UNIVERSITY OF CALIFORNIA SAN DIEGO

Mechanisms of neuronal dysfunction in a genetic model of prion disease

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by

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The Thesis of Maxwell Gruber is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2024

DEDICATION

I dedicate this work to my family, friends, and mentors, who have all took a chance with their time on me and have made me into who I am today. I would like to especially dedicate my work to my father—he is my greatest role model and friend, and I am here in large part because of him!

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I am incredibly grateful for my family and friends who have supported and encouraged me in my goals to pursue science as a career and cannot thank them enough for pushing me to where I am today.

ABSTRACT OF THE THESIS

Mechanisms of neuronal dysfunction in a genetic model of prion disease

by

Maxwell Gruber

Master of Science in Biology

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Professor Gulcin Pekkurnaz, Co-Chair

Prion diseases are fatal neurodegenerative disorders characterized by neuronal loss and the accumulation of misfolded prion protein aggregates (PrP^{Sc}) in the brain. PrP^{C}, the normal cellular prion protein encoded by the *Prnp* gene, has been implicated in binding oligomers, including PrP^{Sc}, Aβ oligomers, and α-synuclein assemblies, that form in patient brains with neurodegenerative disease. However, the mechanism by which PrP^{C} interacts with PrP^{Sc} and

causes neuronal death is poorly defined. The *Prnp*^{92N} point mutation may be a tool to help to elucidate the neurotoxic pathway, as it has been shown to result in spontaneous spongiform degeneration and necrosis of hippocampal pyramidal neurons in mice despite the absence of PrP^{Sc} and prion infectivity. Autophagy may be one cellular mechanism affected by this mutation, as *Prnp*^{92N} mice exhibit increased levels of p62 in whole brain lysate and autophagosome accumulation 20 days after birth, consistent with dysregulated autophagy in other neurodegenerative disease models. Western blot results from *Prnp*^{92N} cerebral cortex show a strong increase in pULK-S757/ULK1 levels at 10 days post-birth. There was also increased p62, a protein degraded by autophagy, and LC3B, an autophagosome marker, consistent with a reduction in autophagic flux. Inhibition of autophagic machinery has previously been shown in prion disease, and understanding how autophagic function changes in *Prnp*^{92N} mice may help to uncover the function of PrP^{C} in prion disease and point towards potential therapeutic avenues.

Additional models are needed to validate *in vivo* results and better substantiate the role of PrP^{C} in biological systems. Due to their low cost, rapid reproduction rate, short life span, and susceptibility to genetic manipulation, *Drosophila melanogaster* are an excellent candidate for studying prion-induced neurodegeneration. To investigate prion disease in a non-mammalian model, we generated transgenic UAS-*Prnp*^{92N} and wild type mouse (WT) PrP control *Drosophila* lines, which were sequence validated by PCR. Western blot analysis revealed that the UAS-*Prnp*^{92N} point mutation is uniquely expressed in the UAS-*Prnp*^{92N} fly lines, and that overall PrP^{C} levels in UAS-*Prnp*^{92N} and WT flies are similar. Lifetime and climbing assays show a reduction in lifespan and motor ability in UAS-*Prnp*^{92N} cohorts, consistent with other prion disease models in *Drosophila*. These experiments show the generation of a mutant prion protein disease model in *Drosophila*, and future experiments will focus on assessing behavior, histopathology, and

signaling pathways towards a goal of understanding how mutant prion protein impacts autophagy and induces neuronal death.

INTRODUCTION

Prion disease

Prion diseases are rare, fatal neurodegenerative diseases characterized by the accumulation and aggregation of abnormally folded proteins called prions (Baldwin & Correll, 2019). Prion diseases occur in animals, such as cattle (bovine spongiform encephalopathy), sheep and goats (scrapie), and cervids (chronic wasting disease), as well as in humans (Johnson, 2005). Human prion diseases can be either sporadic, genetic, or acquired. Sporadic Creutzfeldt-Jakob disease (sCJD), the most common form of the disease, accounts for 85-90% of cases. Genetic prion diseases (e.g. familial CJD, familial fatal insomnia, and Gerstmann-Sträussler-Scheinker disease) account for 10-15% of cases, and acquired prion diseases (e.g. kuru, iatrogenic CJD, and variant CJD) account for less than 1% of cases (Takada et al., 2018). sCJD is characterized by rapidly progressive dementia, behavioral abnormalities, ataxia, and myoclonus, with hallmark histopathological changes including neuronal loss, gliosis, vacuolation, and accumulation of prion protein (Geschwind, 2015).

Prions diseases are prototypic neurodegenerative diseases that have given insight into concepts such as strains, aggregate accumulation and spread, aggregate transmission, and pathogenesis. Animal models serve to accurately represent the disease, providing a robust context to better understand neurodegenerative diseases. The replication and propagation of other neurodegenerative disease-related proteins such as tau, amyloid-β, and α-synuclein show similar pathological mechanisms to prions, including the ability to aggregate in different conformations (Scialò, 2019). Prion diseases are caused by a conformational change of the cellular prion protein (PrP^{C}) into infectious conformers (PrP^{Sc}), with evidence of involvement of the N-terminal domain of PrP^{C} in PrP^{C} trafficking (Prado et al., 2004; Foliaki et al., 2020). PrP^{C} has several known interactors relevant to neurodegenerative diseases, with interaction between residues of

the PrP^C N-terminus and amyloid-β oligomers having been shown to be critical for neurotoxicity (Zhang et al., 2019). Thus, a clearer understanding of PrP^C function and its role in neurodegeneration is necessary in order to generate effective therapeutic strategies and treatments for these diseases, of which none are curative.

PrP^C structure and function

Prion protein (PrP) exists in two forms: a normal glycosylphosphatidylinositol (GPI)-anchored membrane bound glycoprotein rich in alpha helical content (PrP^C) and a pathogenic, conformationally altered isoform (PrP^{Sc}) (Imran & Mahmood, 2011; Westergard et al., 2007). PrP^C consists of a highly structured, globular C-terminus (residues 121-231) that possesses two glycosylation sites as well as cysteine residues that act as sites for a disulfide bridge. The C-terminus also has been shown to facilitate interaction of PrP^C and PrP^{Sc} (Deignan et al., 2004). The N-terminus (residues 23-120) is referred to as the "flexible tail," and is an intrinsically disordered region that contains the metal-binding octarepeat domain critical to PrP^C endocytosis and trafficking (Evans & Millhauser, 2017).

PrP^{Sc} is thought to induce conformational conversion of PrP^C to PrP^{Sc} through a seeded protein polymerization mechanism that propagates PrP^{Sc} (Prusiner, 1982; Igel-Egalon et al., 2019). Interestingly, PrP^C null mice lines have been shown to be resistant to prions, indicating that PrP^C is necessary for PrP^{Sc} formation and PrP^C concentration is the rate-limiting step in disease development (Aguzzi et al., 2000). Mice hemizygous for the *Prnp* gene show partial resistance to scrapie infection (Büeler et al.,1994). The role of PrP^C is still not entirely understood, although it has been implicated in a number of processes including oxidative stress, circadian cycle, neurogenesis, neurite outgrowth, neuronal survival, olfactory behavior, cell

synaptic function, apoptosis, and as a potential receptor for amyloid beta in Alzheimer's disease (Guerrero et al., 2017). Due to its range of potential involvement in cellular function, further research regarding PrP^C function and how PrP^C function is dysregulated in disease is of broad interest.

The role of PrP^C in neurotoxicity

Genetic depletion of PrP^C in mice has been shown to have reverse early spongiform change, prevent neuronal loss, and mitigate progression to clinical disease even in the presence of neuroinvasive prion infection, implicating PrP^C as a major contributor to disease pathology (Mallucci et al., 2003). Studies have also implicated the disordered N-terminal domain of PrP^C as a mediator of PrP^{Sc} toxicity (Resenberger et al., 2011). The amino terminus has previously been implicated in neurotoxicity, as additional N-terminal octapeptide repeats have been shown to cause human prion disease (Goldfarb et al., 1991) and neurotoxicity in mice (Chiesa et al., 1998), with octapeptide repeat deletions preventing neurotoxicity (Sonati et al., 2013). N-terminal PrP^C mutations have been shown to generate abnormal ionic currents, sensitize neurons to glutamate-induced, calcium-mediated death (Biasini et al., 2013), and contribute to degeneration of dendrites of murine hippocampal neurons (Wu et al., 2017),

One mechanism by which excitotoxicity may occur is through N-methyl-D-aspartic acid receptors (NMDARs), as NMDAR-mediated calcium influx yielding increased extrasynaptic glutamate has been shown to be excitotoxic to neurons (Rivas-Santisteban et al., 2023). PrP^C modulates NMDAR in a Cu2+-dependent manner, with misfolded PrP^C inducing abnormally large ionic currents in NMDARs (Mahabadi & Taghibiglou, 2020). PrP^C has been implicated in binding NMDARs, with one study showing that an amino-terminally truncated recombinant PrP

fragment that assumed a toxic conformation leads to calcium influx and cerebellar granule neuron apoptosis. Pretreatment of these neurons with NMDA antagonists reduced these effects, suggesting that PrP^{C} toxicity is mediated by NMDAR (Thellung et al., 2013).

Glycosylation of the N-linked glycosylation sites of the prion protein have been shown to vary between prion strains, and could contribute to the variation in the disease. PrP^{Sc} in familial CJD and protease-sensitive prionopathy, for example, is unglycosylated at N181 (Schilling et al., 2023). Prion protein exists *in vivo* in unglycosylated, mono-glycosylated, and di-glycosylated forms that vary in molecular composition and size. As such, a model is needed to better understand how PrP^{C} interacts with NMDARs, and how PrP^{C} glycosylation could impact this interaction.

Protein glycosylation has also been shown to impact autophagy, with many soluble extracellular glycoprotein ligands activating autophagy whereas extracellular matrix-associated glycoprotein ligands appear to repress autophagy (Neill et al., 2021; Fung et al., 2008). *N*-glycans specifically have been shown to provide assistance in correctly folding proteins in the endoplasmic reticulum (ER), label misfolded proteins for ER-associated degradation, and route glycoproteins from the ER toward the Golgi and beyond (Reggiori et al., 2021), underscoring the potential role of glycosylation in affecting autophagic machinery. However, much of this area remains unexplored, necessitating further work that interrogates how PrP^{C} glycosylation could affect autophagic activity.

<u>A G92N knock-in mouse model used to characterize PrP^{C} toxicity</u>

Further experimentation is needed to better characterize how PrP^{C} impacts autophagic machinery. To better understand the role of the N-terminus of PrP^{C} in neurodegeneration, a

knock-in mouse line was generated expressing an extra glycan via a glycine to asparagine point mutation (G92N) at residue 92. These mice develop seizures, severe and progressive neurodegeneration, and necrosis of hippocampal neurons similar to mice with prion disease, but without PrP^{Sc}. *Prnp*^{92N} mice reach terminal disease around 25-30 days postnatal (p25-30). Using this model, preliminary evidence has shown increased p62 levels and an accumulation of autophagosomes via electron-microscopy in hippocampus. The model uncouples protein aggregation from the autophagy in prion disease and rather points toward PrP^{C} altering the signaling through which autophagy is regulated. More evidence is needed to understand if and at what point in the autophagic pathway potential disruptions occur, as well as if and how different brain regions are affected. By altering normal PrP^{C} function, this model may help to illuminate the role PrP^{C} plays in disease pathology.

Chapter 1 Autophagy and neuroinflammation in a prion disease model

Basic mechanisms of autophagy

Autophagy is the main intracellular degradation system through which cytoplasmic materials are recycled to produce new metabolites and energy for cellular renovation and homeostatic maintenance (Mizushima & Komatsu, 2011). It is one of the main mechanisms through which excess or damaged organelles are eliminated in response to various stressors, including nutrient deprivation, ischemia, hypoxia, metabolic stress, and aggregated/misfolded proteins, making it an important mechanism in development, growth, aging, and waste removal (Nie, Zhu, and Yang, 2021). Autophagic dysfunction has been linked to several pathological conditions, including cancers (Lei et al., 2017), neurodegenerative diseases (Chen et al., 2017; Hwang et al., 2017), and metabolic disorders (Miettinen & Björklund, 2016). Autophagy is a well-conserved catabolic pathway that exists from yeast to humans (Reggiori & Klionsky, 2002), with three primary types of autophagy present in mammalian cells: macroautophagy (herein referred to as autophagy), microautophagy, and chaperone-mediated autophagy (Parzych & Klionsky, 2014). Further, there are five distinct phases of autophagy: initiation, nucleation of the autophagosome, expansion and elongation of the autophagosome membrane, closure and fusion with the lysosome, and degradation of intravesical products (Mulcahy & Thorburn, 2020).

Autophagy is initiated through the Atg1 complex, in which nutrient-sensing pathways such as 5' AMP-activated protein kinase (AMPK) and the target of rapamycin complex 1 (mTOR) detect stimuli necessary for autophagic induction. AMPK acts as a cellular energy sensor that promotes autophagy through direct phosphorylation of upstream autophagy-related proteins such as mTOR, ULK1, and PIK3C3/VPS34 complexes. AMPK can also upregulate autophagic degradation of mitochondria through induction of fragmentation of and promotion of

translocation of autophagic machinery to damaged mitochondria (Li & Chen, 2019). mTOR is considered the main negative regulator of autophagy, and under normal conditions maintains autophagy at a basal level through phosphorylation of key Atg1 kinase complex proteins such as ULK1 (He & Klionsky, 2009). Active mTOR specifically phosphorylates ULK1 Ser 757 (ULK1 S757) to disrupt ULK1 activation and ULK1/AMPK interaction, inhibiting autophagy (Kim et al., 2011). During starvation or autophagy induction through compounds such as rapamycin, mTOR is inhibited and ULK1 auto-phosphorylates at Thr-226, increasing kinase activity and recruiting other autophagy-related proteins to localize at the phagophore assembly site (PAS) to begin nucleation (Ariosa & Klionsky, 2016).

Nucleation is the process by which proteins needed for phagophore expansion into a fully formed autophagosome are recruited to the PAS. The ATG14-containing class III phosphatidylinositol 3-kinase (PtdIns3K) complex consists of PIK3C3/VPS34, PIK3R4/p150, and beclin-1, which in tandem generate PtdIns3P, a required phospholipid for autophagy that further recruits nucleation-related proteins (Parzych & Klionsky, 2014). Beclin-1 specifically is an autophagic mediator that regulates endocytic trafficking, LC3-associated phagocytosis, and other membrane trafficking processes (Tran, Fairlie, and Lee, 2021). Once all proteins necessitated by nucleation are present at the PAS, expansion, and maturation of the autophagosome begins.

To expand and fully form the autophagosome, two ubiquitin-like (Ubl) conjugations systems function to elongate the phagophore using microtubule-associated protein 1 light chain 3 (LC3). The ATG-4 processed form of LC3 is referred to as LC3-I (or LC3-A), and the phosphatidylethanolamine (PE)-conjugated form is called LC3-II (LC3-B) (Parzych & Klionsky, 2014). In the first Ubl reaction, Atg12 associates with Atg5 to then form a multimeric complex

with Atg16. In the second Ubl reaction, LC3 is cleaved by Atg4, then recognized and processed by Atg7 and Atg3 (López et al., 2020). The proteolyzed LC3-A protein then covalently attaches to LC3-B, facilitated by an E3-like Atg12-Atg5-Atg16 complex (Feng et al., 2014). Necessary remaining membrane is delivered from peripheral sites to the PAS by shuttling of Atg9, which ultimately results in the completion of the autophagosome (López et al., 2020).

After completion, autophagosomes are able to target, dock, and fuse with degradative organelles (Yin, Pascual, and Klionsky, 2016). LC3 is present on the inner and outer autophagosome membranes, with the outer side being released by Atg4-dependent cleavage (a necessary step for the fusion phase), and the inner population is degraded in the degradative organelle (López et al., 2020). LC3 has widely been used as a marker for autophagic activity, as it is associated with close, hemi-fusion, and transport of autophagosomes (the number of autophagosomes) and is also degraded by autophagy as a substrate (monitor of autophagic activity) (Lee & Lee, 2016). Cargo is delivered inside the degradative organelle, or autolysosome, which is the result of the fusion of an autophagosome with a lysosome, otherwise called an autolysosome. The autolysosome will degrade autophagic cargo and the inner autophagosomal membrane, and metabolites made from the process are released back into the cytosol to be reutilized by the cell (Lőrincz & Juhász, 2020).

Other proteins integral to the autophagic process are LAMP1, p62, and Rab7. LAMP1, or lysosomal associated membrane protein 1, is a lysosomal marker that can be used to assay for LAMP1-positive organelles and/or LAMP1 distribution as an indicator of functioning degradative machinery, with absence of LAMP1 and LAMP2 being associated with increased accumulation of autophagic vacuoles (Cheng et al., 2018; Eskelinen, 2006). LAMP1 is required for tethering and fusion in generating the autophagolysosome, and thus can be used as a marker

for the autophagic tethering and fusion process (Briones-Herrera et al., 2022). p62 is a ubiquitin-binding scaffolding protein that recognizes and shuttles ubiquitinated proteins to autophagosomes for degradation. As p62 accumulates with inhibited autophagy and decreases when autophagy is induced, it has been used as a marker for autophagic malfunction (Bjørkøy et al., 2009). Finally, Rab7 is a late endosome/lysosome-associated small GTPase that participates in endosomal sorting, biogenesis of lysosomes, and phagocytosis. Rab7 has been shown to regulate autophagosome-lysosome fusion and is essential for autolysosome maturation, and mutations or dysfunctions in Rab7 are associated with a number of traffic disorders (Kuchitsu & Fukuda, 2018; Zhang et al., 2009).

Autophagy in prion disease

Data on autophagy on prion disease was limited until fairly recently, with many recent experiments measuring autophagy being conducted in cell culture (Abdelaziz et al., 2019). *In vitro* results do not always translate to *in vivo* effects, and it is still not entirely clear how autophagy functions and how it is dysregulated in prion diseases. Autophagic induction has been shown to interfere with prion infection, with varying effects: treating prion-infected mice with rapamycin, tacrolimus, and Gleevec, for example, prolonged survival (Cortes et al., 2012; Nakagaki et al., 2013; Ertmer et al., 2007), whereas treating with resveratrol, trehalose and lithium had no effect on survival despite showing promise in cell culture (Wang et al., 2016; Aguib et al., 2009; Heiseke et al., 2009). There is strong evidence that autophagy is involved in prion diseases, however, as autophagosomes have been observed in patients brains and experimentally inoculated mice. Enhanced autophagy has been shown to increase PrP^{Sc} degradation and confer neuroprotectivity (Abdelaziz et al., 2019).

PrP^C has been implicated in autophagic function, with *Prnp* knockout mice showing increased LC3-B and autophagosomes in hippocampal neurons under serum starvation. This increase was inhibited by transfection of the wild-type *Prnp* gene (Oh et al., 2008). Another study demonstrated that H_2O_2-induced oxidative stress in hippocampal neurons activated autophagic flux in $Prnp^{+/+}$ cells and impaired autophagic flux in $Prnp^{0/0}$ cells at early stages. Autophagic flux activity remained in $Prnp^{+/+}$ cells at later stages, and interestingly was reactivated in $Prnp^{0/0}$ cells at later stages (Oh et al., 2012).

PrP^C has also demonstrated several roles related to cellular metabolism, signal transduction, neuroprotection, anti-stress, and anti-apoptotic functions. In experimental mouse models, PrP^C expression has been shown to maintain cellular homeostasis through autophagy, whereas PrP^C deficient mice show signs of impaired autophagic flux (e.g. increases in LC3B, p62, and LAMP1 levels) and autophagic accumulation that may ultimately contribute to autophagic cell death (Shin, Oh, and Kim, 2013). The mechanism behind autophagic alteration in prion diseases is largely unknown.

The role of neuroinflammation in neurodegeneration

Neurodegenerative disease research has also illuminated neuroinflammation as a key player in the neurodegenerative process, with chronic inflammation because of immune signaling having been shown to release neurotoxic factors that exacerbate disease (Zhang et al., 2023). Dysregulated autophagy has also been implicated in the initiation and progression of neuroinflammation, with autophagic deficiency underlying synaptic deficits in neurodegenerative disorders (Bai et al., 2024).

In prion disease, neuroinflammation typically manifests as microglial activation and astrogliosis accompanied by transcriptomic alterations (Li, Chen, and Zhu, 2021). Microglia have been shown to be significantly increased and in an active state in CJD and in prion-infected animals (Sasaki, Hirato, and Nakazato, 1993; Williams et al., 1994) and closely associated with plaques (Guiroy et al., 1994; Mühleisen, Gehrmann, and Meyermann, 1995). Microglial activation occurs at an early disease stage preceding neuronal death and onset of clinical symptoms (Betmouni et al., 1996; Giese et al., 1998), with genes upregulated being expressed mainly by activated microglia (Vincenti et al., 2015). Thus, microglial activation is implicated in driving prion-induced neurodegeneration. Ionized calcium-binding adapter molecule 1 (Iba1) is a reliable cytoplasmic microglial marker (Schwabenland et al., 2021) that has previously been shown to be significantly upregulated in terminal prion-infected animals (Hartmann et al., 2019), and can be used to monitor microglial activity.

Astrogliosis is a common and well-documented phenomena in prion disease. During astrogliosis, astrocytes become activated upon stimulation and exhibit morphological changes and upregulation of glial fibrillary acidic protein (GFAP) expression (Escartin et al., 2021). Astrogliosis is pronounced and widespread in prion disease, as indicated by significantly increased astrocyte cell numbers and associated GFAP signals in multiple prion disease models (Li, Chen, and Zhu, 2021). PrP^{Sc} has been shown to deposit in astrocytes (Na et al., 2007), with evidence of isolated primary astrocytes from ovine PrP-overexpressing transgenic mice being able to sustain efficient replication and propagation of sheep scrapie (Cronier, Laude, and Peyrin, 2004). Dysregulated p-PERK signaling in astrocytes turns astrocytes into reactive states, causing synaptic trophism (i.e. neuronal growth and synapse formation) and neuroprotective function loss. Blocking of p-PERK signaling in prion-infected mice reverses this activation state and

restores synaptic trophism and neuroprotective function, improves behavioral deficits, and increases survival (Smith et al., 2020). Overall, reactive astrocytes replicate and accumulate prions in the brain, and their aberrant phenotype along with dysregulated signaling may play a neurotoxic role in prion pathogenesis.

N-myc downstream-regulated gene 2 (NDRG2) is a member of the NDRG gene family that plays an important role in cell proliferation, differentiation, and apoptosis (Li et al., 2020). Much of the research surrounding NDRG2 has been done in the context of cancer, with loss of NDRG2 causing decreased survival and increased proliferation, migration, and invasiveness *in vitro* as well as tumor growth and metastasis *in vivo* in a gallbladder carcinoma model (Lee et al., 2015). Downregulation of NDRG2 has also been associated with worse prognosis in gastric cancer patients (Ling et al., 2015), higher malignancy in glioma tumor progression (Skiriutė et al., 2014), and the lowest survival rate amongst lung adenocarcinoma patients (Wang et al., 2012).

In the CNS, NDRG2 has been found to be highly expressed in astrocytes (Flügge et al., 2014). Silencing NDRG2 increased 5-bromo-2'-deoxy-uridine-incorporated and proliferating cell nuclear antigen-positive cells while adenovirus-mediated overexpression displayed the opposite, suggesting a regulatory role of NDRG2 in astrocyte activation (Takeichi et al., 2011). Deletion of NDRG2 resulted in lower GFAP expression levels in astrocytes, indicating a potential role of NDRG2 mediating reactive astrogliosis (Takarada-Iemata et al., 2014). NDRG2 has also been found to be expressed in neurons, with neuronal NDRG2 overexpression promoting neurite sprouting and elongation (Takashi et al., 2005).

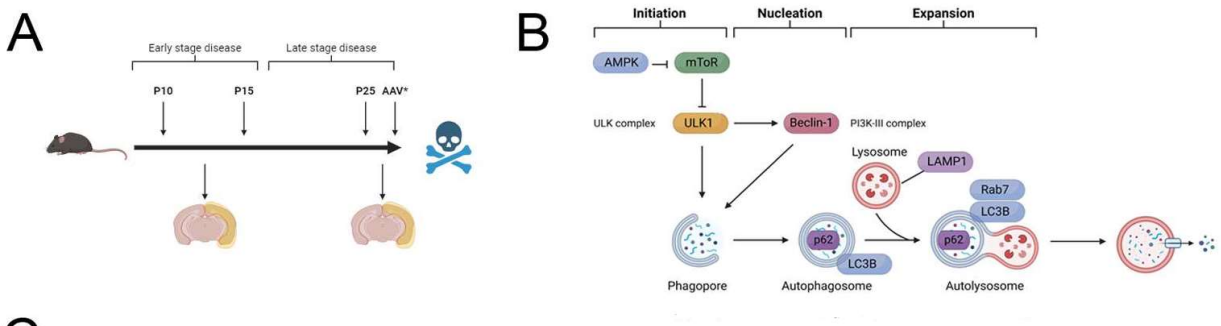
NDRG2 has been shown to stabilize Na^+/K^+-ATPase β1 and thus Na^+ current, and NDRG2 deletion increases neuronal death via disruption in Na^+-dependent glutamate clearance

that results in glutamate excitotoxicity. Increasing NDRG2 expression in *Ndrg2*$^{-/-}$ astrocytes effectively rescues glutamate clearance and attenuates neuronal death (Yin et al., 2020). No studies have been done that examine the role of NDRG2 in the context of prion disease. Taken together, these results underscore the potential importance of NDRG2 as a therapeutic target, as well as the overall importance neuroinflammation plays in the progression of neurodegenerative diseases such as prion disease.

Results

To understand how autophagy may change in the *Prnp*^{92N} model, *Prnp*^{92N} mice were studied at different time points throughout the disease progression. *Prnp*^{92N} mice become terminal 25-30 days post-birth (P25-P30), with clinical symptoms worsening by P20. Thus, two early disease time points (P10 and P15) and one late-stage time point (P25) were established (Figure 1A). Key proteins in the autophagic cascade were selected and probed at each stage (Figure 1B).

Figure 1. *Prnp*^{92N} cerebral cortex shows blockage of autophagic initiation at early-stage disease. (A) Schematic diagram of time points in disease progression at which samples of *Prnp*^{WT} and *Prnp*^{92N} mice brains were collected. **(B)** Schematic diagram of autophagic phases and associated proteins that were probed. Western blots and quantification of phosphorylated AMPKα-T172, phosphorylated mTOR-S2448, and phosphorylated ULK-S757 at **(C)** P10, **(D)** P15, and **(E)** P25 in cerebral cortex. The animal denoted with an asterisk was an outlier and was not included in the quantification. Bar graphs represent the mean ± standard deviation. Welch's unpaired, 2-tailed *t*-test. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.


A
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P10
P15
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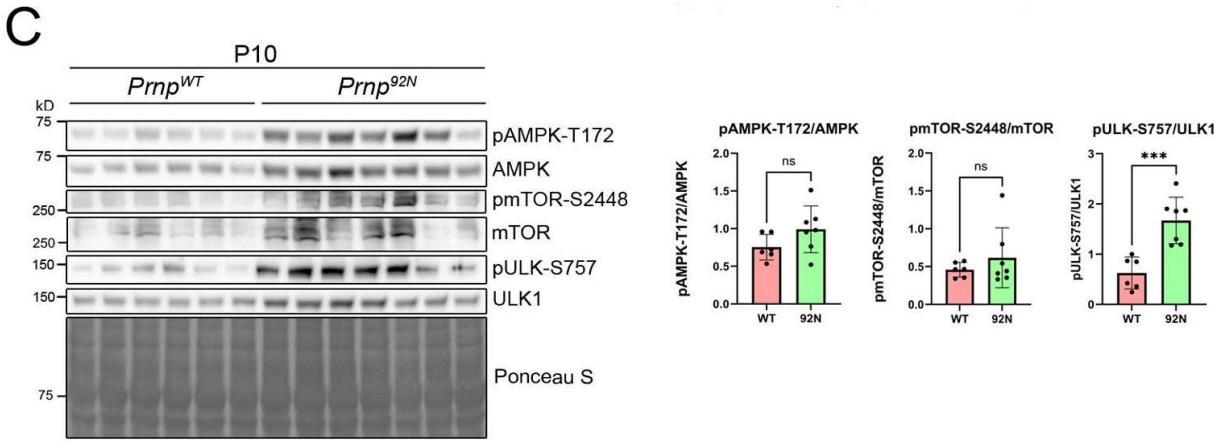
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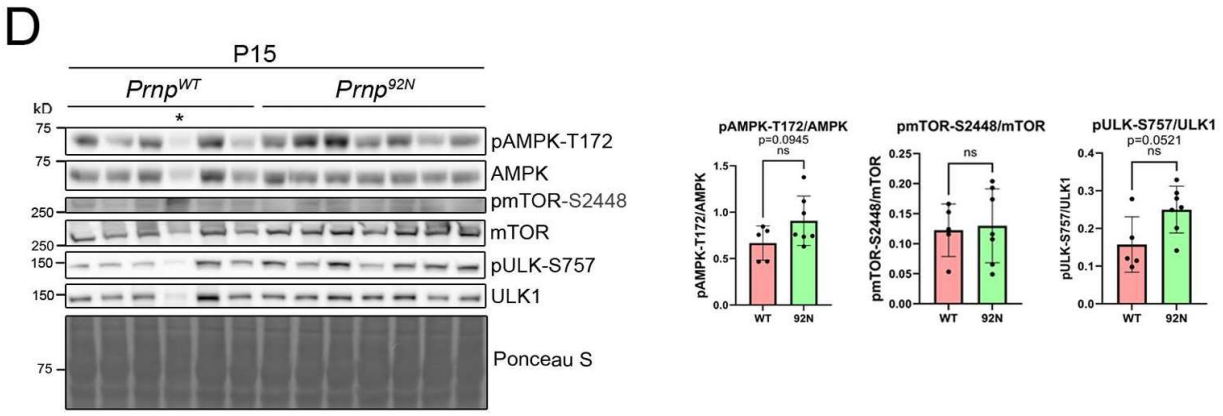
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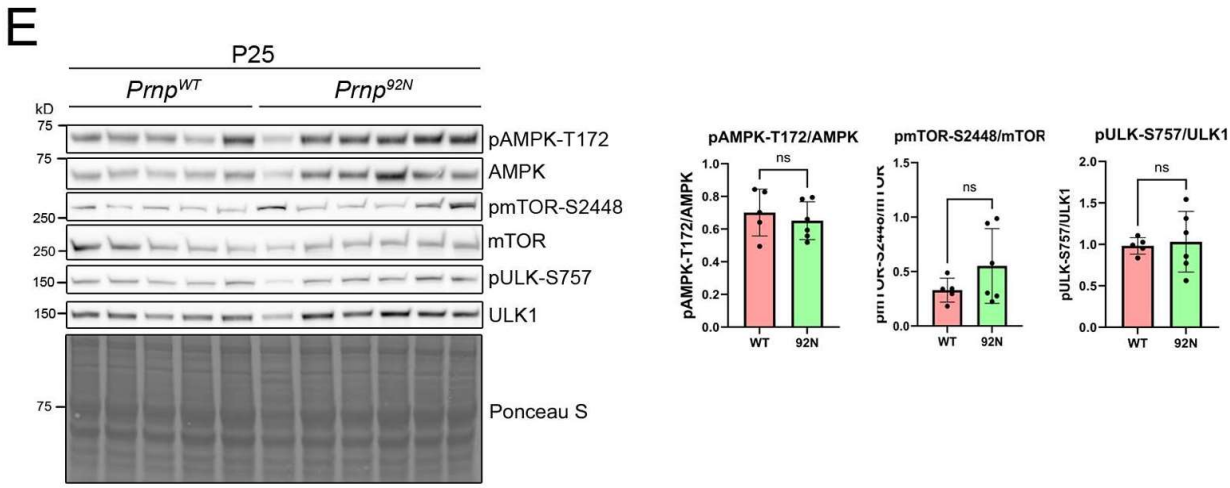
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pmTOR-S2448/mTOR
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WT
92N

Proteins involved in the initiation-phase of autophagy were probed by western blot of cerebral cortex at each time point. At P10, the *Prnp*^{92N} cortices showed a strong (2.68-fold) increase in pULK-S757/ULK1, indicating that ULK1 is inactive (Figure 1C). By P25, the levels were highly variable, potentially due to the different stage of disease (Figure 1E). At P15, there is a trending increase in pAMPK-T172/AMPK, indicating AMPK may be more active at this disease stage (Figure 1D). Collectively, these findings suggest an early inhibition of autophagic initiation.

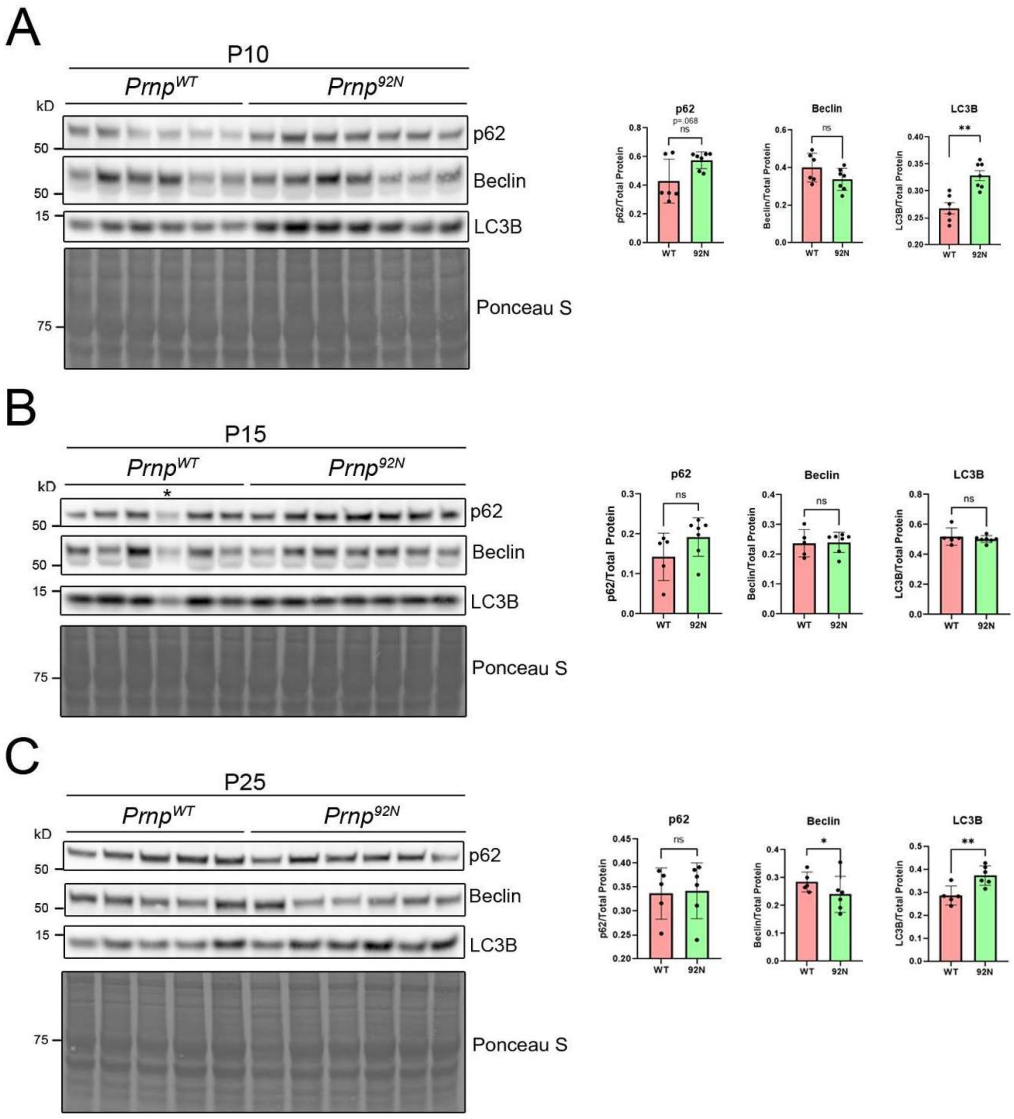


Figure 2. *Prnp*^{92N} cerebral cortex shows no change in levels of autophagy nucleation proteins at early-stage disease. Western blots and quantification of p62, beclin-1, and LC3B at **(A)** P10, **(B)** P15, and **(C)** P25 in cortex. Bar graphs represent the mean ± standard deviation. Welch's unpaired, 2-tailed *t*-test. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.

Autophagy nucleation-related proteins were then probed by western blot of cerebral cortex. At P10, LC3B was increased and there was a trend for increased p62 in the *Prnp*^{92N} mice (Figure 2A). By P15, the p62 levels were similar to *Prnp*^{WT} mice (Figure 2B). By P25, beclin-1 levels were decreased and LC3B levels were increased (Figure 2C). These results suggest an

early-stage increase in autophagosomes that correlates with blocked autophagic initiation, and loss of cytoprotective ability and autophagic homeostasis by late stage disease.

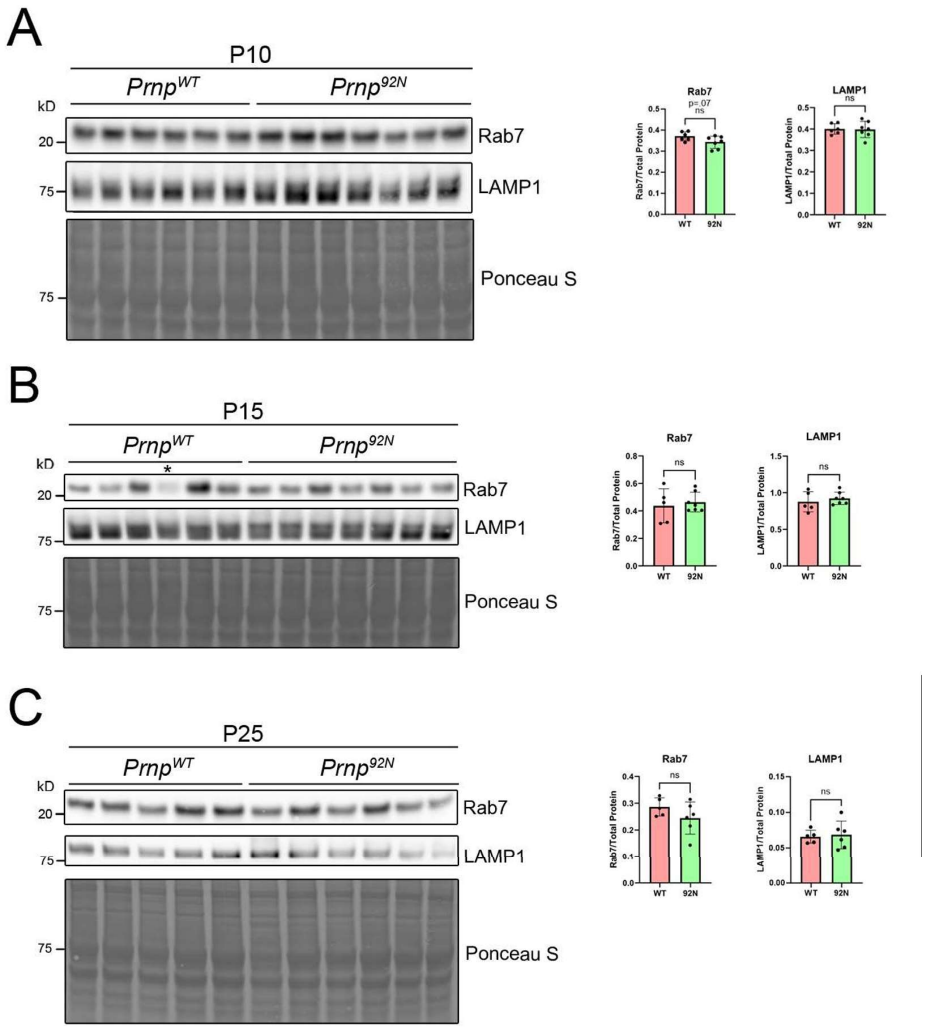


Figure 3. *Prnp^{92N}* cerebral cortex shows no change in autophagosome expansion proteins at early or late-stage disease. Western blots and quantification of Rab7 and LAMP1 at **(A)** P10, **(B)** P15, and **(C)** P25 in cortex. Bar graphs represent the mean ± standard deviation. Welch's unpaired, 2-tailed *t*-test. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.

No significant changes in autophagosome expansion-related proteins (Rab7 and LAMP1) were seen throughout the disease progression (Figure 3A-C). These results substantiate that autophagic alterations occur upstream in the autophagic cascade.

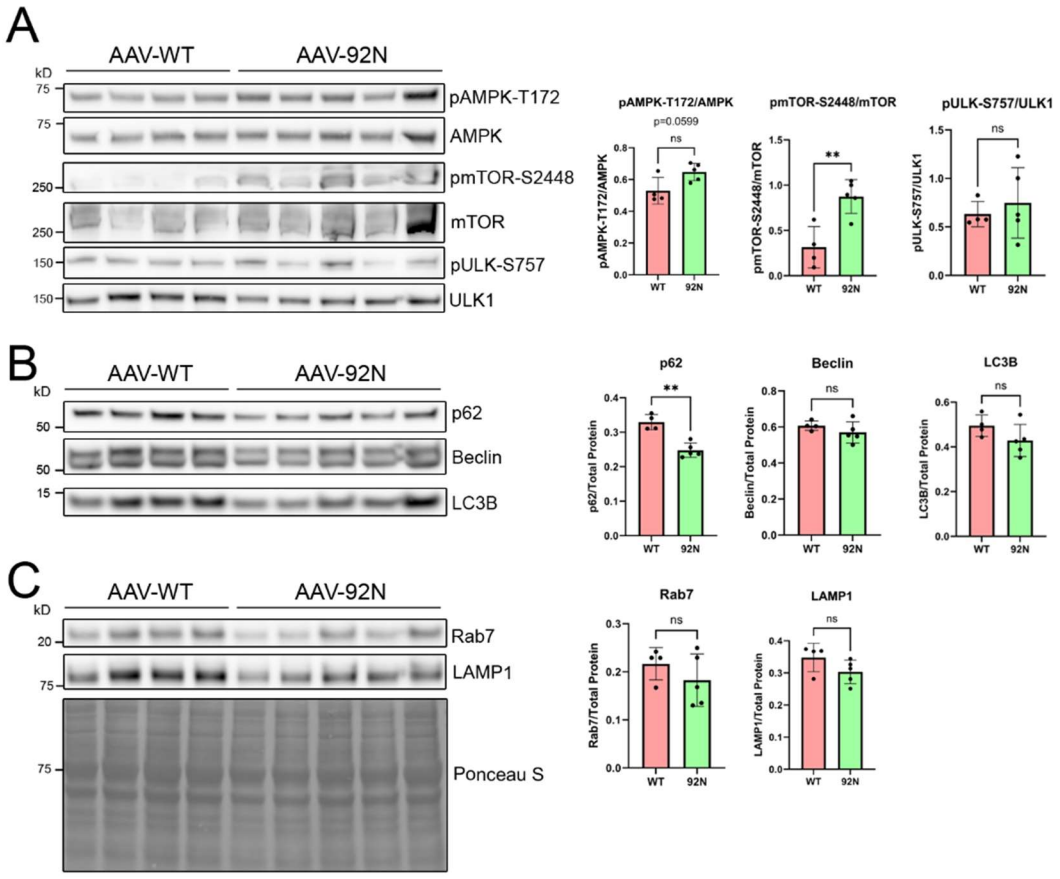
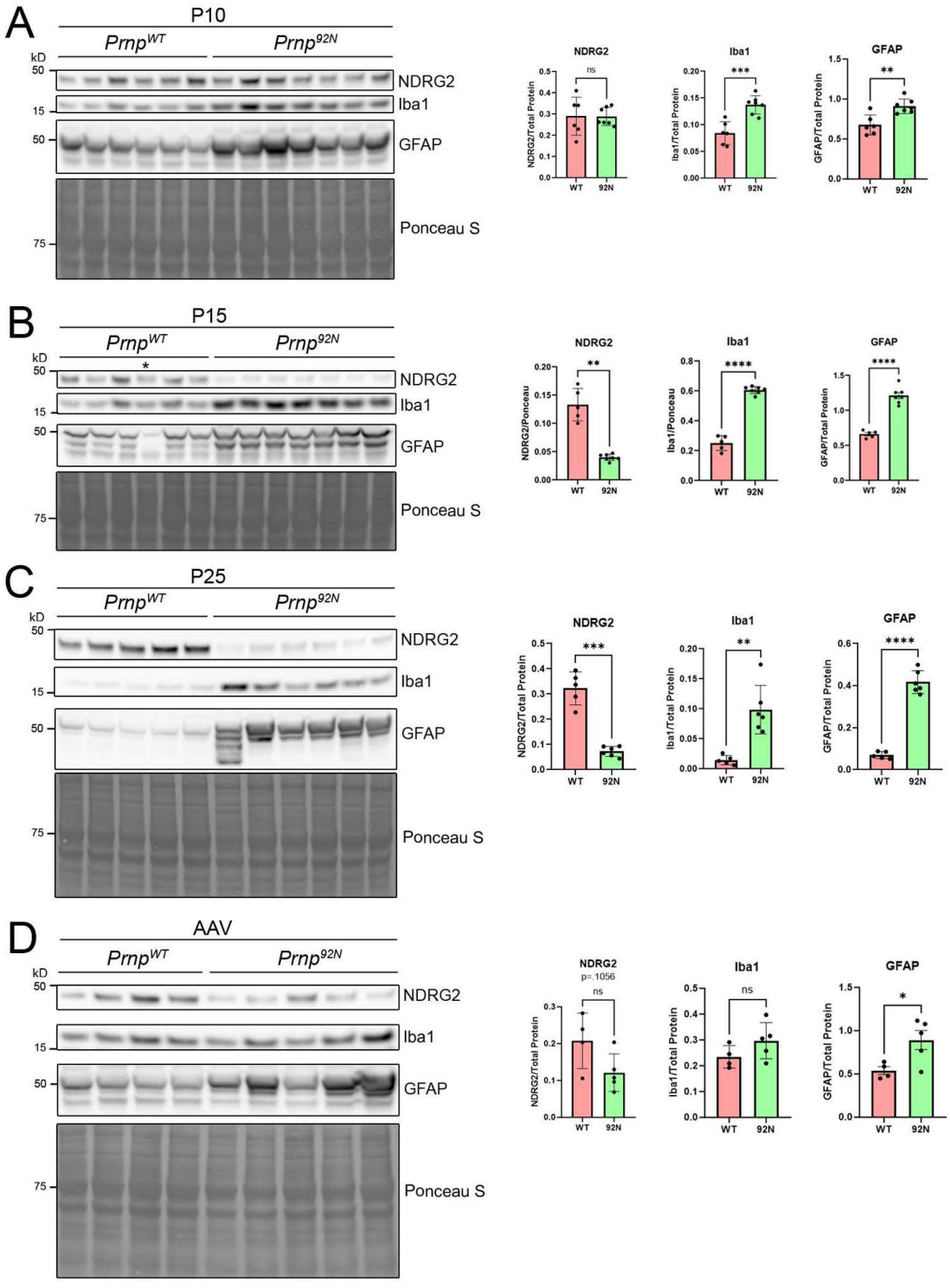


Figure 4. AAV-*hSyn1Prnp*^{92N} cerebral cortex shows aberrant levels of autophagic proteins in late-stage disease. Western blots and quantification of **(A)** phosphorylated AMPKα-T172, phosphorylated mTOR-S2448, phosphorylated ULK-S757, **(B)** p62, beclin-1, LC3B, **(C)** Rab7 and LAMP1 in cortex lysates. Bar graphs represent the mean ± standard deviation. Welch's unpaired, 2-tailed *t*-test. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.

We inoculated AAV-*hSyn1Prnp*^{92N} into *Prnp*$^{-/-}$ mice to specifically express *Prnp*^{92N} in neurons. Mice injected as neonates were collected at terminal disease and probed for autophagy-related proteins to understand how neuron-specific *Prnp*^{92N} compared to the knock-in *Prnp*^{92N} mice. AAV-*hSyn1Prnp*^{92N} cerebral cortices show a trending increase in pAMPK-T172/AMPK and a significant increase in pmTOR-S2448/mTOR (Figure 4A), as well as significantly decreased p62 (Figure 4B). Decreased p62 levels are typically associated with increased autophagy. Here the increase in mTOR activity, trending decrease in other autophagy proteins (e.g. beclin-1, LC3B, Rab7, and LAMP1), and terminal disease stage at which the mice were

collected suggest an inhibition of autophagy with decreased p62 levels likely being caused by a loss of autophagosome production. There was no change in autophagosome expansion-related proteins, similar to the *Prnp*^{92N} knock-in results, further suggesting an early-inhibition of autophagy.

Figure 5. *Prnp*^{92N} cerebral cortex shows a progressive increase of microglial and astrogliosis marker levels correlating with a significant loss of NDRG2. Western blots and quantification of NDRG2, Iba1, and GFAP at **(A)** P10, **(B)** P15, **(C)** P25, and **(D)** in AAV-*hSyn1Prnp*^{92N} in cortex lysates. Bar graphs represent the mean ± standard deviation. Welch's unpaired, 2-tailed *t*-test. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.


A
P10
Prnp^WT
Prnp^92N
kD
50
15
50
75
NDRG2
Iba1
GFAP
Ponceau S
NDRG2
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NDRG2/Total Protein
Iba1
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Iba1/Total Protein
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P15
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Iba1/Ponceau
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NDRG2/Total Protein
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GFAP/Total Protein
D
AAV
p=.1056
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*

Finally, microgliosis (Iba1) and astrogliosis (GFAP) markers were probed to better understand how neuroinflammation changes in the *Prnp*^{92N} mice expressing neurons only. Both Iba1 and GFAP are significantly increased throughout the disease time course (Figure 5A-C), correlating with a loss of NDRG2 at P15 (Figure 5B). AAV-*hSyn1Prnp*^{92N} mice show significantly increased GFAP levels and a trending decrease in NDRG2 levels (Figure 5D) at terminal disease, although this is less severe than in *Prnp*^{92N} knock-in mice at the same disease stage. These results suggest that neuroinflammation is significantly increased throughout the disease and could potentially contribute to the disease progression and autophagic dysregulation.

Discussion

PrP expression allows for maintenance of cellular homeostasis via autophagy whereas PrP-deficient cells exhibit impaired autophagic flux that leads to autophagic cell death (Shin et al., 2013). The exact mechanism by how this cellular maintenance of autophagy occurs, however, is still unclear. Autophagy is thought to be related to protein aggregation, in that autophagic induction is responsible for clearing aggregated PrP^{Sc} which ultimately acts as a neuroprotective mechanism against prion-induced toxicity (López-Pérez et al., 2019). Here we show a genetic model of prion disease that exhibits an early block in autophagic induction in the absence of protein aggregates, evidenced by a significant increase in pULK-S757/ULK1, p62, and LC3B protein at P10. This data is the first demonstration of a blockage of autophagic initiation in prion disease, and has been shown in relation to purely signaling and suggests a PrP^{C}-mediated signaling mechanism driving the inhibition independent of protein aggregation.

The accumulation of protease-resistant misfolded and aggregated proteins is a common feature of many neurodegenerative diseases, such as Alzheimer's disease (amyloid-beta),

Parkinson's disease (alpha-synuclein), amyotrophic lateral sclerosis (TDP-43), and prion diseases (PrP^{Sc}). This is largely due to proteins prone to aggregation into β-sheet-enriched oligomers being resistant to proteolytic pathways, allowing for growth into inclusion bodies or extracellular plaques (Ciechanover & Kwon, 2015). These endogenous, typically soluble proteins with established or putative physiological roles assemble into insoluble, fibrillar species that contribute to the various symptoms and progression rates of these diseases (Davis, Leyns, and Holtzman, 2018). Generally, these aggregates appear to be toxic and lead to cell injury or death, with disease severity often correlating with the abundance of accumulated protein (Voisine, Pedersen, and Morimoto, 2010; Williams et al., 2006). As such, many therapeutics have focused on improving protein clearance pathways such as autophagy that target these seemingly toxic protein aggregates.

Excessive or insufficient autophagic activity in neurons leads to altered homeostasis and neurodegeneration in several neurodegenerative disease models (Kesidou et al., 2013). Previous studies have shown impaired autophagosome clearance and thus autophagosome accumulation in Alzheimer's disease (Lee et a., 2010), deficits in autophagosome formation and autophagosome-lysosome fusion in Parkinson's disease (Winslow et al., 2010; Dehay et al., 2012), and reduction in dynein-mediated transport leading to impaired autophagosome-lysosome fusion and autophagosome accumulation in amyotrophic lateral sclerosis (Kimura, Noda, and Yoshimori, 2008; Ravikumar et al., 2005). In prion disease, autophagosomes have been observed in experimental rodent models and scrapie prion-infected cultured cells (Boellaard, Schlote, and Tateishi, 1989; Schätzl et al., 1997). The $Prnp^{92N}$ mice have a similar accumulation of lysosomes by P20 and autophagosomes by P25, but this accumulation occurs in the absence of misfolded protein aggregation.

This study highlights an alternative signaling-based mechanism that underscores the neurotoxic pathway in prion disease. In normal autophagic function, phosphorylation of AMPK at T172 stimulates AMPK activity, promoting autophagy (Li & Chen, 2019). Phosphorylation of mTOR at S2448 promotes mTOR activity, which inhibits autophagy (Jung et al., 2010). Autophagic inhibition by mTOR is regulated by phosphorylation of ULK1 at S757, a major inhibitory site that disrupts ULK1-AMPK interactions and prevents autophagic initiation (Egan et al., 2011). *Prnp*^{92N} mice cortices demonstrate high levels of pULK-S757/ULK1 in early-stage disease, pointing toward autophagic inhibition early in the disease progression. Increases in p62 and in LC3B at P10 suggest an accumulation of substrate that should normally be degraded by autophagy, as p62 accumulates when autophagy is inhibited (Islam, Sooro, and Zhang, 2018).

Proteins associated with autophagosome expansion (Rab7 and LAMP1) show no change throughout the disease time course, suggesting that this blockage of autophagy occurs upstream in the autophagic cascade. *Prnp*^{92N} mice demonstrate NMDA-triggered calcium influx, which could be related to autophagic inhibition as elevation in intracellular calcium levels has been shown to potently inhibit autophagy as well as inhibit autophagosome biogenesis in the initiation phase (Engedal et al., 2013). This shunting of the initiation of autophagy could lead to the incomplete autophagosomes or impaired autophagosome-lysosome fusion that ultimately leads to autophagic vesicle accumulation, as normal degradation processes are not functional. This initial initiation block may affect autophagy even into late-stage disease, as beclin-1, a key cytoprotective protein, levels decrease while LC3B levels increase, providing further evidence of autophagic loss. Beclin-1 levels are also reduced in Alzheimer's disease brain tissue (Pickford et al., 2008) and cleavage of beclin-1 by caspase-3 has been shown to impair autophagosome formation (Rohn et al., 2011).

Mice that express *Prnp*^{92N} specifically in neurons (AAV-*hSyn1Prnp*^{92N} mice) reached terminal disease stages at approximately 11 days post-injection, which is a more rapid time course compared to the normal 25-30 day lifespan of *Prnp*^{92N} mice. These mice exhibit significantly decreased p62 levels but increased mTOR activity at terminal disease. Although decreased p62 is associated with increased autophagic activity, this decrease is likely due to cell death lowering overall p62 levels rather than a salvatory autophagic mechanism. Many of the other autophagy-related protein levels probed in this model (e.g. beclin-1, LC3B, Rab7, and LAMP1) were trending down, suggesting an autophagic shutdown in which autophagosomes are not able to fully form or form less frequently. mTOR activity would inhibit autophagosome formation and thus corroborate these results.

Further, microgliosis (Iba1) and astrogliosis (GFAP) markers are significantly elevated by P10 and remain at significantly high levels throughout the disease, with both preceding major neuronal loss. This is consistent with other prion disease models (Bourgognon et al., 2021) and in other neurodegenerative diseases (Kwon & Koh, 2020), providing further evidence of a link between neuroinflammation and disease progression. Increases in Iba1 and GFAP correlate with a loss of NDRG2, which occurs between by P15. Further loss of NDRG2 corresponds with an elevation in Iba1 and GFAP levels by P25, suggesting a strong correlation between NDRG2 expression, microgliosis, and astrogliosis.

The mechanism behind the phenotypic changes of microglia and astrocytes when reactive, their loss of neuroprotective function, and their gain of neurotoxic functions are complicated and still not entirely understood. However, protein aggregation in early-stage disease may influence immune signaling that further aggravates protein aggregation and ultimately advances neurodegeneration (Zhang et al., 2023). Here we show evidence of

significantly increased neuroinflammation without the presence of aggregates, suggesting an alternative signaling mechanism that influences neuroinflammation.

Interestingly, in AAV-*hSyn1Prnp*^{92N} mice, NDRG2 is not as significantly altered at terminal disease and Iba1 levels are consistent with wild-type mice. GFAP is still significantly elevated, suggesting that neuronal *Prnp*^{92N} expression may be sufficient to trigger astrogliosis. This lack of microglial activity and thus absence of reactive microglia in the presence of elevated astrogliosis levels suggests that NDRG2 activity is more linked to astrocyte reactivity, which is consistent with previous studies (Flügge et al., 2014; Takeichi et al., 2011). This data underscores the potential role of neuronal PrP in influencing astrocyte activity, but not microglial activity.

Neuronal damage primarily and initially occurs in the hippocampus of *Prnp*^{92N} mice, eventually radiating outward to the thalamus and cortex by end-stage disease. Improving autophagic induction early in the disease progression prior to severe damage could potentially aid in preventing neuronal loss and may be a potential therapeutic mechanism, as pharmacological activation of autophagy in previous prion disease models has prevented neuronal death (Cortes et al., 2012; Thellung et al., 2018). This study supports the hypothesis that autophagy is involved in prion-mediated neurodegeneration, and that autophagic initiation in early-stage disease may contribute to the rapid disease progression.

Methods

Animals

All mice are kept in a 12-hour light/dark cycle with unlimited access to food and water. *Prnp*^{92N} mice were backcrossed onto the C57/BL6 background and were observed daily for

clinical signs. All mouse handling complies with the Institutional Animal Care and Use Committee's animal use protocols.

Brain tissue collection, homogenization, and lysis

Cerebral cortex samples from P10, P15, P25, and AAV mice were collected and snap-frozen in liquid nitrogen. Cortex samples from all mice were weighed and then homogenized in RIPA buffer (50 mM Tris-HCL pH 7.4, 150 mM NaCl, 1mM EDTA, and ultrapure water) with a 1:10 dilution of 10x detergent (1% NP40, 0.5% DOC, 0.1% SDS) and benzonase $MgCl_2$ (weight (g) x 400). Protease inhibitors and phospho-stop were also added, and a bead beater tissue homogenizer was used to create 10% brain homogenate. Samples were then lysed with RIPA buffer at 4-degrees Celsius with rotation for 30 minutes, then kept at -80-degrees Celsius.

Western blot analysis

Equal protein levels for each mouse sample were quantified using a bicinchoninic acid protein assay (BCA). Mouse brain samples were loaded on 4-12% Bis-Tris (P10 and P15) or 10% Bis-Tris (P25 and AAV) gels, and samples were with either MES or MOPS 1X running buffer and transferred to a nitrocellulose membrane by wet blotting. Membranes were incubated overnight at 4-degrees Celsius in primary antibodies: pAMPKα-T172 (Cell Signaling Technology (CST), Cat #: 2535), AMPKα (CST, Cat #: 2532), pmTOR-S2448 (CST, Cat #: 5536), mTOR (CST, Cat #: 2983), pULK-S757 (CST, Cat #: 14202), ULK1 (CST, Cat #: 8054), p62 (ABNOVA, Cat #: H00008878-M01), beclin-1 (BD Biosciences, Cat #: 612113), LC3B (Novus Biologicals, Cat #: NB100-2220), Rab7 (CST, Cat #: 9367), LAMP1 (CST, Cat #: 46843), NDRG2 (CST, Cat #: 5667), Iba1 (CST, Cat #: 17198), and GFAP (Dako, Cat #: W20334) in 1x casein blocking buffer or 1% BSA (bovine serum albumin). Membranes were

washed with 1x TBST the following day, followed by one hour of incubation in respective HRP-conjugated IgG secondary antibody at room temperature. Membranes were then imaged using a chemiluminescent substrate (ECL Supersignal West Dura or Fempto, ThermoFisher Scientific) and visualized using a Fuji LAS 4000 imager. Images were quantified using Multi Gauge version 3.0 software.

Statistical analysis

A Student's *t*-test (two-tailed, unpaired) with a Welch's post hoc correction was used to analyze $Prnp^{WT}$ and $Prnp^{92N}$ mice for protein levels of pAMPKα-T172, AMPKα, pmTOR-S2448, mTOR, pULK-S757, ULK1, p62, beclin-1, LC3B, Rab7, LAMP1, NDRG2, Iba1, and GFAP. Statistical analysis was completed on GraphPad Prism 10 software for western blot quantification. A $p \leq 0.05$ was considered statistically significant, and data represent mean ± standard deviation.

Chapter 2 Establishing a prion disease model in *Drosophila melanogaster*

Drosophila melanogaster and neurodegenerative disease

Drosophila melanogaster has been used for decades as a biological model to understand fundamental and evolutionarily conserved genetic and cellular processes (Verheyen, 2022). Roughly 75% of all human genes implicated in disease have functional *Drosophila* homologs (Rubin et al., 2000), and their low genetic redundancy aids in resolving questions regarding human disease models in cell culture and vertebrates that are inconclusive (Bier, 2005). While small rodents have been used extensively as experimental models of neurodegenerative diseases such as prion disease (Groschup & Buschmann, 2008), mammalian models come with a host of issues, including long incubation times prior to onset of clinical disease and large financial costs (Bujdoso et al., 2023). *Drosophila* offer several additional advantages as a model system, including low cost, rapid reproduction rate, short life span, and ease of genetic manipulation.

Drosophila have been used to model various major neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, dementia with lewy bodies, amyotrophic lateral sclerosis, frontotemporal dementia, and Huntington's disease (Bolus et al., 2020). Prion disease has also been modeled in *Drosophila*, with the first report of a transgenic *Drosophila* expressing wild-type hamster prion protein. Interestingly, PrP expressed in *Drosophila* show a lower molecular mass compared to mammalian species expression (Raeber et al., 1995). Other studies show locomotor dysfunction and neurotoxicity in *Drosophila* transgenic for wild-type hamster or mouse PrP (Fernandez-Funez et al., 2009) as well as in murine PrP transgenic *Drosophila* (Sanchez-Garcia et al., 2016). *Drosophila* transgenic for mutated mammalian PrP have also been generated, with a toxic phenotype related to locomotor dysfunction and decreased survival also present in these models (Gavin et al., 2006; Choi et al., 2010; Murali, Maue, and Dolph, 2014).

The NMDAR of *Drosophila melanogaster*

Drosophila do not normally express the prion protein and their genome do not contain a mammalian prion protein gene ortholog (Rivera-Milla et al., 2006), and as such the use of transgenic *Drosophila* offers unique insights into how prions affect biological systems. *Drosophila* have two NMDAR subunit genes: dNR1 and dNR2. dNR1 contains 15 exons and yields two different transcripts after alternative splicing, which differ in the 5' untranslated region but have the same coding sequence (Ultsch et al., 1993; Xia et al., 2005). dNR2 seems to be the only gene encoding the *Drosophila* NR2 homolog and generates eight different transcripts that encode three different proteins, all of which bear highest homology to NR2D and NR2B in rats (Ultsch et al., 1993; Xia et al., 2005).

The size and structure of dNR2 show high homology to NR2, but the active pharmacological and physiological sites only moderately mimic mammalian counterparts. This is mainly because the two asparagine residues present in the hydrophobic pore-forming segment in other iGluRs (TM2, one of the four hydrophobic regions in the carboxyl terminal half of the protein) are not conserved in dNR2, which normally controls Ca^{2+} permeability and Mg^{2+} blockade (Sheng & Sala, 2001; Nourry, Grant, and Borg, 2003; Burnashev et al., 1992). The type I PDZ binding motif is also missing in dNR2 whereas it is present in vertebrates, suggesting that *Drosophila* NMDARs may interact with PDZ domain-containing proteins through dNR1 but not dNR2 (Xia et al., 2009). dNR1 does, however, have a putative type II PDZ domain-binding motif at its C-terminus as well as a potential type I PDZ-binding motif preceding this type II motif, suggesting that fly NMDARs may interact with PDZ domain-containing proteins via dNR1 subunits.

Overall, however, co-expression of dNR1 and dNR2 yields most of the distinguishing characteristics of mammalian NMDARs (Xia & Chang, 2009), making *Drosophila* an interesting model organism to test the NMDAR hypothesis of neurotoxicity.

Using *Drosophila melanogaster* to better characterize the G92N knock-in model

While transgenic *Drosophila* prion disease models exist, the mechanism by which PrP generates this neurotoxic phenotype is still not entirely known. Further, few previous studies have focused on the role of PrP^C itself in knock-in disease pathogenesis. As such, the G92N knock-in model serves to both better characterize the model and potentially validate results seen in mice, as well as to clarify what the role of PrP^C in neurotoxicity is in a biological system that does not normally express PrP. By establishing this experimental knock-in in *Drosophila melanogaster*, this model may aid in understanding the role PrP^C in disease pathology.

Results

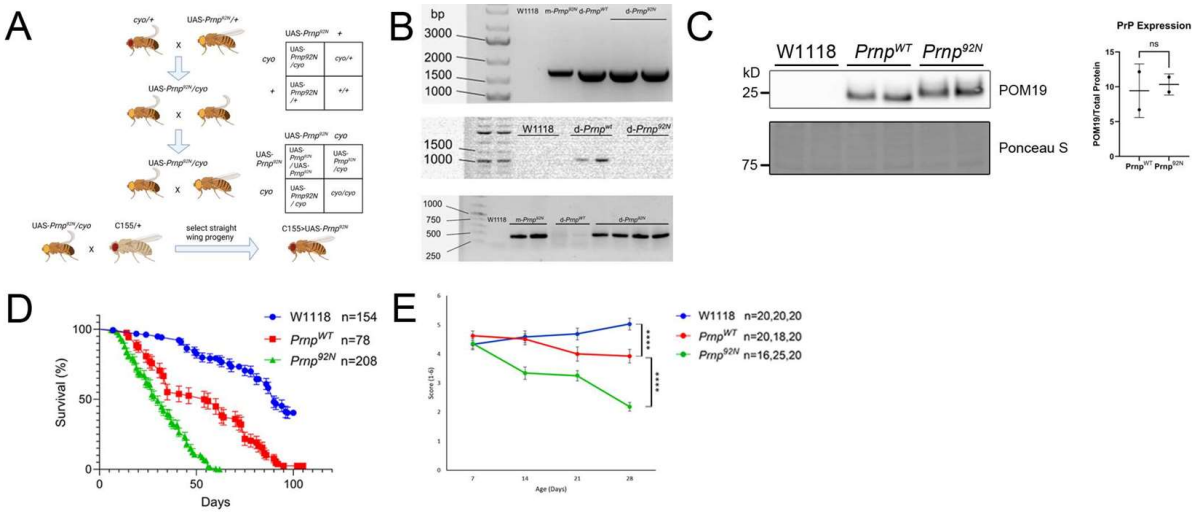

Figure 6. Knock-in *Prnp^{92N} Drosophila* uniquely express *Prnp^{92N}* and exhibit similar PrP expression, decreased lifespan, and decreased motor ability compared to wild-type and non-transgenic control.

A knock-in *Drosophila* model was generated by first crossing the initial knock-in UAS-*Prnp*^{92N} fly line with a balancer *cyo* line. These UAS-*Prnp*^{92N}/*cyo* flies were then crossed with GAL4-expressing flies (C155/+) to overexpress the *Prnp*^{92N} gene. The same process was followed for a *Prnp*^{WT} fly line (Figure 6A). Successful inclusion of the *Prnp*^{WT} and *Prnp*^{92N} gene in each respective fly line was validated by PCR (Figure 6B), and PrP expression levels between each of these fly lines was confirmed to be similar by western blot (Figure 6C). Overall lifespan of *Prnp*^{92N} flies was significantly decreased compared to the *Prnp*^{WT} and non-transgenic control (W1118) groups (Figure 6D). The motor ability was also monitored via a negative geotaxic climbing assay. Here we found that motor ability was significantly and progressively decreased in *Prnp*^{92N} (2.19 ± 0.15) flies compared to *Prnp*^{WT} (3.92 ± 0.23) and W1118 (5.03 ± 0.20) flies by 28 days ($p \leq .0001$ at this stage), with differences in the motor ability of *Prnp*^{92N} flies compared to *Prnp*^{WT} and W1118 flies presenting as early as 14 days of age (Figure 6E). These results suggest the successful generation of a prion disease model in *Drosophila melanogaster*.

Discussion

Further models of prion diseases are needed to better understand the disease pathology, and *Drosophila melanogaster* offers one model through which PrP^{C} function can be better understood. UAS-*Prnp*^{92N} flies specifically express the mutated *Prnp* gene and do not show differences in PrP^{C} protein expression compared to the wild type. Further, UAS-*Prnp*^{92N} flies exhibit markedly decreased lifespans and motor ability compared to both the *Prnp*^{WT} and W1118 non-transgenic control, suggesting a UAS-*Prnp*^{92N} mediated mechanism causing these deficits.

Decreased lifespan and motor ability have previously been shown in *Drosophila* prion disease models (Thackray et al., 2021), although the focus of these studies has predominantly

been on PrP^{Sc} with few studies having examined the role of mutated PrP^{C} in *Drosophila*. Accelerated loss of survival has been shown in classical BSE-exposed bovine PrP *Drosophila* models, with increasing concentrations of inoculum yielding shorter lifespans (Thackray et al., 2021). The average lifespan of UAS-$Prnp^{92N}$ flies is significantly less than all of the measured *Drosophila* cohorts inoculated with various BSE concentrations.

Drosophila in a VRQ ovine PrP transgenic model exhibited decreased performance index of 50-60% from 0 to 30 days of age, which was significantly lower than the non-transgenic control (Thackray, A. M., Andréoletti, O., & Bujdoso, 2018). UAS-$Prnp^{92N}$ flies show a similar 50.2% reduction in average climbing score at 28 days compared to 7 days of age, although differences in climbing ability are present as early as 14 days. Taken together, the lifespan and climbing assay results from this study demonstrate a significant phenotype linked to UAS-$Prnp^{92N}$ that is comparable if not more severe than those demonstrated in PrP^{Sc} *Drosophila* models.

Excitatory-inhibitory imbalance, spongiform degeneration, and neurotoxicity in $Prnp^{92N}$ mice are hypothesized to occur via aberrant PrP^{C}-NMDAR signaling, and as NMDARs are conserved in *Drosophila*, a similar mechanism may underlie the observed behavioral deficits. Further experiments are needed to better understand the mechanisms by which PrP^{C} affects *Drosophila*, but these studies demonstrate the establishment and validation of a model system that can be utilized to better understand PrP^{C} function.

Methods

Animals

All flies are kept at room temperature in 10mL glass vials containing freshly prepared food supply (Texas/Old, a cornmeal-based food containing agar, yeast, sucrose, cornmeal, and water). Flies used for experiments are flipped into new vials with fresh food every three days. All fly handling complies with the Institutional Animal Care and Use Committee's animal use protocols.

Establishing a *Drosophila* line

A transgenic *Drosophila* line was created using the $Prnp^{92N}$ gene with a UAS promoter. To generate a self-sustaining stock of *Drosophila*, UAS-$Prnp^{92N}$ flies were generated, and the UAS-$Prnp^{92N}$ sequence was paired with a pigmented eye phenotype. Flies that exhibited the desired straight wing, pigmented eye phenotype were selected and crossed with "curly" balancer flies. Balancer flies are flies with a balancer chromosome, which have recessive lethal mutations, thus ensuring that all progeny produced have at least one copy of the gene of interest (Hales et al., 2015), in this case being UAS-$Prnp^{92N}$. "Curly" flies also have a curly wing phenotype, allowing for phenotypic identification without the need to genotype each fly. Curly wing flies with pigmented eyes were selected, ensuring these flies expressed one UAS-$Prnp^{92N}$ and one *cyo* containing chromosome.

Flies from this F1 generation were then crossed with one another to generate a self-sustaining stock. As *cyo/cyo* genotypes are lethal in the embryonic stage, resulting progeny are either heterozygous or homozygous for UAS-$Prnp^{92N}$. Homozygotes and heterozygotes can be identified by phenotype (heterozygotes have curly wings while homozygotes do not), and straight wing progeny were selected.

Flies from this self-sustaining stock were selected and crossed with GAL4-expressing driver flies. GAL4 is a yeast-based transcription factor that activates transcription of its target genes by binding to UAS cis-regulatory sites, allowing for tissue-specific expression of a gene of interest under control of UAS sites (Busson & Pret, 2007). As the UAS-$Prnp^{92N}$ flies were engineered to include a UAS site just prior to the *Prnp* gene, crossing UAS-$Prnp^{92N}$ flies with GAL4-expressing flies (C155/+) allows for UAS-$Prnp^{92N}$ overexpression. The same process was followed with UAS-$Prnp^{WT}$ and W1118 flies. W1118 flies are a non-transgenic control.

PCR protocol:

gDNA was prepared via whole body collection of 20 anesthetized flies from each genotype. Flies were ground in 300 uL Buffer A (100 mM Tris-Cl, 100mM EDTA, 100 m M NaCl, 0.5% SDS) using a motorized pestle for 2 minutes. Samples were incubated at 65°C for 30 minutes. 600 uL of Buffer B (200mL 5M potassium acetate and 500 mL 6M lithium chloride) were then added to the samples. Samples were left on ice for 10 minutes. Samples were centrifuged at 12,000 rpm for 15 minutes at room temperature and supernatant was transferred to a new microtube. 480 uL isopropanol was added to each sample. Samples were centrifuged at 12,000 rpm for 15 minutes at room temperature. The generated pellet was washed with 70% ethanol, air-dried for 10 minutes, and resuspended in 100 uL of TE buffer.

For the PCR presented first (top of figure 6B), 25 uL samples for each genotype were generated using 12.5 uL Accustart II PCR Supermix, 1 uL gDNA, 0.5 uL of Xbal and 0.5 uL of EcoR1m primers, and 10.5 uL of sterile water. Amplification was performed on a Bio-Rad CFX96 system under the following conditions: 10 min at 95 °C and 30 cycles of 30 s at 95 °C, 1 min at 59 °C, and 1 min at 72 °C. The PCR presented second (middle of figure 6B) followed the same protocol, but with 0.5 uL of $Prnp^{WT}$ specific primers. The PCR presented third (bottom of

figure 6B) followed the same protocol, but with 0.5 uL of *Prnp*^{92N} specific primers. A 1.5% agarose gel was used for each set of samples.

Brain tissue collection, homogenization, and lysis

15 *Drosophila* whole brains from each genotype were collected and added to 20 uL of 4x NuPage LDS Sample Buffer, 20 uL 4% N-lauryl sarcosine in PBS, and 1.0mm of both Pierce Phosphatase and cOmplete Mini protease inhibitors. The samples were spun down, homogenized on ice with a motorized pestle, boiled at 95°C for 5 minutes, then centrifuged at 14,500g for 5 minutes at 4°C. The supernatant was pipetted off and stored in separate tubes at -80-degrees Celsius.

Western blot analysis

Equal protein levels for each *Drosophila* sample were quantified using a bicinchoninic acid protein assay (BCA). 10 uL of each sample was loaded, electrophoresed through a 10% Bis-Tris gel (Invitrogen) with 1X MES running buffer, and transferred to a nitrocellulose membrane by wet blotting. Membranes were incubated with primary antibodies overnight at 4-degrees Celsius: POM19 (amino acids 201-225) in 1x casein blocking buffer. Membranes were washed with 1x TBST the following day and were then incubated with an HRP-conjugated IgG secondary antibody for one hour at room temperature. The immunoblots were developed using a chemiluminescent substrate (ECL Supersignal West Dura or Fempto, ThermoFisher Scientific) and visualized on a Fuji LAS 4000 imager. Images were quantified using Multi Gauge version 3.0 software.

Lifespan assay

Flies of each GAL4-driven genotype were collected and separated into separate vials, with three repeats for each genotype. An *n* of 15-20 per vial was used. Lifespans of experimental flies not used specifically for this assay were also tracked. Flies were scored based on how many died per day until all flies in a particular vial had died. A Kaplan-Meier curve was generated in GraphPad Prism 10 for each genotype to compare the lifespans.

Climbing assay

The climbing assay was conducted in a graduated cylinder. The flies began to be assayed at seven days old. 24 hours before the assay, flies were transferred to new and clean food vials to allow flies to groom any excess food from themselves overnight. Flies were then assayed every seven days, beginning at 7 days old and ending at 28 days old. The general procedure for this assay is as described below.

1. Transfer a vial of flies from its respective food vial to the climbing assay graduated cylinder.
2. After one minute, gently tap the flies down and immediately begin a timer for 10 seconds.
3. After the 10 seconds, let the flies rest again for one minute.
    a. A white background can be set behind the climbing graduated cylinder to make the flies more visible.
    b. A video recording of the 10 second assay was taken, as it is easier to count the number of flies at each section of the cylinder at exactly 10 seconds.

c. The number of flies in each score range (equal sections of the graduated cylinder to which the flies climb, with 1 being the bottom and 6 being the top) were recorded and the sum of all the flies in each section of the graduated cylinder should amount to the total number of flies that was started with.

4. Repeat the assay for a total of 5 trials.

For all assays, data for each genotype was aggregated and figures were generated using Excel.

Statistical analysis

A Student's *t*-test (two-tailed, unpaired) with a Welch's post hoc correction was used to analyze W1118, UAS-$Prnp^{WT}$, and UAS-$Prnp^{92N}$ flies for POM19 protein levels. A Wilcoxon signed-rank test was used to assess lifespan assay differences. A two-way ANOVA with Tukey's post-hoc test was used to assess climbing assay differences (****$P \leq 0.001$). Statistical analysis was completed on GraphPad Prism 10 software for western blot quantification, lifespan assays, and climbing assays. A $p \leq 0.05$ was considered statistically significant. Data represent mean ± standard deviation for western blot and climbing assays, and mean ± SEM for climbing assays.

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