The Prion Protein:

Modulation of Potassium Channels and a Novel Mouse Model of a Disease-Causing Hydrophobic Domain Insertion Mutation

by

Robert Corrigan Curtis Mercer

A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Experimental Medicine

Department of Medicine
University of Alberta

Abstract

Prion diseases are invariably fatal neurodegenerative diseases of humans and other mammals. While they can manifest as sporadic, infectious or genetic etiologies, the central event in prion disease is the structural conversion of the prion protein (PrP^{C}) to an alternative conformer PrP^{Sc}. PrP is a highly enigmatic molecule with a wide range of proposed functions and disease associated phenotypes. A molecular understanding of the physiological function of PrP and the pathological characteristics of PrP^{Sc} is essential to uncover the means by which these diseases may be combatted. Herein, I describe two sets of analysis: i) the consequences of the physiological interaction between PrP and a potassium channel modulating protein, dipeptidyl aminopeptidase-like protein 6 (DPP6) and ii) the characterization of a novel mouse model of a genetic form of prion disease, Gerstmann–Sträussler–Scheinker disease (GSS).

i) PrP, in a DPP6 dependent manner, enhances the properties of Kv4.2 voltage gated potassium channels such that there is an increase in peak amplitude, a rightward shift of the voltage-dependent steady-state inactivation curve, a slower inactivation, and a faster recovery from steady-state inactivation.

ii) A patient presenting with GSS was found to harbor a novel insertion mutation of the hydrophobic domain of PrP. We created transgenic mice expressing this allele and present a biochemical and histopathological workup of these animals. They recapitulate many of the features of GSS, in particular a defining low molecular weight proteinase K resistant fragment of the prion protein. Brain extracts of affected animals can be used to accelerate disease in mice expressing the same insertion allele but wild type mice are refractory to this treatment.

Preface

Chapter 2 of this thesis has been published:

Mercer, R. C. C., Ma, L., Watts, J. C., Strome, R., Wohlgemuth, S., Yang, J., Cashman, N. R., Coulthart, M. B., Schmitt-Ulms, G., Jhamandas, J. H., & Westaway, D. The prion protein modulates A-type K+ currents mediated by Kv4. 2 complexes through dipeptidyl aminopeptidase-like protein 6. ***Journal of Biological Chemistry***. 2013; *288*(52), 37241-37255.

The electrophysiological assays found in Chapter 2 were performed by Dr. Li Ma in the laboratory of Dr. Jack Jhamandas. Reconstitution of the potassium channel components in cells and all other analysis are my original work with the exception of Figures 2.1, 2.2 and 2.5 which include data prepared by Dr. Joel Watts. The design of the experiments, interpretation of the data and preparation of the manuscript was undertaken by myself, Dr. Ma, Dr. Jhamandas and Dr. Westaway.

Chapter 3 is my original work with supporting histopathological analysis performed by Hristina Gapeshina and Dr. Nathalie Daude and brain inoculations performed by Dr. Charles Mays.

The literature review presented in Chapter 1 and the concluding analysis presented in Chapter 4 are my original work.

The molecular dynamics simulations presented in Appendix A were performed in the laboratory of Dr. Maria Stepanova by Dr. Lyudmyla Dorosh.

Acknowledgements

First and foremost, I would like to thank my supervisor, Dr. David Westaway for allowing me to pursue prion research in his laboratory. His breadth of knowledge and unwavering patience provide a benchmark that I someday hope to reach. I would like to thank our collaborators Drs. Li Ma and Jack Jhamandas without which some of the work presented would not have been possible. I would also like to thank other individuals who have directly contributed to this work; Hristina Gapeshina and Dr. Nathalie Daude for immunohistochemical work, Dr. Charles Mays for help with inoculations, Dr. Serene Wohlgemuth for the transgenic constructs and assistance in other aspects of molecular biology and Dr. Jing Yang and the entire animal staff for maintaining the mouse colony. Many thanks to my supervisory committee, Drs. Joseph Casey and Howard Young for their helpful advice and stimulating discussions over the last six years. I would also like to acknowledge financial support from PrioNet Canada, the Alberta Prion Research Institute, the University of Alberta and Alberta-Innovates Health Solutions.

My time at the Centre for Prions and Protein Folding diseases was greatly enhanced by all past and present members and to them I give thanks. In particular, for many stimulating conversations about neurodegenerative diseases I would like to thank Camilo Duque Velásquez, Drs. Allen Herbst, Tim Revett and Debbie McKenzie.

For unwavering support throughout this endeavor I must thank my Brother, Dr. Jamison Mercer, my sister-in-law Kate Mercer and my nephew (who has only ever known me as a Ph.D. student) Holden Mercer. I had to miss a lot of special moments, but they didn't hold it against me. Also, thank you to my friends from Newfoundland who were always

there when I needed a return to my roots and my newfound friends that I've met here in Alberta who accepted me as one of their own.

My most profound appreciation and thanks are directed to my parents, Bob and Marie Mercer, for always extolling the limitless value of learning.

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List of Abbreviations

3D – Three-dimensional

ALS – Amyotrophic Lateral Sclerosis (Lou Gehrig's disease)

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

Aβo – Amyloid β oligomer

BCA - Bicinchoninic acid

BSE – Bovine spongiform encephalopathy

C1 – Carboxy terminal prion protein fragment

CA1 – First region of the hippocampus proper (latin: *cornus ammonis*)

Ca^{2+}- Calcium ion

$CaCl_2$- Calcium chloride

cAMP - Cyclic adenosine monophosphate

CCD – Charge-coupled device

cDNA – Complementary deoxyribonucleic acid

CJD – Creutzfeldt-Jakob disease

CMV- Cytomegalovirus

CNS – Central nervous system

CO2 – Carbon dioxide

Cu^{2+}- Copper ion

CWD – Chronic wasting disease

DMEM - Dulbecco's modified eagle medium

DNA - Deoxyribonucleic acid

DPP6 - Dipeptidyl aminopeptidase-like protein 6

DPP6-E – Embryonic isoform of DPP6

DPP6-S- Short isoform of DPP6

DPPX- Alternative name for DPP6

DTT – Dithiothreitol

EF1 α – Elongation factor 1 α

EGTA- Ethylene glycol tetraacetic acid

FBS – Fetal bovine serum

fCJD- Familial Creutzfeldt-Jakob disease

FFI- Fatal familial insomnia

G – Needle gauge

GABA - Gamma-aminobutyric acid

GFAP- Glial fibrillary acidic protein

GFP – Green fluorescent protein

GPI- Glycosylphosphatidylinositol

GSS - Gerstmann–Sträussler–Scheinker disease

GT1 - Hypothalamic neuronal mouse cell line

H&E - Hematoxylin and eosin

HA - Human influenza hemagglutinin

HD – Hydrophobic domain

HDdup – Partial hydrophobic domain duplication

HEK293T - Human embryonic kidney 293 cells

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HRP - Horseradish peroxidase

iCJD – Iatrogenic Creutzfeldt-Jakob disease

iPSC - Induced pluripotent stem cells

K^+- Potassium ion

kb - Kilobase

KChIPs – Potassium channel interacting protein

KCl – Potassium chloride

kDa - Kilodalton

kHz - Kilohertz

M – Moles/liter

MALDI-TOF - Matrix assisted laser desorption/ionization – time of flight

$MgCl_2$ – Magnesium chloride

mGluR5 - Metabotropic glutamate receptor 5

ml – Milliliters

mM – Millimole/liter

M_r – Relative molecular mass

MRI – Magnetic resonance image

mRNAs – Messenger ribonucleic acid

ms – Millisecond

mV – Millivolt

MΩ - Milliohm

N-CAM – Neural cell adhesion molecule

N2a - Mouse neuroblastoma cell line

nA - Nanoamp

Na_2ATP – Sodium adenosine triphosphate

NaCl – Sodium chloride

NaGTP – Sodium guanosine triphosphate

NMDA - N-methyl-D-aspartate

NMS – Normal mouse serum

NR2D – Subunit of the NMDA receptor

°C – Degrees Celsius

ORF – Open reading frame

PAGE – Polyacrylamide gel electrophoresis

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PMSF - Phenylmethanesulfonyl fluoride

PNGase F - Peptide -N-glycosidase F

Prnd – Gene encoding for doppel in mice

Prnp – Gene encoding for the prion protein in mice

PRNP – Gene encoding for the prion protein in humans

PrP – Prion protein

PrP^{C} – Cellular prion protein

PrP^{Ctm} – Alternative topology of the prion protein

PrP^{res} – Prion protein remaining after treatment with strong acid

PrP^{Sc} – Scrapie prion protein

PVDF - Polyvinylidene fluoride

RFP – Red fluorescent protein

RIPA - Radioimmunoprecipitation assay buffer

RK13 – Rabbit kidney cells

RML – Rocky mountain labs mouse adapted scrapie isolate

RNA - Ribonucleic acid

sCJD – Sporadic Creutzfeldt-Jakob disease

SD – Standard deviation

SDS – Sodium dodecyl sulfate

SE – Standard error

SEM – Standard error of mean

siRNA – Small interfering ribonucleic acid

Sprn – Gene encoding shadoo in mice

STI1 - Stress inducible protein 1

$t_{1/2}$ – Half life

TBS – Tris buffered saline

TBST Tris buffered saline – tween 20

TM - Transmembrane

TME – Transmissible mink encephalopathy

U - Units

vCJD – Variant Creutzfeldt-Jakob disease

VGLUT1 - Vesicular glutamate transporter 1

VPSPr - Variably protease-sensitive prionopathy

wt – Wild type

g - Gravity

Zn^{2+} - Zinc ion

μg - Microgram

μl - Microliter

μ m - Micrometer

Amino acid single letter code:

G - Glycine

P - Proline

A - Alanine

V - Valine

L - Leucine

I - Isoleucine

M - Methionine

C - Cysteine

F - Phenylalanine

Y - Tyrosine

W - Tryptophan

H - Histidine

K - Lysine

R - Arginine

Q - Glutamine

N - Asparagine

E - Glutamic Acid

D - Aspartic Acid

S - Serine

T - Threonine

Chapter 1

Introduction to Prions:

History, Function and Disease

Prion diseases are invariably fatal neurodegenerative disease of animals. In the history of medicine, they are peerless in two ways; i) that their etiology can be either sporadic, genetic or infectious and ii) that, referring specifically to the infectious nature of the pathogen, a nucleic acid component has never been identified. Before an introduction to these diseases, a brief history of the discovery of the molecule responsible, the prion protein, will be provided.

1.1 Historical perspective

Scrapie, the prototypical prion disease, had been known for hundreds of years and was thought to be caused by a "slow virus" (Eklund *et al*., 1967). Creutzfeldt-Jakob disease (CJD) and Kuru were both known to affect humans and it was appreciated that the two diseases shared neuropathological similarities in 1959 (Creutzfeldt, 1920; Klatzo *et al*., 1959). Around the same time, William Hadlow recognized that these pathological features were also found in the brains of scrapie infected sheep and, as it had been known that scrapie was a transmissible disease, this led to the proposal that Kuru and CJD were transmissible also, which turned out to be the case (Hadlow, 1959; Gajdusek *et al.*, 1966; Gibbs *et al.,* 1968). This understanding, paired with the realization that the scrapie and CJD agents were highly resistant to ultraviolet and ionizing radiation, procedures that were known to destroy nucleic acid, had set the stage; the first steps toward the realization of the existence of an unknown class of pathogen had been taken (Alper *et al.*, 1966; 1967; Latarjet *et al*., 1970; Gibbs *et al.*, 1978).

The adaptation of the scrapie agent for replication in mice was an important advance towards the elucidation of the nature of the agent (Chandler, 1961). Though these assays took almost half a year to complete, they were much cheaper than investigations using

sheep due to the ease of housing the animals and more amenable to systematic investigation as the time needed to transmit the disease between sheep required about 500 days. The use of Syrian hamsters shortened this period to about 70 days and allowed for the rapid ascertainment of infectivity in biochemically-fractionated samples (Marsh and Kimberlin, 1975; Prusiner, 1982). This allowed for the identification of preparations of the infectious agent that were devoid of most other macromolecules through digestion with proteinase K, detergent extraction and centrifugation. Through this, a polypeptide was identified in the brains of infected hamsters that was absent in healthy controls (Bolton *et al.*, 1982). As treatments that denatured or destroyed proteins such as SDS, guanidinium thiosulfate and phenol also abolished infectivity while treatments that destroyed nucleic acid had no effect, it started to become clear that the long sought after agent was composed solely of protein (Prusiner, 1982). It was at this point that the term prion was first brought forth; an amalgamation of *pro(teinaceous)* and *in(fectious particle)* (Prusiner, 1982). Following Edman degradation sequencing of highly infectious material, the N-terminal sequence was used to create degenerate oligonucleotide primers that could then be used as hybridization probes to screen a cDNA library made from the brains of animals in the log phase of prion replication. A corresponding cDNA clone was isolated and the predicted ORF aligned with the peptide sequences. This hybridized to genomic DNA but not phenol extracted extracts of infectious prions. mRNA that coded for the prion protein (PrP) was found in uninfected animals, leading to the discovery of a corresponding protein expressed by healthy individuals (Oesch *et al.*, 1985; Basler *et al.*, 1986; Kretzschmar *et al.*, 1986).

The “protein only hypothesis”, as it came to be called, was bolstered by subsequent findings. The identification of the gene encoding PrP in mice, *Prnp*, led to the identification of mutations in families whose prion diseases appeared to be hereditary

and that when expressed in mice caused spontaneous disease (Hsiao *et al.*, 1989; 1990; Yang *et al.*, 2009). Additionally, mice lacking any expression of *Prnp* through genetic ablation were found to be completely resistant to the disease (Büeler *et al.*, 1993). Finally, in recent years prions have been created *in vitro* using recombinant PrP and have been shown to be infectious (Wang *et al.*, 2010). Despite overwhelming (and frequently mutually complementary) evidence in support of the protein only hypothesis, there remain those who advocate for a cryptic virus as causative agent (Manuelidis *et al.*, 2007).

1.2 Introduction to prion disease

The central event in prion disease is the structural conversion of cellular PrP (PrP^{C}) to PrP^{Sc} (scrapie), which has a significantly increased proportion of β-sheet secondary structure (Prusiner, 1982; Pan *et al.*, 1993; Nguyen *et al.*, 1995; Wille *et al.*, 2002; Requena & Wille, 2014). This three-dimensional transformation renders PrP^{Sc} resistant to digestion by proteinase K and detergent insoluble; properties that accommodate the biochemical identification of this disease associated conformer from its normal cellular precursor. As PrP^{Sc} accumulates, it induces spongiform change in the central nervous system (Prusiner 1982). Astrocytic gliosis is another prominent feature of these diseases. Amyloid plaques formed from PrP^{Sc} can also be present, but are not a ubiquitous feature of these diseases (Figure 1.1).

1.2.1 Prion replication

The mechanism by which PrP^{Sc} replicates itself from PrP^{C} remains one of the most pressing questions in prion biology. Two models have been proposed that differ in their

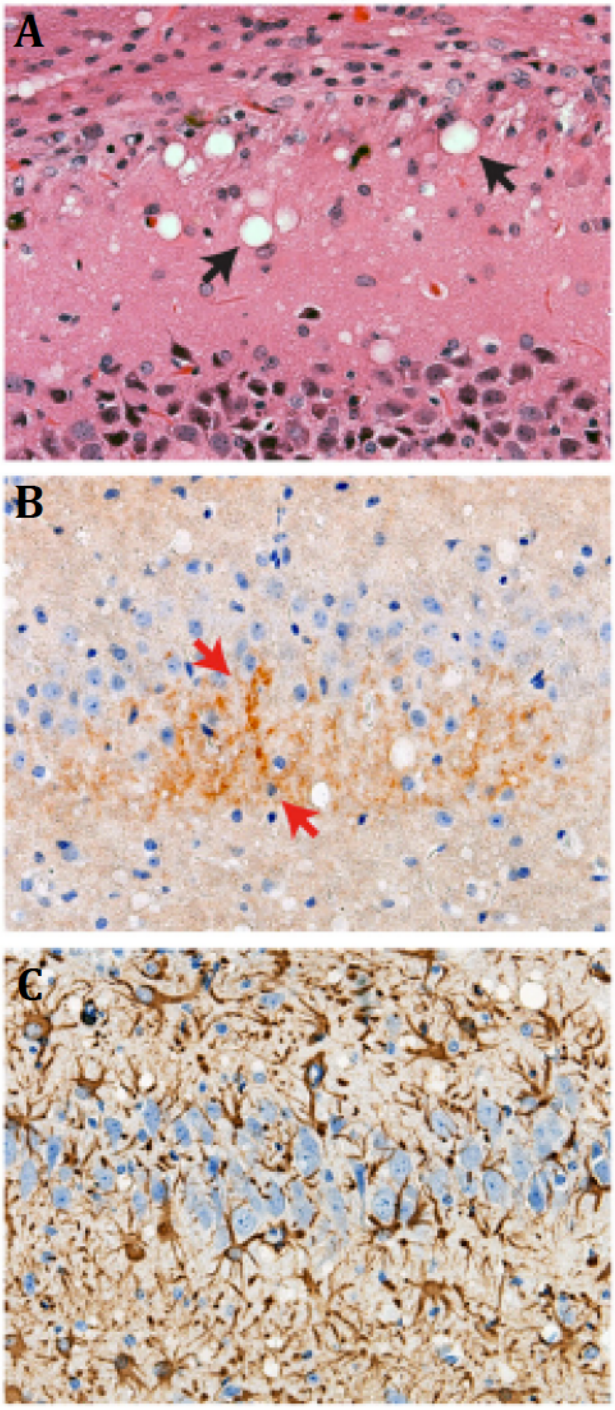
A
B
C

Figure 1.1: Neuropathological features of prion disease.

A) Spongy change revealed by staining with hematoxylin and eosin. **B)** PrP immunoreactive deposits **C)** Astrocytic gliosis revealed by immunodetection of GFAP. Figure taken with permission from Watts & Prusiner, 2014.

assumptions about the initial seed. In the first, referred to as template directed misfolding, postulates that once spontaneous conversion from PrP^{C} to PrP^{Sc} occurs, or after introduction of PrP^{Sc} through infection, the energy barrier for conversion is lowered through an interaction between PrP^{Sc} and PrP^{C}. In this scenario, there are now two molecules of PrP^{Sc} that continue to convert PrP^{C} in an exponential manner (Prusiner *et al.*, 1990; Cohen *et al*, 1994). While there is a significant energy barrier towards the spontaneous conversion of PrP^{C} to PrP^{Sc}, this would have to occur in the context of sporadic disease. The second model is known as the non-catalytic nucleated polymerization model (Jarrett & Lansbury, 1993, Come *et al.*, 1993, Lansbury & Caughey, 1995). Here, there is the underlying assumption that conversion of PrP^{C} to PrP^{Sc} is a ubiquitous process and is always present at very low levels but is undetectable and inherently unstable. Upon the formation of a PrP^{Sc} seed of sufficient size, it would be stabilized which would allow for the recruitment of PrP^{C} at a much faster rate upon fragmentation of the resulting amyloid.

1.2.2 Prion strains

The existence of prion strains is an accepted phenomenon in the field that thus far eludes description at the atomic level (Aguzzi *et al.*, 2007). This notwithstanding, a prion strain can be simply defined as a prion isolate that when used to inoculate identical hosts, produces phenotypes divergent from other isolates. Such phenotypes can be defined not only clinically but also by histopathological analysis of lesion profiles in various brain regions as well as incubation times. Biochemical analyses can also be used as markers of different strains though are not in and of themselves sufficient to define them. In many instances, alterations in the glycosylation pattern of proteinase K resistant PrP are noted in addition to differences in the size of the proteinase K resistant fragments. Strains are

thought to arise from structural differences in PrP^{Sc}; a simple demonstration of this can sometimes be seen in the form of size variations of proteinase K resistant fragments, implying that the conformation of PrP^{Sc} is different despite being generated from the same primary structure (Bessen & Marsh, 1994; Parchi *et al.*, 1996). A highly sensitive method to discriminate between PrP^{C} and PrP^{Sc} through the use of differential antibody binding, the conformation dependent immunoassay, has also been used to demonstrate structural differences between prion strains without the need for proteolytic digestion (Safar *et al.*, 1998). High-resolution structural information of multiple PrP^{Sc} strains, which has yet to be accomplished, will provide insights into the molecular basis of these phenomena (Requena & Wille, 2014).

1.2.3 The species barrier

Before an overview of the various prion disease that affect mammals, a final biological property of prions that must be introduced is the species barrier. This is caused by alterations in the amino acid sequence of PrP such that prions derived from one animal may or may not be able to infect a novel host, or may be able to do so after a period of adaptation that can be observed as decreasing incubation times of successive passages of a given agent into the new host. This can be overcome by matching the PrP expressed in the host with the PrP in the inoculum. For example, transgenic mice that express hamster or human PrP are susceptible to prions derived from these species while wild type mice are not (Prusiner *et al.*, 1990; Collinge *et al.*, 1995).

1.3 Prion diseases of non-human animals

1.3.1 Prion disease of sheep and goats

As mentioned, the first identified prion disease was scrapie; so called because infected animals scratch their coats off due to excessively itchy skin. This disease affects sheep and goats and, interestingly, may have been observed by humans for millennia though this is based on an interpretation of Chinese characters and, as such, rests firmly in the realm of speculation (Wickner, 2005). Scrapie has certainly been known hundreds of years (McGowan, 1922). There are three polymorphisms of sheep PrP that alter the susceptibility of the animal to scrapie: (resistance-codon-susceptible) A136V, H154R and R171Q (Westaway *et al.*, 1994; Goldmann *et al.*, 1994; Hunter *et al.*, 1996). Interestingly, Nor98, an atypical form of scrapie with an altered glycosylation profile is known to primarily affect sheep with the so-called resistant genotypes (Benestad *et al.*, 1998). The existence of atypical scrapie calls into question the proposal to selectively breed for resistant genotypes in endemic areas. To date, there is no data to suggest that scrapie can be transmitted to humans (Wadsworth *et al.*, 2013).

1.3.2 Prion disease of cattle

Perhaps the most widely known example of a prion disease is Bovine Spongiform Encephalopathy, BSE or 'mad cow disease' that was famously epidemic in the United Kingdom in the late 1980's (Wells *et al.*, 1987). BSE represents the dangers of agricultural cannibalism; that is, the feeding of deceased livestock to the living. Although this practice is now banned, the disease was transmitted through meat and bone meal that had been contaminated with brain derived material (Wilesmith *et al.*, 1991).

Horrifyingly, it was discovered that BSE could be transmitted to humans through the consumption of beef (Collinge *et al.*, 1996; Bruce *et al.*, 1997; Scott *et al.*, 1999; see section 1.4.3).

1.3.3 Prion disease of cervids

Chronic Wasting Disease (CWD) is a unique prion disease of non-human animals in that it is found in free ranging as well as captive populations. CWD primarily affects deer (both mule and white-tail) and elk (Williams, 2005). Experimental transmission of CWD has been accomplished in other cervids, namely moose and caribou (Kreeger *et al.*, 2006; Mitchell *et al.*, 2012). Furthermore, the disease has been detected in a free ranging moose (Baeten *et al.*, 2007). There is currently no published experimental or epidemiological evidence to suggest that CWD is transmissible to humans (Belay *et al.*, 2004; Kong *et al.*, 2005; Sandburg *et al.*, 2010).

1.3.4 Prion disease of mink

Transmissible Mink Encephalopathy (TME) affects farm raised mink and was first described in the mid 1960's; (Hartsough & Burger, 1965; Marsh *et al.*, 1969). The last documented outbreak occurred in 1985 (Marsh *et al.*, 1991). Two well-studied strains have been isolated from the adaptation of TME to Syrian golden hamsters: hyper, characterized by hyper excitability and a short incubation period and drowsy, which has a long incubation period with progressive lethargy. Proteinase K treatment of these agents revealed a shift in the molecular weights of the resistant fragments and biophysical studies of PrP demonstrated altered levels of β-secondary structure. These comparative analyses of hyper and drowsy provided the first clues toward understanding

the molecular basis of prion strain diversity (Bessen & Marsh, 1992; 1994).

1.4 Prion Diseases of Humans

1.4.1 Kuru

Kuru was a prion disease endemic to the northern highlands of Papua New Guinea and found in the Fore linguistic group that inhabits the region. This now eradicated disease was caused by the consumption of community members during ritualistic mortuary feasts and was successfully wiped out by the cessation of such practices (Collinge *et al.*, 2006). Recently, in an astonishing example of rapid human evolution under selection pressure of a common disease, it was found that the Fore propagated a *PRNP* mutation, G127V that prevents infection with kuru. The authors hypothesize that even without the cessation of cannibalism, the continued transmission of the disease may well have been prevented because of this (Mead *et al.*, 2009; Asante *et al.*, 2015).

1.4.2 Sporadic Creutzfeldt-Jakob disease

Sporadic CJD (sCJD) is the most common prion disease of humans with a worldwide occurrence of one case per million people per year and was identified at the start of the 20th century (Creutzfeldt, 1920; Masters *et al.*, 1979). The cause of sCJD is unknown; though as the name suggests it is thought to arise from the spontaneous generation of PrP^{Sc} that then propagates within the host. A common polymorphism of *PRNP* (the gene encoding PrP in humans) plays an important role in CJD manifestation. Codon 129 can code for either a methionine or a valine and individuals that are heterozygous at this position are protected relative to homozygotes (Palmer *et al.*, 1991). Iatrogenic CJD

(iCJD) is acquired in a nosocomial manner through exposure to improperly cleaned surgical tools or contaminated dura mater grafts. Children who received cadaver derived human growth hormone contaminated with sCJD have also developed iCJD. This practice was halted in 1985 in favour of recombinant hormones (Brown *et al.*, 2000). It has recently been suggested that Alzheimer's disease, a much more common neurodegenerative disease of humans, may have been concurrently transmitted to some of these patients (Jaunmuktane *et al.*, 2015).

1.4.3 Variant Creutzfeldt-Jakob disease

Variant CJD (vCJD) is due to the consumption of BSE infected cattle but, thankfully, has remained quite rare (Collinge *et al.*, 1996; Bruce *et al.*, 1997; Scott *et al.*, 1999). Evidence suggests that vCJD can be horizontally transmitted through blood transfusions and, because of this, strict policies regarding blood donation have been put in place throughout the world (Llewelyn *et al.*, 2004; Peden *et al*, 2004; Wroe *et al.*, 2006). The polymorphism at position 129 also plays a role in the manifestation of vCJD. All individuals who have thus far presented with vCJD are homozygous for methionine at this position. This has been taken to suggest that individuals that are heterozygous or homozygous for valine at this position may have yet to present with disease. In support of this notion, an analysis of the removed appendixes of people in the United Kingdom demonstrated that one in two thousand tested positive for prions. Of these, a disproportionate number of positive appendixes belonged to people with the valine polymorphism (Gill *et al.*, 2013). Interestingly, the mutation identified in the Fore was also found to prevent the transmission of sporadic, iatrogenic and variant CJD to mice homozygous for valine at position 127 (Asante *et al.*, 2015).

1.4.4 Variable Protease-Sensitive Prionopathy

Variable Protease-Sensitive Prionopathy (VPSPr) is the most recent addition to the spectrum of human prion disease and affects all three *PRNP* codon 129 genotypes, though valine at this position appears to be a risk factor (Gambetti *et al.*, 2008; Rodriguez-Martinez *et al.*, 2010; Zou *et al.*, 2010). As the name suggests, depending on the genotype at codon 129, the resistance to protease is altered; though all are more sensitive to such treatment than the more common human prion diseases and analysis by western blot reveal a ladder like fragmentation pattern after exposure to proteinase K (Zou *et al.*, 2010). When transgenic mice expressing human PrP were challenged with VPSPr from all three genotypes, no clinical signs were observed though one group noted vacuolation in some animals (Diack *et al.*, 2014; Notari *et al.*, 2014). Small granular like deposits that were immunoreactive for PrP were noted, but this does not necessarily represent transmission of VPSPr (Diack *et al.*, 2014; Notari *et al.*, 2014). Surprisingly, no animals were found to have any pathological abnormalities upon second passage. This led the authors to conclude that while VPSPr is transmissible, human PrP (or the mouse brain environment) is not suitable to sustained replication of the strain (Notari *et al.*, 2014).

1.5 Inherited prion diseases

Inherited prion diseases represent 10-15% of all human cases of prion diseases and are caused by autosomal dominant mutations in *PRNP*. There are more than 50 mutations known to cause inherited prion disease and they are subdivided into three groups based upon clinical presentation: Familial CJD (fCJD), Fatal familial Insomnia (FFI) and Gerstmann–Sträussler–Scheinker Disease (GSS) (Figure 1.2; Lloyd *et al*, 2013).

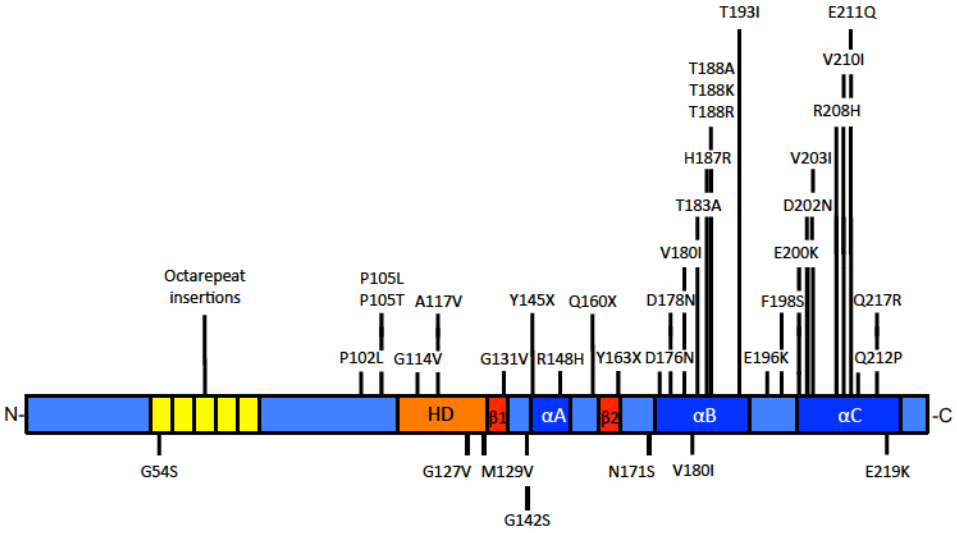
T193I
E211Q
V210I
T188A
T188K
T188R
R208H
H187R
V203I
T183A
D202N
V180I
E200K
Octarepeat
insertions
P105L
P105T
A117V
Y145X
Q160X
D178N
F198S
Q217R
P102L
G114V
G131V
R148H
Y163X
D176N
E196K
Q212P
N
HD
β1
αA
β2
αB
αC
-C
G54S
G127V
M129V
N171S
V180I
E219K
G142S

Figure 1.2: Mutations and polymorphisms of PrP.

Mutations are displayed above the line diagram of PrP and polymorphisms are displayed below. 'X' denotes a premature stop codon. Posttranslational modifications have been omitted for clarity. Repeat regions are yellow, hydrophobic domains are orange and β and α secondary structure are indicated by red and dark blue, respectively. Adapted from Lloyd *et al.*, 2013.

1.5.1 Familial Creutzfeldt-Jakob disease

fCJD is characterized by a rapid progressive dementia and myoclonus. The first identified mutation was an expansion of the octapeptide repeats (Owen *et al.*, 1989; 1990; Goldfarb, *et al.*, 1991a). The most common cause of fCJD is the E200K mutation that is found in a number of clusters worldwide and is particularly prominent in a community of Libyan Jews (Goldgaber *et al.*, 1989; Goldfarb *et al.*, 1990; Hsiao *et al.*, 1991). A third mutation associated with fCJD is D178N (Goldfarb *et al.*, 1991b; Nieto *et al.*, 1991). As will be discussed in the next section, the presenting phenotype of this mutation is dependent upon the identity of residue 129 in *cis* to the mutated amino acid residue.

1.5.2 Fatal Familial Insomnia

FFI is clinically characterized by a progressive and profound inability to sleep which leads to hallucinations and eventual death (Lugaresi *et al.*, 1986). This disease has only a single known causative mutation of *PRNP*, D178N (Medori *et al.*, 1992). Interestingly, the clinical phenotype of this mutation is dependent upon the common polymorphism at position 129. If the mutated allele encodes for a methionine at position 129 the patient presents with FFI, a valine at this position leads to fCJD (Goldfarb *et al.*, 1992). Pathologically, the thalamus is primarily affected and this disorder is transmissible to laboratory rodents (Lugaresi *et al.*, 1986; Medori *et al.*, 1992; Tateishi *et al.*, 1995). Two lines of transgenic mice were recently developed that readily recapitulate these findings, further underscoring the importance of the common polymorphism at position 129 (Dossena *et al.*, 2008; Jackson *et al.*, 2009; Bouybayoune *et al.*, 2015). Fatal insomnia has also been found to occur sporadically, with very similar clinical findings (Mastrianni

et al., 1999; Parchi *et al.*, 1999; Blase *et al.*, 2013). Interestingly, an fCJD associated mutation (E200K) has also been documented to initially present with sleep disturbances and it has been suggested that this may be a more common feature of patients harbouring the E200K mutation than had been previously appreciated (Chapman *et al.*, 1996; Taratuto *et al.*, 2002).

1.5.3 Gerstmann-Sträussler-Scheinker Disease

GSS was first identified in Austria in the early nineteen hundreds as a primarily cerebellar syndrome and, as such, presents with an ataxic phenotype which can also be accompanied by pyramidal signs and cognitive decline (Gerstmann, 1928). There are a number of mutations associated with GSS, P102L being the most common (Hsiao *et al.*, 1989; Doh-ura *et al.*, 1989). Other common GSS causing mutations are A117V and F198S (Doh-ura *et al.*, 1989; Hsiao *et al.*, 1992). Octarepeat expansions have also been shown to cause GSS (Laplanche *et al.*, 1999). The primary pathological feature is the presence of widespread multi-centric amyloid plaques composed of PrP. Gliosis and spongiform change are less consistent and, interestingly, neurofibrillary tangles, composed of tau protein, have been observed in patients harbouring the F198S mutation and in a single patient with the A117V mutation (Ghetti *et al.*, 1995; Tranchant *et al.*, 1997).

1.6 Prion protein structure

PrP is composed of ~253 amino acids (human protein, numbering varies depending upon the animal in which it is derived) and while found in various tissues throughout the body, is primarily expressed by neuronal cells (Oesch *et al.*, 1985; Ford *et al.*, 2002). In humans, it is derived from the gene *PRNP* located on chromosome 20. The immature

protein is subjected to extensive post-translational processing; the first 22 residues direct the polypeptide through the endoplasmic reticulum (where they are subsequently cleaved) and ultimately to the cell surface (Turk *et al.*, 1988). As the protein transits the secretory system, it can be glycosylated at two N-linked glycosylation sites at amino acids N181 and N197 (Haraguchi *et al.*, 1989). The protein can be found as either un- mono- or di- glycosylated, perhaps reflecting a snapshot of molecules in stages of biogenesis towards a mature di-glycosylated form. A disulfide bond is formed between C179 and C214 (helices 2 and 3 respectively) that further stabilizes the structure (Turk *et al.*, 1988). The 23 C-terminal residues serve as a signal for the addition of a glycosyl phosphoinositol (GPI) moiety to anchor the protein to the outer leaflet of the cell membrane and are also removed from the mature protein (Stahl *et al.*, 1987).

PrP is composed of two domains, a so-called disordered N-terminus and a globular C-terminus (Figure 1.3A). The globular domain is composed of three α-helices and two β-sheets arranged in a β1- α1- β2- α2- α3 orientation (Riek *et al.*, 1996; 1997; Zahn *et al.*, 2000). The structure of this domain of the protein has been resolved for numerous species and has a remarkable structural conservation even between species with significantly divergent amino acid sequences (Calzolai *et al.*, 2005). Because of its disordered nature, the structure of the N-terminus of the protein (residues 23-~120) has not been obtained. Regardless, this region of PrP contains a number of important sequences. First is the octapeptide repeat region, the number of which depends on the organism under examination (there are 5 in the human protein). Each repeat is capable of binding divalent metal cations using histidine residues, notably Cu^{2+} (Hornshaw *et al.*, 1995; Brown *et al.*, 1997; Millhauser, 2007). It was recently demonstrated that the amount of Cu^{2+} bound to the repeats inversely correlates with incubation period in transgenic mice challenged with RML prions, a mouse adapted strain of scrapie.

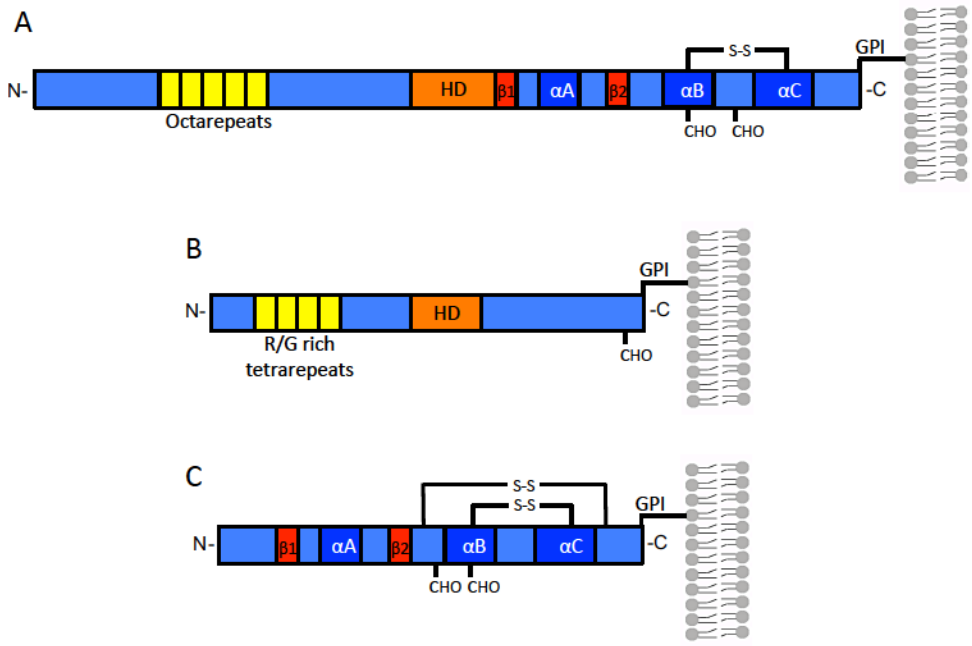
A
N-
Octarepeats
HD
β1
αA
β2
αB
αC
S-S
GPI
-C
CHO
CHO
B
N-
R/G rich
tetrarepeats
HD
GPI
-C
CHO
C
N-
β1
αA
β2
αB
αC
S-S
S-S
GPI
-C
CHO
CHO

Figure 1.3: Line diagrams of the prion protein family members.

Repeat regions are yellow, hydrophobic domains are orange and β and α secondary structure are indicated by red and dark blue, respectively. **A)** The prion protein **B)** Shadoo **C)** Doppel.

In this experimental paradigm, animals expressing a version of PrP that mimics the state of most bound Cu^{2+} have prolonged incubation times relative to wild type animals. The opposite effect, that is a shortened incubation period, was observed when the animals expressed a PrP representing the least Cu^{2+} bound state (Lau *et al.*, 2015). Further underscoring the physiological importance of the octarepeats is that expansion and deletion mutations cause hereditary prion disease; fCJD (Owen *et al.*, 1989) and GSS (Laplanche *et al.*, 1999), as mentioned above (Sections 1.5.1 and 1.5.3). A second important segment of the N-terminus of PrP is the hydrophobic domain (residues 112-135). This is the most highly conserved region of the protein across mammals and birds and has been identified as important for many of the interactions between PrP and its ligands (Wopfner *et al.*, 1999). Additionally, the hydrophobic domain is required for the formation of alternative topologies of PrP (Hay *et al.*, 1987a, 1987b; Yost *et al.*, 1990; Lopez *et al.*, 1990; Hegde *et al.*, 1998; 1999). In this case, the hydrophobic domain can act as a transmembrane domain; one such orientation, PrP^{Ctm} has been associated with neurodegeneration (Hegde *et al.*, 1999; Stewart *et al.*, 2005; Chakrabarti & Hegde, 2009). Further, a mutation of the hydrophobic domain (A117V) that increases the generation of PrP^{Ctm} causes GSS in humans and neurodegeneration in transgenic mice (Hegde *et al.*, 1998; Yang *et al.*, 2009). Finally, experimental N-terminal truncations and internal deletions of PrP have been expressed in mice and induce a spontaneous neurodegeneration that is remarkably similar to that induced by Doppel expression (See section 1.7) in the absence of PrP (Shmerling *et al.*, 1998; Li *et al.*, 2007). Surprisingly, expression of full length PrP rescues this neurodegeneration as well as that induced by Doppel expression, demonstrating an important role of the N-terminal region of PrP in neuroprotection (Shmerling *et al.*, 1998; Drisaldi *et al.*, 2004; Li *et al.*, 2007).

1.7 Prion protein family

There are two other proteins in the prion protein family; Shadoo and Doppel. Shadoo, encoded by the gene *Sprn* on chromosome 7 in mice was identified through a comparative genomics approach and bears a structural similarity to the N-terminus of PrP. This includes a repeat region (although in shadoo they are arginine rich tetrarepeats) and a hydrophobic domain. The C-terminus of shadoo bears less resemblance to PrP though a glycosylation site is present and the protein is GPI anchored (Figure 1.3B; Premzl *et al.*, 2003). The function of shadoo, like PrP, is unknown. It was briefly thought to be required for embryogenesis but was later disputed (Young *et al.*, 2009; Daude *et al.*, 2012). Over the course of prion disease, the levels of shadoo are decreased in a posttranslational manner that correlates with the increase in proteinase K resistant PrP (Watts *et al.*, 2007; 2011; Westaway *et al.*, 2011). It was proposed that this might represent a host response to prion infection and it was later demonstrated that over the course of disease, PrP^{C} is also down-regulated in a putative protective response to decrease the substrate required for PrP^{Sc} propagation (Mays *et al.*, 2014).

The other member of the prion protein family, Doppel, was discovered during early work with *Prnp* knockout animals. Initial efforts to generate mice that did not express PrP resulted in two distinct phenotypes; one group remained phenotypically normal to middle age (Bueler *et al.*, 1992; Manson *et al.*, 1994) while the other group developed a cerebellar ataxia and Purkinje cell loss (Sakaguchi *et al.*, 1996; Moore *et al.*, 1999). Doppel is encoded by *Prnd* which is located on chromosome 2 in mice; 16 kb downstream of *Prnp* (Moore *et al.*, 1999). During some attempts to generate *Prnp* knockout mice, *Prnd* was unwittingly placed under the control of the *Prnp* promoter by

deletion of a splice acceptor site in exon 3 of *Prnp*. This caused ectopic expression of *Prnd* in the brains of these animals that led to the observed toxicity (Moore *et al.*, 1999; Rossi *et al.*, 2001; Moore *et al.*, 2001). Where Shadoo structurally resembles the N-terminus of PrP, Doppel bears a striking structural similarity to the C-terminus of PrP though only shares ~25% sequence identity (Silverman *et al.*, 2000; Mo *et al.*, 2001; Lührs *et al.*, 2003). Mouse Doppel is a 179 residue polypeptide; like PrP, Doppel has a N-terminal signal peptide (25 residues) that is cleaved posttranslationally and a C-terminal hydrophobic domain of 25 residues that constitutes a signal for GPI anchoring (Moore *et al.*, 1999; Silverman *et al.*, 2000). Doppel possesses two disulfide bonds, between cysteine residues 109 and 143 and residues 95 and 148 (Mo *et al.*, 2001). The protein also has two N-linked glycosylation sites at residues 99 and 111 (Figure 1.3; Silverman *et al.*, 2001). Unlike PrP, mice that do not express Doppel have a clear phenotype; the males are sterile due to its role in spermatogenesis and sperm-egg interaction (Behrens *et al.*, 2002; Peoc'h *et al.*, 2002; Paisley *et al.*, 2004; Pimenta *et al.*, 2012).

1.8 Function and interactome of PrP

While *Prnp* knockout mice possess no overt phenotype, many studies have been undertaken to identify more subtle outcomes of the loss of PrP. The first such report demonstrated defective synaptic function due to an impairment of GABA receptor mediated fast inhibition and long term potentiation in the CA1 pyramidal neurons of the hippocampus that could be rescued by the reintroduction of PrP (Collinge *et al.*, 1994; Whittington *et al.*, 1995). Although this finding was soon disputed, it appears that PrP does have a role to play in synaptic function (Lledo *et al.*, 1996; Mallucci *et al.*, 2002; Curtis *et al.*, 2003; Maglio *et al.*, 2004). Disrupted Ca^{2+}-activated K^+ currents and the reorganization of mossy fibres in the hippocampus have been observed in brain slices of

Prnp knockout animals (Colling *et al.*, 1996; 1997). Alterations in circadian rhythm have also been noted; this is a particularly tantalizing finding due to the phenotype in humans carrying the causative mutation for Fatal Familial Insomnia (Tobler *et al.*, 1996). Other studies have exposed knockout mice to various convulsive agents and determined that they are more susceptible to drug induced seizures than their wild type counterparts (Walz *et al.*, 2005). PrP also appears to play a role during stroke; following an ischemic event, PrP levels in plasma and the tissue surrounding the insult increase (McLennan *et al.*, 2004; Weise *et al.*, 2004; Mitsios *et al.*, 2007) and animals lacking the protein have larger infarct sizes (Spudich *et al.*, 2005). PrP has also been reported to be involved in the self-renewal of neural and hematopoietic stem cells (Zhang *et al.*, 2006; Steele *et al.*, 2006; Lee & Baskakov, 2013; Prodromidou *et al.*, 2014). The normal processing of sensory information by the olfactory system is another proposed function of PrP. Mice devoid of PrP have an increased latency period when finding a food source and cannot discriminate between odours (Le Pichon *et al.*, 2009). Finally, PrP is required for the maintenance of myelin. In addition to the proteins expression in neurons, the proteolytic fragmentation of the protein was also found to be required. When either was impaired, through genetic ablation or mutation, a chronic demyelinating polyneuropathy was initiated (Nishida *et al.*, 1999; Bremer *et al.*, 2010). It is important to note that there are caveats associated with the interpretation of phenotypes found in knockout animals. It is possible that mutations can become fixed in linkage disequilibrium with the knockout allele and become selected for during breeding. To ensure that a phenotype is an authentic consequence of the lack of PrP, it is important to reintroduce the ablated gene and observe a restoration of function, that is to say, to perform a genetic complementation assay. It is also useful to observe the same candidate phenotype in independent null alleles maintained on different mouse genetic backgrounds. In practice, these sorts of analyses are lacking for many of the candidate phenotypes described above.

The one phenotype that is universally agreed upon is that the loss of PrP renders an animal completely refractory to prion disease (Büeler *et al.*, 1993).

1.9 Prion protein ligands

The interaction between the neural-cell adhesion molecule (N-CAM) and PrP was discovered using a mild formaldehyde crosslinking in murine neuroblastoma (N2a) cells and later confirmed *in vivo* (Schmitt-Ulms *et al.*, 2001; 2004). While it was shown that this interaction had no effect upon incubation times following intracranial challenge with prions (Schmitt-Ulms *et al.*, 2001), it was later demonstrated that the activation of fyn kinase by PrP occurs through this interaction with N-CAM and promotes neurite outgrowth (Mouillet-Richard *et* al., 2000; Chen *et al.*, 2003; Santuccione *et al.*, 2005). It was recently discovered that PrP deficient cells fail to undergo N-CAM polysialylation during the epithelial-to-mesenchymal transition (Mehrabian *et al.*, 2015). The polysialylation of N-CAM has been demonstrated to play a role in many of the biological process that have been linked to PrP including the organization of mossy fibres (Angata *et al.*, 1997; 2004), induction of long term potentiation and long term depression (Muller *et al.*, 1996), the differentiation of hematopoietic stem cells (Drake *et al.*, 2009), circadian rhythm (Prosser *et al.*, 2003), neurogenesis (Gascon *et al.*, 2010) and myelin repair (Mehanna *et al.*, 2009). While it is tempting to assign the molecular basis of these phenotypes to this novel function of PrP, much more work must be undertaken before such a statement can be made outside of the realm of speculation.

The interaction between PrP and the 37-kDa/67-kDa laminin receptor has also extensively studied (Rieger *et al.*, 1997). It has been demonstrated that upon binding to PrP this receptor mediates its reuptake into cells and that it is required for the

propagation of PrP^{Sc} (Gauczynski *et al.*, 2001; 2006; Leucht *et al.*, 2003). Indeed, expression of a mutant "decoy" laminin receptor in transgenic mice significantly increased incubation time and decreased proteinase K resistant PrP species (Pflanz *et al.*, 2009). However, polymorphisms of the laminin receptor do not appear to have any effect upon susceptibility to sCJD (Yun *et al.*, 2011). Stress-inducible protein 1 (STI1) is a third PrP binding partner that has received much attention (Zanata *et al.*, 2002). This interaction activates the cAMP/protein kinase A and Erk1/2 pathways and has been associated with neuroprotection (Chiarini *et al.*, 2002; Zanata *et al.*, 2002). Additionally, short term memory formation and long term memory consolidation were found to be diminished by preventing this interaction through an intra-hippocampal administration of antibodies directed towards either protein (Coitinho *et al.*, 2007).

Recent investigations of the physiological function of PrP have focused heavily upon its reported interaction with amyloid β oligomers (Aβo) and a subsequent deregulation of the effect of PrP upon glutamate receptors and potassium channels (Laurén *et al.*, 2009; Gimbel *et al.*, 2010; Alier *et al.*, 2011; Um *et al.*, 2012; 2013; Kudo *et al.*, 2012; Hass *et al.*, 2014; Hu *et al.*, 2014). The interaction of PrP with Aβo had been demonstrated to result in the activation of fyn kinase (Larson *et al.*, 2012; Um *et al.*, 2012). Using this as an experimental output, cells in which the PrP:Aβo interaction and fyn phosphorylation were disconnected were used to uncover the transmembrane mediator of this biological outcome (Um *et al.*, 2013). mGluR5, a G-protein coupled glutamate receptor, was unambiguously identified as the mediator of this effect of Aβo and PrP interacting at the cell surface (Um *et al.*, 2013). Further studies demonstrated that allosteric modulators of mGluR5 prevent the observed activation of fyn, providing a possible therapeutic target (Hass *et al.*, 2014). PrP has also been shown to diminish excitotoxicity driven by NMDA receptors by way of a direct interaction (Khosravani *et al.*, 2008; You *et al.*, 2012).

Additionally, the effects of Aβo upon NMDA receptor kinetics and subsequent activation of fyn kinase were shown to require PrP (You *et al.*, 2012; Um *et al.*, 2012). These interacting proteins are listed in Table 1.1.

Many questions remain regarding the role of PrP in normal physiology and pathogenesis. The following chapters address aspects of these questions through i) investigation of the consequences of the recently identified interaction between PrP and dipeptidyl aminopeptidase-like protein 6 and ii) the development and characterization of a newly identified GSS causing mutation of *PRNP*.

Table 1.1: PrP interacting channels and electrophysiological outcomes.

Protein name	Binding sites	Outcome	Reference
mGluR5	PrP residues 91-153 interact with the receptor	PrP is required to transduce neurotoxic Aβ signals through mGluR5	Um *et al.*, 2013; Hass *et al.*, 2014
NMDA receptor	PrP interacts with the NR2B subunit	Attenuated excitotoxicity that is disrupted by the Aβ-PrP interaction	Khosravani *et al.*, 2008; You *et al.*, 2012; Um *et al.*, 2012

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Chapter 2

The prion protein modulates A-type K^+ currents mediated by Kv4.2 complexes through Dipeptidyl Aminopeptidase-like protein 6

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Mercer, R. C. C., Ma, L., Watts, J. C., Strome, R., Wohlgemuth, S., Yang, J., Cashman, N. R., Coulthart, M. B., Schmitt-Ulms, G., Jhamandas, J. H., & Westaway, D.
The prion protein modulates A-type K+ currents mediated by Kv4. 2 complexes through dipeptidyl aminopeptidase-like protein 6. ***Journal of Biological Chemistry***. 2013; *288*(52), 37241-37255.

2.1 Introduction

Prion diseases involve the structural conversion of the primarily α-helical, *cellular* prion protein (PrP^{C}) to an infectious, β-sheet enriched form, PrP^{Sc}. The high degree of primary to tertiary structural conservation of mammalian PrP^{C} (Wopfner *et al.*, 1999; Calzolai *et al.*, 2005) leads to an expectation of an explicit phenotype in *Prnp*$^{o/o}$ mice but, aside from a total resistance to prion disease, this is not the case (Bueler *et al.*, 1992; Manson *et al.*, 1994). That said, within the inventory of subtle or disputed phenotypic changes in these mice are reports of impairment in $GABA_A$ receptor-mediated inhibition and long-term potentiation (Collinge *et al.*, 1994; Whittington *et al.*, 1995; Manson *et al.*, 1995; Curtis *et al.*, 2003). The diversity of altered endpoint measures is, to a certain extent, paralleled in the large number of reported PrP^{C} interacting proteins: The laminin receptor (Rieger *et al.*, 1997; Gauczynski *et al.*, 2001; Hundt *et al.*, 2001), the neural cell adhesion molecule (Schmitt-Ulms *et al.*, 2001; Santuccione *et al.*, 2005), stress-inducible protein-1 (Zanata *et al.*, 2002; Roffe *et al.*, 2010) and NMDA receptors (Khosravani *et al.*, 2008; You *et al.*, 2012) to name but a few (reviewed in Watts and Westaway, 2007).

In addition to previously described interacting proteins, dipeptidyl aminopeptidase-like protein 6 (DPP6, also known as DPPX) was identified using time-controlled transcardiac perfusion crosslinking while probing the PrP^{C} interactome (Schmitt-Ulms *et al.*, 2004). DPP6 is an auxiliary subunit of pore-forming Kv4.2 channels (Nadal *et al.*, 2003; Kim *et al.*, 2008) and, together with most KChIP (K^+ Channel Interacting Protein) isoforms (An *et al.*, 2000), DPP6 increases Kv4.2 trafficking to the cell surface and is required for the reconstitution of the properties of the native channel complex in heterologous cells (Seikel and Trimmer, 2009). A type II transmembrane protein, DPP6 has a number of splice variants differing in the length of the cytoplasmic, N-terminal portion (DPP6-S;

short) (Maffie *et al.*, 2009). KChIPs are a family of intracellular Ca^{2+} binding proteins with four major isoforms (1-4) and at least 16 splice variants (Pruunsild and Timmusk, 2005). Interactions between Kv4.2, KChIPs and DPP6 have been confirmed by proteomic analyses demonstrating the pulldown of KChIPs 1-3 in comparable ratios (Marionneau *et al.*, 2009). Assembled Kv4 channel complexes mediate sub-threshold-operation somatodendritic transient outward K^+ currents (A-type K^+ currents), which play important roles in the regulation of neuronal membrane excitability, somatodendritic signal integration and long-term potentiation (Birnbaum *et al.*, 2004; Johnston *et al.*, 2003). Given the interplay between Kv4.2 channels and DPP6 in neuronal function (Sun *et al.*, 2011) and the ability to crosslink DPP6 and PrP^C *in vivo* (Schmitt-Ulms *et al.*, 2004), we sought to delineate the nature and repercussions of a DPP6 - PrP^C interaction. To this end we investigated the impact of PrP^C upon the properties of A-type K^+ currents mediated by Kv4.2 channel complexes derived from co-expression of Kv4.2, KChIP2 and DPP6-S (Lundby *et al.*, 2010; Witzel *et al.*, 2012).

2.2 Experimental procedures

2.2.1 Plasmid construction

cDNAs encoding Kv4.2 (MMM1012-9498428 clone 30356567, pYX-Asc) and KChIP2 (MMM1013-7511937 clone 4503251, pCR4-TOPO) (Open Biosystems) were inserted behind the CMV and EF1α promoters respectively of pBud.CE4 with Quick Ligase (New England Biolabs) to create pBud.CE4.Kv4.2.KChip2. To construct pBud.DPP6-S.RFP (co-expression driven from the CMV and EF1α promoters respectively), total RNA was isolated from half mouse brains using acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987) and mRNA purified using the Oligotex mRNA Mini Kit (Qiagen). cDNA synthesis was performed in the presence of SUPERase-In RNase inhibitor (Ambion) using 0.5 μg mRNA, Omniscript reverse transcriptase (Qiagen), and an oligo-dT primer. PCR amplification of DPP6-S and DPP6-E was conducted using *Pfu* Turbo DNA polymerase (Invitrogen) with a nested PCR strategy and inserted between the *BamHI* and *XbaI* sites of the pcDNA3 mammalian expression vector (Invitrogen). A *PstI* site was added to the pcDNA3.DPP6-S for insertion into pBud.empty.GFP with Quick Ligase (New England Biolabs) after gel purification (QIAquick gel extraction kit (Qiagen)). GFP was replaced with RFP by digestion of pBud.DPP6-S.GFP and pBud.empty.RFP with *NdeI* and *NheI*, isolation of the desired fragments and ligation. HA-tagged DPP6-S, DPP6Δcyto, and DPP6 deletion mutants were generated by standard PCR-based techniques. The secreted DPP6 ectodomain construct was generated by fusing the DPP6 ectodomain to the PrP N-terminal signal sequence using an introduced *BsrGI* site. The Thy-1 plasmid (Thy-1.2 isoform) was generated by amplification of the Thy-1 open reading frame from the MGC:62652 cDNA clone by PCR and then insertion into pcDNA3. PrP A116V and M128V HD dup PrP were

created using the QuikChange (Stratagene) site-directed mutagenesis procedure with *Pfu* Turbo DNA polymerase. Other PrP mutants and Doppel plasmids were generated as previously described (Drisaldi *et al.*, 2004). All plasmids used for transfection were enriched using the EndoFree Plasmid Maxi kit (Qiagen).

2.2.2 Transfections

HEK293T cells were maintained in DMEM with 10% FBS (Gibco). Transfections were performed with Lipofectamine 2000 (Invitrogen) according to manufactures instructions. Cells used for electrophysiological recording were transfected with the following plasmids (fluorescent proteins co-expressed): pBud.DPP6-S.RFP, pBud.Kv4.2/KChIP2, pBud.wtPrP.GFP, pBud.octa13PrP.GFP and pBud.empty.GFP. These transfection mixtures included (as noted) siRNA directed against the 3' non-coding region of human *PRNP* mRNA (OriGene Trilencer-27 siRNA duplex; rGrGrCrUrUrArCrArArUrGrUrGrCrArCrUrGrArArUrCrG TT) or a scrambled control siRNA. 24 hours after transfection, cells were trypsinized and plated on cover slips for electrophysiological recording the following day. Cells used for complex formation assays were transfected as above using constructs based on a pcDNA plasmid vector backbone (with identities indicated in the figure legends) except for Figure 3C which used a pBud vector.

2.2.3 Generation of DPP6 antibodies

Peptides were synthesized (containing an N-terminal cysteine for KLH conjugation), conjugated to maleimide-activated KLH (Pierce) and then injected into New Zealand White rabbits. 03J2 was raised against residues 507-522 (DKRRMFDL EANEEVQK),

and 03K1 was raised against residues 788-803 (QDKLPTATAKEEEEED). Polyclonal antibodies were precipitated from serum using ammonium sulfate and affinity purified using the corresponding immunogenic DPP6 peptide conjugated to a SulfoLink column (Pierce).

2.2.4 Cell-surface biotinylation assay

Scaled down to a 6-well format and performed according to manufacturers (Pierce) instructions.

2.2.5 Formaldehyde crosslinking of intact cells

24 hours after transfection, cells (HEK293T, RK13 or N2a) were washed twice with PBS and incubated for 15 minutes at room temperature with 2% or 0.4% formaldehyde in PBS. The crosslinking reaction was quenched with 1.25M glycine for 10 minutes. Lysis was performed with RIPA buffer (50 mM Tris base, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% triton x100) containing protease inhibitors (Roche) at 4°C.

2.2.6 Immunoprecipitations

120 µg of cell lysate was incubated overnight at 4°C with 0.7 µg of α-HA antibody (Sigma) in a total volume of 50 µL. 100 µL of protein A/G agarose beads were washed three times with RIPA buffer containing protease inhibitor (Roche) before adding the overnight incubation before incubation at room temperature for 2 hours rotating end over end. After three washes with RIPA buffer, the beads were washed a final time with water and

the complexes were eluted from the beads (and crosslinks reversed) by incubation in sample buffer at 95°C for 30 minutes.

2.2.7 Animal husbandry

All animal protocols were in accordance with the Canadian Council on Animal Care and were approved by the Institutional Animal Care and Use Committees at the University of Alberta and the University of Toronto.

2.2.8 Mouse lines and preparation of mouse brain homogenates

Prnp$^{o/o}$ mice (*ZrchI* strain) were maintained on a C57/B6 background. DPP6$^{df5J/Rw}$ mice were a generous gift from John Schimenti and were maintained on a C3H background. Mice were perfused with saline, half brains were extracted and then either homogenized directly in nine volumes of 0.32 M sucrose with protease inhibitors (Roche) or snap frozen and stored at -80 °C.

2.2.9 Time-controlled transcardiac perfusion crosslinking

Mice (either C57/B6 or FVB strains) were subjected to time-controlled transcardiac perfusion crosslinking as described previously (19). Brains were homogenized in 0.32 M sucrose containing protease inhibitor (Roche).

2.2.10 Western blotting

Following cell lysis with RIPA or brain homogenization with 0.32 M sucrose and a BCA assay to determine protein concentration (Pierce), after boiling in sample buffer (with the exception of samples to be used for Kv4.2 detection due to protein aggregation) samples were electrophoresed with either Tris-glycine gels (8 or 12%) or 4-12% NuPAGE gradient gels (Invitrogen) and transferred to PVDF membranes (Millipore). Membranes were blocked with either 3% (Kv4.2) or 5% milk (all others except SHA31 blots which are not blocked) in TBS with 0.1% tween (Fisher). Membranes were incubated overnight in the indicated primary antibody (DPP6 clones 03K1 and 03J2 created in house; actin, Sigma; Kv4.2 clone K57/1 and KChIP2 clone K60/73, NeuroMab; SHA31, Spi-bio; 8H4 and 7A12, generous gifts from Man-Sun Sy; HA, Sigma; Thy-1 graciously provided by Roger Morris) and after washing were incubated in the appropriate HRP-conjugated secondary antibody for two hours at room temperature (Goat α-mouse or Goat α-rabbit, BioRad), washed again, exposed to ECL (Pierce) and visualized with light sensitive film (Fujifilm). Quantification was performed using ImageJ. The intensity of the ~191 kDa species detected by western blotting was normalized to intensity of "monomeric input" PrP in the same gel lane and plotted using an arbitrary scale. p values were determined using the student's t-test.

2.2.11 Immunocytochemistry

HEK293T cells were transfected with mouse PrP and HA tagged DPP6-S (both on a pcDNA backbone) and an siRNA against endogenous human PrP. 24hrs after transfection, cells were re-plated on glass coverslips and given 24hrs to recover. After rinsing twice with PBS and fixation with 4% paraformaldehyde, cells were washed 3

times with PBS and incubated in Sha31 (1:5000) overnight at 4 °C with rocking. Following 3 PBS washes, cells were blocked with 2% goat serum (Invitrogen) and incubated with goat α-mouse Alexa Fluor 594 (Invitrogen, 1:300) for 2 hours at room temperature. After washing and permeabilization with 0.2% triton x-100, cells were blocked and incubated overnight in 1:500 α-HA (Sigma) as above, washed 3 times, blocked, and incubated for 2 hours with 1:300 goat α-mouse Alexa Fluor 488 (Invitrogen) at room temperature. Nuclei were stained with 1μg/mL Hoechst and visualized using a Nikon Eclipse 90I motorized upright microscope (Nikon) and a CFI PL 40X/ N.A. 0.75 lens (Nikon) using the following excitation/emission filter properties: 325 – 375/500 – 575 nm with a 495 nm long-pass filter (blue channel), 440-510/475-575 nm with a 495 nm long-pass filter (green channel) and, 505-615/570-720 nm with a 595 nm long-pass filter (red channel). Images were acquired with a Retiga 2000R mono cooled camera, fast 1394 using NIS-Elements AR advanced research software at room temperature.

2.2.12 Recordings from HEK293T cells

Whole-cell recordings were always applied to two groups of HEK293T cells in a same day to minimize variation resulting from cell manipulation prior to recordings. Fluorescence-positive individual HEK293T cell were visualized (by way of the GFP and RFP encoded in the bigenic plasmids) and selected for recording under an Axioscope 2 Fs microscope (Zeiss) at 60X magnification. Oxygenated external solution was bath perfused at a rate of 1.0 mL/min. The external solution contains (in mM) 140 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 1.5 $CaCl_2$, 10 HEPES and 10 glucose; pH 7.4. Recording pipette solution was composed of (in mM) 140 potassium methylsulfate, 5 $MgCl_2$, 10 HEPES, 2 Na_2ATP, 0.2 NaGTP, and 0.5 EGTA; pH 7.3. Borosilicate glass capillaries (thin wall with filament, 1.5 mm; World

Precision Instruments) were pulled with a Narishige (PP-83) puller to yield recording pipettes with resistances of 2–4 MΩ. Series resistance of 4-7 MΩ in whole-cell configuration was compensated by 60%. Three different stimulation protocols in our experiments were employed to evoke Kv4.2-mediated A-type K^+ currents, which were used for building relationship of voltage-dependent activation (activation curve), steady-state inactivation (inactivation curve) and rate of recovery time from steady-state inactivation (curve of recovery time). A-type K^+ currents for making the activation curves were evoked during 300 ms depolarizing membrane potential ranging from -60 to 50 mV following a conditioning pulse of -110 mV for 400 ms. The A-type K^+ currents used to build the inactivation curves were evoked during a fixed depolarizing potential of 30 mV following 400 ms conditioning pulses of various membrane potentials, ranging from -110 to -20 mV. A-type K^+ currents for plotting the curve of recovery rate were elicited during a fixed 30 mV depolarizing membrane potential for 300 ms following a series of increasing time intervals of conditioning hyperpolarizing potential at -110 mV. All A-type currents presented were subject to subtraction of the outward currents (endogenous currents) evoked by a conditioning pulse of -20 mV from that evoked by any protocols mentioned above. The current signals were acquired at a bandwidth of 10 kHz using pClamp 9.2 software, and filtered with a 5 kHz low-pass Bessel filter using an Axopatch 200B amplifier (Molecular Devices).

2.2.13 Data analysis

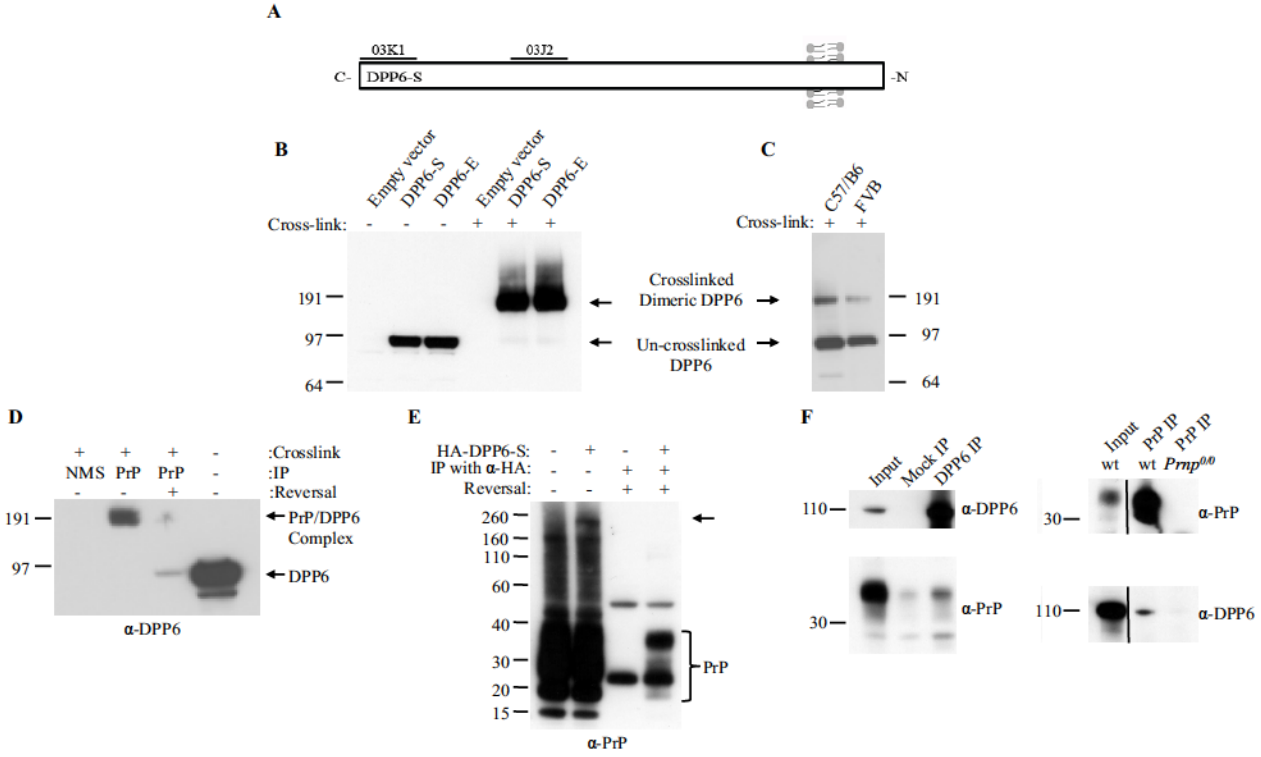
Time to half inactivation ($t_{1/2}$) is the time at which 50% of peak amplitude is inactivated at the indicated voltage. The activation curve was obtained by plotting normalized conductance of peak amplitude of A-type K^+ currents against its corresponding depolarizing membrane potential. The inactivation curve was built by plotting

normalized peak amplitude of A-type currents against its corresponding conditioning membrane potential. The curve of recovery rate from steady-state inactivation was obtained by plotting peak amplitude of A-type currents against its corresponding time intervals at conditioning hyperpolarizing membrane potential of -110 mV. The time constant (t_{rec}) of recovery rate was measured by fitting the curve to a single exponential function. Average values were expressed as mean ± SE, and statistical significance was evaluated by means of the two-tailed Student's unpaired t-test. The significance level for the t-tests was set at $p < 0.05$.

2.3 Results

2.3.1 Determinants of complex formation between PrP^C and DPP6

To facilitate our analyses of DPP6 and DPP6-containing protein complexes, two polyclonal peptide antisera, 03K1 and 03J2, were raised in rabbits (Figure 2.1A). Western blot analysis of brain homogenate from wt and DPP6-deficient mice ($DPP6^{df5J/Rw}$) (Hough *et al.*, 1998) assessed specificity (not shown), revealing immunoreactive species of ~110 kDa in accord with glycosylation of full-length DPP6 (~91 kDa) (Strop *et al.*, 2004). Immunohistochemistry demonstrated the widespread expression of DPP6 in the mouse brain (not shown), confirming previous reports (Clark *et al.*, 2008). The crystal structure of recombinant DPP6 is dimeric (Strop *et al.*, 2004) and protein species with an electrophoretic mobility consistent with dimers were observed in transfected cells and mouse brain homogenate after crosslinking and analysis by western blot (Figure 2.1B, C). When N2a cells expressing endogenous PrP^C were transfected with plasmids encoding DPP6 (DPP6-S and DPP6-E) (Seikel and Trimmer, 2009), crosslinked, immunoprecipitated with 7A1 (α-PrP) and membranes probed with 03K1, a novel species was observed at with a mobility of ~ 190 kDa (Figure 2.1D). These data indicate that PrP^C is located in membrane sub-domains that harbor dimeric DPP6, or that PrP and DPP6 exist in direct physical contact in a protein complex with a stoichiometry that totals to a M_r of ~190 kDa. Reversal of crosslinks yielded a species that co-migrated with full-length glycosylated DPP6-S. In a reciprocal analysis, following crosslinking, cells expressing HA-tagged DPP6-S yielded high M_r species and, after reversal, a signal compatible with glycosylated PrP^C (Figure 2.1E). Co-immunoprecipitation was also achieved in wt mouse brain without crosslinking using either 8B4 (α-PrP) or 03K1 for pull-down (Figure 2.1F).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03K1
03J2
C-
DPP6-S
-N
B
Empty vector
DPP6-S
DPP6-E
Empty vector
DPP6-S
DPP6-E
Cross-link: - - - + + +
191
97
64
Crosslinked Dimeric DPP6
Un-crosslinked DPP6
C
C57/B6
FVB
Cross-link: + +
191
97
64
D
+ + + -
NMS PrP PrP -
- - + -
:Crosslink
:IP
:Reversal
191
97
PrP/DPP6 Complex
DPP6
α-DPP6
E
HA-DPP6-S: - + - +
IP with α-HA: - - + +
Reversal: - - + +
260
160
110
60
40
30
20
15
PrP
α-PrP
F
Input
Mock IP
DPP6 IP
110
α-DPP6
30
α-PrP
Input
PrP IP
PrP IP
wt
wt
Prnp0/0
30
α-PrP
110
α-DPP6

Figure 2.1: DPP6 exists as a dimer and forms high M_r complexes with PrP^C.

A) Schematic representation of DPP6-S with the approximate locations of antibody epitopes indicated. DPP6-S is a type II membrane protein and is presented in the form – HOOC-ectodomain, TM domain, cytoplasmic tail-NH_2 - from left to right. **B)** Transfected membrane-anchored DPP6 exists as a dimer in N2a cells. N2a cells were transfected with plasmids encoding the indicated proteins, crosslinked with 2% formaldehyde as indicated, lysed, and then analyzed by western blotting with 03J2. Crosslinked dimeric DPP6 is the main species observed. **C)** DPP6 exists as a dimer in wt mouse brains. Brains from mice of the indicated strain were crosslinked *in vivo* using the tcTPC procedure and analyzed by western blotting 03J2. A signal at ~191kDa is apparent, indicative of dimeric DPP6. **D)** Immunoprecipitation of DPP6 by 7A12 (α-PrP). N2a cells were transfected with DPP6-S, crosslinked with 2% formaldehyde, lysed, and then immunoprecipitations performed with either normal mouse serum (NMS) or 7A12. Following pull-down, a band reactive to DPP6 antibodies was observed, indicative of complex formation between PrP^C and DPP6-S. After crosslink reversal, a signal was obtained compatible with full-length glycosylated DPP6 monomers. **E)** Immunoprecipitation of PrP^C by α-HA. N2a cells were co-transfected with HA-tagged DPP6-S and PrP^C. Following crosslinking with 2% formaldehyde, high M_r complexes are observed (arrowhead). PrP^C/DPP6 complexes were isolated by immunoprecipitation using α-HA and then crosslinks were reversed**. F)** Reciprocal co-immunoprecipitation of DPP6 and PrP^C from non-crosslinked mouse brain. Detergent-extracted wild-type mouse brain homogenates were subjected to immunoprecipitation with either 03K1 (left panels) or 8B4 (right panels).

Subsequently, mutant alleles were used to map regions within PrP^C and DPP6 required for association (Figure 2.2A, 3A, 4A). Deleting the intracellular, N-terminal portion of DPP6 ("DPP6Δcyto") had no effect upon complex formation with PrP^C (Figure 2.2A, B, E). "secDPP6", where DPP6 residues 56-803 are prefaced by the PrP N-terminal signal peptide causing secretion from the cell, was not associated with complex formation with PrP^C (Figure 2.2A, C, E, F). As this secDPP6 allele was readily detected in the culture medium, this implies a requirement for membrane association for complex formation rather than presence in the extracellular milieu. Also, anchoring to the membrane by a glycosylphosphatidylinositol moiety was not sufficient for complex formation, as the assay failed to detect DPP6/Thy-1 complexes (Homans *et al.*, 1988) (Figure 2.2D).

To delineate the role of DPP6's ectodomain (Strop *et al.*, 2004) in complex formation with PrP^C we used a series of DPP6 deletions with an N-terminal HA tag (Figure 2.3A). Control experiments confirmed that these DPP6 constructs were expressed at the cell surface (Figure 2.3B) and following crosslinking and immunoprecipitation with an α-HA antibody, crosslinks were reversed before analysis by western blot. PrP^C was recovered in conjunction with all DPP6 deletion mutants tested (Figure 2.3C), leading to the inference that DPP6 residues 56-80 contribute to complex formation. This was investigated further by performing the crosslinking assay with an internally deleted form of DPP6 Δ56-81 (this deletion covers the same interval but extends one residue further to isoleucine 81 to maintain the length of the hydrophobic TM region). For DPP6 Δ56-81 the ability to form complexes was lower than for wt DPP6 (Figure 2.3D), underscoring a role for the juxtamembrane region.

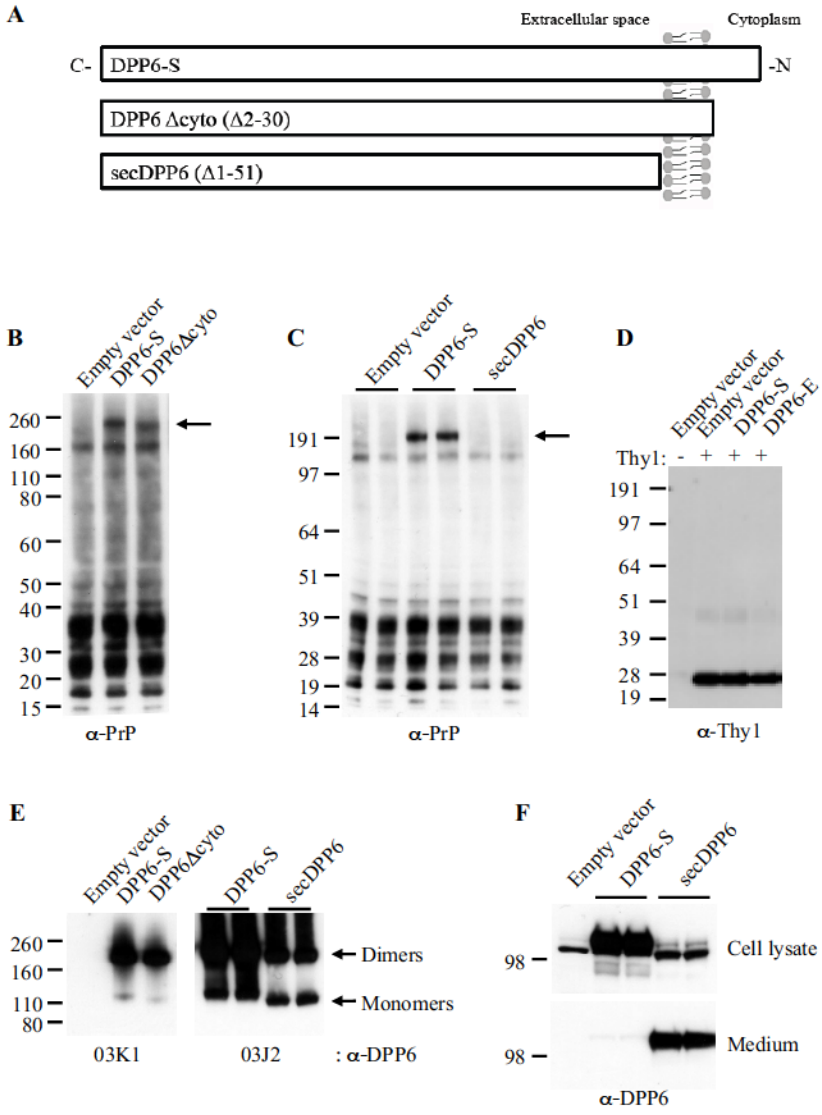

A
Extracellular space
Cytoplasm
C-
DPP6-S
-N
DPP6 Δcyto (Δ2-30)
secDPP6 (Δ1-51)
B
Empty vector
DPP6-S
DPP6ΔCyto
260
160
110
80
60
50
40
30
20
15
α-PrP
C
Empty vector
DPP6-S
secDPP6
191
97
64
51
39
28
19
14
α-PrP
D
Empty vector
Empty vector
DPP6-S
DPP6-E
Thy1: - + + +
191
97
64
51
39
28
19
α-Thy1
E
Empty vector
DPP6-S
DPP6ΔCyto
DPP6-S
secDPP6
260
160
110
80
Dimers
Monomers
03K1
03J2
: α-DPP6
F
Empty vector
DPP6-S
secDPP6
98
Cell lysate
98
Medium
α-DPP6

Figure 2.2: Membrane anchorage of DPP6 is required for complex formation with PrP^C.

(A) Schematic representation of DPP6 mutants. DPPXΔcyto lacks all but three residues of the cytoplasmic domain whereas secDPPX directs the secretion of the DPP6 ectodomain into the lumen/extracellular space. (B-F) N2a cells were transfected with the indicated plasmids, crosslinked with 2% formaldehyde, lysed, and complexes (arrows) analyzed by western blotting. (B-C) The cytoplasmic domain of DPP6 is not required for complex formation with PrP^C whereas the secreted DPP6 ectodomain fails to form a complex with PrP^C. (D) No high M_r complexes are observed between Thy-1 and DPP6. (E) Both DPP6Δcyto and secDPP6 are capable of forming dimers. (F) Minimal amounts of wild-type DPP6-S are present in the conditioned medium whereas large amounts of secDPP6 are found in this fraction. Accordingly, a smaller proportion of secDPP6 is found in the cell lysate.

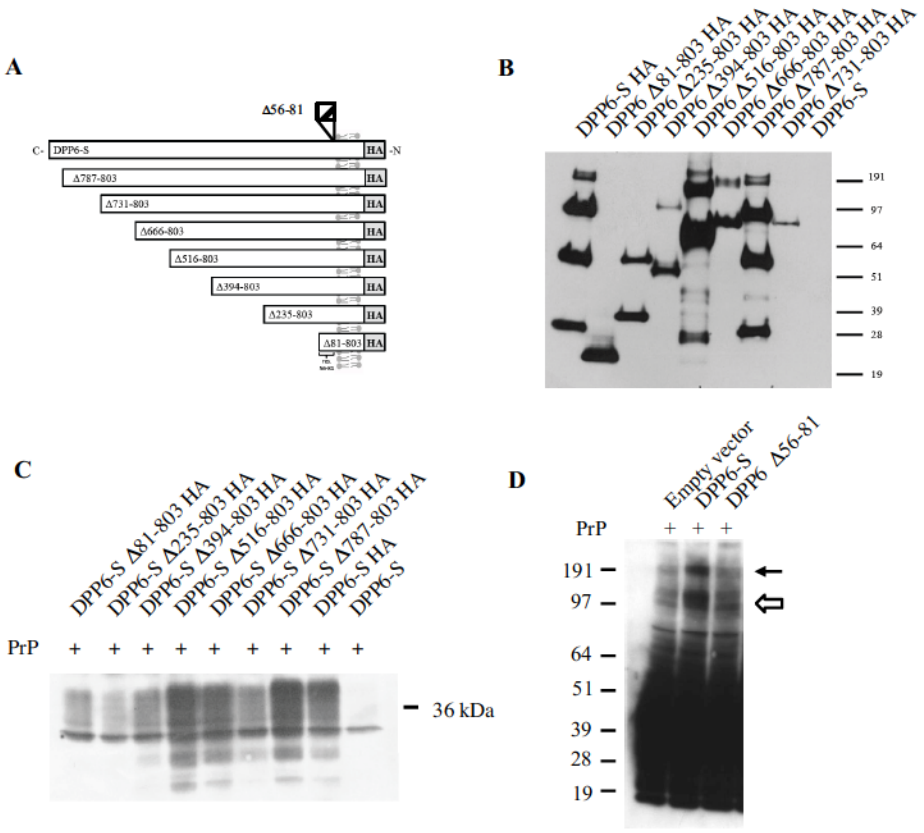
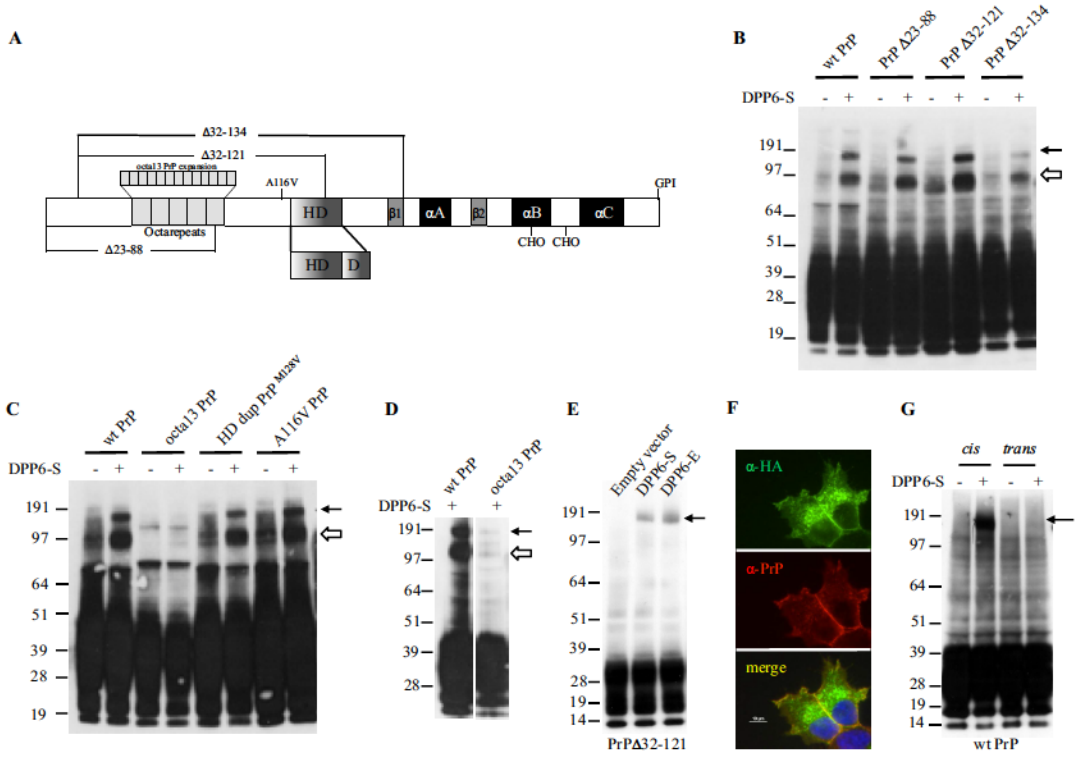

A
Δ56-81
C-
DPP6-S
HA
-N
Δ787-803
Δ731-803
Δ666-803
Δ516-803
Δ394-803
Δ235-803
Δ81-803
B
DPP6-S HA
DPP6 Δ81-803 HA
DPP6 Δ235-803 HA
DPP6 Δ394-803 HA
DPP6 Δ516-803 HA
DPP6 Δ666-803 HA
DPP6 Δ787-803 HA
DPP6 Δ731-803 HA
DPP6-S
191
97
64
51
39
28
19
C
DPP6-S Δ81-803 HA
DPP6-S Δ235-803 HA
DPP6-S Δ394-803 HA
DPP6-S Δ516-803 HA
DPP6-S Δ666-803 HA
DPP6-S Δ731-803 HA
DPP6-S Δ787-803 HA
DPP6-S HA
DPP6-S
PrP
+ + + + + + + + +
36 kDa
D
Empty vector
DPP6-S
DPP6 Δ56-81
PrP
+ + +
191
97
64
51
39
28
19

Figure 2.3: DPP6-S determinants of complex formation with PrP^C.

Schematic of sequential C-terminal DPP6-S deletion intervals. The internal deletion interval D56-81 inserted into wt DPP6-S is also shown (cross-hatching). "HA" denotes the hemagglutinin epitope tag. (B) DPP6 truncations are expressed on the cell surface. Transfected cells were treated with a commercial cell surface biotinylation kit and avidin captured proteins run on a 4-12% NuPAGE gel. Western blots were performed with an α-HA antibody. Lower M_r bands are inferred to represent fragments that retain the HA tag but not the C-terminal epitopes that were used for polyclonal antibody generation. (C) HA-tag immunopurification assay to assess co-precipitation of PrP^C. (D) Western blot demonstrating a lack of complex formation between DPP6 Δ56-81 and PrP^C. RK13 cells were transfected with the PrP and the indicated form of DPP6, crosslinked with 0.4% formaldehyde, lysed and analyzed using Sha31 (α-PrP). The ~191 kDa complex is indicated by a black arrow, the ~100kDa complex is indicated by an open arrow.

With regards to PrP, N-terminal deletions Δ23-88 and Δ32-121, which encompass most or all of the octarepeats, had subtle effects on complex formation (Figure 2.4A, B, E). The contribution of an interval that encompasses the first β-strand of the PrP structure (Riek *et al.*, 1996; 1997) to complex formation with DPP6 is illustrated by the performance of the Δ32-134 PrP allele (Figure 2.4A, B). We also created PrP alleles with more C-terminal deletion intervals; aside from theoretical caveats (see "Discussion"), these were also limited by lower expression levels than wt PrP and were not examined further. A survey of PrP alleles from genetic prion diseases defined a striking result associated with the octarepeat region (Laplanche *et al.*, 1999; Drisaldi *et al.*, 2004), but not with mutations C-terminal to this position (Doh-ura *et al.*, 1989; Hsiao *et al.*, 1991; Hinnell *et al.*, 2011). Octa13 PrP, an expansion of the octarepeats to a total of 13, was less efficient than wt PrP at complex formation with DPP6 (Figure 2.4A, C, D). Densitometry revealed that octa13 PrP formed ~191 kDa complexes at the level of 23.3 ± 16.4% that of wt PrP (100 ± 12.4%; n=3, $p<0.002$). As a preface to the functional studies described below, this result was confirmed in crosslinking studies of HEK293T cells (Figure 2.4D). Furthermore, we confirmed that full-length HA tagged DPP6-S and PrP^C co-localize at the cell surface of the HEK293T cells used for electrophysiology (Figure 2.4F). Lastly, interactions between PrP^C and DPP6 were found to take place in *cis* in a cell biological sense, as demonstrated by the lack of complex formation when cells expressing PrP^C or DPP6 are co-cultured before crosslinking and lysis (Figure 2.4G). Interestingly, Doppel, with a 3D fold similar to that of the PrP^C C-terminus (Mo *et al.*, 2001), can also form complexes with DPP6 isoforms (Figure 2.5).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Δ32-134
Δ32-121
octa13 PrP expansion
A116V
GPI
HD
β1
αA
β2
αB
αC
Octarepeats
CHO CHO
Δ23-88
HD D
B
wt PrP
PrP Δ23-88
PrP Δ32-121
PrP Δ32-134
DPP6-S
191
97
64
51
39
28
19
C
wt PrP
octa13 PrP
HD dup PrP M128V
A116V PrP
DPP6-S
191
97
64
51
39
28
19
D
wt PrP
octa13 PrP
DPP6-S
191
97
64
51
39
28
E
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DPP6-S
DPP6-E
191
97
64
51
39
28
19
14
PrPΔ32-121
F
α-HA
α-PrP
merge
G
cis
trans
DPP6-S
191
97
64
51
39
28
19
14
wt PrP

Figure 2.4: PrP^C determinants of complex formation with DPP6-S.

A) Schematic representation of PrP indicating deletion intervals, point mutations, the octa13 expansion and a partial duplication of the hydrophobic domain found in a Canadian GSS patient ("HD Dup PrP"). **B-C)** Western blots demonstrating complex formation between DPP6 and allelic forms of PrP^C. RK13 cells were transfected with the indicated PrP forms and DPP6-S, crosslinked with 0.4% formaldehyde, lysed and analyzed using Sha31 (α-PrP). The ~191 kDa complex is indicated by a black arrow, the ~100kDa complex is indicated by an open arrow. Intensity of ~191kDa band expressed as a percentage, relative to wt PrP (value ± SD). **B)** – wt PrP: 100 ± 28.0%; PrP Δ22- 88: 70.3 ± 24.2%. n=3, $p > 0.1$; PrP Δ32-121: 102.6 ± 18.4%. n=3, $p > 0.4$; PrP Δ32-134: 43.4 ±31.1%. n=3, $p < 0.05$. **C)** – wt PrP: 100 ± 12.4%; octa13 PrP: 23.3 ± 16.4%. n=3, $p < 0.002$. M128V HD dup PrP: 85.0 ± 15.3%. n=3, $p > 0.1$; PrP A116V: 94.4 ± 13.4%. n=3, $p > 0.3$. **D)** HEK 293 T cells were co-transfected with DPP6-S and the indicated PrP plasmids and crosslinked with 0.4% formaldehyde. Intensity of ~191kDa expressed in arbitrary units – wt PrP: 100 ± 8.3%; octa13 PrP: 6.7 ± 3.7%. **E)** HEK293T cells were co-transfected with PrP Δ32-121 and the indicated DPP6 plasmids and crosslinked with 2% formaldehyde; PrP Δ32-121 was capable of forming complexes with both DPP6 isoforms. **F)** Co-localization of PrP^C and HA-DPP6 in HEK293T cells as assessed by immunofluorescent labeling with Sha31 (α-PrP) and α-HA. Scale bar = 10 μm. **G)** PrP^C/DPP6 complexes result from interactions occurring within the same cell. HEK293T cells were either co-transfected with PrP^C and DPP6-S (in "*cis*" with respect to cellular disposition) or singly transfected with either PrP^C or DPP6-S (in "*trans*" with respect to cellular disposition). 24 hours post-transfection, cells were trypsinized and either re-plated alone (*cis*) or with PrP^C and DPP6-S singly transfected cells mixed together (*trans*). After an additional 24-hour incubation (at which time the cells were confluent), cells were crosslinked with 2% formaldehyde and lysates analyzed by western blotting.

PrP^C - DPP6 complexes (arrow) are only formed when PrP^C and DPP6 are present in the same cell (i.e. co-transfected).

2.3.2 PrP^C modulates A-type K^+ currents mediated by Kv4.2 channel complexes

Following co-expression of the components of the Kv4.2 channel complex (Kv4.2, KChIP2 and DPP6-S; Figure 2.6A), whole-cell recordings were performed on transiently transfected HEK293T cells. Two series of A-type K^+ currents mediated by the Kv4.2 channel complex were investigated in the presence and absence of exogenous PrP^C (the isolate of HEK293T cells used here express endogenous human PrP^C; Figure 2.6A, 2.8A, 2.9A, 2.10A). As seen in Figure 2.6B, an A-type outward K^+ current was generated in response to depolarizing potentials. Following a rapid rise to peak amplitude, the current rapidly decayed despite a continued depolarizing step command. In the presence of exogenous PrP^C, the peak amplitude of the A-type K^+ currents at 20 mV was larger (14.5 ± 0.9 nA; average ± SEM, n=17) than that mediated by the Kv4.2 channel complex in its absence (10.2 ± 1.0 nA; n=21, $p<0.05$) (Figure 2.6C). The curve of voltage-dependent activation of A-type K^+ currents mediated by Kv4.2 channel complexes in the presence (triangles) and absence (squares) of exogenous PrP^C was created by plotting averaged normalized conductance (G/G_{max}) against corresponding depolarizing potential (Figure 2.6D). There was no significant difference in the voltage-dependent activation between the two groups.

To establish voltage-dependent steady-state inactivation, A-type K^+ currents were evoked by another voltage stimulus protocol (see Experimental Procedures). To highlight differences, we have shown two traces of A-type K^+ currents mediated by the Kv4.2 channel complex alone (left panel) and with exogenous PrP^C (right panel) at conditioning potentials of -110 and -60 mV respectively (Figure 2.6E). The ratio of normalized peak amplitudes of the currents evoked at conditioning potentials of -60 mV and -110 mV was

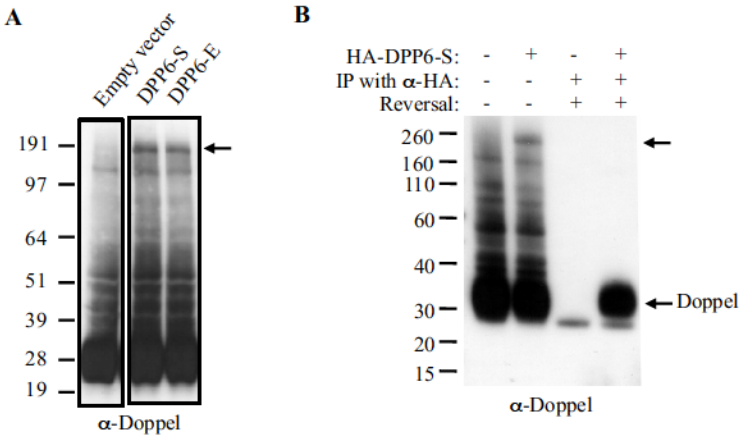

A
Empty vector
DPP6-S
DPP6-E
191
97
64
51
39
28
19
α-Doppel
B
HA-DPP6-S: - + - +
IP with α-HA: - - + +
Reversal: - - + +
260
160
110
60
40
30
20
15
Doppel
α-Doppel

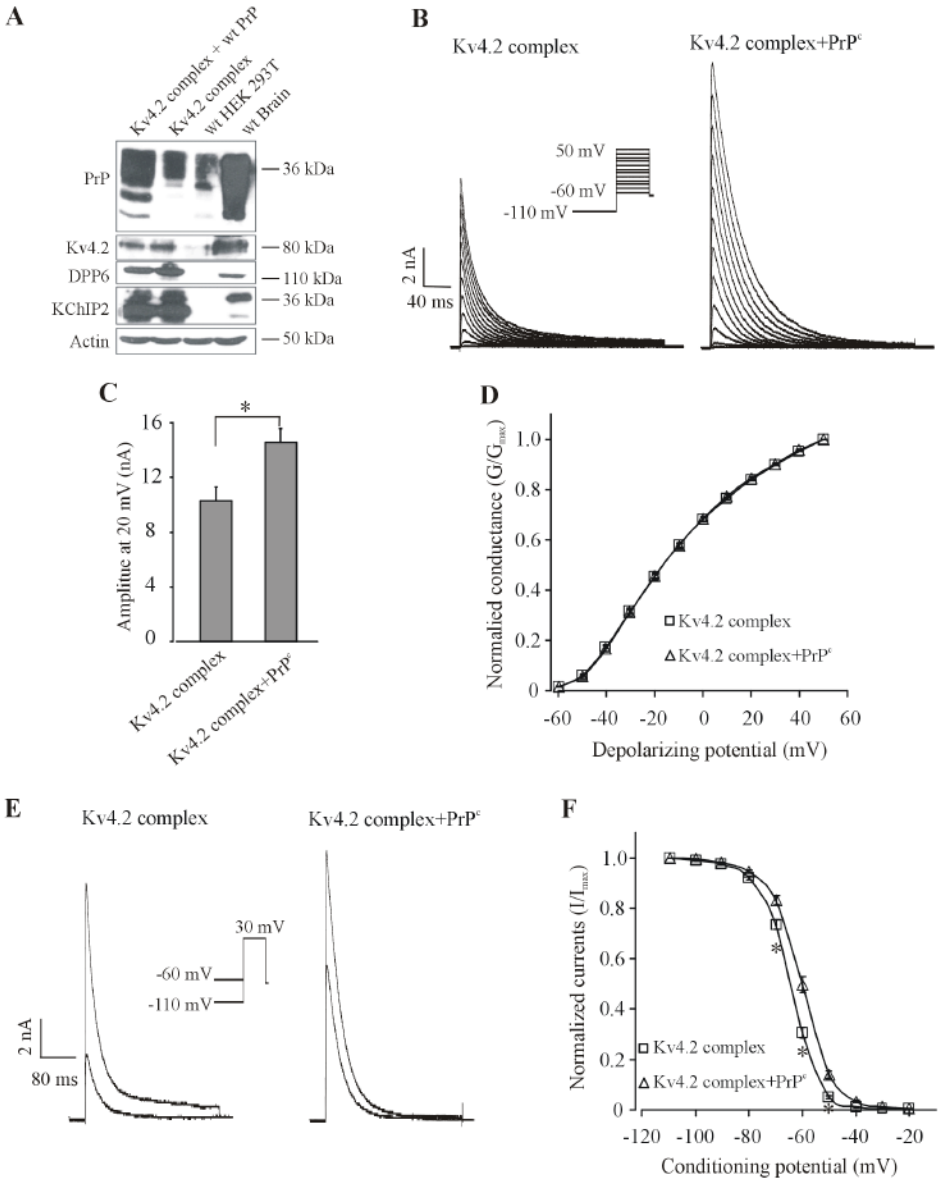
Figure 2.5: The prion protein family member Doppel can also form complexes with DPP6.

A) N2a cells were co-transfected with Doppel and the indicated plasmids and crosslinked with formaldehyde. **B)** Analysis Doppel in high M_r complexes with DPP6. N2a cells were co-transfected with DPP6-S-HA and Doppel. Following crosslinking, high M_r complexes are observed (arrow). Complexes were isolated by immunoprecipitation using α-HA and then crosslinks were reversed (by boiling).

0.29 in the absence of PrP^C versus 0.56 in its presence. The plot for voltage-dependent steady-state inactivation was obtained by plotting corresponding average normalized currents (I/I_{max}) against conditioning potential in the presence (triangles) and absence of exogenous PrP^C (squares) (Figure 2.6F). Significant differences between these two groups were noted at conditioning potentials of -50, -60 and -70 mV (n=10, $p<0.05$).

To measure decay of A-type K^+ current from peak amplitude to baseline, we used the time at which 50% of peak amplitude was inactivated at a given depolarizing potential (half-inactivation time) to quantitatively describe the time course for inactivation (Nadal *et al.*, 2003). Two representative traces of A-type K^+ currents evoked by a depolarizing potential of 50 mV in Figure 2.6B are shown with an expanded time-scale and correspond to the Kv4.2 channel complex with or without exogenous PrP^C (Figure 2.7A). The half-inactivation time was increased from 17.6 ms to 31.2 ms by the presence of exogenous PrP^C. On average, the half-inactivation time of A-type K^+ currents mediated by Kv4.2 channel complexes in the presence of exogenous PrP^C (n=8) was significantly longer than that mediated by the Kv4.2 channel complex alone (n=10) at all but two depolarizing potentials (Figure 2.7B; $p<0.05$).

We noted above (Figure 2.6F) for HEK293T cells expressing Kv4.2 complexes that when the conditioning potential applied is more positive than -40 mV, it is not possible to evoke an A-type K^+ current by stepping to a depolarizing potential of 30 mV. This status can be referred to as complete voltage-dependent steady-state inactivation of Kv4.2 channels and can be removed by first stepping to a conditioning hyperpolarizing potential, for example -110 mV, for a short duration. Consequently, to measure how fast A-type currents can recover from complete voltage-dependent steady-state inactivation, we recorded A-type K^+ currents evoked by a different stimulus protocol (Figure 2.7C).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Kv4.2 complex + wt PrP
Kv4.2 complex
wt HEK 293T
wt Brain
PrP
36 kDa
Kv4.2
80 kDa
DPP6
110 kDa
KChIP2
36 kDa
Actin
50 kDa
B
Kv4.2 complex
Kv4.2 complex+PrP^c
50 mV
-60 mV
-110 mV
2 nA
40 ms
C
Amplitue at 20 mV (nA)
16
12
8
4
0
*
Kv4.2 complex
Kv4.2 complex+PrP^c
D
Normalied conductance (G/G_max)
1.0
0.8
0.6
0.4
0.2
0
-60 -40 -20 0 20 40 60
Depolarizing potential (mV)
Kv4.2 complex
Kv4.2 complex+PrP^c
E
Kv4.2 complex
Kv4.2 complex+PrP^c
30 mV
-60 mV
-110 mV
2 nA
80 ms
F
Normalized currents (I/I_max)
1.0
0.8
0.6
0.4
0.2
0
-120 -100 -80 -60 -40 -20
Conditioning potential (mV)
Kv4.2 complex
Kv4.2 complex+PrP^c

Figure 2.6: PrP^C modulates the voltage-dependent properties of Kv4.2-mediated A-type currents in HEK293T cells.

A) Western blots demonstrating the presence or absence of the channel components. **B)** A series of A-type K^+ currents evoked by a stimulation protocol (insert) in cells transfected with the Kv4.2 channel complex with (right panel) and without exogenous PrP^C (left panel). **C)** Average peak amplitude of A-type K^+ currents recorded at depolarizing potential of 20 mV with (n=21) and without exogenous PrP^C (n=17). **D)** Average voltage-dependent activation of A-type K^+ currents with (triangles, n=8) and without (squares, n=10) exogenous PrP^C. **E)** Sample traces of A-type K^+ currents evoked by a stimulation protocol (inset) with (right panel) and without exogenous PrP^C (left panel). **F)** The curves of averaged voltage-dependent steady-state inactivation of A-type currents with (triangles, n=10) and without (squares, n=10) exogenous PrP^C . *indicates significant difference at a given depolarizing or conditioning potential between the two groups, $p<0.05$.

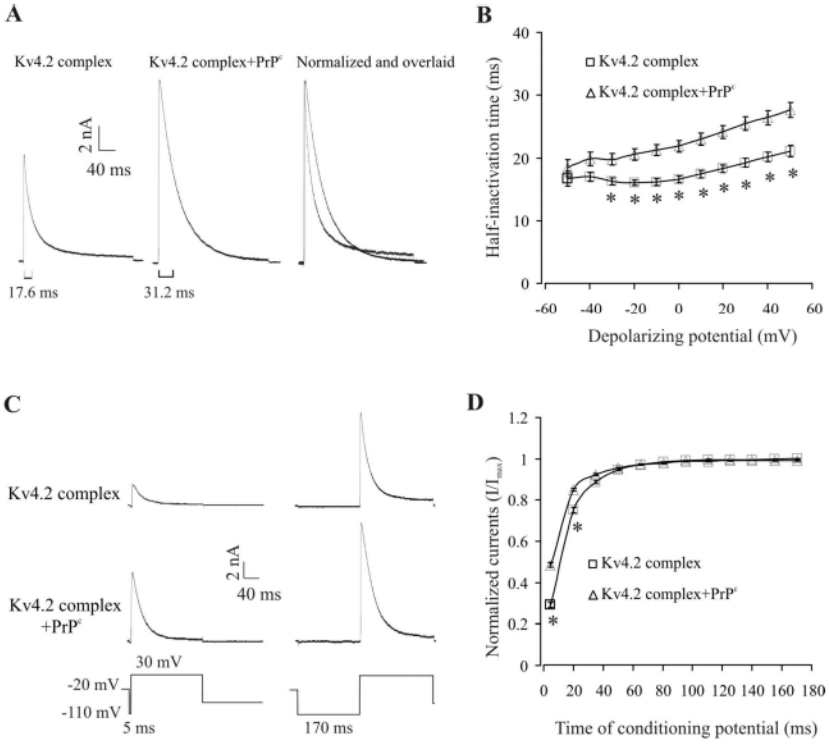
A
Kv4.2 complex
Kv4.2 complex+PrP^c
Normalized and overlaid
2 nA
40 ms
17.6 ms
31.2 ms
B
Kv4.2 complex
Kv4.2 complex+PrP^c
Half-inactivation time (ms)
Depolarizing potential (mV)
C
Kv4.2 complex
Kv4.2 complex +PrP^c
2 nA
40 ms
30 mV
-20 mV
-110 mV
5 ms
170 ms
D
Normalized currents (I/I_max)
Kv4.2 complex
Kv4.2 complex+PrP^c
Time of conditioning potential (ms)

Figure 2.7: PrP^C modulates time-dependent properties of Kv4.2-mediated A-type K^+ currents in HEK293T cells.

A) Re-plotted traces of A-type K^+ currents obtained from Figure 2.6B evoked by a depolarizing potential of 50 mV in cells without exogenous PrP^C (left panel), with exogenous PrP^C (middle panel), normalized and overlayed (right panel). Note that the time to half-inactivation is increased in the presence of PrP^C. **B)** Average half-inactivation time of A-type K^+ currents recorded at different depolarizing potentials with (triangles, n=8) and without (squares, n=10) exogenous PrP^C. **C)** Traces of A-type K^+ currents evoked by a stimulus protocol (bottom of panel), consisting of conditioning hyperpolarizing potential of -110 mV for 5 and 170 ms respectively with (middle panel) and without (upper panel) exogenous PrP^C. Note that the normalized amplitude of the current recorded with a 5 ms conditioning hyperpolarizing potential to that with 170 ms is smaller in the absence of exogenous PrP^C. **D)** Average curves of recovery rate from steady-state inactivation were created by plotting normalized peak currents (I/I_{max}) against corresponding time interval of conditioning hyperpolarizing potential in the presence (triangles, n=8) and absence (squares, n=10) of exogenous PrP^C. *indicates significant difference at giving depolarizing potential or time interval of conditioning hyperbolizing potential in the two groups ($p<0.05$). In both groups, recovery from steady-state was virtually complete after 100 ms following a conditioning hyperpolarizing potential of -110 mV.

Representative traces of the resulting A-type K^+ currents are shown for the presence and absence of exogenous PrP^C (Figure 2.7C) with conditioning potentials of -110 mV for 5 and 170 ms, respectively. The ratio of the amplitudes of the currents measured with 5 ms conditioning potentials of -110 mV and 170 ms were 0.24 (without) and 0.58 (with exogenous PrP^C). Average recovery rate from steady-state inactivation was obtained by plotting time of conditioning hyperpolarizing potential at -110 mV against corresponding normalized currents (I/I_{max}) (Figure 2.7D). The recovery rate was significantly faster in the presence of exogenous PrP^C (n=8) than in its absence (n=10) at the first two time intervals of 5 and 25 ms

2.3.3 PrP^C complex formation with DPP6-S is required for modulation of Kv4.2 channel properties

To ascertain whether or not the effects of PrP^C in this system are mediated through DPP6-S, we characterized the modulatory properties of PrP^C upon Kv4.2 mediated A-type K^+ currents in the absence of co-expressed DPP6-S (Figure 2.8A). These A-type K^+ currents showed smaller peak amplitudes, a right shift of the activation curve, a right shift of the steady-state inactivation curve, a longer half-inactivation time and a slower recovery rate from steady-state inactivation. These changes in the properties of A-type K^+ currents without DPP6-S are consistent with the previous findings observed by another group (Maffie *et al.*, 2009). However, exogenous PrP^C did not have a significant effect upon any A-type K^+ current properties in the absence of DPP6-S (Figure 2.8B-F).

Next, we studied the impact of co-expression of octa13 PrP (Figure 2.4A) with Kv4.2 complexes that include DPP6-S. While the stimulus protocols for these studies remained the same as previous recordings (with the exception of those used to investigate recovery

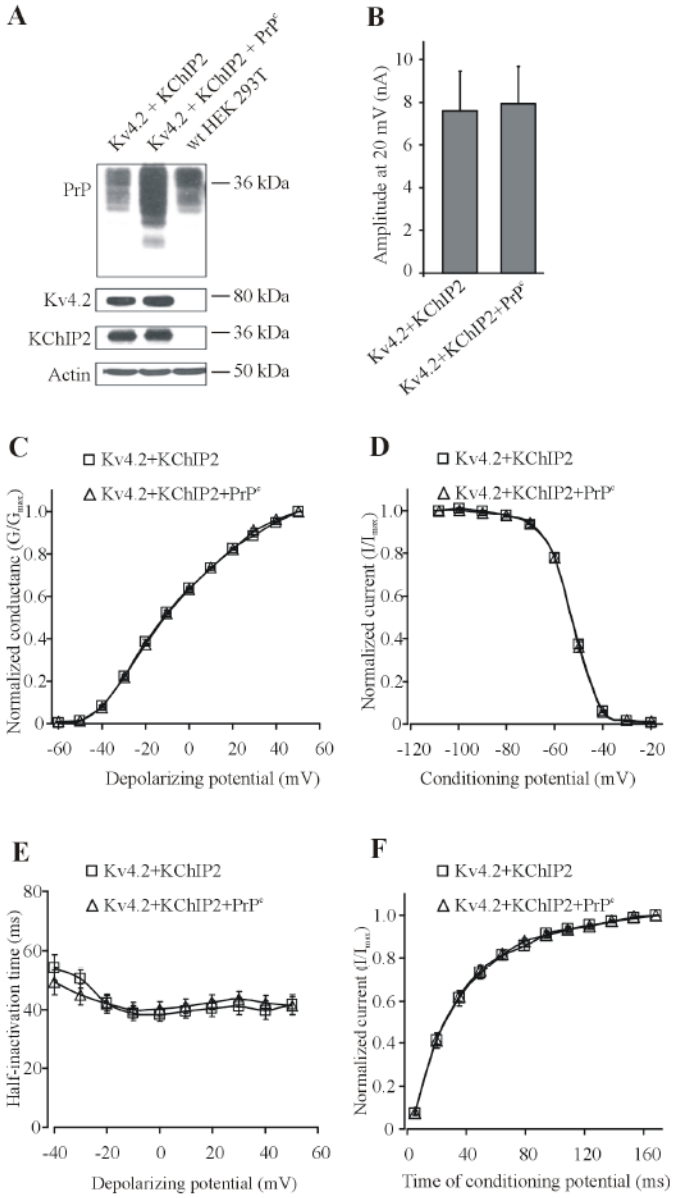
A
Kv4.2 + KChIP2
Kv4.2 + KChIP2 + PrP^c
wt HEK 293T
PrP
36 kDa
Kv4.2
80 kDa
KChIP2
36 kDa
Actin
50 kDa
B
Amplitude at 20 mV (nA)
Kv4.2+KChIP2
Kv4.2+KChIP2+PrP^c
C
Kv4.2+KChIP2
Kv4.2+KChIP2+PrP^c
Normalized conductance (G/G_max)
Depolarizing potential (mV)
D
Kv4.2+KChIP2
Kv4.2+KChIP2+PrP^c
Normalized current (I/I_max)
Conditioning potential (mV)
E
Kv4.2+KChIP2
Kv4.2+KChIP2+PrP^c
Half-inactivation time (ms)
Depolarizing potential (mV)
F
Kv4.2+KChIP2
Kv4.2+KChIP2+PrP^c
Normalized current (I/I_max)
Time of conditioning potential (ms)

Figure 2.8: Modulation of Kv4.2 mediated A-type K^+ a current by PrP^C is dependent upon the presence of DPP6-S.

A) Western blots demonstrating the presence or absence of channel components. **B)** Average peak amplitude of A-type K^+ currents mediated by Kv4.2+KChIP2 and by Kv4.2+KChIP2+exogenous PrP^C. **C)** Average curves of voltage-dependent activation of A-type currents. **D)** Averaged curves of voltage-dependent state-state inactivation half-inactivation time of A-type currents. **E)** Averaged half-inactivation times of A-type currents. **F)** Averaged curves of recovery rate from steady-state inactivation of A-type currents. For panels B-F data are from Kv4.2+KChIP2 transfected cells (squares, n=8) versus cells expressing Kv4.2+KChIP2+ exogenous PrP^C (triangles, n=9)

rate from steady-state inactivation, Figure 2.7C, D), we adjusted two aspects of the experimental design. First, the time interval for conditioning hyperpolarizing potential was reduced from the previous 20 ms to 5 ms to acquire more data points. Secondly, we used siRNA against human PrP mRNA to reduce the levels of endogenous PrP^C (Figure 2.9A); by this means we reduced crosstalk from wt PrP^C that could complicate the interpretation of data associated with transgene-encoded PrP. In this experimental context, wt PrP^C was found to modulate the Kv4.2 channel complex in the same manner as our previous data (Figure 2.9B-F). However, the properties of A-type K^+ currents in the presence of octa13 PrP showed no significant difference from those mediated by the Kv4.2 channel complex alone (Figure 2.9B-F, Table 1). These data indicate that octa13 PrP is a loss-of- function with regards to modulation of Kv4.2, a finding that parallels its >75% loss in efficiency at forming crosslinked complexes with DPP6-S (Figure 2.4C, D).

In a final set of analyses, we further investigated the inference that residues 56-80 of DPP6 are required for complex formation with PrP^C. A-type K^+ currents mediated by the Kv4.2 complex composed of Kv4.2, KChIP2 and DPP6 D56-81 showed smaller peak amplitudes, a right shift of the activation curve, a right shift of the steady-state inactivation curve, a longer half-inactivation time and a slower recovery rate from steady-state inactivation (Figure 2.10). These changes in the properties of A-type K^+ currents are similar to those observed in the absence of DPP6-S (Figure 2.8), indicating that DPP6 D56-81 is not fully functional. However, exogenous PrP^C did not modulate the properties of these A-type K^+ currents, further supporting the idea that this juxtamembrane region of DPP6 is required for complex formation with PrP^C (Figure 2.3, 2.10).

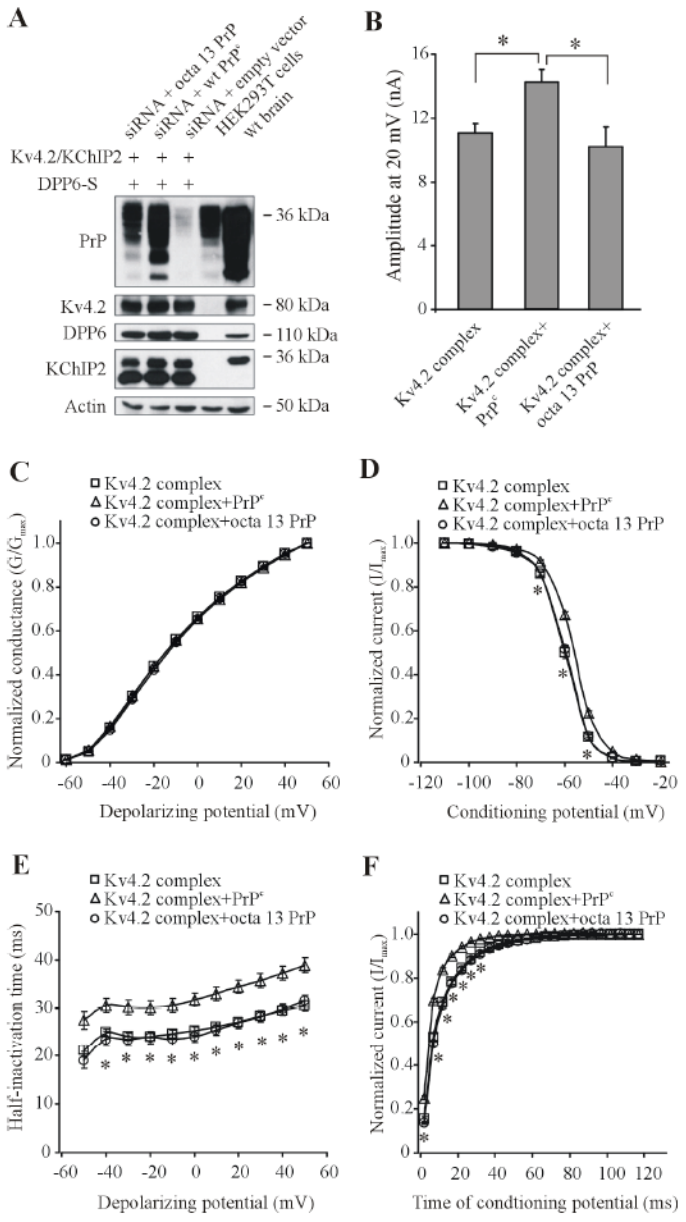
A
siRNA + octa 13 PrP
siRNA + wt PrP^c
siRNA + empty vector
HEK293T cells
wt brain
Kv4.2/KChIP2 + + +
DPP6-S + + +
PrP
36 kDa
Kv4.2
80 kDa
DPP6
110 kDa
KChIP2
36 kDa
Actin
50 kDa
B
Amplitude at 20 mV (nA)
Kv4.2 complex
Kv4.2 complex+ PrP^c
Kv4.2 complex+ octa 13 PrP
C
Kv4.2 complex
Kv4.2 complex+PrP^c
Kv4.2 complex+octa 13 PrP
Normalized conductance (G/G_max)
Depolarizing potential (mV)
D
Normalized current (I/I_max)
Conditioning potential (mV)
E
Half-inactivation time (ms)
F
Time of condtioning potential (ms)

Figure 2.9: Expansion of PrP octarepeats is loss-of-function for effects upon Kv4.2 mediated A-type currents.

A) Western blots demonstrating the presence or absence of channel components. **B)** Average peak amplitude of A-type K^+ currents at a depolarizing potential of 20 mV mediated by different Kv4.2 channel complexes, as indicated **C-F)** Averaged activation curves, steady-state inactivation curves, half-inactivation time, and recovery curves from steady-state inactivation of A-type currents mediated by Kv4.2 channel complexes. For data in panels C-F, for Kv4.2 complexes n=13, for Kv4.2 channel complex plus PrP^C n=9, and for Kv4.2 channel complex plus octa13 PrP, n=12. Channel compositions in traces are denoted within insets. There is no significant difference between the properties of A-type K^+ currents of the Kv4.2 channel complex alone and in the presence of octa13 PrP. Endogenous PrP^c was reduced by application of siRNA directed against human *PRNP*. *indicates significant difference (group of Kv4.2 channel complex plus exogenous PrP^C versus Kv4.2 channel complex plus octa13 PrP and/or Kv4.2 channel complex alone, $p<0.05$.)

Table 2.1: The properties of Kv4.2 mediated A-type currents in HEK 293T cells

	Activation (mV)			Inactivation (mV)		Kinetics (ms)	
	V_{on}	$V_{0.5}$	k	$V_{0.5}$	k	$t_{0.5}$ at 40 mV	τ_{Rec}
siRNA + Kv4.2 +KChIP2 + DPP6-S	-58.7	-21.5±2.6	26.2±2.7	-60.1±0.2	5.2±0.2	29.5±7.3	11.7±0.5
siRNA + Kv4.2 +KChIP2 + DPP6-S + PrP^C	-58.8	-22.6±3.1	28.3±3.1	-56.3±0.2	5.1±0.2	37.2±8.8	7.3±0.3
siRNA +Kv4.2 +KChIP2 + DPP6-S + octa13 PrP	-59.1	-20.7±2.1	25.2±2.2	-59.8±0.2	5.2±0.2	29.7±7.2	12.2±0.5

Table 2.1: The properties of Kv4.2-mediated A-type currents in HEK293T cells.

Activation: V_{on}, depolarizing membrane potential at which significant currents first become apparent; $V_{0.5}$, depolarizing membrane potential, in which 50% conductance of A-type currents is activated, obtained by fitting the activation curve to a first-order Boltzmann function; K, slope at midpoint of the curve. Inactivation: $V_{0.5}$, conditioning membrane potential, in which 50% of the Kv4.2 channels were inactivated, obtained by fitting the inactivation curve to a first-order Boltzmann function; K, slope at midpoint of the curve. Kinetics: $t_{1/2}$ is the time at which 50% of peak amplitude is inactivated at the indicated membrane potential; t_{rec} is time constant of recovery curve, obtained by fitting the curve to a single exponential function.

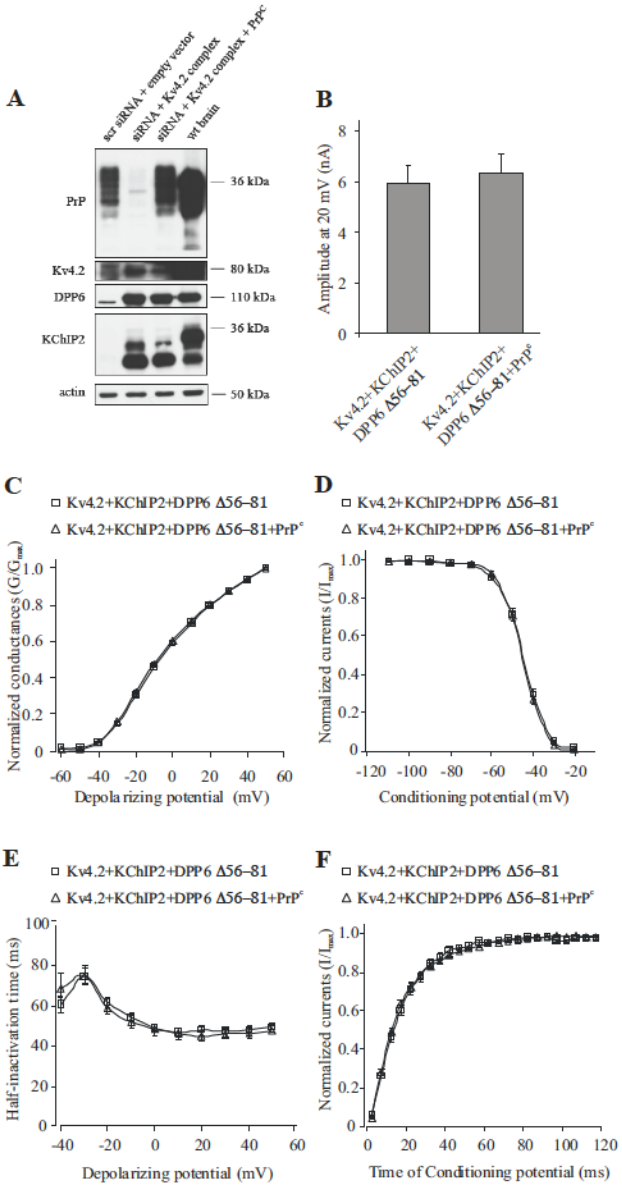
A
scr siRNA + empty vector
siRNA + Kv4.2 complex
siRNA + Kv4.2 complex + PrP^C
wt brain
PrP
36 kDa
Kv4.2
80 kDa
DPP6
110 kDa
KChIP2
36 kDa
actin
50 kDa
B
Amplitude at 20 mV (nA)
Kv4.2+KChIP2+ DPP6 Δ56–81
Kv4.2+KChIP2+ DPP6 Δ56–81+PrP^C
C
Kv4.2+KChIP2+DPP6 Δ56–81
Kv4.2+KChIP2+DPP6 Δ56–81+PrP^C
Normalized conductances (G/G_max)
Depolarizing potential (mV)
D
Kv4.2+KChIP2+DPP6 Δ56–81
Kv4.2+KChIP2+DPP6 Δ56–81+PrP^C
Normalized currents (I/I_max)
Conditioning potential (mV)
E
Kv4.2+KChIP2+DPP6 Δ56–81
Kv4.2+KChIP2+DPP6 Δ56–81+PrP^C
Half-inactivation time (ms)
Depolarizing potential (mV)
F
Kv4.2+KChIP2+DPP6 Δ56–81
Kv4.2+KChIP2+DPP6 Δ56–81+PrP^C
Normalized currents (I/I_max)
Time of Conditioning potential (ms)

Figure 2.10: PrP^C fails to modulate Kv4.2 mediated A-type K^+ currents reconstituted with DPP6 D56-81.

A) Western blots demonstrating the presence or absence of channel components. **B)** Average peak amplitude of A-type K^+ currents at a depolarizing potential of 20 mV. **C)** Average curves of voltage-dependent activation of A-type currents. **D)** Averaged curves of voltage-dependent steady-state inactivation of A-type currents. **E)** Averaged half-inactivation times of A-type currents. **F)** Averaged curves of recovery rate from steady-state inactivation of A-type currents. For panels B-F data are from HEK293T cells expressing Kv4.2, KChIP2 and DPP6 D56-81 (squares, n=8) versus cells expressing Kv4.2, KChIP2 and DPP6 D56-81 with exogenous PrP^C (triangles, n=9)**.** Endogenous PrP^c was reduced by application of a siRNA directed against human *PRNP*.

2.4 Discussion

2.4.1 PrP^C and ion channels

PrP^C is highly expressed in the central nervous system (Oesch *et al.*, 1985; Kretzschmar *et al.*, 1986; Peralta *et al.*, 2009) and, consequently, its role in regulating neuronal excitability is of great interest. Prior studies have centered upon perturbations in electrophysiological recordings made from brain slices of *Prnp*$^{o/o}$ mice (Collinge *et al.*, 1994; Whittington *et al.*, 1995; Manson *et al.*, 1995; Herms *et al.*, 2001; Carleton *et al.*, 2001; Mallucci *et al.*, 2002; Curtis *et al.*, 2003; Khosravani *et al.*, 2008; Prestori *et al.*, 2008; You *et al.*, 2012). Here we have investigated the influence of PrP^C upon Kv4.2 channel complexes by their reconstitution in HEK293T cells to study the currents produced by these complexes in isolation. These cells have been reported to have small endogenous delayed rectifier K^+ currents (Yu *et al.*, 1998). We found identical currents in our HEK 293T cell isolate (generally smaller than 200 pA) but did not detect A-type K^+ currents as previously reported by another group (Jiang *et al.*, 2002). Therefore, the A-type K^+ currents produced here (and measuring in the nA range) by reconstitution of the Kv4.2 channel complex are not contaminated by the presence of endogenous currents. More recently, expression of PrP Δ105-125 in transfected cells has been reported to induce spontaneous non-selective, cation-permeable ion currents (Solomon *et al.*, 2010; 2011). In this instance, because of the striking nature of the effect observed in HEK293T cells we undertook parallel studies with the same allele inserted in our expression vectors; but none displayed a spontaneous ionic current (n=10). To observe the currents, the cells were held at either -80 or +80 mV for several minutes. Under these conditions it would be unlikely to miss any spontaneous current activity (Solomon *et al.*, 2010; 2011). These experiments were performed both at 23 °C and 34 °C, and with pipette solutions containing either 0.5 mM EGTA or 10 mM EGTA (Solomon *et al.*, 2010;

2011). While these data (not shown) did not support the concept of a solitary action of PrP for this particular allele under our designated experimental conditions, wt PrP^C impacted the performance of co-expressed DPP6/Kv4.2 complexes, as elaborated below.

In prior analyses PrP^C has been implicated in the modulation of a variety of ion channels, including $GABA_A$ receptor/channels (Collinge *et al.*, 1994; Whittington *et al.*, 1995; Curtis *et al.*, 2003), calcium-dependent K^+ channels and NMDA receptors/channels (Colling *et al.*, 1996; Herms *et al.*, 2001; Khosravani *et al.*, 2008; Powell *et al.*, 2008; You *et al.*, 2012), stress-inducible protein-1-dependent intracellular Ca^{2+} fluxes mediated by the α7 nicotinic acetylcholine receptor (Zanata *et al.*, 2002; Roffe *et al.*, 2010; Beraldo *et al.*, 2010), and an AMPA dependent Zn^{2+} re-uptake phenomenon (Watt *et al.*, 2012). Remarkably, these studies convey a diversity of mechanisms whereby PrP^C modulates ion channels and neuronal excitability. For instance, enhanced and drastically prolonged NMDA-evoked currents in PrP^C knock out mouse neurons were the result of a functional up-regulation of NMDA receptors containing NR2D subunits (Khosravani *et al.*, 2008). On the other hand, impaired and depressed Ca^{2+}-dependent after-hyperpolarization potential in PrP^C knock out mouse neurons arises from an increased intracellular Ca^{2+} buffering capability (Herms *et al.*, 2001; Powell *et al.*, 2008). In this case, the free Ca^{2+} through influx of activated voltage-gated Ca^{2+} channels is decreased, and in turn depresses after-hyperpolarization potential. Here, the modulation of Kv4.2 properties by PrP^C requires interaction with DPP6-S and indicates that yet another, presently unknown, mechanism is employed. One possibility is increased trafficking of DPP6-S in the presence of PrP^C. A larger current amplitude and a faster recovery time from steady-state inactivation can be attributed to an increase of DPP6-S at the cell surface (Nadal *et al.*, 2003). However, the modulation of the voltage-dependence of inactivation and half-inactivation time by PrP^C in this study counters the effect of DPP6-S. This suggests that

the modulation of these channels by PrP^C occurs, at least partially, in a manner distinct from trafficking. Before addressing the puzzle presented by the pleiotropic actions of PrP^C, we will first consider molecular and mechanistic aspects of the PrP^C/A-type current paradigm.

2.4.2 PrP^C and the Kv4.2 channel complex

In genetic mapping of determinants necessary for PrP^C - DPP6 interactions (Figure 2.1-2.5), wt N-terminal sequences up to residue 121, which are considered natively unstructured in the un-metallated form of PrP (Reik *et al.*, 1997; Hornemann *et al.*, 1997), were not required. Deletions that begin to encroach on the C-terminal globular domain diminished the interaction (Figure 2.4B). While there are certain caveats concerning expression levels, chaperone interactions and global folding that apply to the use of C-terminal deletions (Muramoto *et al.*, 1997; Watts *et al.*, 2009), as yet we have been unable to find a crucial, common segment of PrP that is required for complex formation. We infer that a natively structured PrP^C globular domain (rather than a linear "epitope") is essential for complex formation. This is supported by the finding that Doppel also forms high molecular weight complexes with DPP6 (Figure 2.5). For DPP6-S, the intracellular portion had no effect on complex formation with PrP^C but there was a requirement for anchoring to the cell membrane (Figure 2.2). With progressive ectodomain deletions we determined that residues 1-81 retained the ability to immunoprecipitate PrP^C following crosslinking (Figure 2.3A-C). As the 55 N-terminal residues of DPP6-S are found either on the cytoplasmic side of, or spanning the membrane (and thus inaccessible to PrP^C) these data lead to an inference that the DPP6-S juxtamembrane region (residues 56-80) either complexes directly with the globular domain of PrP or is retained in PrP-enriched membrane domains by the action of an intermediary protein. This inference was bolstered by analyses of cells expressing an

internal deletion (residues 56-81) incorporated into full-length DPP6-S (Figure 2.3A, D). These findings have a potential parallel in analyses and complement data indicating that the extracellular portion of DPP6 is not necessary for modulation of Kv4.2 channel properties (Ren *et al.*, 2005).

2.4.3 Membrane protein assemblies and pleiotropic actions of PrP^C

To explain the curiously diverse actions of PrP^C in different experimental paradigms Linden and co workers (Linden *et al.*, 2008) hypothesized its action as a dynamic scaffold at the cell surface - one that can not only assemble membrane proteins in a cellular signaling microenvironment, but also one that can impact or adjust function. Findings presented here seem compatible with this proposal as can the loss-of-function phenotype of the octa13 allele in electrophysiological assays (Figure 2.9). Aside from the inefficiency of octa13 PrP at forming DPP6-S containing complexes (Figure 2.4C, D), our studies revealed it is incapable of forming another type of complex. This effect is apparent in both RK13 and HEK293T cells (Figure 2.4C, D), where a complex of ~110 kDa containing wt PrP (indicated by open arrows) is absent for octa13 PrP. The ~110 kDa complex could represent a PrP^C/DPP6-S monomer complex or interaction with a different protein, as suggested by failure to detect endogenous DPP6 in HEK293T cells (Figure 2.6A, 2.9A, 2.10A). In terms of potentially analogous effects for protein: protein interactions, it is notable that PrP with 14 octarepeats was loss-of-function for inhibiting β-cleavage of APP, whereas PrP^C without the octarepeat region retained activity (Parkin *et al.*, 2007). While a direct physical interaction between PrP^C and the β-cleaving enzyme has been questioned (McHugh *et al.*, 2012), the concept of plasma membrane signaling microdomains orchestrated by PrP^C may have considerable merit.

2.4.4 PrP^C, DPP6 and neurologic diseases

The >75% reduction in formation of ~191 and ~110 kDa complexes by octa13 PrP is notable (Figure 2.4C, D) and serves as useful control to measure against the performance of wt PrP^C. However, two other GSS alleles tested here did not behave in the same way and GSS pathogenesis is normally considered as "gain-of-function" due to misfolding of PrP. Whether the loss-of-function effect is due to a ~one third reduction in protein at the cell surface (50.1 ± 7.5% versus 78.1 ± 3.9% for octa 13 versus wt as measured by a biotinylation assay; not shown) is not clear. The electrophysiological observations made here apply to non-neuronal HEK293T cells and it remains possible that excitable cells may behave differently, but given prominent expression of DPP6 and PrP^C in the CNS, where they are located in close proximity (Schmitt-Ulms *et al.*, 2004), our findings do broach the question as to how modulation of A-type K^+ currents by wt PrP^C *via* DPP6-S might feature within a broader spectrum of neurological diseases. While the verdict may still be out on the DPP6 locus as a significant risk factor for autism spectrum disorders (Marshall *et al.*, 2008; Noor *et al.*, 2010) and ALS (van Es *et al.*, 2008; Cronin *et al.*, 2008; Fogh *et al.*, 2011), other possibilities remain. Wt PrP^C modifies the A-type K^+ currents from reconstituted Kv4.2 channel complexes by increasing peak amplitude, shifting the voltage-dependent steady-state inactivation curve to the right (more positive membrane potential), slowing inactivation and decreasing recovery time from steady-state inactivation. This overall impact of enhancement prompts two speculations. First, enhancement plays a critical role in the down-regulation of neuronal membrane excitability and is associated with a decreased susceptibility to seizures (Johnson *et al.*, 2003; Brinbaum *et al.*, 2004; Fransen and Tigerholm, 2010); interestingly, a reported phenotype of $Prnp^{o/o}$ mice is an increased vulnerability to drug-induced seizures (Walz *et al.*, 1999; Rangel *et al.*, 2007). Second, our previous work has established that PrP^C is

essential for the modulation of neuronal excitability by Aβ oligomers in cholinergic basal forebrain neurons (Alier *et al.*, 2011). Thus, the present findings implicate PrP^C regulation of Kv4.2 channels as a mechanism that could contribute to the observed effects of oligomeric Aβ – and perhaps other types of protein aggregate assemblies (Resenberger *et al.*, 2011) - on neuronal excitability and viability.

2.5 References

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Chapter 3

A Novel Gerstmann-Sträussler-Scheinker Disease insertion mutation causes disease in transgenic mice.

3.1 Introduction

3.1.1 Gerstmann-Sträussler-Scheinker Disease

Gerstmann-Sträussler-Scheinker Disease (GSS) is an autosomal dominant disorder with an onset from the second to seventh decade of life (Masters *et al.*, 1981; Goldfarb *et al.*, 1992; Kitamoto *et al.*, 1993; Michele *et al.*, 2003). This inherited amyloidosis is caused by a number of point mutations in the human gene *PRNP* that is located on the short arm of chromosome 20 that codes for the cellular prion protein (PrP^C) (Liao *et al.*, 1986). While the clinical presentation of GSS is highly variable and partially dependent on the causative mutation, the most common clinical phenotypes are cerebellar ataxia and pyramidal signs with eventual cognitive decline before inevitable death (Collins *et al.*, 2001). Multicentric plaques composed of truncated PrP fragments are found in the brains of these patients and used for neuropathological diagnosis (Ghetti *et al.*, 1995). Spongiosis may or may not be present (Mastrianni *et al.*, 1995; Ghetti *et al.*, 1995; Parchi *et al.*, 1998; Mallucci *et al.*, 1999).

3.1.2 GSS-P102L

The mutation causing a substitution of proline 102 with leucine was the first discovered and is by far the most common GSS mutation; indeed, it is the mutation found in the original family that was reported by Gerstmann (Hsiao *et al.*, 1989; Doh-ura *et al.*, 1989; Kretzschmar *et al.*, 1991; Young *et al.*, 1995). This mutation is overwhelmingly found in *cis* to methionine at codon 129 although there are rare reports of it being found in patients that are homozygous for valine at this position (Young *et al.*, 1997; Bianca *et al.*, 2003).

The most consistent pathological findings in the brains of GSS-P102L patients are PrP-immunoreactive deposits in the cerebral and cerebellar parenchyma with variable spongiform change (Hainfellner *et al.*, 1995; Piccardo *et al.*, 1998; 2007). Clinically, GSS-P102L presents as a progressive cerebellar syndrome with ataxia and difficulty with both speaking and coordinating eye movements. Dementia is noted at advanced stages of the disease. Like all forms of GSS, there can be significant divergence in both pathological and clinical phenotypes amongst family members (Hainfellner *et al.*, 1995). Interestingly, a Japanese family was identified that also carried a glutamic acid to lysine substitution at codon 219 in *cis* to the disease causing mutation at codon 102. While this polymorphism appears to be protective against sCJD, it modified the clinicopathological findings in this family such that one member had very few PrP-immunoreactive deposits and two others clinically presented with dementia with no cerebellar signs (Furukawa *et al.*, 1995; Shibuya *et al.*, 1998).

There are two main proteinase K resistant fragments associated with GSS-P102L, one of ~21 kDa and another of ~ 8kDa (Parchi *et al.*, 1998; Piccardo *et al.*, 1998). The presence of the 21 kDa fragment was found to correlate with spongiform change and the 8 kDa fragment with PrP-immunoreactive amyloid deposits (Parchi *et al.*, 1998). Sequencing analysis demonstrated that both of these fragments have a similar amino terminus (~residue 80) and that the 8 kDa fragment is C-terminally truncated near the first alpha helix (Parchi *et al.*, 1998). It was originally reported that the proteinase K resistant PrP found in the brains of GSS-P102L patients was derived solely from the mutant allele but the involvement of the wild type allele has since been demonstrated and may contribute to the variability seen in clinical and pathological phenotypes (Parchi *et al.*, 1998; Wadsworth *et al.*, 2006; Monaco *et al.*, 2012).

3.1.3 GSS-A117V

Another common GSS causing mutation is a substitution of alanine for valine at residue 117. To date, this mutation has only been found in *cis* with valine at position 129 (Doh-ura *et al.*, 1989; Hsiao *et al.*, 1991; Mastrianni *et al.*, 1995; Mallucci *et al.*, 1999). Pathologically, PrP-immunoreactive plaques are found throughout the brain though there is significant pathological divergence, both spatially and morphologically, within and between families. Spongiform change and gliosis are not consistently observed (Hsiao, *et al.*, 1991; Mastrianni *et al.*, 1995; Mallucci *et al.*, 1999). Neurofibrillary tangles composed of hyperphosphorylated tau protein have been noted in a single member of an affected family (Tranchant *et al.*, 1997).

The major proteinase K resistant fragments found in the brains of GSS-A117V patients are ~14 kDa and ~7 kDa (Piccardo *et al.*, 2001). The amyloid fibrils are composed primarily of the ~7 kDa species. This fragment was found to be truncated at the amino and carboxy termini and to be derived solely from the mutant allele (Tagliavini *et al.*, 2001). While the primary clinical feature of GSS is ataxia, GSS-A117V can also present as a telencephalic variant where dementia dominates over the more common ataxic phenotype (Hsiao *et al.*, 1991; Mallucci *et al.*, 1999). This mutation can adopt the alternative topology, PrP^{Ctm}, which may account for some of its associated pathology (Hegde *et al.*, 1999).

3.1.4 GSS-F198S

This mutation was identified as the cause of GSS in a family known as the Indiana Kindred. Similarly to the other GSS causing mutations discussed, only patients with

valine at position 129 in *cis* to the mutation have presented with disease (Ghetti *et al.*, 1989; Dlouhy *et al.*, 1992; Hsiao *et al.*, 1992). Patients homozygous for valine at residue 129 present with disease more than ten years before heterozygotes (Dloughy *et al*, 1992). GSS-F198S initially presents with a gradual loss of short-term memory and difficulty walking that later progress to dementia and severe movement problems including poor gait, rigidity and bradykinesia (Ghetti *et al.*, 1995). Pathologically, PrP-immunoreactive amyloid is noted in the cerebral and cerebellar parenchyma. Spongiform change is mild but astrocytic gliosis is noted. Neurofibrillary tangles composed of hyperphosphorylated tau protein are routinely observed in the brains of patients harbouring this mutation (Giaccone *et al.*, 1990; Ghetti *et al.*, 1994; Ghetti *et al.*, 1995). The amyloid core found in GSS-F198S is composed of 7 and 11 kDa N- and C-terminally truncated fragments that are derived from the mutant allele (Tagliavini *et al.*, 1991; 1994).

3.1.5 Insertion mutations

Mutations that cause an expansion of the octarepeats of PrP cause a variable clinicopathological phenotype and presents as CJD or GSS depending on the number of additional octarepeats that are inserted (Goldfarb *et al.*, 1991; 1992; 1993; Krasemann *et al.*, 1995; Laplanche *et al.*, 1995; 1999; Pietrini *et al.*, 2003). Considering GSS cases, even amongst patients with the same number of inserts there can be considerable variation in the levels of spongiform change but PrP immunoreactive plaques are a consistent feature (Laplanche *et al.*, 1999). Attempts to detect proteinase K resistant PrP in the brains of these patients has not been reported. A GSS presenting case with 8 additional octarepeats was successfully transmitted to a chimpanzee (Goldfarb *et al.*, 1992). The polymorphism at residue 129 of PrP also appears to play an important role in the clinical

course of these mutations. Patients who were homozygous for methionine died at younger ages than heterozygotes (Poulter *et al.*, 1992; Collinge *et al.*, 1992).

3.1.6 Mouse models and transmission properties of GSS

Transgenic animal models for GSS have been developed using two strategies: i) imposing the mutation found in humans upon mouse PrP and ii) developing mice expressing the mutated human allele. For reasons that are unclear, the latter strategy has failed to produce animals that develop spontaneous disease though they succumb to disease when intracerebrally inoculated with brain homogenate from patients with the corresponding mutation (Asante *et al.*, 2009; 2013). The former strategy has proven to be more successful. Transgenic mice expressing mouse PrP with the mutations P101L and A116V (mouse numbering) succumb to a spontaneous neurologic syndrome when the transgene is expressed at high levels (Hsiao *et al.*, 1990; Telling *et al.*, 1996; Nazor *et al.*, 2005; Yang *et al.*, 2009). One study introduced the P102L mutation into mice through a gene targeting approach. These mice failed to develop a spontaneous disease, perhaps due to the wild type expression levels of PrP that are not adequate to cause disease over the course of the animals relatively short life span (Manson *et al.*, 1999). It is noteworthy that these outcomes are not due to the overexpression of $Prnp^{a}$; animals with very high expression of this allele do not develop neurological abnormalities (Karapetyan *et al.*, 2009). Transgenic mice expressing a PrP with a total of 14 octarepeats (PG14PrP) also succumb to a spontaneous neurodegenerative disease (Chiesa *et al.*, 1998; Senatore *et al.*, 2012).

Mice with the P101L mutation that succumb to disease have not been reported to have any proteinase K resistant PrP even following inoculation with brain homogenate from

sick mice to accelerate disease (Hsiao *et al.*, 1990; 1994; Telling *et al.*, 1996; Nazor *et al.*, 2005). However, sick PG14 expressing animals display proteinase K resistant PrP at lower concentrations of enzyme; it is unknown if P101L animals would reveal proteinase K resistant fragments using these milder digestion conditions (Chiesa *et al.*, 1998). In the case of A116V mice, the tell-tale 7 kDa proteinase K resistant fragment is observed following exposure of brain homogenate to proteinase K (Yang *et al.*, 2009). Interestingly, following inoculation with brain homogenate from GSS patients, mouse lines expressing the human version of P101L (P102L) succumb to prion disease and have proteinase K resistant PrP, though the fragmentation pattern is different than observed in patients and a low molecular weight fragment is not present (Asante *et al.*, 2009).

The transmission of GSS-P102L to non-human primates and laboratory rodents has been achieved but is not consistent (Masters *et al.*, 1981; Baker *et al.*, 1990; Tateishi *et al.*, 1996). Attempts to transmit GSS-A117V and GSS-F198S to wild type mice were not successful (Ghetti *et al.*, 1995; Tateishi *et al.*, 1996). However, the use of transgenic mice expressing PrP with GSS associated mutations have been used to demonstrate that while GSS is not transmissible *per se*, brain homogenates of human samples or from mice that spontaneously develop disease can be used to accelerate the disease process in recipient mice; mice expressing wild type PrP are refractory to this treatment (Hsiao *et al.*, 1994; Telling *et al.*, 1996; Nazor *et al.*, 2005; Asante *et al.*, 2009; 2013). However, this disease initiation is unlikely to represent classical transmission; animals that do not succumb to disease within their lifetime due to low expression of the transgene are not susceptible to inoculation. This led to the conclusion that the previous observations of transmission in these transgenic mice represent acceleration of an inevitable disease as opposed to a *bona fide* transmission (Nazor *et al.*, 2005). It is unknown if brain homogenates from A116V mice are capable of accelerating disease (Young *et al.*, 2009).

3.1.7 A Novel PRNP allele found in a GSS patient

Recently, the Canadian Creutzfeldt-Jakob Disease Surveillance System discovered a novel *PRNP* mutation in a GSS patient (Hinnell *et al.*, 2011). A 34-year-old male presented with pronounced seizures, ataxia and cognitive decline. MRI revealed mild diffuse cortical atrophy and mild cerebellar vermian atrophy. Histopathological examination of a right middle frontal gyrus biopsy showed mild neuronal loss, microspongiosis and anti-PrP immunoreactive multicentric plaques that were consistent with GSS. Sequencing of *PRNP* revealed that the patient was heterozygous for a partial internal duplication of the hydrophobic domain. The 24 base-pair insertion between residues 129 (homozygous for valine) and 130 corresponds to the amino acid sequence LGGLGGYV and is predicted to disrupt the first β-strand of PrP (residues 128-131; Zahn *et al.*, 2000; Hinnell *et al.*, 2011). The patient died after a seven-year disease course at the age of 36 (Gerard H. Jansen, *personal communication*). The HD is a particularly interesting region as it is the most conserved area of PrP across animals and capable of adopting alternative membrane topologies which have been associated with the A117V allele in cell free translation systems (Hegde *et al.*, 1998; 1999; Wopfner *et al.*, 1999). Additionally, recent work has suggested that this region may be involved in the early structural rearrangements during the transition between PrP^{C} and PrP^{Sc}. Here, the authors co-crystalized human PrP with a nanobody that inhibits prion propagation; potentially stabilizing the protein in an intermediate structure. They found that the palindrome motif of the HD immediately preceding the described insertion adopted a β-structure forming a three-stranded antiparallel β-sheet with the already present two-stranded β-sheet (Abskharon *et al.*, 2013). Because of the novel nature of this allele and the interest in the hydrophobic domain in prion disease, we created transgenic mice in

order to characterise the neuropathological and biochemical changes induced by this partial duplication of the hydrophobic domain.

3.2 Experimental Procedures

3.2.1 Plasmid construction

pcDNA3.moPrP.wt was subjected to site directed mutagenesis to create pcDNA3.moPrP128V and pcDNA3.moPrPHDdup128V using the Agilent QuikChange II XL kit. 128V primers: SW132 5'-GGGGGGCCTTGGTGGCTACGTGCTGGGGAGCGCCATGAGC-3', SW133 5'-GCTCATGGCGCTCCCCAGCACGTAGCCACCAAGGCCCCCC-3'. The sequences were then verified using the universal T7 and SP6 primers before excision and relegation into pcDNA3 that had not been subjected to site directed mutagenesis.

3.2.2 Generation of transgenic mice

For generation of transgenic mice, sequences encoding PrP^{128V} and $PrP^{HDdup128V}$ were inserted into the MoPrP.Xho half genomic vector that had undergone modification to insert Aat II, Fse I and Mlu I recognition sites at the 5' end, allowing for directional cloning (Borchelt et al., 1996). PCR primers used: SW112 5'-AAAAACTCGAGAGTCCAATTTAGGAGAGCCAAG-3' (Xho I site added), SW134 5'-ACGCGTGCCCCTCATCCCACGATCAGG-3' (Mlu I site added). The Qiagen QIAquick Gel Extraction Kit was used to purify PCR products which were ligated into pCR2.1TOPO (Invitrogen) using New England Biolabs T4 DNA Ligase. Sequences were verified using universal T7 and M13 REV primers. TOPO constructs and MoPrP.Xho.mod were then digested with Xho I and Mlu I. Restriction Enzyme digest products were gel purified and ligated into MoPrP.Xho.mod. Primers used: SW96 5'-GGACTCCTGAGTATATTTCAG-3', SW97 5'-GCTACAGGTGGATAACCCCTC-3'. The resulting plasmids were purified using

the Qiagen EndoFree Plasmid Maxi Kit and digested with Not I, electrophoresed on 1% agarose gel without stain and purified with UltraClean GelSpin DNA Extraction Kit (Mo Bio Laboratories Inc.). This DNA was injected into the pronuclei of mouse FVB/N *Prnp* $^{+/+}$ embryos by the University of Calgary Transgenic Services. TgCRND8, TgTauP301L and Tga20 mice have been described previously (Fischer *et al.*, 1996; Chishti *et al.*, 2001; Murakami *et al.*, 2006)

3.2.3 Animal husbandry and inoculations

Animals were housed in groups of up to five under a twelve-hour light/dark cycle with food and water *ad libitum*. All protocols were in accordance with the Canadian Council on Animal Care and were approved by the Animal Care and Use Committee at the University of Alberta. Hemi brains of mice were flash frozen on dry ice upon collection or fixed in 4% paraformaldehyde for immunohistochemistry. Homogenates were made in cold PBS by successive passage through 18, 20, 21 and 25G needles or with an automatic homogenizer (Omni International). Inoculations were performed by intracerebral injection of 30 µl of 1% (wt/vol) brain homogenate.

3.2.4 Western blotting

Indicated amounts of brain homogenate or cell lysate were subjected to 10% Tricine-SDS-PAGE or 12% Tris-glycine SDS-PAGE (Bio-Rad) and transferred to PVDF membranes (Millipore). Membranes were equilibrated with 0.5% TBST and placed immediately into primary antibody in the case of Sha31 or equilibrated in 0.1% TBST and blocked in 5% milk before overnight incubation with the primary antibody at 4 °C. Membranes were washed with either 0.5 or 0.1% TBST and incubated with the appropriate HRP-conjugated secondary antibody for 2 hours at room temperature before

incubation with ECL (Pierce) and visualized with light sensitive film (Fujifilm) or a CCD camera (General Electric). Quantification was performed using ImageJ. Primary antibodies used: Sha31 (α-PrP; 1:30000; Spi-bio), 12B2 (α-PrP; 1:5000; a gift from Dr. Jan Langeveld; Langeveld *et al.*, 2006), VRQ61 (α-PrP; 1:5000; a gift from Dr. Human Rezaei; Moudjou *et al.*, 2004) and PrP248 (α-PrP; 1:1000; a gift from Dr. Human Rezaei; Moudjou *et al.*, 2001). α-actin (Sigma). Secondary antibodies used: horseradish peroxidase conjugated goat α-mouse and goat α-rabbit (Bio-Rad). Quantification was performed using ImageJ.

3.2.5 Proteinase K Digestion

250 µg of protein from a brain homogenate was incubated for 1 hour at 37°C with 10 µg/ml proteinase K (Roche) in a final volume of 250 µl (100:1 total protein:PK). Following incubation with 5mM (final concentration) PMSF for 5 minutes on ice, samples were centrifuged at 20,800 x g for 1 hour at 4°C. Pellets were resuspended in sample buffer containing 50 mM dithiothreitol (DTT) and western blots were performed.

3.2.6 PNGase F digestion

20 µg of protein from a brain homogenate was incubated overnight at 37°C with 100 U PNGase F (New England Biolabs) in a final volume of 20 µl according to manufactures instructions. Samples were precipitated in 5 volumes of methanol at -30 °C overnight. Following centrifugation at 20,800 x g for 30 minutes at 4 °C, pellets were resuspended in sample buffer containing 50 mM dithiothreitol and western blots were performed.

3.2.7 Sucrose Gradient Ultracentrifugation

Linear 10-45% sucrose gradients were prepared by layering 375 µl of increasing concentrations (5% steps) of sucrose (in PBS, pH 7.4 and 1% Sarkosyl) in OptiSeal™ polypropylene tubes (Beckman Coulter). Gradients were linearized by incubation overnight at 4°C. 250 µg of brain homogenate was brought to 300 µl in PBS (pH 7.4) containing 2% Sarkosyl and layered on top. Ultracentrifugation was performed at 268, 000 x g for 73 minutes at 4°C using a swinging bucket rotor and eight fractions were collected from the bottom of the tube. Equivalent volumes of each fraction were then interrogated for the presence of PrP by western blot.

3.3.8 Histopathological analysis

Sagittal sections were fixed in 10% phosphate buffered formalin and embedded in paraffin before sectioning to 4.5-6µm thick. Hematoxylin and Eosin staining was performed by incubation with Mayer's hematoxylin (Sigma), washed and incubated with Eosin Y (Sigma) before dehydration and subsequent mounting with cytoseal (Fisher). For immunodetection, slices were heated to 121 °C in 10 mM citrate buffer and allowed to cool to room temperature. Staining for PrP^{res} was accomplished by subsequent incubations in formic acid and 4 M guanidine thiocynate, respectively, and the slices were thoroughly rinsed and incubated overnight with biotinylated SAF83 (Cayman Chemicals). Glial fibrillary acidic protein immunodetection was also accomplished by heating to 121 °C in 10 mM citrate buffer followed by incubation in 3% peroxide, washing and an overnight incubation in biotinylated primary antibody (BD Biosciences). These slices are counterstained with Mayer's hematoxylin.

3.3.9 Cell culture

Rabbit kidney epithelial (RK13) cells were maintained in Dulbecco's Modified Eagles Medium (DMEM; Invitrogen) that had been supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen) at 37°C and 5% CO2.
Cells were transfected at a confluence of 80-90% using lipofectamine 2000 (Invitrogen) according to the manufacturers instructions.

3.3.10 Immunocytochemistry

RK13 cells were transfected with the indicated pcDNA3 PrP vector. 24hrs after transfection, cells were re-plated on glass coverslips and given 24hrs to recover. After rinsing twice with PBS and fixation with 4% paraformaldehyde, cells were washed 3 times with PBS before permeablilzation with 0.2% Triton X-100 (Sigma). Cells were rinsed 3 times with PBS and incubated in Sha31 (1:5000) overnight at 4 °C with rocking. Following 3 PBS washes, cells were blocked with 2% goat serum (Invitrogen) and incubated with goat α-mouse Alexa Fluor 488 (Invitrogen, 1:300) for 2 hours at room temperature. Nuclei were stained with 1µg/mL Hoechst and visualized using a Nikon Eclipse 90I motorized upright microscope (Nikon) and a CFI PL 40X/ N.A. 0.75 lens (Nikon) using the following excitation/emission filter properties: 325 – 375/500 – 575 nm with a 495 nm long-pass filter (blue channel) and 440-510/475-575 nm with a 495 nm long-pass filter (green channel). Images were acquired with a Retiga 2000R mono cooled camera, fast 1394 using NIS-Elements AR advanced research software at room temperature.

3.3.11 Cell Surface Biotinylation Assay

The Pierce cell surface protein isolation kit was scaled down to a 6-well format and

performed according to manufacturers instructions. Cells were washed twice with 1x PBS and incubated at 4 °C for 30 minutes with Sulfo-NHS-SS-Biotin in PBS. The reaction was ceased with 100 µl of the provided quenching solution and cells were scraped from the well and added to a 15 ml tube. The well was washed with 2 ml 1x TBS and added to the tube. The cell suspension was centrifuged at 500 x g for 3 minutes and the supernatant was discarded. The cells were washed a second time with 1x TBS and centrifuged again before discarding the supernatant. Cell lysis was performed with RIPA buffer (50 mM Tris base, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% triton x100) containing protease inhibitors (Roche) on ice with sonication on low power for 5 seconds ever 5 minutes for 30 minutes. Following centrifugation at 10,000 x g for 2 minutes at 4 °C, the clarified lysate was transferred to a new tube. At this point, an aliquot was removed to be analyzed in parallel as total protein. Biotinylated proteins were isolated using spin columns containing NeutrAvidin Agarose with 60 minutes of end over end mixing at room temperature. The column was washed with three cycles of provided wash buffer with added protease inhibitors and centrifugation for 2 minutes at 1000 x g. Protein was eluted using 1X sample buffer with 50 nM DTT and heating to 95 °C for 5 minutes and a 2 minute centrifugation at 1000 x g.

3.3 Results

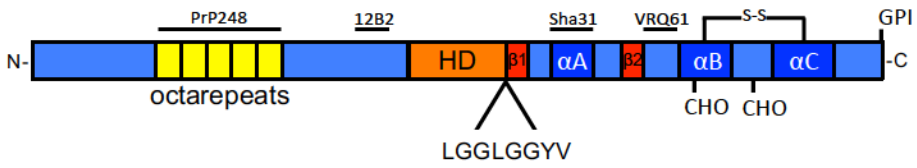
3.3.1 Generation of transgenic PrP^{HDdup} mice

We created two lines of transgenic mice expressing mouse PrP with the 8-residue insertion in the hydrophobic domain between amino acids V128/L129 (mouse numbering scheme; LGGLGGYV) dubbed PrP^{HDdup} (Hinnell *et al.*, 2011; Figure 3.1A). We also created two control lines expressing a mouse *Prnp*^{a} allele that incorporates the equivalent of the human valine 129 polymorphism, PrP^{128V}. Because mutations causing genetic prion disease are dominant in their natural setting (in the presence of wild type PrP), all animals used in the study are based on a FVB/N *Prnp* $^{+/+}$ background. Mice expressing PrP^{HDdup} succumbed to a spontaneous neurologic syndrome, with earliest time of onset in animals with the highest transgene expression levels (Figure 3.1B, Table 3.1). The primary clinical feature of this syndrome was slowly progressive ataxia. As the disease progresses, weight loss is apparent and the animals were euthanized when showing kyphosis and hypokinesia. Transgenic animals expressing PrP^{128V} at comparable levels to the mutated transgene did not present with any neurologic abnormalities and were used throughout the study as controls (Figure 3.1B, Table 3.1).

3.3.2 Biochemical signature of PrP^{HDdup} in murine brain

A proteinase K resistant PrP fragment of 6-11 kDa is observed in the brains of GSS patients, the size of which depends upon the causative mutation (Parchi *et al.*, 1998; Tagliavini *et al.*, 2001; Piccardo *et al.*, 2001). The smallest amyloid subunit extracted from the brains of GSS patients corresponds to a N- and C- insoluble, proteinase K resistant and thought to be exclusively derived from the mutant

A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Sha31
VRQ61
S-S
GPI
N-
HD
β1
αA
β2
αB
αC
-C
octarepeats
LGGLGGYV
CHO
CHO

B

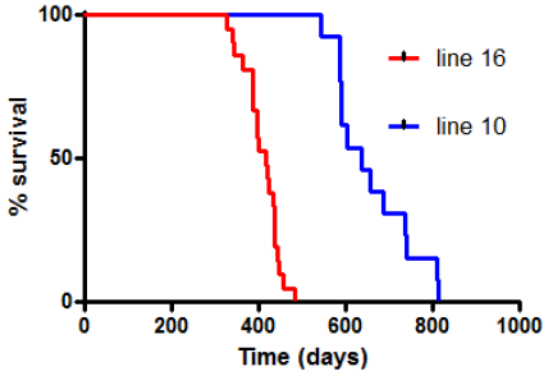

100
50
0
% survival
0
200
400
600
800
1000
Time (days)
line 16
line 10

Figure 3.1: Structural and biochemical features of PrP^{HDdup}.

A) Line diagram of PrP demonstrating the site of insertion. A recently discovered human PrP allele with a 24 base pair insertion that is believed to cause GSS was created in a murine context with a M128V mutation corresponding to the M129V polymorphism. Approximate epitopes of α-PrP antibodies used are indicated. **B)** Kaplan-Meier survival plot of the transgenic lines expressing PrP^{HDdup}. Line 16; 410 ± 40 days (SD; n=23) and line 10; 662 ± 76 days (SD; n=16).

Table 3.1: Expression level and disease onset of transgenic mice.

Line	16-PrP^{HDdup}	10-PrP^{HDdup}	39-PrP^{128V}	25-PrP^{128V}
Expression level relative to WT	1x	0.4x	0.9x	1x
Age at sacrifice (days)	410 ± 40	662 ± 76	N/A	N/A

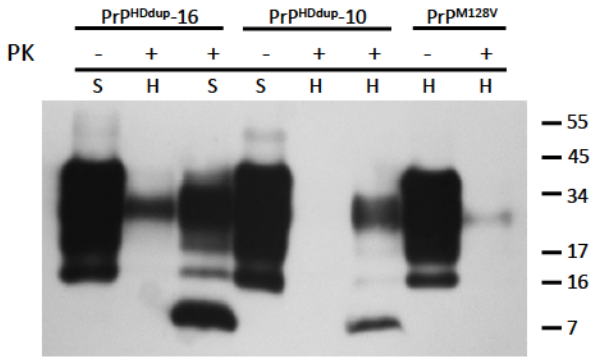
Table 3.1: Expression level and disease onset of transgenic mice.

Expression level of total PrP was determined relative to wild type FVB/N mice by western blot using the helix 1 antibody Sha31. All transgenic mice used in this study are based on a FVB/N *Prnp* $^{+/+}$ background; reported expression level is correct for the contribution of wild type mouse PrP and reflects the expression level of the indicated transgene only. Age of sacrifice is reported as ± SD. PrP^{HDdup}-16, n=23; PrP^{HDdup}-10, n=16.

allele (Tagliavini *et al.*, 1991; 1994; 2001). In the current study, exposure of brain homogenates derived from sick animals expressing PrP^{HDdup} to proteinase K revealed an ~7 KDa PrP fragment that is absent in both pre-symptomatic PrP^{HDdup} expressing animals and PrP^{M128V} control animals (Figure 3.2A). In these assays, residual full-length PrP is present at higher levels in sick animals than observed in both pre-symptomatic and control animals. This is likely due to a combination of two factors; firstly, the amount of proteinase K (100:1 total protein:proteinase K) is lower than typically used for this assay. Secondly, increased levels of PrP in the brains of animals over the course of disease has been noted in other models which is reflected in the amounts of residual PrP seen in the brains of sick animals in this study (Figure 3.2A; Yang *et al.*, 2009; Mays *et al.*, 2014). Assays utilizing PNGase F to remove the carbohydrate moieties from PrP were unremarkable (Figure 3.2B). Using α-PrP antibodies that recognize epitopes from various regions of the molecule demonstrates the presence of 12B2 (residues 97-115) and Sha31 (helix 1; residues 145-155) epitopes (Figure 3.3). Through sequence analysis using these epitopes as N- and C-termini predicts the fragment to consist of at least residues 97-155 which, including the eight additional amino acids, corresponds to a predicted molecular weight of ~6 kDa. This is in agreement with other studies of GSS associated 7 kDa fragments that were mapped to residues 90-153 (Tagliavini *et al.*, 2001).

In order to probe the quaternary state of PrP^{HDdup}, ultracentrifugation assays utilizing a linear gradient of 10-45% sucrose were performed in the presence of 1% sarkosyl. These studies revealed that following a high-speed spin, PrP^{HDdup} populates lower fractions (i.e. higher M_r) of the gradients in comparison to PrP^{M128V} (Figure 3.4). Comparing sick animals to their pre-symptomatic counterparts reveals a similar profile with the exception that the lowest fractions are under-populated in the samples from younger

A


PrP^HDdup-16
PrP^HDdup-10
PrP^M128V
PK
- + + - + + - +
S H S S H H H H
55
45
34
17
16
7

B

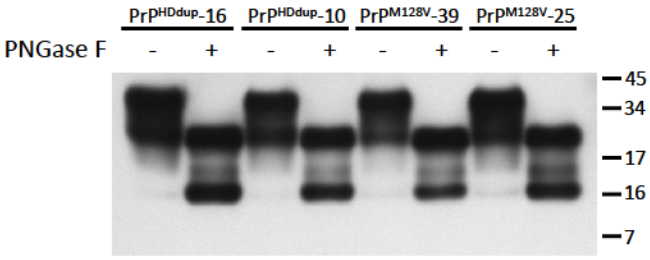
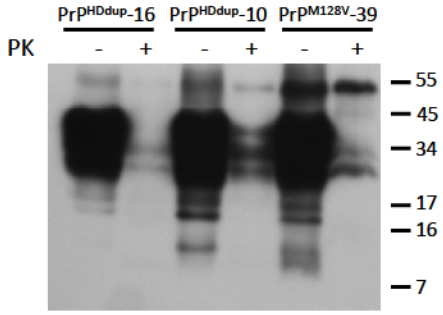

PrP^HDdup-16
PrP^HDdup-10
PrP^M128V-39
PrP^M128V-25
PNGase F
- + - + - + - +
45
34
17
16
7

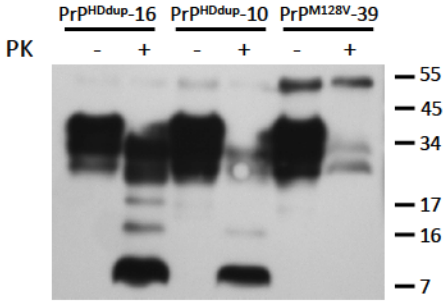
Figure 3.2. Proteinase K resistance and biological processing of PrP^{HDdup}.

A) At the terminal stage of disease with multiple neurologic signs, HDdup prions are resistant to digestion by 10 μg/ml proteinase K at 37 °C for 1 hour and reveal a 7 kDa fragment. This fragment is unique to symptomatic animals. It is not detected in the brain of pre-symptomatic or control animals. **B)** PNGase F digestions of PrP^{HDdup} and PrP^{M128V} expressing animals. The formation of the C1 fragment is unaffected by the insertion mutation.

A


PrP^HDdup-16
PrP^HDdup-10
PrP^M128V-39
PK
- + - + - +
55
45
34
17
16
7

B


PrP^HDdup-16
PrP^HDdup-10
PrP^M128V-39
PK
- + - + - +
55
45
34
17
16
7

C

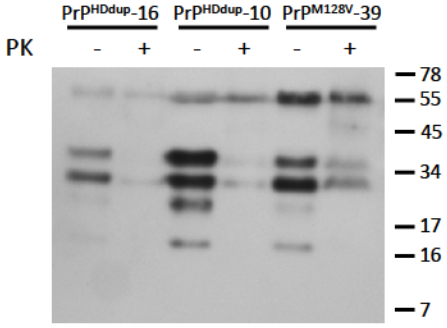

PrP^HDdup-16
PrP^HDdup-10
PrP^M128V-39
PK
- + - + - +
78
55
45
34
17
16
7

Figure 3.3. Identification of a 7 kDa PrP fragment.

Brain homogenates from sick PrP^{HDdup} and PrP^{M128V} control animals were subjected to 10 μg/ml proteinase K at 37 °C for 1 hour. The immunoreactivity of the 7 kDa fragment to various α-PrP antibodies was determined. Figure 1.1 A shows the epitopes for the antibodies used. **A)** PrP248 recognizes the octarepeat region of PrP and fails to detect the 7 kDa fragment **B)** 12B2: residues 97-115 is reactive with the 7 kDa fragment **C)** VRQ61 antibody that recognizes the β2-α2 loop of PrP is not immunoreactive to the 7 kDa peptide. Combined with the recognition of the 7 kDa fragment by Sha31 (Figure 3.2; residues 145-155), these data lead to the inference that the 7 kDa fragment is both N- and C-terminally truncated and composed of, roughly, residues 97-155.

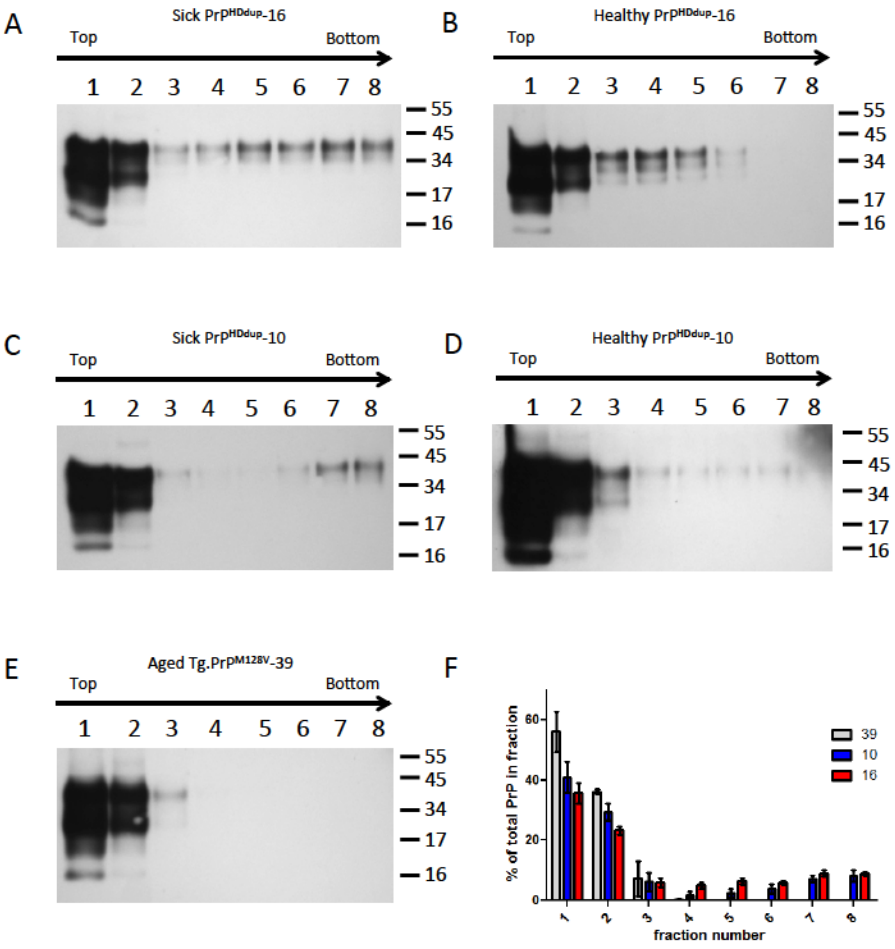

A
Sick PrP^HDdup-16
Top
Bottom
1 2 3 4 5 6 7 8
55
45
34
17
16
B
Healthy PrP^HDdup-16
Top
Bottom
1 2 3 4 5 6 7 8
55
45
34
17
16
C
Sick PrP^HDdup-10
Top
Bottom
1 2 3 4 5 6 7 8
55
45
34
17
16
D
Healthy PrP^HDdup-10
Top
Bottom
1 2 3 4 5 6 7 8
55
45
34
17
16
E
Aged Tg.PrP^M128V-39
Top
Bottom
1 2 3 4 5 6 7 8
55
45
34
17
16
F
% of total PrP in fraction
60
40
20
0
39
10
16
1 2 3 4 5 6 7 8
fraction number

Figure 3.4: Altered quaternary state of PrP^{HDdup}.

Pathogenic PrP is found in lower fractions of linear 10-45% sucrose gradients in 1% Sarkosyl than non-pathogenic PrP in both young and sick PrP^{HDdup} expressing animals. PrP immunoreactivity emerges in the lowest fraction at end-stage of disease, when PrP becomes proteinase K resistant. Assays using animals expressing equivalent levels of PrP^{M128V} show that non-pathogenic PrP remains in the top fractions of these gradients. **A)** Sick PrP^{HDdup}-16 animal. **B)** Healthy PrP^{HDdup}-16 animal. **C)** Sick PrP^{HDdup}-10 animal. **D)** Healthy PrP^{HDdup}-10 animal. **E)** Aged PrP^{M128V}-39 animal. The PrP helix 1 antibody Sha31 was used for all blots. **F)** Quantification of amount of PrP in each fraction of sick animals (Table 3.2).

Table 3.2: Quantification of total PrP in each fraction of sucrose gradients of sick animals.

	Fraction number							
	1	2	3	4	5	6	7	8
PrP^{HDdup}-16	35.7 ± 6.0	23.3 ± 2.2	5.8 ± 2.5	5.0 ± 1.8	6.5 ± 1.6	6.0 ± 1.2	9.0 ± 1.8	8.8 ± 1.0
PrP^{HDdup}-10	40.9 ± 8.8	29.3 ± 4.9	6.1 ± 5.2	2.0 ± 1.9	2.4 ± 2.5	3.9 ± 2.7	7.1 ± 2.1	8.2 ± 3.4
PrP^{M128V}-39	56.1 ± 11.4	36.2 ± 1.2	7.3 ± 10	0.4 ± .7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Table 3.2: Quantification of total PrP in each fraction of sucrose gradients of sick animals.

Each fraction of the sucrose gradient fractionations presented in Figure 3.4A, C, and E were quantified using ImageJ software. The percentage of total PrP in each fraction is presented (± SD, n=3). For PrP^{HDdup} expressing animals, the majority of PrP (~70% for PrP^{HDdup}-16 and ~78% for PrP^{HDdup}-10) is found in the four upper fractions of the gradients, while PrP^{M128V}-39 animals have 100% of total PrP in these fractions.. Less than 30% of total PrP is incorporated into the detergent insoluble aggregates observed in the lower fractions derived from brain homogenates of PrP^{HDdup} expressing animals.

animals (Figure 3.4A vs. B, 3.4C vs. D). While the profile of pre-symptomatic animals is clearly divergent from aged PrP^{M128V} expressing animals, the presence of PrP in a given lower fraction is less consistent. This is likely a consequence of younger animals being at different points in the clinical spectrum of disease, which is reflected by the standard deviation of end point times (Figure 3.1B, Table 3.1). As pre-symptomatic animals at these time points do not possess proteinase K resistant material, these analyses suggest that the development of proteinase K resistant PrP, in the context of this mutation, is associated with the largest detergent insoluble aggregates that are observed in the lowest fraction of the gradient. The amount of total PrP in the lower fractions from sick animals is less than the contribution of transgene expressed PrP^{HDdup} to total PrP (Figure 3.4F, Table 3.2). This suggests that, even at end stage, only a small proportion of mutant PrP forms these detergent insoluble aggregates.

3.3.3 Histopathology induced by PrP^{HDdup}

Histopathological analysis of these mice at end stage reveals prominent vacuolation and gliosis as well as punctate PrP immunoreactive deposits (Figure 3.5). PrP immunoreactivity following chemical hydrolysis is most prominent in the hippocampus, thalamus and cortex. The cerebellum displays faint PrP^{res} immunoreactivity in both the molecular and granular layers (Figure 3.5J). The most intense vacuolation and gliosis are also noted in these regions (Figure 3.5). The medulla is mildly affected, showing PrP^{res} deposition, vacuolation and GFAP immunoreactivity. Other areas in the brains of these animals show less consistent pathology, including the midbrain, pons, anterior commissure and olfactory bulb (not shown).

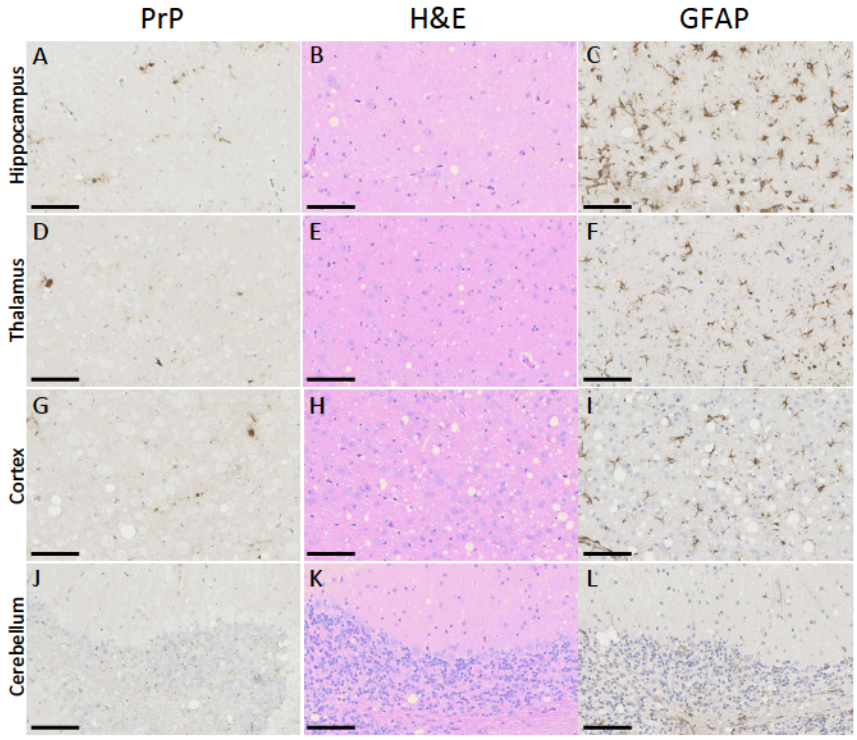

PrP
H&E
GFAP
Hippocampus
Thalamus
Cortex
Cerebellum
A
B
C
D
E
F
G
H
I
J
K
L

Figure 3.5: Histopathological examination of PrP^{HDdup}-16 animals.

Photomicrographs of sagittal sections of sick PrP^{HDdup}-16 mice. Immunostaining for PrP was performed after treatment of the slices with formic acid, slices destined for examination with H&E or GFAP were left untreated. The hippocampus **(A-C)**, thalamus **(D-F)**, cortex **(G-I)** and cerebellum **(J-L)** are shown. PrP^{res} immunostaining is present in all regions and least intense in the cerebellum of these mice. Vacuolation and GFAP immunostaining are present in all four regions. Scale bar = 100 μm.

The histopathological findings in the brains of PrP^{HDdup}-10 animals diverge from that observed in PrP^{HDdup}-16 mice. Firstly, vacuolation in PrP^{HDdup}-10 animals is not as wide spread and is restricted to the hippocampus, thalamus and cerebellum (Figure 3.6B, E, H, Figure 3.7B, E, H). The intensity of spongy change is also less consistent. Curiously, PrP^{res} immunoreactivity is prominent in the cerebellum of PrP^{HDdup}-10 animals and absent in other regions of the brain; the opposite of what is observed in the brains of PrP^{HDdup}-16 animals (Figure 3.5, 3.7). PrP^{res} deposition is also less consistent in these animals both in terms of intensity and location; some animals have deposition throughout the cerebellum while for others it is observed in patches (Figure 3.7). The reasons for this are unclear, but could be due to the overall lower expression level of the transgene in PrP^{HDdup}-10 animals or to a regional difference in expression levels between lines, which has been observed in the case of Tga20 animals (Karapetyan *et al.*, 2009). Aged-matched PrP^{M128V} expressing animals did not present with these histopathological changes (Figure 3.8).

Two pathological features set GSS apart from other prion diseases: i) PrP amyloid deposition that can be detected using the amyloid specific Congo red dye and ii) neurofibrillary tangles of hyperphosphorylated tau protein in the case of the F198S mutation (Ghetti *et al.*, 1995). Because of their higher expression level of PrP^{HDdup} and more intense pathology, we used sagittal sections of the brains from PrP^{HDdup}-16 animals in order to ascertain for the presence of amyloid deposits and hyperphosphorylated tau. Using the Aβ amyloid in TgCRND8 mice as a control, attempts to detect Congo red binding deposits in the brains of PrP^{HDdup}-16 animals were unsuccessful (Chishti *et al.*, 2001; Figure 3.9). TgTauP301L mice were used as controls to determine the phosphorylation state of tau using the AT8 antibody; no hyperphosphorylated protein

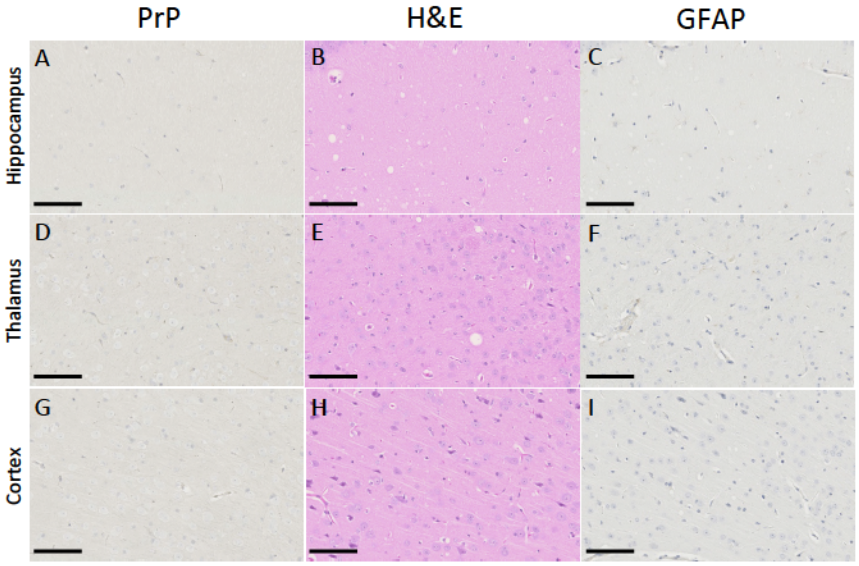

PrP
H&E
GFAP
Hippocampus
Thalamus
Cortex
A
B
C
D
E
F
G
H
I

Figure 3.6: Histopathological examination of PrP^{HDdup}-10 animals.

Photomicrographs of sagittal sections of sick PrP^{HDdup}-10 mice. Immunostaining for PrP was performed after treatment of the slices with formic acid, slices destined for examination with H&E or GFAP were left untreated. The hippocampus **(A-C)**, thalamus **(D-F)** and cortex **(G-I)** are shown. PrP^{res} and GFAP immunostaining is absent in these regions. Vacuolation is restricted to the hippocampus and thalamus. Scale bar = 100 μm.

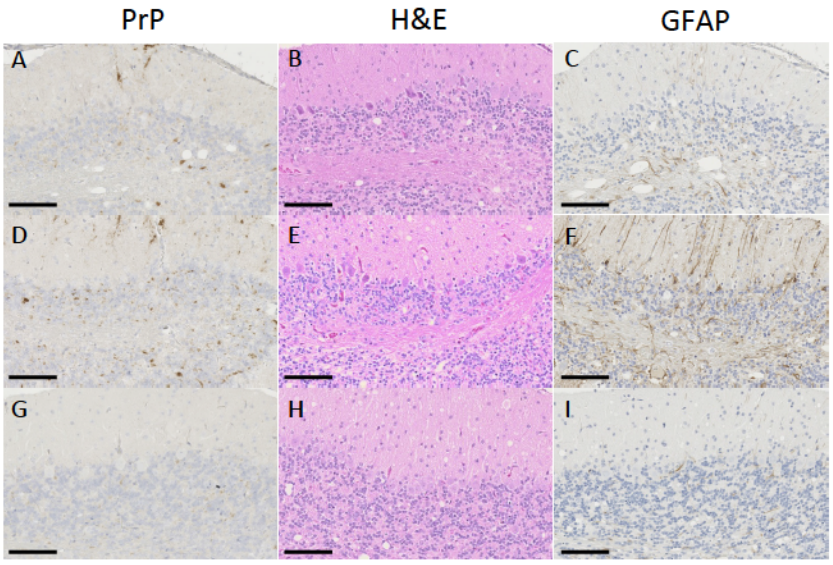

PrP
H&E
GFAP
A
B
C
D
E
F
G
H
I

Figure 3.7: Histopathological examination of the cerebellum of PrP^{HDdup}-10 mice.

Photomicrographs of sagittal sections of sick PrP^{HDdup}-10 mice. Immunostaining for PrP was performed after treatment of the slices with formic acid, slices destined for examination with H&E or GFAP were left untreated. The cerebellum from three mice **(A-C, D-F and G-I)** are shown. The intensity of PrP^{res} and GFAP immunoreactivity and vacuolation are variable across individual animals. Scale bar = 100 μm.

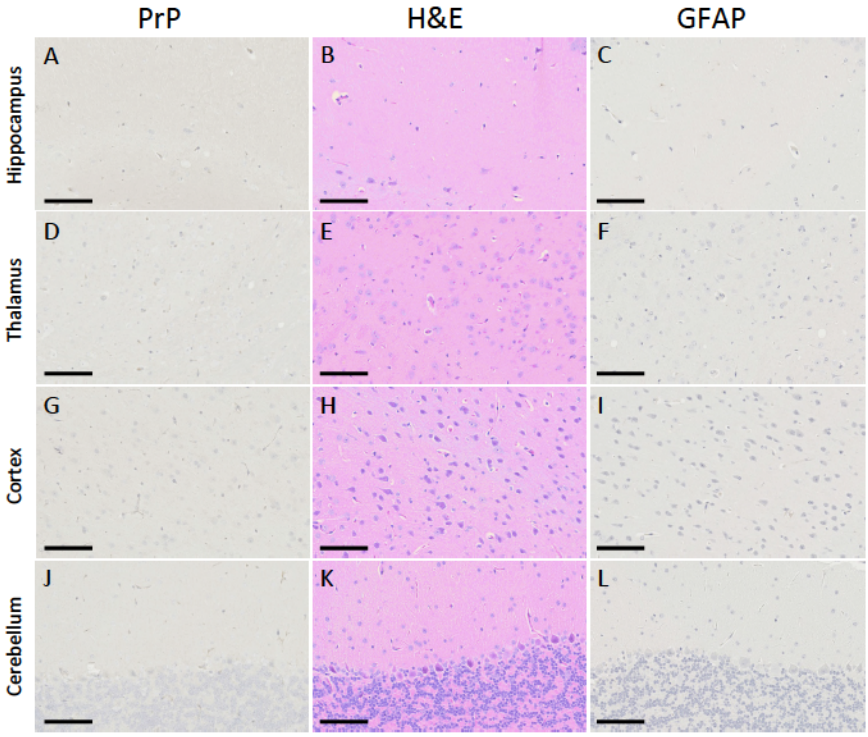

PrP
H&E
GFAP
Hippocampus
Thalamus
Cortex
Cerebellum
A
B
C
D
E
F
G
H
I
J
K
L

Figure 3.8: Histopathological examination of PrP^{HDdup}-39 animals.

Photomicrographs of sagittal sections of sick PrP^{HDdup}-16 mice. Immunostaining for PrP was performed after treatment of the slices with formic acid, slices destined for examination with H&E or GFAP were left untreated. The hippocampus **(A-C)**, thalamus **(D-F)**, cortex **(G-I)** and cerebellum **(J-L)** are shown. Vacuolation, PrP^{res} and GFAP immunostaining are absent in all regions in the brains of these mice. Scale bar = 100 μm.

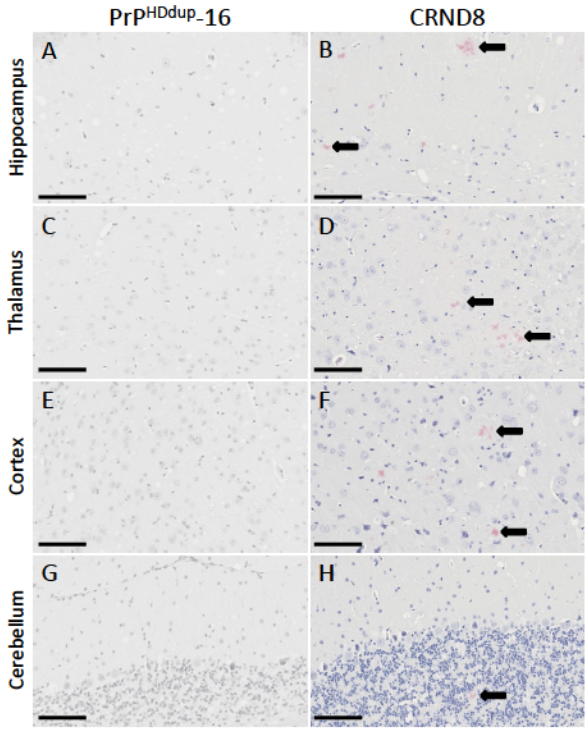

PrP^HDdup-16
CRND8
Hippocampus
Thalamus
Cortex
Cerebellum
A
B
C
D
E
F
G
H

Figure 3.9: PrP^{HDdup}-16 mice do not have Congo red binding deposits.

Photomicrographs of sagittal sections of sick PrP^{HDdup}-16 and TgCRND8 mice stained with Congo red. The hippocampus **(A-B)**, thalamus **(C-D)**, cortex **(E-F)** and cerebellum **(G-H)** are shown. Congo red staining can be seen in the hippocampus, thalamus cortex and cerebellum TgCRND8 mice due to the presence of Aβ plaques (indicated by black arrows). There is no Congo red staining in the sections of PrP^{HDdup}-16 animals, indicating that the observed PrP immunoreactive deposits have not formed amyloid. Scale bar = 100 μm.

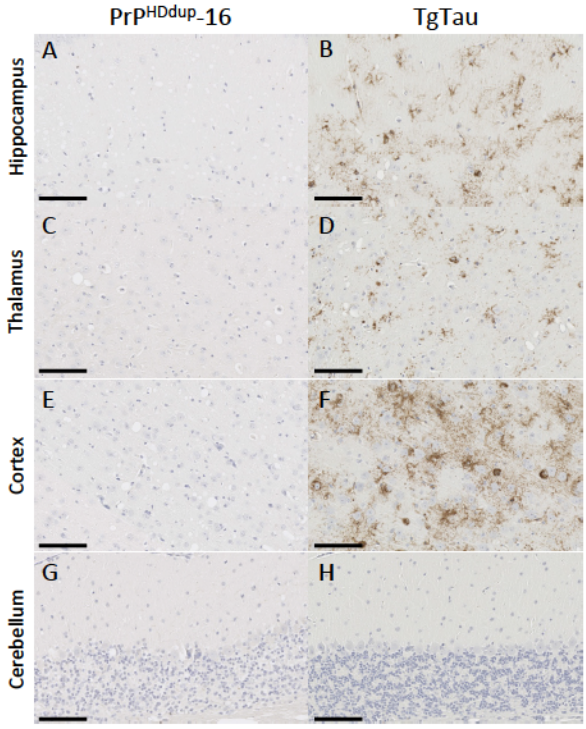

PrP^HDdup-16
TgTau
Hippocampus
Thalamus
Cortex
Cerebellum
A
B
C
D
E
F
G
H

Figure 3.10: The phosphorylation state of tau in the brains of PrP^{HDdup}-16 mice.

Photomicrographs of sagittal sections of sick PrP^{HDdup}-16 and TgTauP301L mice immunostained with AT8. The hippocampus **(A-B)**, thalamus **(C-D)**, cortex **(E-F)** and cerebellum **(G-H)** are shown. Hyperphosphorylation of tau is noted in the hippocampus, thalamus and cortex of TgTauP301L mice. No hyperphosphorylated tau was detected in the brains of PrP^{HDdup}-16 mice. Scale bar = 100 μm.

was revealed in the brains of PrP^{HDdup} expressing animals (Murakami *et al.*, 2006; Figure 3.10).

3.3.4 Cellular localization of PrP^{HDdup}

It has been observed that pathogenic mutations of PrP cause the protein to undergo improper biogenesis resulting in an impaired delivery to the cell surface and a partial retention in the endoplasmic reticulum (Ivanova *et al.*, 2001). To address this possibility for PrP^{HDdup}, a cell surface biotinylation assay was undertaken using transiently transfected RK13 cells. The amount of PrP^{HDdup} that is biotinylated in this assay is comparable to that of wild type, suggesting that it's delivery to the cell surface is unencumbered (Figure 3.11). A mutant PrP with a total of 13 octarepeats, octa13PrP, which is known to accumulate inside cells, was used to demonstrate the utility of the assay. Utilizing this same cell culture system, permeabilized cells showed no qualitative differences in the intracellular location of PrP^{HDdup} compared to controls, providing further evidence that the proteins movement through the secretory system progresses in a typical fashion (Figure 3.12). These data, paired with the observation that the glycosylation pattern of PrP^{HDdup} is indistinguishable from that of wild type PrP suggest that there are no alterations in the biogenesis of PrP^{HDdup} which may account for its observed toxicity in the mammalian brain (Figure 3.2).

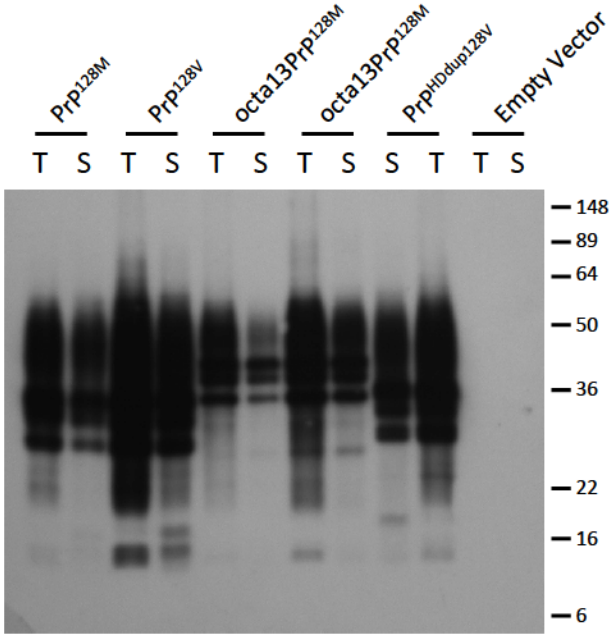

PrP128M
PrP128V
octa13PrP128M
octa13PrP128M
PrPHDdup128V
Empty Vector
T S T S T S T S S T T S
148
89
64
50
36
22
16
6

Figure 3.11: Cell surface biotinylation assay of PrP expressing RK13 cells.

RK13 cells were transiently transfected with wild type mouse PrP, PrP^{M128V}, octa13PrP^{128M}, octa13PrP^{128v} and PrP^{HDdup} using a pcDNA vector. The biotinylation reagent is impermeable to the cell membrane. Total PrP and biotinylated PrP are loaded side by side. PrP^{HDdup} is comparably accessible to the biotinylation reagent as control PrP. Reduced levels of octa13PrP at the cell surface is used as a control.

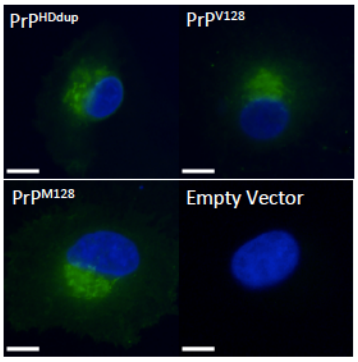

PrP^HDdup
PrP^V128
PrP^M128
Empty Vector

Figure 3.12: Immunocytochemistry of permeabilized RK13 cells.

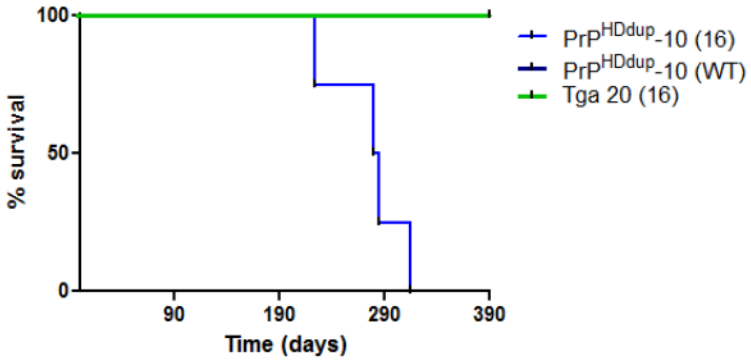
RK13 cells were transiently transfected with wild type mouse PrP, PrP^{M128V} and PrP^{HDdup} using a pcDNA vector. The cells were permeabilized after fixation to analyze intracellular PrP. No qualitative difference in the localization of the three proteins was observed (n=6). Scale bar = 10 µm

3.3.5 Acceleration of disease

Attempts to transmit GSS isolates to primate and rodent models have yielded inconsistent results (Tateishi & Kitamoto, 1995; Collins *et al.*, 2001). Here, we used PrP^{HDdup}-10 and Tga20 animals as recipients for brain homogenate from sick PrP^{HDdup}-16 animals (Fischer *et al.*, 1996). Through inoculation of PrP^{HDdup}-10 animals with brain homogenate from sick PrP^{HDdup}-16 animals, the end stage of disease was shortened from 662 ± 76 days to 276±38 days (Figure 3.1B, 3.13A). This abbreviated disease course was accompanied by the development of the 7 kDa proteinase K fragment (Figure 3.13B). Pathologically, PrP^{res} and GFAP immunoreactivity as well as vacuolation was prominent in the cerebellum of only PrP^{HDdup}-10 mice inoculated with brain homogenate from clinical PrP^{HDdup}-16 mice (Figure 3.14). Importantly, PrP^{HDdup}-10 animals inoculated with non-transgenic, healthy brain homogenate were culled at 399 days post inoculation with no signs of disease (Figure 3.13, 3.14). Tga20 mice which express PrP at ~5x wild type levels (higher than any mouse in our study) remained healthy and displayed none of these disease associated changes when inoculated with the same brain homogenates when culled 460 days post inoculation (Figure 3.13, 3.14). Examination of animals inoculated with PrP^{HDdup}-16 brain homogenate for Congo red staining was negative (Figure 3.15). Together, these data demonstrate that HDdup prions are not transmissible to animals expressing wild type PrP, as has been reported for other forms of GSS modeled in mice (Hsiao *et al.*, 1994; Telling *et al.*, 1996; Nazor *et al.*, 2005). As PrP^{HDdup}-10 animals would have eventually developed disease, the findings of these analyses reflect an acceleration of disease as opposed to the induction of disease (Figure 3.1B, 3.2B, 3.7). That HDdup prions are not transmissible *per se*, but can accelerate pathology in mice expressing homologous PrP is in line with the findings from analyses of other

forms of GSS (Hsiao *et al.*, 1994; Telling *et al.*, 1996; Nazor *et al.*, 2005; Asante *et al.*, 2013; 2015).

A


100
50
0
% survival
90
190
290
390
Time (days)
PrP^HDdup-10 (16)
PrP^HDdup-10 (WT)
Tga 20 (16)

B

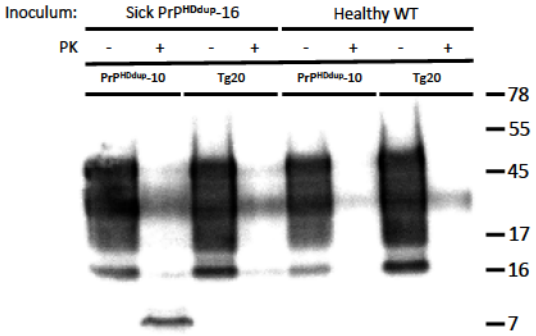

Inoculum:
Sick PrP^HDdup-16
Healthy WT
PK
- + - + - + - +
PrP^HDdup-10
Tg20
PrP^HDdup-10
Tg20
78
55
45
17
16
7

Figure 3.13: Brain homogenate from sick PrP^{HDdup} animals can accelerate disease in animals expressing homologous PrP.

A) Kaplan-Meier death curve for animals in the study. Source of inoculum is indicated in brackets; 16 = sick PrP^{HDdup}-16 brain homogenate, WT= healthy WT brain homogenate. n=4 for all groups. Tga20 animals, with the exception of one that had to be euthanized due to intercurrent illness, were sacrificed after 460 days. PrP^{HDdup} line 10 animals inoculated with healthy brain homogenate were sacrificed after 400 days. Line 10 animals inoculated with brain homogenated from sick line 16 animals succumbed to disease at 276±38 days (± SD). **B)** Proteinase K treatment of brain homogenates from inoculated animals. Only animals exposed to brain homogenate from sick PrP^{HDdup} expressing animals display a 7 kDa fragment after exposure to proteinase K.

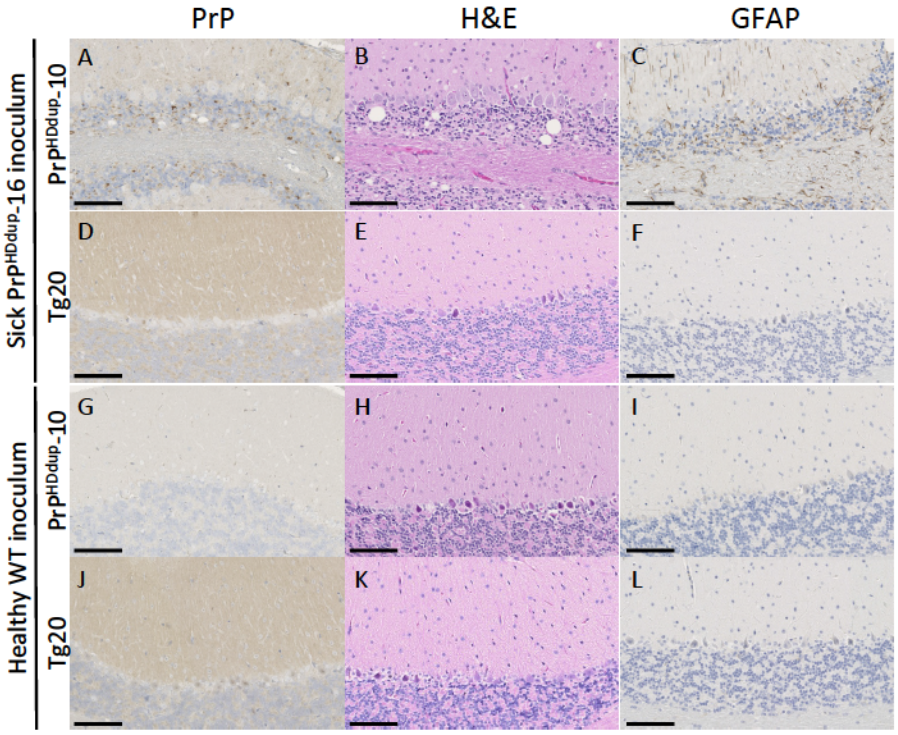

PrP
H&E
GFAP
Sick PrP^HDdup-16 inoculum
PrP^HDdup-10
Tg20
Healthy WT inoculum
PrP^HDdup-10
Tg20
A
B
C
D
E
F
G
H
I
J
K
L

Figure 3.14: Brain homogenate from sick PrP^{HDdup} animals can initiate pathological changes in animals expressing homologous PrP.

Low expressing PrP^{HDdup}-10 animals and Tga20 animals that highly express wild type PrP received an intracranial inoculation with brain homogenate from a sick PrP^{HDdup}-16 or a healthy wild type FVB/N *Prnp* $^{+/+}$ animal. The onset of pathological changes was accelerated in the animals receiving the PrP^{HDdup}-16 brain homogenate. **(A-C)** GFAP immunoreactivity in the cerebellum. **(D-F)** H&E staining in the cerebellum. **(G-I)** PrP^{Sc} staining in the cerebellum. Only PrP^{HDdup}-10 animals inoculated with brain homogenate from sick PrP^{HDdup}-16 displayed symptoms and pathology consistent with prion disease. All other animals remained behaviourally and pathologically normal. Scale bar = 100 µm.

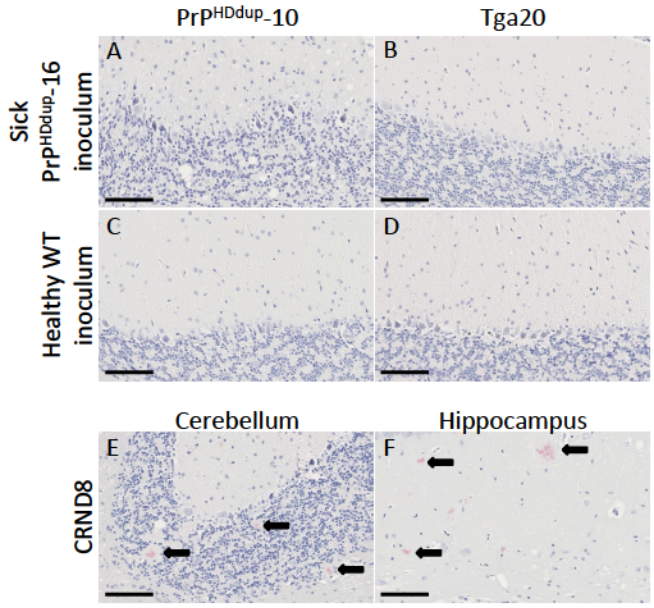

PrP^HDdup-10
Tga20
Sick PrP^HDdup-16 inoculum
Healthy WT inoculum
A
B
C
D
Cerebellum
Hippocampus
CRND8
E
F

Figure 3.15: Inoculation with HDdup prions does not induce amyloid formation in recipient mice.

Photomicrographs of sagittal sections of the cerebellum of PrP^{HDdup}-10 and Tga20 mice **(A-D)**. TgCRND8 mice are used as controls **(E-F)**. Congo red staining can be seen in the cerebellum and hippocampus of TgCRND8 sections due to the presence of Aβ plaques (indicated by black arrows). There is no Congo red staining in the sections of inoculated animals. Scale bar = 100 μm.

3.4 Discussion

The present work presents a third mouse model of GSS (joining TgP101L and TgA116V; Hsiao *et al.*, 1990; Yang *et al.*, 2009) with characterization including histopathological and biochemical profiling on transgenic animals expressing the novel allele (Hinnell *et al.*, 2011). During the study it was noticed that the standard deviation of the end stage of disease increases as the expression level decreases (Table 3.1). It is possible that as the expression level of PrP^{HDdup} decreases, so too does the likelihood of a pathological trigger that may initiate disease in these animals. This concept is supported by the observation that upon inoculation with the brain homogenate from sick PrP^{HDdup}-16 animals, the standard deviation of end point of disease in PrP^{HDdup}-10 animals is narrower by 50% (± 76 days to ±38 days; Table 3.1, 3.3). Indeed, such a scenario is reflected in the varying age of onset in other transgenic models of GSS and also a report of monozygotic twins carrying the GSS causing P102L mutation with a seven-year separation in age of onset (Hsiao *et al.*, 1990; Telling *et al.*, 1996; Chiesa *et al.*, 1998; Webb *et al.*, 2009).

3.4.1 The 7 kDa proteinase K resistant fragment

Proteinase K resistant PrP fragments of 7-8 kDa are noted in human GSS-P102L and A117V and are present in the brains of sick A116V mice (Yang *et al.*, 2009). Though proteinase K resistant PrP has not been observed in the brains of P101L mice, detection of abnormally folded PrP in brain homogenates from sick animals has been achieved using a PrP^{Sc} specific antibody (Hsiao *et al.*, 1990; Korth *et al.*, 1997; Nazor *et al.*, 2005). Unfortunately, it is not known if such a fragment can be detected in brain homogenate from the PrP^{HDdup} patient, though its ubiquitous presence in human cases of GSS and in PrP^{HDdup} mice would suggest that it is (Figure 3.2A, 3.3B; Tagliavini *et al.*, 1991; Parchi *et al.*, 1998; Piccardo *et al.*, 2001). In the context of GSS, it had been thought that the

Table 3.3: Disease onset of inoculated animals.

Line	PrP^{HDdup}-10	Tga20	PrP^{HDdup}-10	Tga20
Innoculum	Sick PrP^{HDdup}-16	Sick PrP^{HDdup}-16	Healthy WT	Healthy WT
Age at sacrifice (days)	276±38 days	N/A	N/A	N/A

Table 3.3: Disease onset of inoculated animals.

Tga20 animals, with the exception of one that had to be euthanized due to intercurrent illness, were sacrificed after 460 days. PrP^{HDdup} line 10 animals inoculated with healthy brain homogenate were sacrificed after 400 days. Line 10 animals inoculated with brain homogenate from sick line 16 animals succumbed to disease at 276±38 days (± SD) n=4 for all groups.

pathogenic PrP was derived solely from the mutant allele (Tagliavini *et al.*, 1991; 1994; Parchi *et al.*, 1998). Recently, for patients harbouring the P102L mutation, it has been demonstrated that wild type PrP can also be converted (Wadsworth *et al.*, 2006; Monaco *et al.*, 2012). These studies utilize an antibody that can detect wild type human PrP but fails to react with PrP^{P102L}. The authors found that wild type PrP became proteinase K resistant and populated lower fractions of sucrose gradients. The 8 kDa proteinase K resistant fragment, however, was found to derive solely from the mutant allele (Wadsworth *et al.*, 2006; Monaco *et al.*, 2012). While the present study cannot address this question directly, quantification of the PrP population in each fraction of the sucrose gradients shows that the majority of PrP remains in the first four fractions where non-pathogenic PrP is found (Figure 3.4, Table 3.2). This finding, paired with the observation that wild type PrP does not convert to a pathogenic state when Tga20 mice are challenged with the brain homogenate from sick PrP^{HDdup}-16 animals, suggests that the pathogenic PrP that is observed in these animals is derived solely from the mutant allele. As the mutation is located within the proteinase K resistant fragment, ongoing studies utilizing MALDI-TOF mass spectroscopy will allow for unambiguous identification of the allelic origin of the proteinase K resistant PrP species. A similar approach could also be applied to sucrose gradient separated material.

3.4.2 Histopathological findings

Histopathological analysis of paraffin embedded blocks of the brain from the patient that presented with the HDdup insertion mutation shows mild spongiosis with diffuse and multicentric plaques. There is a high multicentric plaque burden in the hippocampus and the temporal and parietal lobes of the cortex of this patient. The frontal cortex shows slightly less plaques. The thalamus displays diffuse and multicentric plaques that are also found in the cerebellum at a higher density (Gerard H. Jansen, *personal*

communication). Between the two lines of PrP^{HDdup} mice, many of these features are recapitulated (Figure 3.5, 3.6, 3.7). However, the reason for the inconsistency of these pathological features between lines remains unresolved. Further studies that examine the pattern of expression of the transgene in the brains of these mice may begin to shed light upon this phenomenon.

A116V and P101L mice develop PrP amyloid plaques as evidenced by their binding to thioflavin S, another amyloid binding compound and Congo red, respectively (Hsiao *et al.*, 1994; Yang *et al.*, 2009). True amyloid was not detected in the brains of sick PrP^{HDdup} mice, though such examination has not been performed upon the brain of the patient so their absence here may be a moot point (Figure 3.9, 3.15). Regardless, it would be prudent to interrogate brain slices from sick PrP^{HDdup} expressing animals with thioflavin S of T, reagents that can detect amyloid with higher sensitivity than Congo red.

Currently, there are no mouse models of the F198S mutation, so it is unknown if the tau pathology observed in humans with this mutation would be recapitulated in a mouse. However, wild type mouse tau does appear capable of hyperphosphorylation in response to pathogenic PrP accumulation. In a mouse model of vCJD, which is similar to GSS in the sense that there is significant PrP plaque deposition, hyperphosphorylated tau was noted (Giaccone *et al.*, 2008). Such changes were also observed in mice expressing bovine PrP infected with BSE and in wild type mice at advanced stages of RML infection (Brion *et al.*, 1987; Bautista *et al.*, 2006). Investigation of the paraffin embedded blocks from the patient revealed mild granular tau staining around the plaques in the hippocampus suggesting that they may be present in the brains of sick PrP^{HDdup} mice (Gerard H. Jansen, *personal communication)*. It would be of interest to cross PrP^{HDdup}

mice with TgTauP301L mice to determine if the mutated PrP can accelerate the wide spread tau pathology that develops in these animals (Murakami *et al.*, 2006).

3.4.3 Validity of mouse models of GSS

Recently, the validity of introducing human mutations into mouse *Prnp* for the purpose of modeling inherited prion disease has been challenged due to altered transmission properties when compared to mice expressing the human versions of these alleles (Asante *et al.*, 2015). With regards to mouse models of GSS, only those that introduce these mutations within the framework of mouse *Prnp* develop spontaneous disease and GSS-like neuropathology (Hsiao *et al.*, 1990; Yang *et al.*, 2009; Asante *et al.*, 2009; 2013). The etiology of GSS as seen in neurology clinics is one of a dominant Mendelian trait; therefore the spontaneous development of disease is more relevant than its experimental transmission properties. Because of this, it can be concluded that modeling GSS using mouse-derived alleles has considerable merit and should be continued.

3.4.4 Toxicity of PrP^{HDdup}

The toxic insult levied by PrP^{HDdup} cannot simply be explained by aberrant biogenesis. This can be ruled out by equivalent glycosylation patterns and cleavage products of PrP^{128V} and PrP^{HDdup} observed by western blot, a similar expression at the cell surface and qualitatively similar localization within cells (Figure 3.2B, 3.11, 3.12). What then can be inferred of a potential mechanism of the neuronal insult levied by this mutation? PrP^{HDdup} could be involved in aberrant protein-protein interactions through two, but not mutually exclusive mechanisms. First, the insertion lengthens the hydrophobic domain, which could conceivably increase the likelihood of PrP^{Ctm} generation. This topological variant of PrP has been proposed to be a mediator of toxicity that is formed upon co-translational insertion of the hydrophobic domain into the endoplasmic reticulum

membrane (Hegde *et al.*, 1998; 1999). The proportion of PrP adopting this topology has been demonstrated to increase due to another GSS causing hydrophobic domain mutation, A117V, and over the course of experimental prion disease (Hegde *et al.*, 1998; 1999). It was later demonstrated that PrP^{Ctm} participated in an aberrant interaction with mahogunin, an E3 ubiquitin ligase, causing neurodegeneration (Chakrabarti & Hegde, 2009). Secondly, within the context of PrP^{Ctm}, the hydrophobic domain of PrP contains three GXXXG motifs that have been identified as mediators of transmembrane helix-helix association (Russ & Engelman, 2000; Barnham *et al.*, 2006). The described insertion extends the chain of GXXXG motifs to a total of five, potentially contributing to aberrant protein –protein interactions that would be inaccessible to other forms of PrP^{Ctm}. However, previous work with PrP^{HDdup} by our lab utilizing formaldehyde crosslinking assays did not reveal any differences in the banding pattern of high molecular weight species that are PrP immunoreactive. While this suggests that there are no alterations in the protein-protein interactions of PrP^{HDdup}, investigations with greater sensitivity are be required to rule out this possibility with certainty (Mercer *et al.*, 2013, Figure 2.3C).

3.4.5 Structural perturbations due to the insertion

Because the insertion occurs at the beginning of the first β strand between residues 129 and 130 (human numbering) there is a potential to modify this structure. A nanobody that inhibits PrP^{Sc} replication in GT1 cells was recently co-crystalized with recombinant human PrP and the resulting structure revealed a third β strand formed by the palindromic sequence N-terminal to this insertion; this is thought to represent an initial step in the conversion of PrP^{C} to PrP^{Sc} (Abskharon *et al.*, 2013). Sequence analysis of PrP^{HDdup} reveals that the first β strand is split by the insertion, creating a second β strand separated by a short linker region (Figure 3.16). As the conversion from PrP^{C} to PrP^{Sc} is

known to result in an increase in the β content of the protein, it is tempting to speculate that PrP^{HDdup} has intrinsically more β structure than its wild type counterparts which could drive its conversion to a β structure rich, detergent insoluble and proteinase K resistant form (Pan *et al.*, 1993; Requena & Wille, 2014). Molecular dynamic simulations have been performed using PrP^{HDdup} and the results have prompted structural studies of recombinant PrP^{HDdup} that are now well underway (See Chapter 4 and Appendix A).

β1

PrP^{128V} 112-AGAAAAGAVVGGLGGYVLGSAMSRPMIHFGND-143

β0 β1

PrP^{HDdup} 112-AGAAAAGAVVGGLGGYV**LGGLGGYV**LGSAMSRPMIHFGND-151

Figure 3.16: Sequence analysis of PrP^{HDdup}.

The first β strand of PrP, β1, is formed by the sequence YVLG (top). The LGGLGGYV insertion interrupts this sequence causing it to occur twice in a short span (β0; below).

3.5 References

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Chapter 4

Discussion and Future Directions

4.1 The interaction between PrP and DPP6

Justifiably, much of the focus upon the biology of PrP has been directed toward its role in prion disease. It has been well established that PrP plays a role in neuronal excitability (Collinge *et al.*, 1994; Whittington *et al.*, 1995; Manson *et al.*, 1995; Herms *et al.*, 2001; Carleton *et al.*, 2001; Mallucci *et al.*, 2002; Curtis *et al.*, 2003; Khosravani *et al.*, 2008; Prestori *et al.*, 2008; You *et al.*, 2012). Many different ion channels have been identified that interact with and are modulated by PrP including $GABA_A$ receptor/channels (Collinge *et al.*, 1994; Whittington *et al.*, 1995; Curtis *et al.*, 2003), Ca^{2+} dependent K^+ channels (Colling *et al.*, 1996; Herms *et al.*, 2001), NMDA receptor/channels (Khosravani *et al.*, 2008; You *et al.*, 2012) and the $\alpha 7$ nicotinic acetylcholine receptor (Beraldo *et al.*, 2010). The interaction with DPP6 described in Chapter 2 may well have implications for other diseases that are distinct from that of prion disease. We have shown that in a heterologous cell system, the interaction of PrP with DPP6 enhances the activity of Kv4.2 channels by increasing the peak amplitude, shifting the voltage-dependent steady-state inactivation curve to a more positive membrane potential, slowing the inactivation time and decreasing the time of recover from steady-state inactivation.

Two questions immediately emerge from these studies of the interaction between PrP and DPP6: i) what are the effects of this interaction *in vivo* and ii) what role, if any, does DPP6 play in prion disease pathogenesis? To address the first question, a systematic breeding program using DPP6 and PrP knockout mice paired with electrophysiological analysis of brain slices could be undertaken and will likely provide unambiguous results (Clark *et al.*, 2008). The influence of DPP6 upon prion disease pathogenesis can also be examined using DPP6 knockout mice (Clarke *et al.*, 2008). Unpublished results from our

lab using mice with a modest decrease in DPP6 levels show a slight, non-significant, increase in incubation time following challenge with RML prions. Studies utilizing knockout animals other than *Prnp* knockouts have mostly shown only a modest, non-significant, reduction in prion disease incubation time (Tatzelt *et al.*, 1996; Schmitt-Ulms *et al.*, 2001; Prinz *et al.*, 2003; Salmona *et al.*, 2005). Alterations of the laminin receptor, through siRNA knockdown or the expression of a mutant "decoy" increased incubation time slightly, though significantly, and decreased proteinase K resistant PrP species (Pflanz *et al.*, 2009a; 2009b).

Turning to the electrophysiological profiles in the brains of prion-infected mice, prolonged epileptiform discharges in cortex and hippocampus have been observed before the onset of clinical signs (Jefferys *et al.*, 1994). A depression of synaptic responses in prion-infected mice that was accompanied by cognitive and behavioural deficits that can be rescued by depletion of neuronal PrP^C has also been noted (Mallucci *et al.*, 2007). Additionally, a diminished ability to maintain long term potentiation and alterations of potassium currents has been reported (Johnston *et al.*, 1998a; 1998b). Due to difficulties associated with obtaining brain slices from aged animals, studies to delineate a role for the PrP:DPP6 interaction over the course of prion disease could be undertaken using the Prion Organotypic Slice Culture Assay (Falsig & Aguzzi, 2008; Falsig *et al.*, 2008). This brain slice culture method has been demonstrated to recapitulate the pathology observed *in vivo* and should prove to be useful in electrophysiological studies of prion infection (Falsig *et al.*, 2012; Campeau *et al.*, 2013; Cortez *et al.*, 2015). It is noteworthy that the octa13PrP mutant was not able to modulate Kv4.2 channel properties presumably because of a decreased capacity to interact with DPP6 (Figure 2.4C). It is important to note that the formation of high molecular weight complexes between DPP6 and mutant PrP molecules does not preclude the possibility

that the subsequent modulation of Kv4.2 channel will distinct from that observed in the presence of wild type PrP. Though labour-intensive, future studies should systematically assess the capability of known PrP mutants to modulate Kv4.2 channels through their interaction with DPP6. Underscoring this point is a report of a disruption in the glutamatergic neurotransmission of the cerebellar granule neurons in the PG14 transgenic mouse. This disruption was shown to be due to impaired trafficking of the $\alpha_2\delta$-1 subunit of the glutamate transporter VGLUT1 that was induced by its interaction with the intracellularly retained mutant PrP (Senatore *et al.*, 2012).

4.2 The PrP:DPP6 interaction and other neurologic diseases

Genome wide association studies have identified single nucleotide polymorphisms in the DPP6 locus that are associated with amyotrophic lateral sclerosis (ALS) (van Es *et al.*, 2008; 2009; Cronin *et al.*, 2008; Blauw *et al.*, 2010). While this finding does not hold in other populations (Li *et al.*, 2009; Fogh *et al.*, 2011), these polymorphisms are located in the intronic region of the gene and it is unknown what effect, if any, they would have on the expression of DPP6. However, recent investigations have utilized a motor neurons differentiated from induced pluripotent stem cells (iPSCs) derived from ALS patients that carry a mutation of *C9ORF72*, the most common cause of familial ALS (Renton et al., 2011; Sareen et al., 2013). This mutation is an expansion of the GGGGCC hexanucleotide repeats found upstream of the *C9orf72* coding region and causes the formation of intracellular inclusions of poly-glycine-alanine and poly-glycine-proline that are generated by the non-ATG–initiated translation of these repeats (Mori *et al.*, 2013). It was reported that the iPSC derived motor neurons had increased expression of DPP6 mRNA and were hyperexcitable when examined electrophysiologically, lending credence

to the genome wide association studies that suggest DPP6 has some role to play in ALS (Sareen *et al.*, 2013; Wainger *et al.*, 2014). Interestingly, this expansion mutation is also associated with frontotemperal lobe dementia, symptoms of which are observed in a sub-set of ALS patients (Renton *et al., 2011;* Robberecht *et al.*, 2013). With regards to PrP, reduced protein levels were noted in a mouse model of ALS utilizing a superoxide dismutase-1 mutation and crossing with *Prnp* knockout animals was shown to exacerbate ALS pathology and shorten lifespan (Dupuis *et al.*, 2002; Steinacker *et al.*, 2010). Fortunately, a mouse model of the expansion mutation of the *C9orf72* coding region has recently been developed that recapitulates the clinical and neuropathological phenotypes observed in patients with this mutation (Chew *et al.*, 2015). It will be of interest to examine the levels of both DPP6 and PrP in these animals. In conjunction with the motor neuron culture system described above, insight into the possible role of the PrP:DPP6 interaction in ALS should be easily ascertained.

Copy-number variation of the DPP6 gene and has been linked to autism (Marshall *et al.*, 2008; Noor *et al.*, 2010; Egger *et al.*, 2014) and so has a mutant form of Kv4.2 that has been demonstrated to modulate the currents such that the time to reach peak amplitude and the inactivation time are increased (Lee *et al.*, 2014). A decrease in DPP6 mRNA due to a gene disruption has also been found in a family with Tourette's syndrome (Prontera *et al.*, 2014). Interestingly, *Prnp* mice have been shown to have learning deficits, though this phenotype is not universally accepted (Lipp *et al.*, 1998; Roesler *et al.*, 1999; Coitinho *et al.*, 2003; 2007; Criado *et al.*, 2005). A haplotype was discovered in three families that suffer from familial idiopathic ventricular fibrillation that caused a 20-fold increase in DPP6 mRNA; while PrP is expressed in the heart, whether it plays any role in this disorder through an interaction with DPP6 is unknown (Oesch *et al.*, 1985; Ford *et*

al., 2002; Alders *et al.*, 2009). Quite curiously, disruption of the DPP6 gene was found in an azoospermic male and while the molecular mechanism underlying the lack of sperm in the ejaculate of this patient is unknown, it is noteworthy that Doppel, which is expressed highly in the testes, can form high molecular weight complexes with DPP6 and is required for spermatogenesis (Figure 2.5; Behrens *et al.*, 2002; Li *et al.*, 2014).

Lately, here has been much interest in the interaction between PrP and Aβ oligomers (Laurén *et al.*, 2009; Gimbel *et al.*, 2010; Freir *et al.*, 2011; Um *et al.*, 2012; 2013; Hu *et al.*, 2014; Kostylev *et al.*, 2015). Recent work by our lab has demonstrated the requirement for PrP for the modulation of potassium currents by Aβ oligomers (Alier *et al.*, 2011). The interaction of DPP6 and PrP may have a role to play here as well, and this too can be determined by electrophysiological examination of brain slices from DPP6 knockout mice in the presence or absence of these oligomeric species (Clark *et al.*, 2008). Additionally, these mice can be crossed with TgCRND8 mice, a well-characterized model of Alzheimer's disease, to probe for improvements in the diminished learning and memory that these mice exhibit (Chishti *et al.*, 2001; Hyde *et al.*, 2005; Hanna *et al.*, 2012).

4.3 A new mouse model of GSS

Chapter 3 presented a biochemical and histopathological workup on a mouse model of a novel GSS causing allele referred to as PrP^{HDdup}. This is the first reported expansion mutation of *PRNP* that does not involve the octarepeat region of the protein (Hinnell *et al.*, 2011). Histopathologically, this mouse model recapitulates the spongiosis and PrP deposition that was observed in the brain of the patient. Mice expressing this mutated allele do not possess amyloid deposits, as judged by Congo red staining, or

hyperphosphorylated tau protein by immunolabeling with the AT8 antibody. No biochemical data has been obtained for the single human report of this mutation, precluding a comparison of such properties with those observed in the brains of PrP^{HDdup} expressing transgenic mice. Nevertheless, the presence of a proteinase K resistant 7 kDa internal PrP fragment in the brains of these mice is a further indication that this mouse model faithfully recapitulates the human disease as it is a hallmark of GSS (Parchi *et al.*, 1998; Piccardo *et al.*, 1998; 2001; Tagliavini *et al.*, 1991; 1994).

While the model presented here and the A116V mice both display the telltale 7 kDa PrP fragment following exposure to proteinase K, this characteristic feature of GSS has not been reported for P101L mice (Hsiao *et al.*, 1990; 1994; Telling *et al.*, 1996; Nazor *et al.*, 2005). Importantly, not all forms of GSS with the P102L mutation present with such a fragment after proteinase K exposure (Parchi *et al.*, 1998). While it remains a possibility that P101L mice might reveal such a fragment if less stringent digestion conditions were used, brain homogenates from these animals can nevertheless be used to accelerate disease in recipient P101L animals (Hsiao *et al.*, 1994; Telling, *et al.*, 1996; Nazor *et al.*, 2005). The results of such investigations have not been reported for A116V mice, which possess the 7 kDa fragment (Yang *et al.*, 2009). However, it has been shown that brain homogenates from A117V patients can accelerate disease in A117V mice with the presence of this fragment noted at end stage (Asante *et al.*, 2013).

When brain homogenates from two P102L patients were used to inoculate P101L mice, a striking disconnect between PrP deposition and induction of disease was uncovered. These human cases could be differentiated biochemically by their proteinase K resistant PrP fragments; one possessed an ~21 kDa fragment and the other had a fragment of ~8 kDa (Piccardo *et al.*, 2007). The case that induced disease signs and spongiosis in

recipient mice possessed only an ~21 kDa proteinase K resistant fragment while the case with the 8 kDa fragment failed to induce clinical signs but resulted in large PrP amyloid plaques in the recipient mice (Piccardo *et al.*, 2007). This study was extended using P101L mice that were generated using a gene targeting approach that do not develop spontaneous disease (Manson *et al.*, 1999). Here, the amyloid deposition found in the brains of mice that were inoculated with the 7 kDa fragment could be serially transmitted over three passages. However, the behavioural and pathological abnormalities observed in the mice inoculated with the brain homogenate from patients with the 21 kDa proteinase resistant fragment were not observed upon subsequent passage (Piccardo *et al.*, 2013). In contrast to this, studies that utilized a β structure enriched peptide containing the P101L mutation (residues 90-144) that roughly corresponds to the amyloid fragment found in the brains of A117V patients was able to accelerate disease in P101L mice while a non-β control peptide was not. These animals displayed signs of disease, spongiosis and PrP deposition. Importantly, the β-enriched peptide could not induce disease in wild type mice (Kaneko *et al.*, 2000; Tagliavini *et al.*, 2001).

These discordant data are difficult to resolve at the current time and more work will be needed to draw firm conclusions about the PrP conformer(s) responsible for disease acceleration and/or toxicity. During the course of the studies presented in Chapter 3, a third line of mice, PrP^{HDdup}-32 was developed. These animals succumbed to the same neurologic syndrome at younger ages due to a much higher expression level of the transgene. Interestingly, these animals did not possess any proteinase K resistant material and when brain homogenates from these animals were used to inoculate PrP^{HDdup}-10 animals, no acceleration of disease was observed. Unfortunately, this line was lost due to poor fecundity and the low number of samples did not allow for a robust analysis, leading to their exclusion from the presented data set. Regardless, PrP^{HDdup}

expressing animals may serve as useful tool to address this question and future studies that attempt to create additional lines of PrP^{HDdup} mice with varied expression levels should be undertaken.

4.4 GSS and neuronal excitability

The question of neuronal excitability as it relates to GSS has not been extensively studied but can be viewed as a combination of the loss of function of PrP and also a gain of toxic function. The demonstration that the disruption in the glutamatergic neurotransmission of PG14 mice was due to the altered subcellular trafficking of mutant PrP relative to wild type mice is an example of a loss of function phenotype (Senatore *et al.*, 2012). This finding is extended by data presented in Figure 2.9 that show octa13PrP is incapable of enhancing A-type K^+ currents, presumably also due to its intracellular retention. P101L PrP has been expressed in the CNS of Drosophila and recapitulates some of the pathological changes observed in GSS. Importantly, this was not observed in flies expressing wild type mouse PrP (Gavin *et al.*, 2006). Further study revealed a loss of function phenotype such that mutant PrP was impaired in its ability to enhance synaptic responses and increase the number of miniature synaptic currents at the neuromuscular junction of these flies (Robinson *et al.*, 2014).

On the other hand, there is a clear example of a gain of toxic function; GSS associated PrP fragments have been shown to be neurotoxic (Fioriti *et al.*, 2007). An experimental paradigm of prion disease utilizes a short PrP peptide composted of residues 105-125 (mouse numbering). These peptides are highly toxic to cultured neurons and were found to form cation permeable channels in cultured cells (Forloni *et al.*, 1993; Lin *et al.*, 1997; Kourie & Culverson, 2000). Interestingly, this region is found in the low molecular

weight peptides associated with GSS. When a 7 kDa peptide that roughly corresponds to this proteinase K resistant fragment was synthesized it was found to form non-selective ion channels in artificial membranes; scrambling the 105-125 region abolished this effect (Bahadi *et al.*, 2003).

The elucidation of the mechanisms of neuronal dysfunction in GSS will require systematic studies whereby wild type PrP is pitted against a disease associated mutant PrP in functional assays like the ones described in Chapter 2. Such studies will provide not only a greater understanding of the role of PrP in normal physiology, but unique insight into disease pathogenesis.

4.5 References

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APPENDIX A

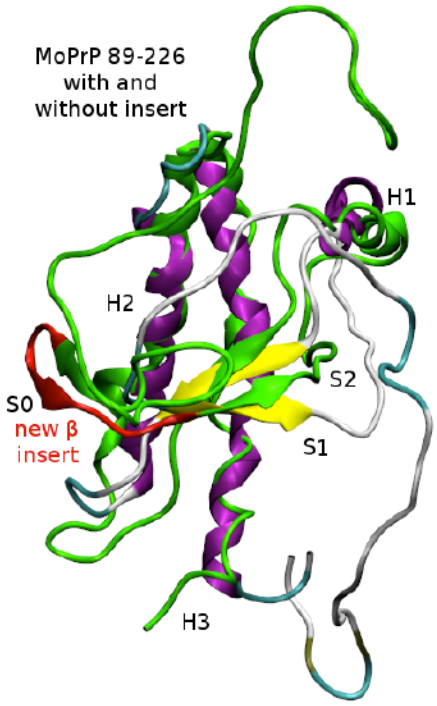
Molecular dynamics simulations of PrP^{HDdup}

These molecular dynamics simulations were performed in the laboratory of Dr. Maria Stepanova by Dr. Lyudmyla Dorosh.

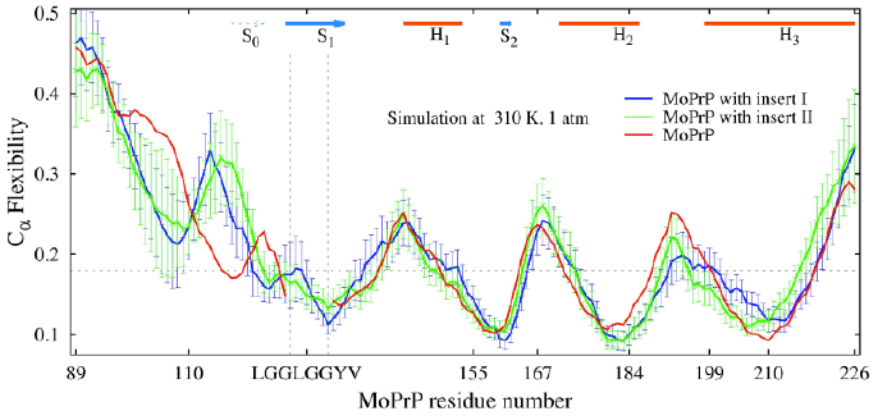
Results

The structure of murine PrP was assessed *in silico* by molecular dynamics simulations with (PrP^{HDdup}) and without insert (PrP^{M128V}) (Figure 5). The PrP molecules were simulated at 310 K and pH 4.5 for 20 ns each. During the 20 ns simulation time PrP^{M128V} retained its secondary structure elements largely without changes. The simulation of PrP^{HDdup} revealed substantial destabilization of α-helices H2 and H3 and the formation of an additional β-strand from the insert sequence (Figure 5A). The main chain flexibility profiles illustrate the extensive structural transformations in PrP^{HDdup} models (Appendix Figure 1).

A


MoPrP 89-226
with and
without insert
H1
H2
S2
S0
new β
insert
S1
H3

B


0.5
0.4
0.3
0.2
0.1
Cα Flexibility
S0
S1
H1
S2
H2
H3
Simulation at 310 K, 1 atm
MoPrP with insert I
MoPrP with insert II
MoPrP
89
110
LGGLGGYV
155
167
184
199
210
226
MoPrP residue number

Appendix Figure 1. Molecular dynamics simulations on murine PrP^{HDdup}.

The PrP^{HDdup} insert destabilizes the structure of murine PrP at the site of the insert and at spatially more distant α-helices. **A)** Alignment of averaged conformations from 20 ns of molecular dynamics simulations for PrP^{M128V} and PrP^{HDdup}. In the PrP^{M128V} control α-helices are indicated in purple and β-strands in yellow. In PrP^{HDdup} structural differences with respect to the control structure are depicted in green and the insert is shown in red. **B)** Main chain flexibility profiles for PrP^{M128V} and PrP^{HDdup}. For the PrP^{M128V} control the flexibility profiles are shown in red and for PrP^{HDdup} the profiles are shown in green and blue. The insert area is marked with vertical dashed lines. The main secondary structure elements are indicated at the top of the plot (orange lines for α-helices and blue arrows for β-strands).

Method

The PrP^{HDdup} and PrP^{M128V} models were built using the SWISS-MODEL homology-modelling server (Arnold *et al.*, 2006), and the global and per-residue model quality were assessed using the QMEAN scoring function (Benkert *et al.*, 2011). The homology search for PrP^{HDdup} produced 285 templates, from which 10 were chosen to build models. These included PDB ID codes 4MA8, 4MA7, 2L39, and 2L1H with a sequence identity of 99.12%-98.21%. The best matching models were based on constructs 2L39 with 98.25% identity, 2L1H with 99.11% identity, and 4MA7 with 99.12% identity. The control PrP^{M128V} structure was based on the murine PrP(89-226) sequence and the NMR-derived structure PDB ID 2L39. The M128V polymorphism and the LGGLGGYV insert were introduced using Accelrys VS.

In all molecular dynamics simulation runs, the N- and C-termini were kept charged ($-COO^-$ and $-NH3^+$), whereas all other titratable amino acids were assigned their canonical state at pH 4.5 with the PropKa server (Søndergaard *et al.*, 2011). All models were subjected to minimizations, equilibrations, and production molecular dynamics simulations in the Gromacs v 4.5.3 package (Berendsen *et al.*, 1995) with OPLS forcefields (Jorgensen *et al.*, 1996). For PrP^{HDdup} we also ran an additional simulation using the same starting positions but different starting velocities of the atoms. The starting models were minimized *in vacuo* for 10,000 steps of steepest descent minimization. Then the models were solvated in a single point charge extended (SPC/E) rectangular periodic water box, after which Cl^- or Na^+ ions were added to neutralize the systems. Subsequent solvent minimizations with decreasing position restraints (K_{posre} = 1×10^5, 1×10^4, 1,000, 100, 10 and 0 kJ $mol^{-1}nm^{-2}$) on non-hydrogen protein atoms were made to relax solvent and protein. Subsequent heating with the Berendsen thermostats from 0 K to 310 K and NPT equilibration with adjustment of solvent density to 1 g/cm^3

followed the minimizations. The last equilibration step and the production simulations were conducted at 310 K temperature and at a pressure of 1 atm with isotropic pressure coupling (NPT ensemble). The bond lengths were restrained with the LINCS algorithm with a fourth order of expansion. The short-range electrostatic and van der Waals interactions cutoff radii were equal to 14 Å each. Long-range electrostatic interactions were treated with the particle-mesh Ewald (PME) summation with grid spacing of 0.135 nm for the fast Fourier transform and cubic interpolation as described elsewhere (Humphrey *et al.*, 1996). The simulations were performed for 20 ns for each system with 1 fs time steps and snapshots that were saved every 20 fs in order to analyze the essential collective dynamics.

To analyze the PrP constructs from the molecular dynamics trajectories, their secondary structure content, numbers of hydrogen bonds and salt bridges, contact maps, and solvent accessible areas were calculated using scripts implemented in the Gromacs (Berendsen *et al.*, 1995) and VMD (Stepanova, 2007) packages. The snapshots of trajectories and graphical representation of models were done in VMD or Accelrys VS (Accelrys).

To analyze and compare the dynamics of the PrP constructs in even greater depth, the essential collective dynamics (ECD) method (Humphrey *et al.*, 1996; Blinov *et al.*, 2009; Santo *et al.*, 2011; Potapov & Stepanova, 2012; Issack *et al.*, 2012; Dorosh *et al.*, 2013) was employed. The method stems from the statistical-mechanical analysis of a protein's generalized Langevin dynamic (Blinov *et al.*, 2009; Santo *et al.*, 2011), according to which persistent correlations between atomic motions in a protein can be determined from principal eigenvectors of the covariance matrix of a protein's molecular dynamics trajectory. A suite of dynamics descriptors has been derived within this framework, including the main-chain flexibility profiles and pair correlation maps (Humphrey *et al.*,

1996; Blinov *et al.*, 2009; Potapov & Stepanova, 2012; Santo *et al.*, 2011; Issack *et al.*, 2012; Dorosh *et al.*, 2013). Previously the method was validated extensively against NMR-derived (Potapov & Stepanova, 2012; Santo *et al.*, 2011; Issack *et al.*, 2012; Dorosh *et al.*, 2013) and X-ray based (Humphrey *et al.*, 1996; Dorosh *et al.*, 2013) structural data, and was demonstrated to predict accurately the main-chain flexibility and other dynamics trends from short fragments of molecular dynamics trajectories.

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