The increased speed of studies combined with the availability of a large number of experimental subjects validated earlier findings in sheep and goats while also providing foundational knowledge that supports more in-depth investigations in the subsequent decades. For example, Chandler conducted transmissions with different dilutions of mouse-infected brains and reported incubation periods of 6 months for 10^{-5} dilutions, while 10^{-8} dilutions led to time to disease onset in 8–9 months, illustrating an inverse relationship between the time to disease onset and the concentration of the inoculum (Chandler 1963). These findings were later confirmed by specifically assaying prion infectivity (Klohn et al. 2003; Prusiner et al. 1981; Prusiner et al. 1980).

Before the discovery of the prion protein, Alan Dickinson and colleagues described a gene—or a quantitative trait locus—that regulated the incubation period of scrapie in mice. Transmission of murine-passaged scrapie ME7 produced different disease kinetics depending on the inbred strain of mice (RIII and VM mice) (Dickinson et al. 1968). The researchers created the first filial generation (f1) by crossing RIII and VM

mice (Figure 2D). When challenged with the ME7 strain, the f1 mice exhibited an intermediate incubation period relative to their progenitors (Figure 2E). In agreement with subsequent findings (Dickinson and Meikle 1971), these data were consistent with an autosomal gene with a single pair of alleles, which they termed the *sinc* gene (referring to the scrapie incubation period). They named the two alleles *s7* and *p7*, which correspond to the short and prolonged incubation periods of ME7, respectively. Furthermore, the authors observed distinct locations and severities of spongiform lesioning in the RIII, VM, and f1 mice, indicating that variations in strain properties could be attributed to genetic differences. Following the discovery of PrP^{Sc} and PrP^{C} (Basler et al. 1986; Mckinley et al. 1983), Stanley Prusiner and colleagues identified the I/Ln mouse strain as a suitable replacement for VM mice due to their common ancestry (Carlson et al. 1988). They found that RIII and I/Ln mice harbored differences in PrP primary structure at amino acid positions 108 (Leu/Phe) and 189 (Thr/Val) (Westaway et al. 1987) (terming these genotypes $Prnp^{a}$ and $Prnp^{b}$, Figure 2D,E). These differences corresponded to shortened or prolonged incubation periods, indicating that changes in the primary structure of PrP^{C} modified the tempo of the disease. Subsequent investigations established that *Prnp* and *sinc* were, in fact, the same gene (Moore et al. 1998).

Early studies showed that multiple different strains could be propagated following scrapie transmission to mice. Importantly, while the initial interspecies adaptation may have occurred in different genotypes/lines of experimental mice, passaging into the same inbred backgrounds resulted in distinct strain outcomes (Fraser and Dickinson 1973). These data demonstrated that the differences in strains could not be due to differences in amino acids. For example, RML prions were generated following the passaging of Chandler (or 139A) scrapie prions by members of Rocky Mountain Laboratories in a closed colony of CD-1 Swiss mice (reviewed in Block and Bartz 2023). RML and ME7 murine-passaged scrapie prions were shown to have different strain properties following transmission to the same inbred C57BL/6 mice (Thackray et al. 2007; Thackray et al. 2002). More recently, high-resolution structural comparisons utilizing cryo-EM revealed that RML and ME7 prion rods possessed distinct molecular architectures (Manka et al. 2023; Manka et al. 2022b). While both prion assemblies exist as predominantly single protofilament fibrils, their helical crossover difference varied by approximately 25 nm. Additionally, while they share a conformationally conserved N-terminal region, there is a large variation in β-sheet distribution and positioning in the C-terminal region. These data indicate that subtle differences between protein conformations can result in prion strain differences.

Seminal findings regarding the pathogenesis of prion diseases have occurred during studies in wild-type mice, such as the demonstration that prion infections can manifest as a 'subclinical' disease (Hill and Collinge 2003). This is characterized by high levels of prion accumulation in the CNS or other tissues without the onset of any neurological clinical signs and has been reported during both intra- and interspecies transmissions involving wild-type mice (Dickinson et al. 1975; Hill et al. 2000; Race et al. 2001; Zlotnik 1965). Advancements in the field of comparative pathology have also significantly enhanced the relevance of prion research conducted in non-transgenic mice for higher mammalian models. For example, analyses of spongiform degeneration of prion-infected mice became a go-to method to discriminate between strains, a systematic evaluation known as lesion profiling (Fraser and Dickinson 1967, 1968). With the subsequent establishment of PrP^{Sc} deposition in the CNS as a defining pathognomonic characteristic of prion diseases, the extensive characterization of the murine CNS by the larger scientific community has facilitated the correlation of neuroanatomy, neural circuitry, and brain function to areas affected by prion diseases.

Studies in murine models also allow for insights into the interactions between PrP^{Sc} and endogenous proteins, cells, and tissues. For example, early pathological studies identified specific neurological responses like astrogliosis (Field and Raine 1964), which paved the way for more in-depth investigations into reactive astrocytes in murine hosts (e.g., Bradford et al. 2019; Makarava et al. 2019). Furthermore, one of the significant early findings is the temporal and spatial progression of prion infectivity during the course of infection, such as the description of early accumulation of scrapie infectivity in the LRS and spleen (Kimberlin and Walker 1988). These findings undoubtedly led to later findings of the critical role that specific cells (e.g., follicular dendritic cells) contribute to the spread of prions from extraneural compartments to cause disease in the CNS (Brown et al. 1999; McBride et al. 1992; Montrasio et al. 2000).

3.3 | Fundamental Advances Using Other Laboratory Rodents

Researching prion disease in hamsters has proven beneficial due to the high levels of infectivity in the CNS and short incubation periods (Kimberlin and Marsh 1975; Kimberlin and Walker 1977; Marsh and Kimberlin 1975). Richard Kimberlin and Richard Marsh found an inverse relationship between the incubation period and the amount of prion-infected brain tissue used for inoculum. While supporting Chandler's results in mice (Chandler 1961), the ~70-day incubation period of scrapie-passaged hamster prions afforded Prusiner the ability to conduct elegant purification schemes with a much more rapid outcome measure for infectivity. He demonstrated that procedures altering proteins decreased infectivity, whereas methods diminishing nucleic acids did not change infectivity, providing compelling evidence for the existence of proteinaceous infectious agents, or prions (Prusiner 1982).

While the existence of different strains initially posed a significant challenge to the "protein-only hypothesis" studies in hamsters were the first to provide clarity to this matter. During the passage of transmissible mink encephalopathy (TME) into Syrian golden hamsters, Marsh and Richard Bessen described two strains, Hyper-HY and Drowsy-DY, which corresponded to different clinical presentations and incubation periods. Upon biochemical analysis of the constituent infectious proteins, they determined that HY and DY prions exhibited distinct protease sensitivities, sedimentation properties in the presence of a detergent, and size/immunoreactivity of the protease-resistant PrP core, indicating that the conformation of the prion

enciphers strain information (Bessen and Marsh 1992; Marsh and Bessen 1994). Because these two strains are biologically cloned and share primary structure homology, more intricate studies of HY and DY in hamsters have resulted in critical insights regarding pathogenesis and the behavior of prion strains (e.g., Ayers et al. 2009; Bartz et al. 2005; Bartz et al. 2007; Caughey et al. 1998; Gunnels et al. 2023).

Bank voles have demonstrated utility in prion research due to their extremely rapid disease kinetics periods following interspecies prion transmission. For example, inoculation with murine-passaged, rat-passaged scrapie resulted in incubation periods of just 3–4 months (Chandler 1971; Chandler and Turfrey 1972). Voles have also been a favorable tool to discriminate between different prion strains of the same species. While mice and hamsters have been shown to be relatively resistant to infection with human prions, voles are highly susceptible to certain human strains and refractory to others, further demonstrating strain variability (Nonno et al. 2006). More recently, experiments in voles were used to differentiate CWD isolates from Norway and North America (Nonno et al. 2020).

Despite the significant findings during the study of prion diseases in mice, hamsters, voles, and other rodents (Chandler and Fisher 1963; Chandler and Turfrey 1972; Zlotnik and Rennie 1965), the inability to study prion strains within their native PrP^C primary structure is a significant drawback. Moreover, the fixed host PrP^C in experimental rodents restricts the investigation of the host-range dynamics of prion strains. Consequently, it has become a high priority to develop models that can accurately represent the characteristics of higher-order mammals within rodent species.

4 | Utilizing Transgenic Mice Expressing Exogenous PrP for Studying Prion Biology

For most of the 20th century, modeling genetic variability in experimental settings was largely limited to breeding mice exhibiting two observable phenotypes. Notably, the discovery of the *s7* and *p7* alleles was significantly influenced by serendipitous experimentation, meticulous observation during prion bioassays, and clever breeding strategies involving mice. In the early 1970s, Rudolf Jaenisch and Beatrice Mintz produced the first transgenic (Tg) mice—i.e., a process by which scientists transplant foreign genes into a laboratory animal—by injecting viral DNA into murine blastocysts during the embryonic stage of development (Jaenisch 1976; Jaenisch and Mintz 1974). Subsequently, this technique was optimized for the microinjection of a recombinant plasmid carrying DNA into the pronuclei of fertilized murine oocytes (Gordon and Ruddle 1981; Gordon et al. 1980). This method revolutionized transgenics, allowing researchers to introduce nearly all types of genomic material into the cells of laboratory animals.

To investigate whether the host PrP^C primary structure of different species influenced prion transmission, Prusiner's group generated mice that artificially expressed Syrian hamster PrP (SHaPrP) (Scott et al. 1989). They found that the expression of SHaPrP facilitated the transmission of hamster prions, abrogating the strong species barrier between hamster prions and murine hosts. The level of SHaPrP expression was also inversely related to the incubation period of hamster prion transmissions in newly generated Tg(SHaPrP) mice (Prusiner et al. 1990). These findings indicated that the species barrier could be ablated by transgenic expression of PrP^C from another mammalian species and that transmission between species was largely, if not solely, reliant on the differences in the primary structure of PrP^C. Additionally, Tg(SHaPrP) mice inoculated with hamster prions and non-Tg mice inoculated with murine prions produced distinct PrP^{Sc} amyloid deposition, indicating that the interaction of the prion strain and the PrP^C host can determine the plaque deposition profile (Prusiner et al. 1990).

Developing Tg mice for studying human prion disease proved to be more complicated because, unlike Tg(SHaPrP) mice that were fully susceptible to hamster prions, Tg(HuPrP) mice were resistant to human prion infection (originally described as Tg(152)/FVB mice; Telling et al. 1994). To circumvent this issue, the chimeric MHu2M cosmid was generated, encoding HuPrP from amino acids 96 to 167, with the flanking regions occupied by MoPrP (originally described as Tg(5378)/FVB mice; Telling et al. 1994, 1995). In contrast to HuPrP, which differs from MoPrP at 28 amino acid residues, MHu2M only differs at nine amino acids, and the resulting Tg(MHu2M) mice were fully susceptible to human prion infection. "Protein X" (Kaneko et al. 1997; Telling et al. 1995), or a species-specific macromolecule, was posited as an explanation for endogenous mouse PrP preventing the transmission of human prion infection. To further address this hypothesis, the HuPrP cosmid was injected into the pronuclei of fertilized oocytes of $Prnp^{-/-}$, or PrP knockout mice, instead of FVB wild-type mice (Figure 2F) (originally described as Tg(152)/$Prnp^{0/0}$ mice; Telling et al. 1995) (for a more in-depth review of PrP knockout mice, please see Weissmann and Flechsig 2003). These Tg(HuPrP)/$Prnp^{-/-}$ mice were fully susceptible to human prions, while their Tg(HuPrP)/FVB counterparts were largely resistant (Figure 2G). These data indicated that endogenous MoPrP could inhibit natural mammalian prion infection in transgenic mice; however, in its absence, prion infection could occur within a murine model with another mammalian PrP. Consequently, most transgenic PrP mice are generated by injecting PrP expression vectors into $Prnp^{-/-}$ mice.

Because of the increased susceptibility of Tg(SHaPrP) mice to hamster prions, Prusiner's group tested if pathogenic *Prnp* mutations could cause spontaneous disease. Prusiner and colleagues had established a familial connection with GSS, a single base change in *PRNP* at codon 102 (proline to leucine) (Hsiao et al. 1989). Tg(GSSPrP) mice were created that harbored the homologous mutation in mouse PrP (P101L), and these mice developed spontaneous disease in contrast to their non-Tg littermates (originally described as Tg(GSSPrP)174 mice; Hsiao et al. 1990). Consistent with the transmission of human prions to Tg mice, endogenous wild-type MoPrP interfered with the spontaneous misfolding event of mutant P101L, as Tg(GSSPrP) mice produced on a $Prnp^{-/-}$ background developed disease more rapidly than Tg(GSSPrP) mice on an FVB background (Telling et al. 1995, 1996a) (for a comprehensive review of models utilized to study genetic prion diseases, read Watts and Prusiner 2017). Collectively, these early findings demonstrate that natural prion diseases can be recapitulated in murine models by the expression of exogenous *PRNP*.

4.1 | Constructing Tg Mice for Prion Disease Research

Cloning vectors are molecular biological tools that facilitate the transfer of DNA sequences into host organisms (Palmiter and Brinster 1985). When selecting a cloning vector, several factors influence successful transgenesis, such as the size of the exogenous DNA, the desired copy number, and the selection marker (Preston 2003). The first PrP Tg mouse was generated via a cosmid expression vector (Scott et al. 1989), which was subsequently refined to produce the cosSHa.Tet vector, derived from ~43 kb of SHaPrP genomic DNA (Scott et al. 1992). Unlike murine *Prnp* (Figure 1A), hamster *Prnp* contains only two exons, with the entire ORF contained within the second exon (Figure 3A). A tetracycline resistance cassette was integrated into the ORF via a single homologous recombination event to create an expression vector suitable for other mammalian PrP genes.

Several other expression constructs have been used to generate Tg mice. Fischer and colleagues injected various PrP-encoding genes into $Prnp^{-/-}$ mice to investigate how the reintroduction of MoPrP influences the prion infection efficiency (Fischer et al. 1996). The "half-genomic construct" plasmid (phgPrP) successfully restored PrP expression in Tg mice and susceptibility to murine prions. The phgPrP expression construct was produced by removing the second intron, resulting in the fusion of the 2nd and 3rd exons (Figures 1A and 3B). Interestingly, Tg mice with the phgPrP construct were shown to succumb to prion disease more rapidly than Tg mice with a cosmid expression vector of the entire murine *Prnp* sequence. While the spatial mRNA expression patterns in phgPrP Tg mice were largely similar to those in wild-type mice, significant differences were observed in some regions of the CNS, such as the molecular layer of the cerebellum and Purkinje cells, suggesting that the second intron of *Prnp* contributes to the normal cellular expression of PrP (Fischer et al. 1996). The MoPrP.Xho expression plasmid was generated by replacing the ORF of the phgPrP expression construct with an Xho I restriction endonuclease site (Borchelt et al. 1996).

The cosSHa.Tet and MoPrP.Xho expression constructs have been extensively utilized by various laboratories to develop several PrP transgenic models, resulting in significant advancements in the field of prion biology (Groschup and Buschmann 2008). Another popular Tg strategy employed a bacterial artificial chromosome (BAC) (Shizuya and Kouros-Mehr 2001), which was first used for modeling prion diseases by harboring ~125 kb of ovine *PRNP* (Figure 3C) (Vilotte et al. 2001). BAC Tg mice were also developed on a $Prnp^{-/-}$ background, and the large DNA insert also included the ovine *Prnd* (doppel) encoding gene located 52 kb downstream of the ovine *PRNP* locus (Essalmani et al. 2002). In contrast to many previous Tg models, the overexpressing mice generated using BACs exhibited the same relative PrP^{C} levels in CNS and peripheral tissues compared to wild-type mice (Beringue et al. 2012).

4.2 | Seminal Advancements in Understanding Prion Diseases Using Tg Mice

Using experimental models that abrogate the species barrier has enabled the investigation of prion strains within hosts that harbor their native PrP^{C} primary structures and understanding how strains evolve through interspecies transmission. Building

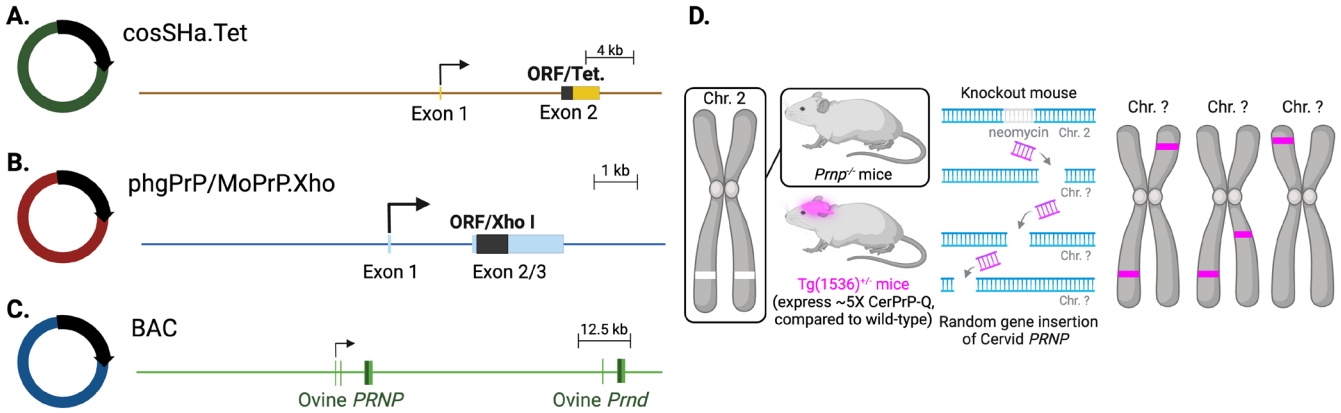

FIGURE 3 | Molecular features of expression constructs and the generation of Tg mice. (A) The cosSHa.Tet cosmid expression construct was generated from ~49 kb of Syrian hamster (SHa) *Prnp* with the cos6EMBL vector (Scott et al. 1992). A single homologous recombination event between the pPrP2Tet and cosSHaPrP (Scott et al. 1989) plasmids resulted in a tetracycline-resistant cassette, which can be replaced by the ORF of mammalian PrP coding sequences. Scale bar = 4 kb. (B) The half-genomic *Prnp* plasmid expression construct was generated from ~12 kb of murine *Prnp* lacking the second intron, resulting in the fusion of the 2nd and 3rd exons from mouse *Prnp* (Fischer et al. 1996). The murine ORF was replaced with a Xho I restriction endonuclease site (Borchelt et al. 1996), which permits the insertion of other mammalian PrP coding sequences. Scale bar = 1 kb. (C) Bacterial artificial chromosomes carry large DNA inserts, such as 125 kb of ovine *PRNP* in the case of the tg3 mice series (Vilotte et al. 2001). The most commonly used clone, tg388 mice, also contained the *PRND* (doppel) loci downstream of *PRNP* (Essalmani et al. 2002). Scale bar = 12.5 kb. Genomic promoters are identified as a black arrow, and ORF is depicted by dark shaded bars. (D) Tg(1536)$^{+/-}$ mice were generated by microinjections of the cosSHaPrP.Tet expression vector carrying deer *PRNP* into the pronuclei of fertilized PrP knockout ($Prnp^{-/-}$) oocytes. Zurich I $Prnp^{-/-}$ mice were generated by replacing amino acids 4–187 of murine *Prnp* with the neomycin phosphotransferase gene (Bueler et al. 1992). The random transgene insertion is visualized by magenta DNA chains' (deer *PRNP*) incorporation into unknown chromosomal locations. These random insertions are visualized by several different genomic locations of magenta *PRNP* (orange bars) on unknown chromosomes (Chr.?), resulting in ~5X CerPrP-Q protein expression compared to wild-type mice. Tg clones were selected by monitoring PrP^{C} expression in the CNS (Browning et al. 2004).

upon the discovery of distinct hamster prion strains HY and DY (Bessen and Marsh 1992), the transmission of two distinct human prion diseases to Tg(MHu2M) mice provided compelling evidence that prion strain diversity is determined by the conformation of PrP^{Sc} (Collinge et al. 1996; Telling et al. 1996b). FFI prions have a protease-resistant PrP core of 19 kilodaltons (kDa) (after deglycosylation), and fCJD prions have a protease-resistant PrP core of 21 kDa (Parchi et al. 1996). While they are caused by different pathogenic mutations within human *PRNP*, after transmission to syngeneic Tg(MHu2M) mice, the resulting diseased mice produced protease-resistant PrP cores that were identical to the inoculum, illustrating that the informational component of the prion is the conformation of the PrP^{Sc}. These data also demonstrated that Tg mice could recapitulate the strain properties of native prions.

One of the significant successes of Tg mice was their application during the outbreaks of BSE and vCJD. BSE was first identified in the UK in 1986 and became endemic within 5–10 years (Prusiner 1997). Initially, there was speculation that the cause of this previously unrecognized cattle prion pathology was linked to scrapie-contaminated meat and bone meal, which led to a feed ban and a subsequent decline in BSE cases. In 1996, the occurrence of CJD in much younger UK patients spurred concern due to its unique neuropathological characteristics, including florid spongiform plaques distinct from those seen in sCJD. This led to the description of a novel disease termed vCJD. The transmission of BSE and vCJD prions to Tg(HuPrP)/$Prnp^{-/-}$ mice revealed similar incubation periods, lesion profiles, and PrP^{Sc} glycosylation profiles that led to a causal connection between the transmission of BSE prions to human patients with vCJD (Hill et al. 1997).

Research conducted on Tg mice has highlighted strain differences among various natural mammalian prions, contributing to risk assessments and management strategies for potential future outbreaks. Notably, L-type and H-type BSE (low molecular weight and high molecular weight BSE, respectively) were discovered during the surveillance of classical BSE (Biacabe et al. 2004; Casalone et al. 2004). Subsequent studies in Tg mice expressing bovine PrP confirmed the existence of distinct biochemical properties with accompanying variations in disease phenotypes (Beringue et al. 2007; Beringue et al. 2006). Similarly, upon the description of Nor98 scrapie (Benestad et al. 2003), the transmission of these scrapie prions, as well as several French incongruous scrapie cases to Tg mice expressing ovine PrP, established the novelty of atypical scrapie prions (Le Dur et al. 2005). Likewise, cases of CWD in Norwegian moose and reindeer (Benestad et al. 2016; Pirisinu et al. 2018) were determined to comprise prion strains distinct from CWD in North America by characterization in Tg mice expressing cervid PrP (Bian et al. 2021).

In addition to abrogating the species barrier for naturally occurring prions, Tg mice have been valuable in identifying the role of specific PrP polymorphisms present in mammalian hosts. For instance, the primary structure of human PrP can differ at residue 129, encoding methionine or valine (Collinge et al. 1991; Palmer et al. 1991). This single amino acid difference at residue 129 also produces distinct strain profiles upon zoonotic or iatrogenic transmissions (Cassard et al. 2014; Collinge et al. 1995; Wadsworth et al. 2004). While the majority of vCJD cases have presented in humans homozygous for methionine at residue 129 (Zerr and Poser 2002), studies in transgenic mice suggest that individuals encoding valine at that position are also susceptible to BSE prions. Another example is studies using Tg models (M129) demonstrated that mice homozygous and heterozygous for valine at amino acid position 127 (G127V) were completely resistant to kuru and sCJD infections (Asante et al. 2015), which is a natural protective polymorphism carried by some humans within kuru endemic regions (Mead et al. 2009). Similar effects of PrP polymorphisms have been observed for other mammals, such as cervid hosts, whose differences in primary structure can affect intra- and interspecies susceptibility, transmission, and strain selection (e.g., Angers et al. 2014; Angers et al. 2010; Green et al. 2008). For a more in-depth review of cervid polymorphisms, please see Arifin et al. (2021).

Prion detection has also been improved following the development of Tg mice. The lack of suitable antemortem detection methods is a significant impediment to successful diagnoses of prion diseases (Altuna et al. 2022). Historically, immunological detection outcomes (e.g., immunoblotting, immunohistochemistry, and enzyme-linked immunoassay) have been the go-to approaches for monitoring prion disease in animals and humans; however, these techniques lack sensitivity to assay infectivity in most peripheral tissues (McNulty et al. 2019). While bioassays in experimental models undoubtedly require more time, Tg mice emerged as a valuable method for monitoring prion infectivity following the direct transmission of peripheral tissues and fluids (e.g., Andreoletti et al. 2011; Angers et al. 2006; Haley et al. 2009). Furthermore, Tg models generated with a BAC allow for the monitoring of replication in the spleen due to transgene overexpression in the LRS (Beringue et al. 2006; Beringue et al. 2008; Beringue et al. 2020; Le Dur et al. 2005). Notably, Vincent Béringue and colleagues found that interspecies transmission following ic injections resulted in a significant proportion of Tg mice with PrP^{Sc}-positive spleens while remaining free of clinical signs and prion accumulation in the CNS, suggesting a much higher occurrence of interspecies transmissions than what was previously assumed (Beringue et al. 2012).

To circumvent the time and cost limitations of bioassays in Tg models, in vitro protocols, such as protein misfolding cyclic amplification (PMCA), have been developed that recapitulate the conversion of PrP^{C} into PrP^{Sc} (Saborio et al. 2001). In this process, uninfected brain homogenate becomes infectious PrP^{Sc} through iterative cycles of sonicating and incubation with small amounts of a prion-infected seed. While the initial descriptions of this technique used wild-type rodent tissues (Castilla et al. 2005), the utilization of brains from Tg mice afforded the possibility of assessing the conversion efficiency of higher mammalian hosts. For example, brains from Tg mice expressing cervid PrP have been utilized in PMCA studies that monitor levels of CWD prion infectivity for different tissues, fluids, and environmental sources (e.g., Haley et al. 2011; Haley et al. 2009; Kramm et al. 2017; Nichols et al. 2009; Selariu et al. 2015). Taken together with bioassays in Tg mice, these findings are critical for managing prion diseases by informing risk analyses and public health protocols.

Tg expression constructs originally designed for exogenous *PRNP* expression have also been adapted to study other neurodegenerative diseases. The cosSHa.Tet construct was the first employed to overexpress the amyloid precursor protein (APP) and cause a spontaneous pathology for studying Alzheimer's disease (Hsiao et al. 1995). Due to its capability to drive global expression to the CNS, the MoPrP.Xho plasmid has also been widely used to create various models expressing mutant and wild-type alpha-synuclein (Emmer et al. 2011; Giasson et al. 2002), mutant and wild-type tau (Lewis et al. 2001; Lewis et al. 2000; Xia et al. 2022; Yoshiyama et al. 2007), mutant and wild-type APP (Borchelt et al. 1996), and other human genes associated with neurodegenerative diseases (e.g., Kim et al. 2009; Lee et al. 1997; Shi et al. 2021).

4.3 | Constructing Gt Mice for Studying Prion Diseases

While any alteration to the mouse genome is considered transgenesis, with the resulting offspring known as transgenic mice, for this review, we will differentiate between two methods of genomic alteration. The generation of mice following the non-homologous insertion—or random integration—of foreign DNA into the murine genome is referred to as transgenic (Tg) models, while mice produced through homologous recombination targeting vector, directing the introduction of exogenous DNA, will be designated as gene-targeted (Gt) models. We acknowledge that some research groups refer to these models as knock-in (ki) mice. While we maintain the original names assigned by each respective research group, for the sake of clarity, we will refer to all of these models as Gt mice.

The optimization of processes for introducing specific genetic alterations through homologous recombination in pluripotent embryonic stem cells occurred in the 1980s (reviewed in Capecchi 1989). Since these advancements, prion disease models have employed molecular biology tools such as the Cre-loxP and CRISPR systems to generate Gt mice (Kuhn and Torres 2002; Navabpour et al. 2020). For example, to more accurately assess the *sinc/Prnp* loci that control the prion disease incubation period in mice, Gt strategies were employed to produce RIII ($Prnp^a$) mice with the $Prnp^b$ dimorphism (Moore et al. 1998; Moore et al. 1995). These modifications to the *Prnp* gene produced disease kinetics and neuropathology consistent with $Prnp^b$ mouse strains, such as VM and I/Ln lines. Gt strategies provide a more precise system to understand polymorphic effects and further support previous evidence that changes in the PrP^C primary structure can control time to disease onset and prion strain outcome. However, in the two decades following these seminal studies, Gt strategies that insert other mammalian PrP sequences proved surprisingly ineffective for developing models to study human and higher mammal prion diseases. These deficiencies have been addressed by novel Gt strategies.

Jean Manson's and Tetsuyuki Kitamoto's groups constructed a Gt targeting vector that replaced amino acids 40–254 in the murine open reading frame with human *PRNP* (Figure 4C,E) (Asano et al. 2006; Bishop et al. 2006; Bishop et al. 2010; Kitamoto et al. 1996). Because the coding sequence from amino acids 23–39 of MoPrP and HuPrP is homologous, the Gt(HuPrP) models from Manson's and Kitamoto's groups putatively express amino acid residues 23–254 of the mature HuPrP coding sequence from the murine *Prnp* locus. Transmissions of sCJD prions to Gt(HuPrP) mice resulted in prolonged incubation periods, often with incomplete attack rates, contrasting with transmissions to Tg(HuPrP) models that succumb to disease with similar inoculum more rapidly and complete attack rates (Asante et al. 2002; Beringue et al. 2008; Collinge et al. 1995; Kong et al. 2005; Telling et al. 1995). Seminal findings using Tg mice demonstrated that while transmissions of human prions to Tg(HuPrP)/$Prnp^{-/-}$ were successful, Tg(MHu2M)/$Prnp^{-/-}$ mice were more susceptible (originally described as Tg(5378)/$Prnp^{0/0}$ mice; Telling et al. 1995). Thus, to further optimize human prion transmission to Gt mice, Kitamoto's group designed a truncated targeting vector that only inserted human PrP from amino acids 40–186, resulting in a flanking murine signal peptide, N-terminal region, C-terminal region, and GPI signal peptide (Figure 4F, Ki-ChM) (Kitamoto et al. 2002; Taguchi et al. 2003). While this model offered slight improvements for some sCJD variants, the inoculation of other sCJD variants and vCJD resulted in low disease susceptibility. These findings suggest that gene dosage could be a reason for the differences in incubation period/attack rate between Tg and Gt mice.

Furthermore, while Tg strategies encoding for bovine (Bov) PrP produced models that were highly susceptible to BSE prions (e.g., Castilla et al. 2003; Scott et al. 1997), Gt(Bov) mice exhibited negligible differences when compared to wild-type mice (Figure 4D) (Asano et al. 2006; Bishop et al. 2006; Bishop et al. 2010; Kobayashi et al. 2007). A consistent feature among these early Gt strategies is the retention of the mouse N-terminal signal peptide (amino acids 1–22) (Figure 4C–F). This prompted the development of novel Gt mice that inserted the entire cervid PrP sequence, including the N-terminal signal peptide and GPI signal peptide (Bian et al. 2019) (Figure 4E). Compared to their Tg model counterparts, these Gt mice expressing cervid PrP proved to be similarly susceptible to CWD prions (Bian et al. 2019; Bian et al. 2021).

While Tg mice harboring pathogenic PrP mutations illustrated a connection to spontaneous neurodegenerative disease, Tg(GSSPrP) mice overexpressed PrP roughly 8 times higher than wild-type mice, and serial transmission of Tg(GSSPrP) prions to wild-type mice did not result in disease (Hsiao et al. 1990). Subsequent findings suggest that transgene overexpression of pathogenic mutations accelerated disease and did not generate a novel prion pathology (Nazor et al. 2005). Additionally, knock-in models expressing physiological levels of MoPrP(P101L) failed to develop any spontaneous neurological disease or pathology (Manson et al. 1999), suggesting that overexpression was required to generate a prion disease model for spontaneous disease.

However, knock-in mouse models for FFI and fCJD were created by expressing the murine analogs D177N and E199K, respectively (Jackson et al. 2009; Jackson et al. 2013). These mutations caused a novel murine pathology, and brain material from spontaneously sick mice was infectious to wild-type mice, thereby confirming that the spontaneous generation of prion infectivity

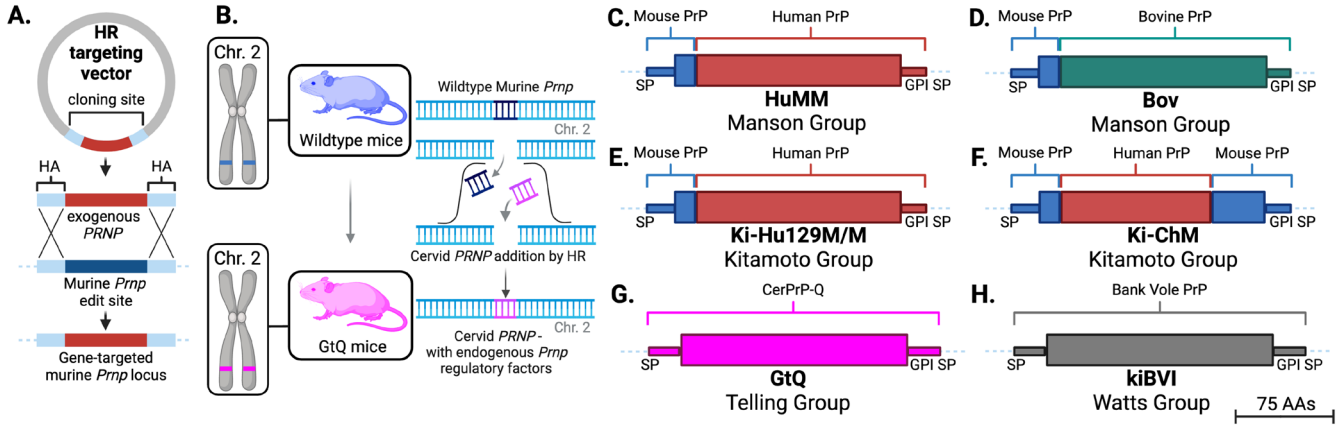

FIGURE 4 | Generation and molecular construction of different gene-targeted mice. (A) Schematic of gene editing using a homologous recombination (HR) targeting vector carrying the gene of interest. The gene of interest, the exogenous *PRNP* gene (red bar), is flanked by upstream and downstream homology arms (HA) of murine *Prnp* (light blue bar) and is introduced into the genome of murine oocytes. Upon HR, exogenous *PRNP* is now expressed at the murine *Prnp* locus. Dotted light blue lines represent upstream and downstream regions of murine *Prnp*. (B) GtQ mice were generated via microinjections of a targeting vector containing deer *PRNP* (magenta DNA chains), homology arms upstream and downstream of the open reading frame, and selectable markers. Wild-type murine embryonic stem cells were electroporated with the targeting vector, and HR events were selected using antibiotic treatment. Successful HR was confirmed by southern blotting and PCR analyses (Bian et al. 2019). Deer *PRNP* is expressed at the murine *Prnp* locus on chromosome 2. (C-H) Schematics of the resulting *Prnp* ORF of Gt mice after homologous recombination. Blue dotted lines represent flanking exon 3 regions; thin rectangles represent N-terminal SP and GPI SP, with the remaining bar representing the coding sequence. Unaffected regions of the coding sequence are shown in blue as Mouse PrP. (C) The Gt HuMM targeting vector inserted the majority of HuPrP (amino acids 40–254) (Bishop et al. 2006). (D) The Gt Bov targeting vector inserted the majority of Bov PrP (amino acids 40–254) (Bishop et al. 2006). (E) The Ki-Hu129M/M targeting vector inserted the majority of HuPrP (amino acids 40–254) (Asano et al. 2006). (F) Ki-ChM mice targeting vector inserted the majority of the human PrP sequence (amino acids 40–186). The central HuPrP sequence is flanked by the murine-specific amino acids N-terminal SP and region (amino acids 1–39), C-terminal region, and GPI SP (amino acids 187–254) (Kitamoto et al. 2002). (G) GtQ mice comprise the entire CerPrP-Q ORF (Bian et al. 2019). (H) kiBVI mice comprise the entire bank vole PrP ORF (Mehra et al. 2024).

could be due to point mutations in *Prnp*. More recently, kiBVI (knock-in bank vole PrP, BvPrP) mice were generated that encode the complete bank vole PrP sequence with isoleucine at amino acid position 109 (Figure 4G) (Mehra et al. 2024). While kiBVI mice remain free of neurological signs and disease, kiBVI^{D178N} and kiBVI^{E200K} mice (comprising homologous mutations for FFI and fCJD, respectively) develop spontaneous disease, demonstrating that modeling spontaneous disease does not require PrP overexpression or homology between the murine native promoter and PrP sequence. While there is still a lack of a Gt model for GSS, collectively, these findings suggest that the expression of full-length exogenous *PRNP* may be necessary to generate an effective gene-targeted model for prion diseases.

4.4 | Comparing Tg and Gt Mice for Studying Prion Diseases

While the development of Tg mice led to innumerable advances in the field of prion biology, there are considerable drawbacks intrinsic to this model system. Generating Tg mice involves introducing an expression construct carrying a gene for the mammalian prion protein of interest into the genome of a *Prnp*$^{-/-}$ mouse. During this process, the investigator(s) cannot control the integration of the *PRNP* transgene or the total copy number (Figure 3D). Surrounding chromosomal elements and epigenetic factors can greatly influence gene expression (reviewed in Jaenisch and Bird 2003; Wilson et al. 1990); therefore, exogenous *PRNP* expression within random murine chromosomes would be expected to differ for each transgene insertion. Furthermore, ligation of exogenous *PRNP* to distinct expression constructs encompassing different mammalian PrP genes could result in wide-ranging genetic promoter effects (e.g., cosSHa.Tet construct encompasses ~49 kb of the Syrian hamster PrP gene, and the MoPrP.Xho construct encompasses ~12 kb of a chimeric mouse PrP gene, Figure 3A,B). Collectively, these limitations complicate comparisons across Tg models. For example, cervids are polymorphic at residue 226: deer, moose, and reindeer encode glutamine (CerPrP-Q), and elk encode glutamic acid (CerPrP-E) at that position. Tg(1536)$^{+/-}$ mice were generated with the cosSHa.Tet construct, expressing CerPrP-Q at ~5X levels compared to wild-type mice (Figure 5A, herein known as TgQ mice) (Browning et al. 2004), while Tg(5037)$^{+/-}$ mice were generated with the MoPrP.Xho construct, expressing CerPrP-E at ~5X levels compared to wild-type mice (Figure 5B, herein known as TgE mice) (Angers et al. 2009). While these two models express approximately the same levels of PrP^{C} in the CNS, the different expression constructs do not allow for accurate comparisons of how the E226Q polymorphism modifies prion susceptibility and strain outcome.

Tg models that utilize different expression constructs have led to distinct responses to prion transmission, even when they express the same exogenous PrP. For example, to evaluate the potential for interspecies transmission, several groups assessed how scrapie transmits to Tg mice expressing elk PrP. Using SSBP/1 prions, ic transmissions to Tg(12577)$^{+/-}$ mice (generated using the cosSHa.Tet construct expressing CerPrP-E at ~2X

wild-type levels) (Tamguney et al. 2006) resulted in a > 90% attack rate and time to disease onset around 270 dpi (Tamguney et al. 2009). By contrast, ic transmissions of SSBP/1 prions to TgE mice resulted in the absence of clinical signs and prion accumulation for greater than 495 dpi (Angers et al. 2014). Because Tg mice generated with the MoPrP.Xho construct have been shown to harbor distinct PrP^C expression profiles compared to wild-type mice and Tg mice generated with the cosmid expression construct (Fischer et al. 1996), it raises the possibility that cell-specific expression in these mice leads to different susceptibilities to prion infection. Moreover, elegant studies in Tg mice that express varying levels of ovine PrP demonstrated that PrP^C dosage is also a determinant of scrapie strain variation (Le Dur et al. 2017). These discrepancies may help explain why several Tg mice expressing human PrP have yielded inconclusive results following transmission with CWD prions (Hannaoui et al. 2022; Kong et al. 2005; Race et al. 2019; Sandberg et al. 2010; Tamguney et al. 2006; Wadsworth et al. 2022; Wang et al. 2021).

In contrast to Tg models, Gt mice are generated by integrating exogenous mammalian PrP sequences at the murine *Prnp* locus through homologous recombination (Figure 4A,B). This event results in *PRNP* transcriptional expression under the endogenous genomic regulatory elements. GtE and GtQ mice encode for CerPrP-E and CerPrP-Q, respectively, and because they are produced on identical FVB backgrounds, they are syngeneic except for the amino acid residue 226, making them suitable for studying the precise effects of primary structural differences of the cervid prion protein (Bian et al. 2019). Additionally, comparisons between *Mus musculus* models developed by different research groups are more appropriate with Gt mice due to the minimal role of other genes during prion pathogenesis (Hummerich et al. 2024; Jones and Mead 2020). For example, TgE mice and $Tg(C594)^{+/-}$ mice (a Tg line expressing bank vole PrP, herein known as TgBv) were developed using the same MoPrP.Xho expression construct, but due to the random transgene integration and difference in PrP expression, it is difficult to compare the effect of elk and bank vole primary structures (Figure 5B,C). In contrast, despite being developed by different research groups, the use of the entire ORF of both elk and bank voles to create GtE and kiBVI mice facilitates a direct comparison of the effects of PrP primary structures with these models (Figure 5E,F).

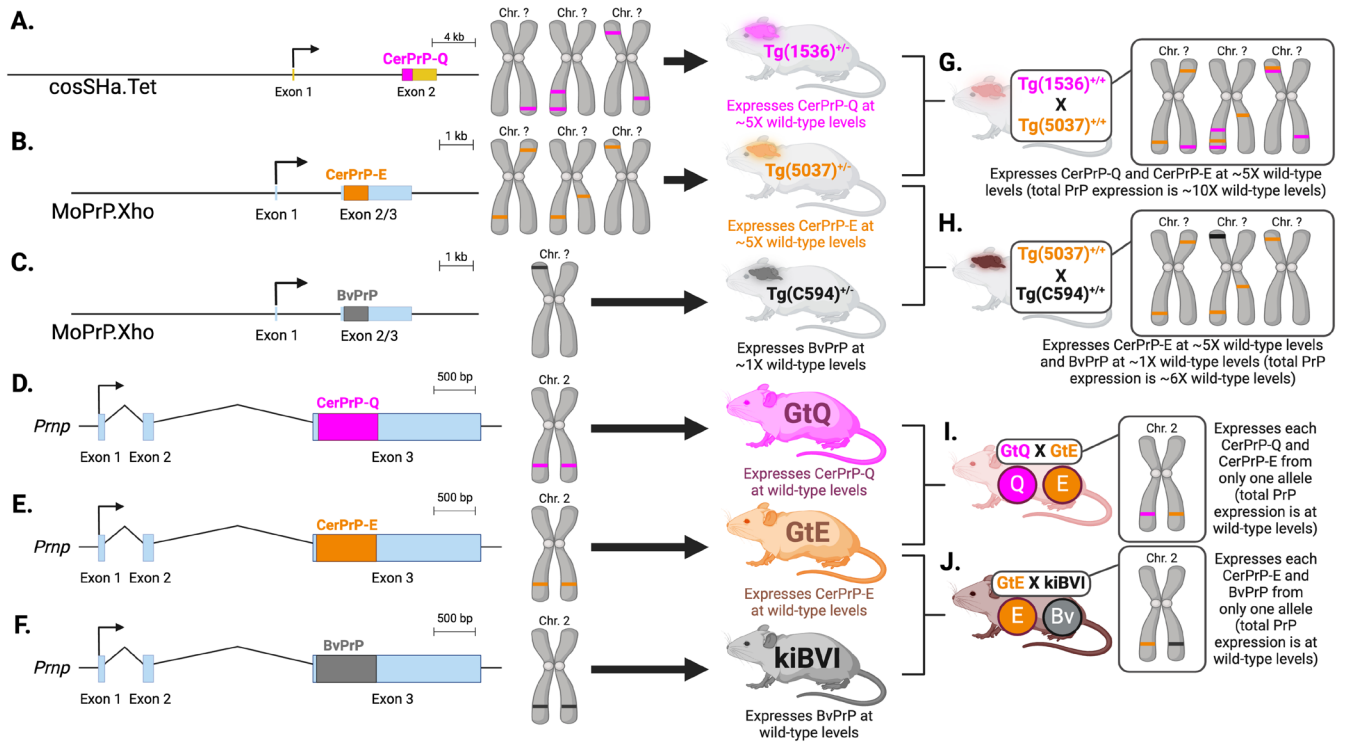

FIGURE 5 | Comparison of exogenous PrP expression between Tg and Gt mice. (A) $Tg(1536)^{+/-}$ (TgQ) mice are engineered to express CerPrP-Q PrP at ~5X higher than wild-type (Wt) levels. Deer *PRNP* is carried by the cosSHa.Tet expression construct (Browning et al. 2004). (B) $Tg(5037)^{+/-}$ (TgE) mice are engineered to express CerPrP-E at ~5X higher than Wt levels. Elk *PRNP* is carried by the MoPrP.Xho expression construct (Angers et al. 2009). (C) $Tg(C594)^{+/-}$ (TgBv) mice are engineered to express bank vole (I109) PrP at ~1X higher than Wt levels. Vole *PRNP* is carried by the MoPrP.Xho expression construct (Erana et al. 2019). Random transgene insertions are visualized by several different genomic locations of *PRNP* (colored bars) on unknown chromosomes (Chr.?), with each bar equating to ~1X prion protein expression compared to wild-type mice. Tg mice are generally maintained at a hemizygous state, and the position of transgenes in the genome/specific chromosomes is randomized for visual effects. (D) GtQ mice express CerPrP-Q at Wt levels under the endogenous *Prnp* promoter (Bian et al. 2019). (E) GtE mice express CerPrP-E at Wt levels under the endogenous *Prnp* promoter (Bian et al. 2019). (F) kiBVI mice express BvPrP (I109) at Wt levels under the endogenous *Prnp* promoter (Mehra et al. 2024). (G) Theorized breeding of $Tg(1536)^{+/-}$ (TgQ) and $Tg(5037)^{+/-}$ (TgE) mice would create Tg mice that express both CerPrP-Q and CerPrP-E. (H) Theorized breeding of $Tg(5037)^{+/-}$ (TgE) and $Tg(C594)^{+/-}$ (TgBv) mice would create Tg mice that express both elk and vole PrP. (I) Breeding of GtQ and GtE mice created GtE/Q mice (Bian et al. 2019). (J) Theorized breeding of GtE and kiBVI mice would create Gt mice that express equal levels of elk and vole PrP.

There are also more subtle drawbacks to using Tg models, such as mice utilized for transmission studies primarily being bred to hemizygosity for the transgene array. This is mainly because Tg mice harboring a high number of PrP transgene copies develop prion disease spontaneously (Castilla et al. 2004; Westaway et al. 1994b). While this further demonstrates the critical role of PrP in prion diseases, it also highlights a limitation of Tg models and the difficulties in modeling heterozygosity. For example, while TgQ and TgE mice express cervid PrP ~5X higher than wild-type mice, an f1 offspring would express both transgene arrays (Figure 5G). Interpreting experimental results would be challenging because it would be difficult to rule out slight differences in CerPrP-E and CerPrP-Q expression and distinct transgene expression constructs that could contribute to allelic interference. The differences in PrP expression are exemplified during a prospective TgE and TgBv breeding scheme, where the f1 offspring would express the elk PrP transgene at five times higher than bank vole PrP (Figure 5H). Additionally, the resulting heterozygous mice would always express higher levels of PrP than the hemizygous versions of the progenitors, potentially resulting in spontaneous disease of the Tg model (e.g., f1 offspring between TgQ and TgE mice would have a total transgene expression of ~10X, Figure 5G).

Gt mice offer a more precise opportunity to study PrP heterozygosity found in nature, such as with GtE/Q mice that model a natural polymorphism found in red deer, *Cervus elaphus* (Figure 5I) (Bian et al. 2019). Because of the expression from the murine *Prnp* locus, GtE/Q mice express CerPrP-E and CerPrP-Q from only one allele, and the total PrP expression is equivalent to that of wild-type mice. The ic transmission of CWD to the resulting GtE/Q offspring produced an intermediate strain with characteristics reminiscent of the prion strains produced in both GtE and GtQ homozygous mice. Similar success has been observed by utilizing Gt mice to model heterozygosity for the cervid S138N polymorphism found in reindeer/caribou, *Rangifer tarandus* (Arifin et al. 2023). While human PrP heterozygosity at residue 129 has been previously modeled in Gt systems, these models were generated with the murine N-terminal signal peptide (Bishop et al. 2006; Bishop et al. 2010). Therefore, it is essential to develop newly refined Gt(HuPrP) mice encoding the entire human *PRNP* sequence to model M129V heterozygosity. Moreover, due to the generation of several Gt models encoding PrP from different mammalian species, this system can examine more intricate PrP interactions during interspecies transmissions. For example, SSBP/1 prions have been experimentally transmitted to mammalian hosts encoding for elk and vole PrP (Tamguney et al. 2006; Vidal et al. 2022). By breeding GtE and kiBVI (Figure 5J), the resulting f1 offspring would create a system to understand how elk and vole PrP interact during the transmission of sheep scrapie prions and could elucidate novel mechanisms of interspecies prion strain evolution.

An essential characteristic of acquired prion diseases, including CWD, is the replication of high infectious titer in peripheral tissues. Generally, Tg mice do not authentically recapitulate peripheral pathogenesis due to murine Tg clone selection usually based on CNS PrP^C expression (Browning et al. 2004), resulting in variable peripheral PrP^C expression. For example, TgQ mice using the cosSHa.Tet expression construct were relatively resistant to extraneural infection with diminished CWD prion replication in peripheral tissues (Bian et al. 2019; Denkers et al. 2010; Denkers et al. 2011). In contrast, Gt mice have been shown to harbor PrP expression in the periphery equivalent to wild-type mice (e.g., in knock-in mice expressing BvPrP with codon 109 expressing methionine, kiBVM, Arshad et al. 2025), and GtQ mice are highly susceptible to peripheral CWD infection with attendant prion replication in LRS tissue (Bian et al. 2019). Recent findings using Gt mice also showed that different routes of inoculation propagate distinct CWD strains, illustrating that selective pressures in CNS and peripheral compartments can modify the conformational properties of the constituent infectious proteins and influence disease outcome (Figure 6C) (DeFranco et al. 2024). Other groups have arrived at similar conclusions in different Gt mice (Chang et al. 2024). These findings demonstrate that not only are peripheral PrP^C expression and extraneural prion replication critical for modeling disease pathogenesis, but that modeling natural infection scenarios is vital for retaining the strain properties of native prions. Consequently, generating Gt mice expressing full-length human PrP and challenging them by authentic routes of inoculation (viz. *per os*) with natural prions is a high priority.

While there will always be limitations with prion model systems—such as physiological differences between murine models and higher mammals (e.g., cervid, bovine, ovine, and human hosts)—Gt models offer a valuable platform for studying natural prions in a controlled and consistent setting. Gt mice express exogenous *PRNP* from the same promoter and genomic location. This system affords the opportunity to study natural prions, such as CWD, sCJD, BSE, and scrapie, and their interactions with their native host PrP (e.g., with existing models such as GtE and GtQ, as well as prospective future models encoding for HuPrP, BovPrP, and ovine PrP) and allows researchers to explore more complex questions regarding the replication of prions within various host compartments. For instance, why are sCJD prions predominantly found in the CNS of human patients, whereas CWD and scrapie prions proliferate readily in the LRS of cervids and ovine species, respectively? What cellular factors enable the replication of BSE prions in the Peyer's patches of BovPrP but not in other tissues? Are there specific strain and host PrP^C combinations whose infections rely more heavily on non-neuronal cells (e.g., astrocytes and microglia)? And if so, are there molecular components responsible for these interactions? These types of studies could elucidate prion strain-specific cofactors (Burke et al. 2020; Simmons and Bartz 2024) and potentially inform future risk management of the transmissibility of other natural prion strains.

The inverse relationship between levels of PrP^C expression and time to disease onset has been repeatedly demonstrated in Tg and Gt mice (Bian et al. 2019; Bueler et al. 1993; Fischer et al. 1996; Prusiner et al. 1990). Following observations of the limited susceptibility of Gt mice expressing bovine and human PrP to their respective prions, it was assumed these models were deficient because they expressed PrP at wild-type levels. Furthermore, comparisons among murine models with different *Prnp* gene constructions indicated that the large intron between exons 2 and 3 might impede prion susceptibility (Fischer et al. 1996). Indeed, Gt strategies have the innate, unavoidable issue of PrP expression at levels equivalent to wild-type mice. This limitation may affect the veracity of the bioassay with Gt

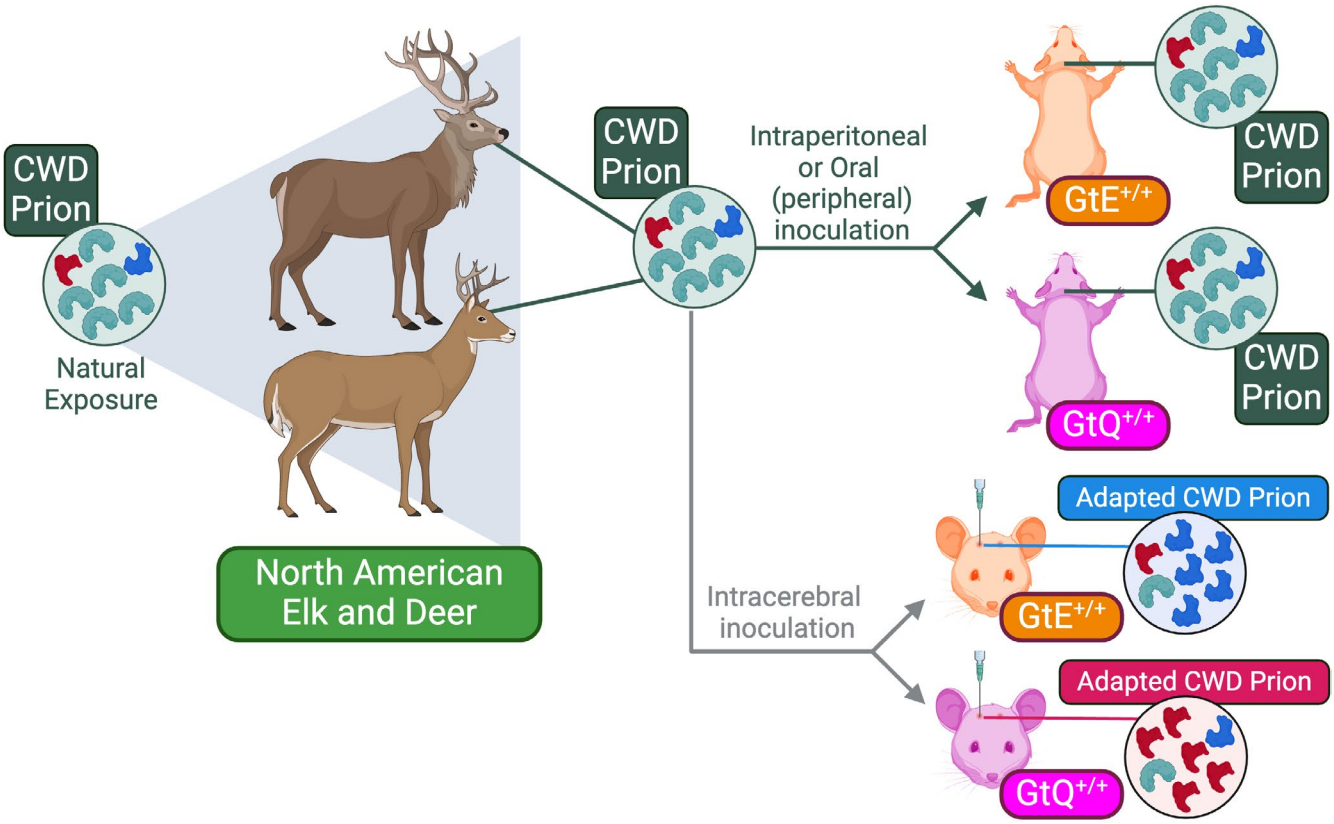

FIGURE 6 | Graphical model of route of inoculation influencing prion strain selection. Based on findings from DeFranco et al. (2024), native CWD prions are represented as a cloud of different protein conformations. Upon natural exposure, the strain properties of elk and deer CWD prions are indistinguishable, represented by the same dominant protein conformer (green) in the prion cloud. CNS tissue from diseased North American elk or deer is intraperitoneally, orally, or intracerebrally inoculated into GtE and GtQ mice. While peripheral inoculations retain the strain properties of native CWD and convergent prion conformations (represented by green protein conformers), intracerebral inoculations result in an adapted CWD strain with divergent prion conformations depending on the host genotype (represented by either blue or red protein conformers).

mice, as various studies suggest that the lifespan of the mouse may not permit sufficient time for prion propagation from the LRS to the CNS and the manifestation of progressive neurological signs (Beringue et al. 2012; Dickinson et al. 1975).

However, interesting findings have been found when comparing CWD transmission between Tg and Gt mice. As previously noted, there is an ~5X difference in overall CNS expression between TgQ/TgE and GtQ/GtE mice (Figure 5A,B,D,E), and North American CWD transmits with equal or slightly higher efficiency to TgQ/TgE mice than GtQ/GtE mice (Bian et al. 2019; Bian et al. 2021). Surprisingly, Norwegian reindeer CWD prions consistently transmitted more readily to GtQ/GtE mice than TgQ/TgE mice (Bian et al. 2021). We speculate that an explanation for this difference could be the preference of Norwegian reindeer CWD prions for replication in specific cell types. Because Tg expression constructs can drive distinct cervid *PRNP* expression profiles differing from GtQ mice, which are controlled by the endogenous regulatory elements of the murine *Prnp* locus, this could explain the difference in disease kinetics. Moreover, the possibility of initial prion replication and subsequent propagation in different cell types caused by these Tg expression constructs could be causing distinct conformational properties and disease outcomes (Supattapone 2020).

Leveraging the strengths of both Tg and Gt models may lead to improved experimental systems. This approach has been spearheaded by Kitamoto's group, who crossed Tg and Ki mice expressing HuPrP, resulting in Tg+Ki mice that exhibited a reduced time to disease onset and prion peripheralization in extraneural compartments, such as splenic follicular dendritic cells (Asano et al. 2006; Kobayashi et al. 2007). Given that certain prion strains are adapted to peripheral transmission routes, such as North American CWD (DeFranco et al. 2024), it is essential to develop experimental models susceptible to natural transmission routes. Additional Tg+Ki models that encode for other mammalian PrP, such as cervid PrP, may decrease disease onset to peripheral challenges, enabling more rapid experimentation while retaining the strain properties of native prions.

5 | Summary and Perspectives

A critical component for the advancement of biological sciences is experimental systems that recapitulate what is found in nature. *Bona fide* model systems are essential for disease research, enabling us to understand pathogenesis, evaluate the efficacy of therapeutics, and analyze how pathogens evolve. While significant progress has been made in understanding the causative agent of prion diseases, there are still significant gaps in

our understanding of how the protein misfolding process occurs, how strains evolve, and how strains contribute to specific phenotypes (e.g., host-range dynamics, transmissibility, tissue tropism). While these knowledge gaps can hinder our ability to make a perfect model, significant progress has been made in studying prions in their native form.

Transmitting scrapie and other natural prions to rodent models generated a rapid system to facilitate the study of several critical aspects of prion biology. Tg models ablated the species barrier of natural prions and allowed for the study of prions within their native PrP^C primary structure. However, Tg models possess several innate issues, such as random transgene genomic integration, uncontrolled PrP^C expression, and limited prion propagation in peripheral tissues. Contemporary Gt mice are generated by replacing the murine *Prnp* coding sequence with the entire exogenous prion protein ORF. Gt models allow for precise assessments of differences in PrP^C primary structure, are highly suited for peripheral challenges, and, in some cases, can be more susceptible than Tg models (Bian et al. 2021).

While bioassay remains the gold standard for evaluating prion strain evolution, host-range dynamics, and the efficacy of therapeutics, it is important to acknowledge the progression of in vitro systems. Assays using recombinant proteins (viz., real-time quaking-induced conversion [RT-QuIC] and protein misfolding shaking amplification [PMSA]) continue to provide novel findings that seemingly are only attainable in these cell-free systems (Atarashi et al. 2007; Erana et al. 2024). However, it is important to note that RT-QuIC does not produce *de novo* infectious prions, and both systems generate products lacking post-translational modifications. Consequently, assessing host-range potential and therapeutic efficacy may lead to erroneous conclusions. PMCA produces infectious prions with post-translational modifications, and prion conformational properties can be conserved during cell-free conversion and PMCA amplification (Bessen et al. 1995; Castilla et al. 2005; Shikiya et al. 2010). However, PMCA cannot recapitulate certain features of in vivo infection, such as prion clearance and distinct strain evolutions produced following different routes of inoculation (DeFranco et al. 2024; Shikiya et al. 2014). Conventional two-dimensional tissue culture systems and three-dimensional organoid models are used extensively and can propagate infectious prions (Falsig and Aguzzi 2008; Groveman et al. 2019; Klohn et al. 2003; Krejciova et al. 2017; Ladogana et al. 1995); however, these systems frequently fail to recapitulate the strain properties of natural prions, and there are lingering questions about the perplexing behavior of prions between different immortal cell lines.

Gt models recapitulate the characteristics of natural prions during infection to appropriate host PrP genotypes (DeFranco et al. 2024). The peripheral expression of exogenous PrP promoted by the murine genomic elements affords the modeling of natural infection scenarios and their potential for intra- and interspecies transmissions. Additionally, due to the shared construction of these models using homologous recombination at the murine *Prnp* loci, experimental results from different research groups using Gt models encoding for different PrP^C primary structures can be more precisely compared. Lastly, because of the direct relationship between PrP and prion diseases, it is important to acknowledge the translational lessons that can be learned from these models and applied to other neurodegenerative disease research (Condello et al. 2024). Therefore, researchers undergoing experimentation in transgenic mice expressing PrP and other proteins with prion-like characteristics responsible for other neurodegenerative diseases should consider the construction of the experimental model and whether it is appropriate for the research question.

Author Contributions

Joseph P. DeFranco: writing – review and editing, writing – original draft, visualization, conceptualization, funding acquisition. **Glenn C. Telling:** funding acquisition, conceptualization, writing – review and editing, project administration.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The authors have nothing to report.

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