STUDY OF THE INTRACELLULAR PROTEIN TAU AND ITS RELATIONSHIP WITH KUNJIN VIRUS AND NEURODEGENERATIVE DISEASES

by

Sarah Kimberley Lotz

A thesis submitted to the faculty of
The University of North Carolina at Charlotte
in partial fulfillment of the requirements
for the degree of Master of Science in
Biology

Charlotte

2022

Approved by:

_____
Dr. Kristen Funk

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Dr. Ian Marriott

_____
Dr. Samantha Furr

ABSTRACT

SARAH KIMBERLEY LOTZ. Study of the Intracellular Protein Tau and its Relationship with Kunjin Virus and Neurodegenerative Diseases.
(Under the direction of DR. KRISTEN FUNK)

Neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis, comprise a family of disorders characterized by progressive loss of nervous system function. Neuroinflammation is increasingly recognized to be associated with many neurodegenerative diseases but whether it is a cause or consequence of the disease process is unclear. Of growing interest is the role of microbial infections in inciting degenerative neuroinflammatory responses and genetic factors that may regulate those responses. Microbial infections cause inflammation within the central nervous system through activation of brain-resident immune cells and infiltration of peripheral immune cells. These responses are necessary to protect the brain from lethal infections but may also induce neuropathological changes that lead to neurodegeneration. The thesis research to follow explores the elaborate relationship between the neuroinvasive Kunjin virus and the intracellular protein Tau. Elucidating these mechanisms is critical for developing targeted therapeutic approaches that prevent the onset and slow the progression of neurodegenerative diseases.

ACKNOWLEDGEMENTS

I would like to say thank you to all who helped guide and support me through this journey. First and foremost, I would like to thank my advisor, the brilliant Dr. Kristen Funk, who paved the groundwork for this project. Without your guidance and encouragement, I would not have accomplished what I have. To Dr. Katie Reagin, for your mentorship throughout my time here; thank you for answering all my questions no matter how many I asked. To Rae Lee, who jumped in to help me with my project in my time of need; your positivity brightened my day. To Dr. Ian Marriott, for sharing your love of immunology through teaching; without this class I would not know where I would be today.

I would also like to say a special thank you to those who have personally changed my life, in the way I view myself and imparting the ability to take responsibility for my own happiness. To Hank Green for teaching me to embrace my innate curiosity and that it is okay to be the 'but why' child. To my Fab Five Friends, for your undoubting friendship and support this past year; you brought joy even on my hardest days. To the incomparable Dr. Samantha Furr, for your friendship, guidance and support these past five years. Your unwavering belief in me to do whatever I was passionate about inspired me to be the person that I am today, and I will always be grateful for that. To Alex Hertzing, for your constant love and support these past four years; you always believed in me even when I lost faith in myself, and I could not be more grateful to have you in my life. Thank you for being my shoulder to cry on through all the failed experiments, rejections, and hardships I have come to face, but for also being by my side to share the some of my happiest memories with. I could not be more excited to see where life takes us next. To Nadia Alvarez, Diana Diaz, and Hayley Popp for your constant emotional support and being my personal hype team; you will never know how much you mean to me. Lastly, to Post Malone, Billie Eilish, Julia Michaels, and The Lumineers for accompanying me through my academic career, who I am listening to as I write this; your music has allowed me to do the impossible at times, allowed me to feel when I needed to, and accomplish my goals when I needed an extra push, so for that, I appreciate your presence throughout this journey.

DEDICATION

For six-year-old me—it was always real, all of it. May you find happiness through the looking glass and peace in the Poppy fields.

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LIST OF ABBREVIATIONS

Aβ	Amyloid beta
AD	Alzheimer's Disease
ALS	Amyotrophic Lateral Sclerosis
APP	Amyloid precursor protein
BBB	Blood brain barrier
BCA	Bicinchoninic acid assay
B. burdorferi	*Borrelia burdorferi*
BHK	Baby hamster kidney
C. albicans	*Candida albicans*
CD33	CD33 gene
CDK5	Cyclin-dependent kinase 5
C. elegans	*Caenorhabditis elegans*
CMV	Cytomegalovirus
CNS	Central nervous system
COVID-19	Coronavirus Disease 2019
C. pneumoniae	*Chlamydia pneumoniae*
CSF	Cerebral spinal fluid

C. spp	*Candida species*
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
DPI	Days post infection
EAAT2	Excitatory amino acid transporter 2
EBV	Epstein Barr virus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
E218A	Attenuated strain of the NY-99 strain of West Nile virus
FBS	Fetal bovine serum
FTD	Frontotemporal dementia
FUS	Fused in sarcoma
GLT-1	Glutamate transporter-1
GSK-3β	Glycogen synthase kinase-3β
HAND	HIV-associated neurocognitive disorder
HBSS	Hank's Balanced Salt Solution
HCl	Hydrochloride
HCV	Hepatitis C virus

HERV-K	Human endogenous retrovirus-K
HHV	Human herpesvirus
HIV-1	Human immunodeficiency virus 1
H. pylori	*Helicobacter pylori*
HPI	Hours post infection
HSE	Herpes simplex encephalitis
HSV	Herpes simplex virus
HTLV-1	Human T-cell leukemia virus type 1
i.c.	Intracranial
ICAM-1	Intracellular adhesion molecule-1
IHC	Immunohistochemistry
IL	Interleukin
IP	Immunoprecipitation
iPSCs	Induced pluripotent stem cells
IRF	Interferon regulatory factors
IFN	Interferon
JEV	Japanese encephalitis virus
KUNV	Kunjin virus

L. monocytogenes	*Listeria monocytogenes*
MAP	Microtubule associated protein
MAPT	Microtubule associated protein Tau gene
MHC I/II	Major histocompatibility complex I/II
μL	Microliter
mL	Milliliter
M. leprae	*Mycobacterium leprae*
M. tuberculosis	*Mycobacterium tuberculosis*
NaCl	Sodium chloride
NC	Non-Carrier
NDD(s)	Neurodegenerative disease(s)
NFDM	Non-fat dry milk
NF-κB	Nuclear factor-kappa B
NFTs	Neurofibrillary tangles
NLP-29	Neuropeptide-like protein 29
nm	Nanometer
NY-99	New York 1999 strain of West Nile virus
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PD	Parkinson's Disease
PFA	Paraformaldehyde
PFU	Plaque forming units
P. gingivalis	*Porphyromonas gingivalis*
PRKN	Parkin gene
PSEN1	Presenilin 1
qPCR	Quantitative polymerase chain reaction
RABV	Rabies virus
RNA	Ribonucleic acid
RSV	Respiratory syncytial virus
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SCNA	SCNA gene
Ser	Serine
S. typhimurium	*Salmonella typhimurium*
Tat	Transcription transactivator protein
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline with Tween-20

TDP-43	TAR DNA-binding protein 43
Thr	Threonine
TMEV	Theiler's murine encephalomyelitis virus
TNE	Tris/NaCl/EDTA
TNF-α	Tumor necrosis factor alpha
TREM2	TREM2 gene
VZV	Varicella zoster virus
WNV	West Nile virus
WNND	West Nile neuroinvasive disease
WK	Week
WT	Wild type

CHAPTER 1: Microbial infections are a risk factor for neurodegenerative diseases

Abstract

Neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis, comprise a family of disorders characterized by progressive loss of nervous system function. Neuroinflammation is increasingly recognized to be associated with many neurodegenerative diseases but whether it is a cause or consequence of the disease process is unclear. Of growing interest is the role of microbial infections in inciting degenerative neuroinflammatory responses and genetic factors that may regulate those responses. Microbial infections cause inflammation within the central nervous system through activation of brain-resident immune cells and infiltration of peripheral immune cells. These responses are necessary to protect the brain from lethal infections but may also induce neuropathological changes that lead to neurodegeneration. This review discusses the molecular and cellular mechanisms through which microbial infections may increase susceptibility to neurodegenerative diseases.

1.1 Introduction

Neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS), are clinically characterized by the progressive decline of cognitive, motor, and behavioral functions. Pathologically, these diseases exhibit significant neuronal death, brain atrophy, protein aggregation, and neuroinflammation. Despite improved understanding of disease progression, the cause or causes that initiate disease processes are not well understood. Recent genome-wide association studies have highlighted the contribution of immune molecules in many neurodegenerative diseases. Several genes with polymorphisms that increase the risk of neurodegenerative diseases, such as *CD33* and *TREM2* in AD, *PRKN, SCNA,*

LRRK2, and *HLA* in PD, and *C9ORF72* in ALS have been linked to various immune functions including phagocytosis, microglial activation, complement activation, MHC class II expression, and hematopoiesis.[1–7] Because these genetic risk factors do not cause disease in all carriers, it is hypothesized that environmental factors that induce inflammation may contribute to the etiopathogenesis of neurodegenerative diseases. Microbial infections have become of increasing interest in inciting neurodegenerative pathology, as they can invade the central nervous system (CNS) and cause significant neuroinflammation through activation of resident immune cells, such as microglia and astrocytes, as well as promote infiltration of peripheral macrophages and T cells.[8–11] Though these immune responses exist to protect the brain, they can cause critical damage in an attempt to clear the invading pathogen (Figure 1).

Infectious agents may contribute to neurodegenerative disease pathology by eliciting an inflammatory response. Following infection, inflammation prevents damaging pathology and promotes tissue repair and regeneration; however, if uncontrolled, inflammation can become lethal to healthy cells.[12] These inflammatory responses originate locally at the site of infection, but can rapidly become widespread, and in some cases, involve the CNS. Increased production of inflammatory cytokines and chemokines, including IL-1β, promote breakdown of the blood brain barrier (BBB), which typically protects the CNS resident cells from harmful agents and inflammatory mediators.[13–15] However, if there is a BBB breach, these soluble mediators can stimulate CNS resident astrocytes and microglia which, upon activation, amplify inflammatory conditions in the CNS that can cause significant damage to both infected and uninfected neurons as well as resident glial cells.[16–18] Importantly, neurotropic infections can lead to harmful neuroinflammation that has been identified as a potential risk factor for neurodegenerative diseases.[19–22] This review discusses recent studies linking microbial infections to

neurodegenerative diseases and the cellular and molecular mechanisms through which they may increase susceptibility to disease (summarized in Table 1).

1.2 Alzheimer's disease

AD is characterized pathologically by the deposition of two proteinaceous lesions in the brain—extracellular senile plaques and intracellular neurofibrillary tangles (NFTs).[23] Senile plaques are extracellular aggregates composed of insoluble amyloid beta (Aβ) peptides, the proteolytic product of amyloid beta precursor protein (APP). Under homeostatic conditions, APP is cleaved by α-secretase and γ-secretase, which is facilitated by presenilin 1 (PSEN1).[24–26] In AD, APP is instead cleaved by β-secretase and γ-secretase, forming the insoluble Aβ peptides, which self-aggregate into senile plaques and are believed to be toxic to neurons.[27–29] Neurofibrillary tangles are intracellular aggregates composed of hyperphosphorylated microtubule-associated protein Tau. Tau can be phosphorylated by a number of kinases, including cyclin-dependent kinase 5 (CDK5) and glycogen synthase kinase-3β (GSK-3β).[30] Under homeostatic conditions, phosphorylation modulates the affinity of Tau for microtubules, allowing their dynamic growth and retraction.[31] In AD, Tau becomes hyperphosphorylated, which decreases its affinity for microtubules and increases its propensity to self-aggregate into pathogenic neurofibrillary tangles.[30,32–34]

While the mechanisms that incite Aβ and Tau aggregation are not fully understood, recent studies have suggested a role for inflammatory cytokines, including tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-1β, IL-6, IL-10, and IL-18.[35–39] For example, IL-1β is an essential mediator of the inflammatory response and has been found to be elevated near Aβ plaques.[40,41] Expression of IFN-γ, a pro-inflammatory cytokine, was elevated in transgenic mice

with AD-related pathology [42], though it does not appear to be significantly elevated in human patients.[43,44] The impact of IFN-γ on AD pathology is apparently diametric, as some reports indicate that IFN-γ treatment promoted Aβ clearance by microglia and macrophages, thus reducing pathological load.[45,46] Also, overexpression of IFN-γ in transgenic mice that develop amyloid and Tau pathologies resulted in a significant decrease in Tau pathology and improved neurogenesis, suggesting elevated levels of IFN-γ can be beneficial for alleviating AD pathology within the brain.[47] However, co-stimulation of primary human astrocytes in culture with IFN-γ and TNF-α induced Aβ production, and deletion of the IFN-γ receptor reduced gliosis and amyloid plaque deposition in APP transgenic mice, which would suggest elevated levels of inflammation within the CNS exacerbates AD pathology.[48,49] These seemingly conflicting observations could be, in part, due to differences in the magnitude of cytokine elevation and timeframe of expression as well as other environmental and genetic factors. Altogether, they suggest that acute episodes of neuroinflammation, such as those caused by infections, may initiate pathological Aβ and Tau deposition.

Infectious microbes have long been suspected to play a role in the onset of AD, though direct evidence is still limited.[50–52] Several cohort studies have examined infectious burden in patients with AD, indicating a correlation between infections and AD pathology.[53–56] Using multiscale networks of AD-associated virome data, integrating genomic, transcriptomic, proteomic, and histopathological information, Readhead *et al.* identified evidence of increased herpesvirus 6A (HHV-6A) and human herpesvirus 7 (HHV-7) in patients with late-onset AD compared to healthy controls.[57] Additionally, a strong association was detected between the presence of herpes simplex virus 1 (HSV-1) antibodies and patients with AD, specifically in women, subjects older than 60 years of age, and when plasma samples were taken at least 6.6

years prior to dementia diagnosis.[58] The authors proposed that this 6.6 year lag between HSV-1 antibody detection and AD diagnosis indicates that HSV-1 plays a role primarily in early AD development.[58] Furthermore, HSV-1 DNA sequences and the functional HSV-1 genome, in its entirety, were detected in the brains of patients with AD.[59] Similarly, the presence of *Chlamydia pneumoniae* was detected in post mortem brain-tissue samples of patients with AD.[53,55]

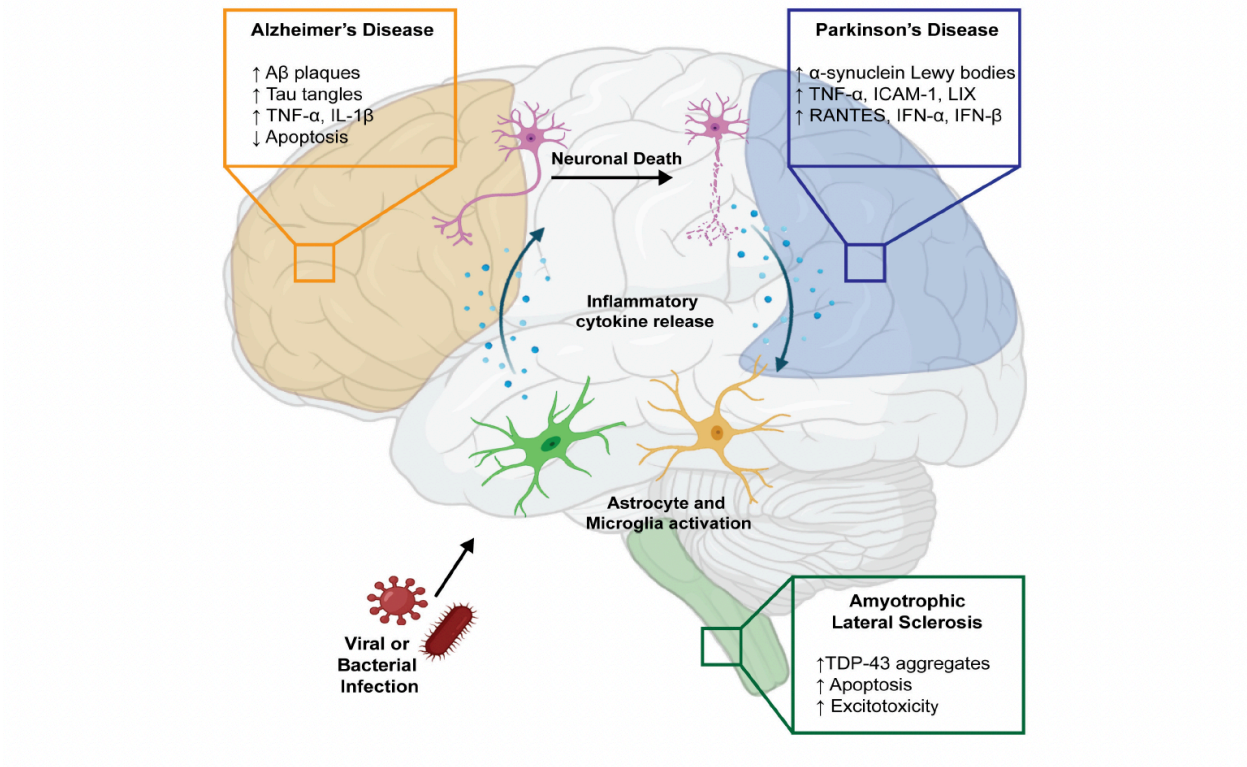

Figure 1: Infectious agents elicit an inflammatory cyclic cascade associated with neurodegenerative diseases. Infectious agents may contribute to neurodegenerative diseases directly or via immune activation. Infection by viral or bacterial pathogens can cause pro-inflammatory activation of CNS resident immune cells, including astrocytes and microglia, resulting in neuronal death. Additionally, cellular death directly caused by infectious agents and the release of damage-associated molecular patterns can exacerbate the inflammatory state through further activation of CNS immune cells, perpetuating a cycle of inflammation. In AD, this is often associated with high levels of pro-inflammatory cytokines, TNF-α and IL-1β, reduced clearance of infected cells, and accumulation of neurotoxic aggregates composed of Aβ and Tau. This pro-inflammatory state has also been documented in the context of PD, where increased accumulation of neurotoxic α-synuclein is accompanied by high levels of TNF-α, ICAM-1, LIX, RANTES, IFN-α, and IFN-β produced by infected and activated astrocytes and microglia. Additionally, some pathogens can directly infect neurons resulting in alterations in metabolism, enhanced neuronal excitotoxicity and enhanced apoptosis, as seen in ALS. Created with BioRender.com.

Additionally, serum antibody levels to common periodontal microbiota were observed to increase risk of developing AD.[60,61] More recent studies have identified *Porphyromonas*

gingivalis in the brains and biofluids of patients with AD.[62,63] Very recently, researchers reported that in a Swedish cohort of people over the age of 50, untreated herpesvirus infection (either HSV-1 or varicella zoster virus (VZV)) increased the risk of dementia by 1.5-fold. Patients diagnosed with herpesvirus infection who took antiviral medication showed reduced risk of dementia by 25% compared to those with untreated herpesvirus infection.[64] Epidemiological data cannot prove causation between infections and AD, but collectively these studies support the hypothesis that pathogens increase the risk of developing AD.

Some of the earliest data regarding the microbial etiology hypothesis of AD implicated HSV-1, a neurotropic enveloped virus that establishes life-long latent infection in the CNS with periodic reactivation cycles. Following resolution of primary infection, HSV-1 can remain dormant, predominantly in the trigeminal ganglion, and upon reactivation induce severe acute encephalitis in the temporal and frontal cortices of the brain, known as herpes simplex encephalitis (HSE).[65] Ball first proposed a link between HSV-1 and AD in 1982, recognizing that similar brain regions are affected by both HSE and AD, and that people who survived HSE exhibited clinical symptoms similar to AD, including memory loss and cognitive impairment.[66] Since then substantial progress has been made to understand the molecular mechanisms by which HSV-1 may contribute to the onset of AD. Zambrano *et al.* showed that infection of primary neurons with HSV-1 caused significant neuronal damage and death via hyperphosphorylation of Tau, increased acetylation and tyrosination of tubulin, disrupted microtubules, and damaged and shortened neurites.[67] Similarly, HSV-1 induced glycogen synthase kinase 3 β- (GSK3-β) and protein kinase A-mediated Tau hyperphosphorylation.[68] All of these findings are synonymous with the pathology seen in AD, suggesting that HSV-1 infection may promote AD onset.

TABLE 1 | Overview of infections associated with neurodegenerative diseases.

Disease	Pathogen	Type of pathogen	Association with disease processes
Alzheimer's disease (AD)	*Chlamydia pneumoniae*	Gram-negative bacteria	• Detected in post-mortem brains and brain tissue samples of AD patients (Balin et al., 1998; Gérard et al., 2006) • Upregulated β and γ secretase in infected astrocytes, promoted Aβ peptide formation *in vitro* (Al-Atrache et al., 2019)
	Porphyromonas gingivalis	Gram-negative bacteria	• Detected in the brains and biofluids of AD patients (Poole et al., 2013; Dominy et al., 2019) • Increased production of TNF-α, IL-6, and IL-1β in mice (Ding et al., 2018; PMID: Ilievski et al., 2018) • Increased Aβ peptide accumulation in the brain of infected or PgLPS-treated mice (Wu et al., 2017; Ilievski et al., 2018)
	Salmonella typhimurium	Gram-negative bacteria	• Increased Aβ peptide deposition in the brain of infected mice to bind and entrap bacteria (Kumar et al., 2016)
	Human herpesvirus 6A (HHV-6A) and 7 (HHV-7)	Herpesvirus	• Identified HHV-6 and HHV-7 in late-onset AD patients (Readhead et al., 2018) • Reduced autophagy, promoted accumulation of hyperphosphorylated Tau and Aβ peptides (Romeo et al., 2020) • Infected microglia enhanced Aβ peptide deposition and Tau phosphorylation (Bortolotti et al., 2019)
	Herpes simplex virus 1 (HSV-1)	Herpesvirus	• Found HSV-1 antibodies in female AD patients over 60 years of age (Lövheim et al., 2015a) • Detected HSV-1 DNA in the brains of AD patients (Wozniak et al., 2009b) • Increased β-amyloidosis as an antimicrobial defense mechanism, which increased senile plaque formation (Eimer et al., 2018) • Promoted Tau hyperphosphorylation and damage in primary neurons (Zambrano et al., 2008)
	Human immunodeficiency virus (HIV-1)	Retrovirus	• Inhibited Aβ degradation in human brain cultures (Rempel and Pulliam, 2005) • Promoted cleavage of APP into Aβ peptides (Kim et al., 2013; Hategan et al., 2017) • Promoted Tau hyperphosphorylation and NFT deposition (Giunta et al., 2009). • Enhanced pro-inflammatory cytokine secretion from microglia, astrocytes, and monocytes (Nookala and Kumar, 2014; Haij et al., 2015; Liu and Kumar, 2015) • Activated immune signaling pathways (Herbein and Khan, 2008; Herbein, 2016) • Promoted Aβ secretion from primary hippocampal neurons (Aksenov et al., 2010) • Inhibited apoptosis in infected human neuroblastoma cells (Thomas et al., 2009) • Increased trafficking of Aβ to neural progenitor cells (András et al., 2017, 2020) • Detected elevated hyperphosphorylated Tau in the hippocampus of HIV-infected patients (Anthony et al., 2006)
	Human T cell leukemia virus type I (HTLV-1)	Retrovirus	• Increased activation of Tau kinases, increased Tau phosphorylation *in vitro* (Maldonado et al., 2011)
Parkinson's disease (PD)	*Helicobacter pylori*	Gram-negative bacteria	• Found in PD patients in high prevalence (Huang et al., 2018; McGee et al., 2018) • Improved motor functions in patients who have cleared *H. pylori* infection (Lahner et al., 2009; Huang et al., 2018)
	Hepatitis C virus (HCV)	Flavivirus	• Increased neuronal death (Weissenborn et al., 2006; Abushouk et al., 2017; Wijarnpreecha et al., 2018) • Increased production of pro-inflammatory cytokines and chemokines from activated microglia (Lyons and Benveniste, 1998; Wu et al., 2015; Abushouk et al., 2017)
	Human immunodeficiency virus (HIV-1)	Retrovirus	• Infected dopaminergic neurons and associated with development of dementia (Nath et al., 2000)
	Cytomegalovirus (CMV)	Herpesvirus	• Elevated levels of circulating pro-inflammatory myeloid cells found in PD patients (Goldeck et al., 2016)
	Theiler's murine encephalomyelitis virus (TMEV)	Picornavirus	• Infected dopaminergic neurons *in vivo* and promoted neurodegeneration (Oliver et al., 1997)
	Japanese Encephalitis virus (JEV)	Flavivirus	• Infected dopaminergic neurons, modulated dopamine signaling, promoted neurodegeneration (Simanjuntak et al., 2017)
	Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)	Coronavirus	• Detected viral RNA and evidence of microglia activation and T lymphocyte infiltration in the post-mortem brain of COVID-19 patients (Matschke et al., 2020)

(Continued)

TABLE 1 | Continued

Disease	Pathogen	Type of pathogen	Association with disease processes
Amyotrophic lateral sclerosis (ALS)	Human immunodeficiency virus (HIV-1)	Retrovirus	• Reduced glutamate transport and increased neuronal excitotoxicity in infected human astrocytes *in vitro* (Wang et al., 2003) • Increased production and mislocalization of Fus in iPSC-derived spinal neurons (Bellmann et al., 2019) • Promoted axonal degeneration (Berth et al., 2016)
	Human endogenous retrovirus K (HERV-K)	Retrovirus	• Regulated activation of TDP-43 (Li et al., 2015)
	Theiler's murine encephalomyelitis virus (TMEV)	Picornavirus	• Promoted TDP-43 phosphorylation, mislocalization, and aggregation following infection *in vitro* and *in vivo* (Masaki et al., 2019)
	Rabies virus (RABV)	Rhabdovirus	• Increased production and mislocalization of Fus in iPSC-derived spinal neurons (Bellmann et al., 2019)

Another virus often associated with AD pathology is human immunodeficiency virus 1 (HIV-1), a retrovirus that can become neuroinvasive and induce severe encephalitic and cognitive changes. Patients with HIV-associated neurocognitive disorder (HAND) demonstrate increased production of Aβ and development of amyloid plaques.[69–71] HIV-1 infection induced the expression of RAGE (the receptor for advanced glycation end products) in brain endothelial cells and the accumulation of Aβ in a RAGE-dependent manner. Aβ aggregates were then transferred from brain endothelial cells to neural progenitor cells, stimulating further aggregation and progenitor cell dysfunction.[72,73] However, while much research has focused on mechanisms of Aβ production and aggregation, the total level of Aβ in the brain also depends on the mechanisms of clearance. One clearance mechanism involves the zinc-metalloprotease neprilysin, which has been shown to cleave and degrade Aβ monomers *in vitro* and *in vivo*.[74–79] In an *in vitro* assay, the HIV-1 transcription transactivator (Tat) protein inhibited activity of neprilysin by 80% and increased the soluble Aβ by 125% when applied to human brain cultures.[80] HIV-1 Tat also recruited APP in lipid rafts and stimulated its cleavage by β-secretase and γ-secretase, yielding higher levels of the Aβ peptides and causing neurotoxicity.[81,82] Furthermore, HIV-1 surface protein, gp120, promoted Aβ secretion in primary embryonic rat

hippocampal neurons [83], inhibited apoptosis of infected human neuroblastoma cells via inhibition of the Fas-pathway [84], and induced neurotoxicity in human neuroblastoma cells through the CXCR4 and CCR5 chemokine receptors.[85,86] Additionally, HIV-1 Tat and Nef proteins exacerbated the secretion of pro-inflammatory cytokines from surrounding microglia, astrocytes and monocytes, causing neurotoxic effects.[87–89] Furthermore Nef can mimic TNF-α signaling by activating inflammatory pathways, such as NF-κB, AP1, JNK and AKT.[90,91] HIV-1 infection can also promote Tau aggregation, as Anthony *et al.* found elevated levels of hyperphosphorylated Tau in the hippocampus of HIV-1-infected individuals compared with age-matched controls.[92] Another study found higher expression levels of the Tau kinase CDK5 in patients with HIV encephalitis versus HIV-positive patients without neuroinvasive disease, which correlated with increased Tau hyperphosphorylation.[93] Furthermore, transgenic mice that express HIV-1 glycoprotein gp120 exhibited increased brain levels of CDK5, Tau hyperphosphorylation, and neurodegeneration, which could be rescued by either genetic knockdown or pharmacological inhibition of CDK5.[93] Additionally, HIV-1 Tat protein was similarly found to induce hyperphosphorylation of Tau in neurons through the CDK5, resulting in accelerated NFT deposition in transgenic mice.[94] Collectively, these data indicate HIV-1 infection may induce AD pathology through several potential mechanisms.

Periodontitis has been associated with increased risk of developing AD as well as other dementias.[95,96] Specifically, the bacteria *P. gingivalis* and its toxic proteases, called gingipains, were identified in the brains of AD patients, and their levels correlated with AD pathology.[63] Studies investigating the mechanism underlying this relationship have identified inflammatory processes, including cytokine expression and complement activation, as well as amyloid production as mediators of *P. gingivalis* pathogenesis.[97] Murine models of *P. gingivalis* infection

resulted in cognitive impairment in middle-aged (12 month), but not young (4 week) mice. Researchers attributed this to elevated production of proinflammatory cytokines including TNF-α, IL-6, and IL-1β in the brains of aged mice following infection.[98] This was supported by additional studies in mice and primary cell cultures of microglia and hippocampal neurons, which indicated that the lysosomal protease Cathepsin B may be critical in initiating the neuroinflammatory response to repeated *P. gingivalis* lipopolysaccharide exposure.[99] Following repeated oral application of *P. gingivalis*, the bacteria was detected in the hippocampus of infected mice, serving as more direct evidence of the role of *P. gingivalis* in AD pathology.[100] This study also showed significantly elevated expression of inflammatory cytokines IL6, TNFα and IL1β, as well as APP and β-secretase, increased Tau phosphorylation, and neurodegeneration.[100] Together, these data propose a mechanistic link between periodontal disease and AD pathology.

Other evidence suggests that common infectious agents may contribute to AD pathology by promoting the deposition of Tau and Aβ. *C. pneumoniae* is an obligate intracellular bacterium that takes residence in the nasal and pulmonary mucosa.[101] It has been proposed that *C. pneumoniae* invades the brain through the lateral entorhinal cortex, then disseminates to the frontal and temporal cortices, the same regions where Aβ plaques and NFTs are found.[102,103] Subsequent *in vitro* studied demonstrated that infection of astrocytes with *C. pneumoniae* decreased activity of α-secretase and increased expression of both β-secretase and γ-secretase, yielding the aggregation-prone Aβ peptide.[104] Similarly, HHV-6A, a neurovirulent pathogen, was shown to promote Aβ secretion along with Tau hyperphosphorylation in primary neurons and astrocytoma cells by reducing protein degradation via autophagy and activating the unfolded protein response.[105] Furthermore, HHV-6A infection of microglial cells *in vitro* induces a pro-

inflammatory activation status, stimulates the production of Aβ peptides, and promotes Tau phosphorylation.[106] Human T-cell leukemia virus type 1 (HTLV-1) has also been shown to increase Tau phosphorylation via CDK5 and GSK3-β activation, which resulted in neurite retraction in a cell culture model.[107] These studies suggest that many infectious agents can contribute to AD pathology, and it is likely that the composite infectious burden is more important than a single microbe.

Antimicrobial peptides are host-defense mechanisms that defend against infectious pathogens and have recently been hypothesized to initiate pathological processes that lead to neurodegeneration. Using *C. elegans* PVD neurons as a model, researchers showed that an epidermally-expressed antimicrobial peptide NLP-29 (neuropeptide-like protein 29) causes age-dependent dendrite degeneration and that fungal infections can induce degeneration through similar mechanisms.[108] This NLP-29-induced degeneration could be similarly stimulated in primary cultured rat neurons, suggesting that this is an evolutionarily-conserved mechanism.[108] A recent hypothesis posits Aβ may act as an antimicrobial peptide, providing innate immune defense against infection. Soscia *et al.* showed that synthetic Aβ exerts antimicrobial activity *in vitro* against eight common, clinically-relevant pathogens, including seven bacterial and one yeast species.[109] Aβ also shows neutralizing activity against seasonal (H3N2) and pandemic (H1N1) strains of influenza A virus *in vitro*, inducing viral agglutination and preventing its infectivity in epithelial cells.[110] Bourgade *et al* showed that Aβ prevented entry of HSV-1 into fibroblast, epithelial, and neuronal cell cultures. They hypothesized that based on the sequence homology between Aβ and a proximal transmembrane region of HSV-1 glycoprotein B, Aβ may directly interfere with HSV-1 replication via insertion into the viral envelope.[111] Kumar *et al.* extended these findings *in vivo* to mouse and nematode models of disease, demonstrating that Aβ

oligomers bind the microbial cell wall of *Salmonella typhimurium* and *Candida albicans* to prevent adhesion to host cells and reduce *S. typhimurium* load in the brains of infected mice.[112] They went on to show a similar effect with both HSV-1 and HHV6 infection in a mouse model of AD, demonstrating that overexpression of Aβ in mice correlated with longer survival from HSE; however, all mice still succumbed to infection within six days, and authors provided no evidence of reduced viral burden in the brains Aβ overexpressing mice.[113] Altogether, these data suggest that Aβ may function in innate immunity against microbial infection. However, its role in agglutination may then seed additional amyloid deposition, initiating pathogenic plaque formation, causing persistent neuroinflammation, and ultimately, lead to neurodegeneration.

1.3 Parkinson's disease

PD is the second most common neurodegenerative disease, following AD, afflicting motor functions.[114,115] It is characterized by prominent dopaminergic neurodegeneration within the substantia nigra pars compacta region of the brain, which is caused by dopamine deficiency, and leads to motor neuron dysfunction.[114,116,117] Patients with PD present with bradykinesia, resting tremors, gait impairment, diminished postural quality, and muscular rigidity.[117,118] Treatments for PD exist to manage symptoms or slow disease progression, but there is no cure. As the disease progresses, cognitive function declines and results in dementia.[117,119,120] Though the mechanisms by which degeneration of dopaminergic neurons occurs are not fully understood, it is well established that the aggregation of misfolded α-synuclein protein in the form of Lewy bodies is a hallmark of the disease.[121] However, whether the α-synuclein aggregates themselves are neurotoxic or may be a protective mechanism to sequester the more neurotoxic protofibrils is still debated.[122] Yet another hypothesis posits that neurodegeneration is due to the loss of

function of α-synuclein when it forms aggregates.[123] The physiological function of α-synuclein is not clear, but it appears to play an important role in dopamine biosynthesis and dopaminergic neurotransmission.[124–126] Genetic variants and post-translational modifications, including oxidation, nitration, and phosphorylation, influence the propensity of α-synuclein to aggregate; however, the physiological factors that incite these aggregation pathways are not well understood.[127]

Genetic factors that cause or increase risk of developing PD include mutations in SNCA (encoding α-synuclein), PRKN, and DJ-1, among others.[128] Interestingly, several of these genetic factors have been shown to contribute to immune defense against infectious agents. Polymorphisms in PRKN, a ubiquitin ligase, have been associated with increased susceptibility to intracellular pathogens, *Mycobacterium leprae* and *Salmonella typhi*.[129,130] Recently PRKN was shown to limit replication of bacterial pathogens *Mycobacterium tuberculosis* and *Listeria monocytogenes* in both mice and flies by targeting them for ubiquitin-mediated autophagy.[131] Also, mice in which SNCA is deleted are more susceptible to West Nile virus and Venezuelan equine encephalitis, possibly by modulating ER stress signaling and thereby limiting viral replication.[132] In contrast, DJ-1 appears to negatively regulate the immune system. When DJ-1 was deleted in a mouse model of polymicrobial sepsis, mice showed improved survival and bacterial clearance. Authors showed this to be due to enhanced phagocytosis and bactericidal activity in DJ-1-deficient macrophages, adoptive transfer of which could rescue septic wildtype mice.[133] Although genetic mutations account for only 5-15% of all PD cases [128], better understanding these genetic causes of disease have informed the pathophysiology of the more common sporadic disease cases.

Multiple environmental factors, including chemical exposure, lifestyle, and

socioeconomic conditions impact the development of PD, and pathogenic infection is increasingly recognized as a possible risk factor for PD.[134,135] The infectious etiology hypothesis of PD was originally proposed following the presentation of PD-like symptoms in individuals infected with influenza.[136] A 1963 cohort analysis identified a striking increase in PD incidence in Guam, which seemed to recede in patients born after 1920. Authors hypothesized that this transient increase in PD incidence may have been due the influenza pandemic of 1918.[136] Another study identified three seemingly random clusters of early-onset PD patients in Canada, in which patients lacked typical genetic markers of early-onset disease.[137] This suggested that environmental factors may have increased risk of PD in these patients, and the authors hypothesized that viral infection or other toxic exposure may be an underlying cause for these clusters of disease.[137] A cohort study examining the antibody titers to common infectious pathogens found higher seropositivity to cytomegalovirus (CMV), Epstein Barr virus (EBV), HSV-1, *Borrelia burdorferi*, *C. pneumoniae* and *Helicobacter pylori* in PD patients compared with healthy controls.[134] A recent meta-analysis of cohort and case-controlled studies revealed that patients with *H. pylori, C. pneumoniae,* HCV, or Malassezia yeast may be at an increased risk of PD.[138] While cohort studies cannot demonstrate that infections caused PD pathogenesis, together, they suggest that infection may be an important environmental risk factor for PD.

Certain viruses directly cause degeneration of dopaminergic neurons, which results in decreased dopamine availability in the CNS. Typically considered a hepatotropic virus, Hepatitis C virus (HCV) has recently been observed to invade the CNS and disrupt dopaminergic neurotransmission, leading to neuronal death.[139–141] HCV patients are affected by neurological complications, including cognitive impairment and peripheral neuropathy.[142] HCV may gain entry to the CNS by interacting with receptors expressed by brain microvascular endothelial cells

at the BBB, including CD68, CD81, and claudin-1.[140,141] Recent studies showed that once in the CNS, HCV activated resident microglia and astrocytes. This activation promoted a pro-inflammatory state through up-regulation of cytokines and chemokines, such as TNF-α and intracellular adhesion molecule-1 (ICAM-1), which caused significant damage to dopaminergic neurons.[140,143,144] Additionally, HCV infection was found to indirectly trigger neurotoxic effects seen in PD through IFN-α therapy. IFN-α treatment of HCV-infected murine models inhibited transmission through the nigrostriatal dopaminergic pathway, thereby reducing the levels of dihydroxyphenylacetic acid and dopamine present in the substantia nigra.[145] Furthermore, IFN-γ, which is transcriptionally upregulated in HCV-infected human brain microvascular endothelial cells [146], caused significant death of dopaminergic neurons in both *in vitro* murine microglia/neuron co-cultures and *in vivo* murine models.[147] PD is generally characterized by chronic low-level systemic inflammation; however, individuals with higher infectious burden have higher levels of circulating inflammatory cytokines, including IL-1β and IL-6.[134] PD patients infected with another Herpesviridae virus, CMV, have higher frequencies of circulating pro-inflammatory myeloid dendritic cells compared with CMV-positive subjects without PD.[148] Furthermore, When HIV-1 becomes neuroinvasive, it shows specific affinity for dopaminergic regions, including the basal ganglia, resulting in their degeneration, decreased availability of dopamine, and the development of dementia associated with acquired immunodeficiency syndrome (AIDS).[149–151] In a mouse model of disease, Theiler's murine encephalomyelitis virus (TMEV) was stereotaxically inoculated into the substantia nigra. TMEV specifically infected dopaminergic neurons with minimal infection or destruction to surrounding brain regions.[152] Japanese encephalitis virus (JEV) is recognized to not only target dopaminergic neuron-rich brain regions, but can also selectively manipulate dopamine signaling to increase the cell surface

expression of the molecules it uses to infect the cell.[153] These studies indicate that certain viruses can specifically impact populations of neurons that can lead to neurodegeneration of dopaminergic neurons directly.

Although the CNS is a primary focus of PD research, pathophysiology affects all levels of the brain-gut axis, including the autonomic and enteric nervous systems. Mulak and Bonaz recently hypothesized that α-synuclein aggregates initiate in the gut and proceed to spread along the brain-gut axis to the CNS, resulting in the motor and neuronal deficits characteristic of PD [154]. One pathogen hypothesized to incite α-synuclein aggregation in the gut is EBV. The C-terminal region of the α-synuclein is molecularly similar to a repeat region of the latent membrane protein 1, encoded by EBV.[155] This led to their hypothesis that in genetically-susceptible individuals, antibodies to the critical repeat region of the EBV latent membrane protein may cross-react with the homologous epitope on α-synuclein and induce α-synuclein oligomerization.[155] Following this initial aggregation event, α-synuclein oligomers may spread trans-neuronally to the CNS, causing PD neuropathology, as initially proposed by Braak.[156] In support of the brain-gut trans-neuronal hypothesis, researchers showed that when pre-formed α-synuclein fibrils were injected into the duodenal and pyloric muscularis layers of a mouse model, phosphorylated α-synuclein spread to regions of the CNS affected by PD, such as the locus coeruleus and substantia nigra pars compacta.[157]

Another gastric microbe that is associated with increased risk of PD is the bacteria *H. pylori.*[134,158] *H. pylori* is found in the intestinal endothelium and afflicts individuals with peptic ulcers, gastritis, gastric adenocarcinoma formation, and mucosal inflammation.[159] Previous studies have linked *H. pylori* to extra-gastrointestinal diseases, such as ischemic heart disease and neurodegenerative diseases, including AD and PD.[158,160–163] A Danish population-based

study found that prescriptions for *H. pylori* eradication treatments and proton pump inhibitors were associated with an increased risk of PD diagnosis 5 or more years later, suggesting either that chronic *H. pylori* infection may contribute to PD etiopathogenesis or gastritis symptoms may precede pathognomonic PD symptoms.[164] The mechanism underlying the role of *H. pylori* in PD onset is not well understood; however, the benefit of treating infections in PD patients is well-documented. A prospective cohort study found that *H. pylori*-IgG positivity in PD patients was associated with higher daily dose of levodopa and more severe symptoms compared with *H. pylori*-negative patients, and were improved after *H. pylori* eradication treatment.[165] Several studies have shown that eradicating *H. pylori* infection improved motor function of PD patients by increasing oral drug absorption.[166,167] A recent cohort study showed that PD patients with successful *H. pylori* eradication therapy exhibited improved clinical PD symptoms, including tremors, mood, and gastrointestinal distress, compared with patients with failed *H. pylori* eradication therapy.[168] Patients with active *H. pylori* infection had longer mean levodopa onset time, suggesting that *H. pylori* may interfere with the bioavailability of levodopa, possibly because of increased gastric inflammation, delayed gastric emptying, and/or impaired active transport of levodopa to the site of absorption.[168] Though much is still unclear of the involvement of *H. pylori* in the etiopathogenesis of PD, these data indicate that it is prevalent in PD patients and may exacerbate the symptoms of PD by interfering with levodopa bioavailability.

The global pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and its resulting disease (COVID-19) emerged as an unprecedented worldwide healthcare crisis. In the flood of viral pneumonia and the overwhelming challenges to the healthcare systems, researchers are just beginning to understand the extent to which patients develop acute or chronic neurologic manifestations. It was reported early in the pandemic that

36% of COVID-19 patients develop neurologic symptoms, but whether these were due to CNS infection, systemic inflammatory response, or intensive care unit delirium was unknown.[169] More recently, a neuropathological study found evidence of viral RNA and/or protein in the brains of 53% of autopsied COVID-19 patients; however, it is important to highlight that this study analyzed only patients who died, and thus, results are probably not generalizable to less severe cases of infection.[170] In fact, a systematic search of the literature revealed that in COVID-19 patients, SARS-CoV-2 RNA was detected in only 6.4% of those who underwent CSF PCR testing, which is likely still not representative of patients with mild infection.[171,172] Nonetheless, autopsies of COVID-19 patients revealed uniform presentation of neuroimmune pathology, including microglial activation and cytotoxic T lymphocyte infiltration in the brainstem and cerebellum. This pathology was independent of the detection of virus in the brain, suggesting that CNS damage and neurological symptoms may be due to cytokine storm and neuroimmune response rather than direct viral infection.[170] Considering the importance of the cerebellum and brainstem in coordinating voluntary movement, gait, posture, and motor functions, the localization of immune cell infiltration and activation may be of particular significance to the parkinsonian symptoms seen in some post-infectious COVID-19 patients.[173–175] Post-encephalitic parkinsonism has been reported previously for other viruses, but whether these symptoms constitute bona fide PD is unclear.[176] The three case reports describing parkinsonism following COVID-19 exhibited impaired dopaminergic nigrostriatal function, but this is not necessarily diagnostic of PD.[177] Rather, these may represent a transient syndrome that eventually resolves spontaneously instead of the progressive neurodegeneration of PD.[178] Alternatively, it is possible that SARS-CoV-2 unmasked previously preclinical PD.[177] However, given the high rate of SARS-CoV-2 infection, especially in the vulnerable aging population, the potential for

developing post-infectious PD is of particular concern.

1.4 Amyotrophic Lateral Sclerosis

ALS is a neurodegenerative disease that is characterized by the loss of upper and lower motor neurons. The decrease in motor function starts as muscle weakness in the limbs and progresses to eventual paralysis of all muscular motor movements in the body.[179] Eventually, the motor neuron degeneration prevents proper functioning of the diaphragm, disrupting the proper respiratory function needed to survive.[180] As there are currently no pathognomonic tests for ALS, diagnosis relies on the identification of concomitant progressive upper and lower motor neuron dysfunction and the exclusion of mimicking conditions.[181] Further complicating ALS diagnosis, is the existence of "ALS-like syndrome," which refers to a heterogenous group of conditions in which their clinical presentation is similar to ALS (*i.e.,* motor neuron dysfunction), but in many cases, the underlying cause of these symptoms is treatable.[182] For example, patients with HIV-1 infection presenting with ALS-like syndrome that were treated with antiretroviral therapy showed partial recovery of their motor deficit.[183] In published reports, ALS-like syndromes cannot always be distinguished from bona fide ALS, so for the purpose of this review, we do not attempt to separate the two conditions.

There is emerging data that suggests infectious agents, including viruses and fungi, may be associated with ALS. Enteroviruses have long been suspected to play a role in ALS due to their ability to infect motor neurons in the CNS and cause meningitis and encephalitis.[184] However, clinical data connecting enterovirus infection and ALS have been inconclusive. Several studies have identified enterovirus RNA in spinal cord tissue of 70-80% of ALS patients [185,186]; however, others have found no detectable viral RNA in ALS patients.[187,188] Therefore,

further investigation into the role of enteroviruses in ALS is necessary in order to clarify this relationship. Other recent studies have identified DNA from several *Candida spp*. of fungi, as well as fungal antigens in the CSF and brain tissue of ALS patients.[189] This, coupled with the detection of fungal hyphae within the motor cortex and spinal cord of ALS patients [190], supports the idea that infection may contribute to or exacerbate ALS pathology. Numerous cellular dysfunctions associated with ALS are impacted by infectious agents, including protein aggregation and mislocalization, and glutamate excitotoxicity.[191] Better understanding of how infectious agents may contribute to these cellular mechanisms that lead to motor neuron deficit will improve our understanding of the progressive neurodegeneration associated with ALS.

The presence of ubiquitinated protein aggregates in affected motor neurons is a central hallmark of disease; however, the composition of those aggregates varies among ALS patients.[192] The molecular characteristics and distribution of these protein aggregates, in many cases, are linked to the genetic mutations that cause the disease. However, proteinaceous aggregates of similar composition are also detected in non-mutation carriers, indicating a convergence of underlying cellular and pathological processes in both familial and sporadic cases of ALS.[192] The identification of ALS-associated mutations in two DNA/RNA binding proteins, TAR DNA-binding protein 43 (TDP-43) and protein fused in sarcoma (FUS), also implicate alterations in RNA processing as a key event in ALS pathogenesis.[193] Furthermore, mutations in TDP-43 lead to misfolded and truncated proteins, such as TDP-25 and TDP-35, as well as mislocalization from the nucleus to the cytoplasm.[194] Because the translocation of TDP-43 from the nucleus to the cytoplasm is tightly linked to the formation of pathological aggregates in the cytoplasm, it is difficult to decouple the consequences of its loss of function as a DNA-binding protein in the nucleus from the potentially toxic gain of function effects of the aggregates in the cytoplasm.[195]

However, a similar cytoplasmic translocation occurs during HIV and fungal infection.[196,197] It is hypothesized that the release of neurotoxins, such as ochratoxin A, during fungal infection causes TDP-43 to mislocalize to the cytoplasm, leading to ALS pathogenesis.[198] The overall structure of TDP-43, along with its propensity to aggregate and mislocalize, is further influenced by post-translational modifications.[199] It has been demonstrated that infection with TMEV, both *in vitro* and *in vivo,* caused TDP-43 phosphorylation and cleavage, resulting in its cytoplasmic mislocalization and aggregation.[200] These data indicate that viral and fungal infections promote the neuropathology associated with ALS.

Interestingly, the relationship between viral infection and TDP-43 aggregation may be reciprocal in nature, as TDP-43 aggregation may enhance expression of endogenous retroviruses in the CNS. In autopsied samples of cortical and spinal neurons from ALS patients, the transcriptional expression of human endogenous retrovirus-K (HERV-K) polymerase was enhanced.[201] Furthermore, in patients with sporadic ALS, HERV-K reverse transcriptase expression was correlated with TDP-43 and HERV-K long terminal repeats have four binding sites for TDP-43, which have been shown to regulate its activation.[202] In a *Drosophila* model of disease, focal glial expression of human TDP-43 triggered gypsy-ERV replication, as well as DNA damage, and neuronal apoptosis.[203] Additionally, TDP-43 harbors binding sites for interferon regulatory factors (IRF) and κB, which are important inflammatory mediators, causing TDP-43 to become transcriptionally upregulated in response to antiviral interferon expression.[201] Together, these data suggest that HERV-K expression may be driven, in part, by TDP-43 as well as in response to local neuroinflammation.[196,201,202] In fact, TDP-43 was originally found to inhibit HIV transcription in cell culture [204], though this function is still debated and may reflect differences in cell types and model systems.[205–208] Together, these studies indicate that TDP-43

aggregation and infectious agents may develop a reciprocal relationship in causing pathogenic changes that lead to ALS.

A second DNA/RNA binding protein that has been associated with familial ALS is FUS, which, when mutated, interferes with RNA metabolism, suppresses protein translation, and decreases the nonsense-mediated decay pathway.[209] ALS-associated genetic mutations result in the formation of stress granules, which are composed, in part, of RNA-binding proteins, including TDP-43 and FUS.[210] The formation of FUS-containing stress granules can be stimulated by respiratory syncytial virus (RSV), as well as by poly(I:C), which is used by laboratory researchers to mimic viral double-stranded RNA.[211] It was also found that infection of induced pluripotent stem cell-derived spinal neurons with either rabies virus (RABV) or HIV-1 increased the production of FUS and promoted its cytoplasmic mislocalization.[212] Furthermore, many other viruses have been shown to promote the formation of stress granules.[213,214] These studies demonstrate a link between viral infection and a key neuropathogenic hallmark of ALS.

Glutamate is a major excitatory neurotransmitter in the brain; however, excessive stimulation due to increased glutamate receptor expression or ligand availability can cause excitotoxicity and lead to neuronal death.[215,216] Perisynaptic astrocytes express glutamate transporters, including excitatory amino acid transporter 2 (EAAT2) and glutamate transporter-1 (GLT-1), which clear glutamate from neuronal synapses.[217] Defects in glutamate transport have been found in synaptosomes prepared from neural tissue from ALS patients.[218] This is likely due to a combined effect of upregulation of genes that transcribe glutamate receptors in the motor cortex of ALS patients and selective loss of glutamate transporters in the motor cortex of ALS patients.[219,220] In transgenic mice expressing mutant *SOD1*, GLT-1 was found to decrease in accordance with disease progression and survival could be extended by increasing expression of

EAAT2.[221,222] During viral infection, exposure of fetal human astrocytes *in vitro* to the HIV-1 envelope glycoprotein, gp120, caused a 40-70% decline in steady-state levels of EAAT2 RNA.[215] This resulted in reduced glutamate transport and may contribute to glutamate excitotoxicity following HIV-1 infection.[215] Furthermore, exposure of neurons to fungal neurotoxins caused the spontaneous release of endogenous glutamate [223], and elevated glutamate levels have been shown to increase the toxicity associated with SOD1, as well as to promote TDP-43 translocation.[224,225] Moreover, EAAT2 expression is downregulated by TNF-α, an important cytokine involved in the antiviral response to many viruses including HIV-1, VZV, EBV, and CMV, among others.[226,227] Thus, this excitotoxic impact from glutamate is likely common among many viral infections. Together, these data indicate that infectious diseases cause changes in glutamate signaling that can lead to excitotoxicity that is symptomatic of ALS.

1.5 Conclusions and Future Directions

Here we have reviewed recent literature linking microbial infections to neurodegenerative diseases, including AD, PD, and ALS. Although epidemiological data indicate an association between infectious agents and neurodegenerative diseases, in many cases the molecular and cellular mechanisms underlying those associations are unclear. Alternatively, patients with neurodegenerative diseases may be at increased risk of being infected with a neurotropic agent, potentially due to compromised immune systems and/or leaky BBB in affected individuals. Further research using *in vitro* and *in vivo* models will help elucidate whether infectious agents increase the risk of developing neurodegenerative diseases on their own, via anti-microbial inflammatory pathways, or other unknown mechanisms. The study of model systems, including both rodent and non-rodent models, will also improve our understanding of post-infectious

neurologic and cognitive dysfunction that occurs following many systemic and neurotropic infections beyond the common neurodegenerative diseases reviewed here. Identification of molecular mechanisms common among these neurologic disorders may lead to new diagnostic biomarkers to identify individuals that may develop progressive neurocognitive or neurodegenerative diseases, as well as new therapeutic options for them.

CHAPTER 2: Study of the Intracellular Protein Tau and its Relationship with Kunjin Virus and Neurodegenerative Diseases

2.1 Introduction

Alzheimer's disease (AD) is the sixth-leading cause of death in the United States—higher than breast cancer and prostate cancer combined.[228] It is a neurodegenerative disease (NDD) characterized by the deposition of two lesions in the brain—extracellular senile plaques and intracellular neurofibrillary tangles (NFTs)—resulting in progressive impairment of cognitive abilities.[229] NFTs are the product of an accumulation of hyperphosphorylated Tau fibrils.[230] Though age and genetic factors have been identified as risk factors for this NDD, microbial infections have become of keen interest as they are thought to contribute to NDD pathology through eliciting inflammatory responses.[231–235] Previous research has identified several inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-1β, IL-6, IL-10, and IL-18 to provoke the aggregation of both Aβ and Tau.[236–239] Though inflammatory processes are essential in clearing microbial challenges, they begin to have severe consequences when they become systemic.[240–242] Widespread activation of inflammatory cytokines and chemokines can promote breakdown of the blood brain barrier (BBB), allowing entry of damaging agents and inflammatory mediators.[242–244] Additionally, breach of the BBB promotes stimulation of CNS-resident astrocytes and microglia, further amplifying inflammatory conditions capable of causing significant damage to both infected and uninfected neurons.[240,245,246]

The microbial etiology hypothesis of AD has been of keen interest to researchers for decades. Recent studies demonstrated herpes simplex virus 1 (HSV-1) infection directly correlated with elevated hyperphosphorylation of Tau.[247,248] Tau is an intracellular microtubule

associated protein (MAP) that aids in the stabilization of microtubules that line the axonal cytoskeleton of neurons.[249] In a normal state, phosphorylation activity of kinases and phosphatases modulate the affinity of Tau for microtubules, allowing for dynamic growth and retraction. However, dysregulation of this activity leads to hyperphosphorylation of Tau resulting in a decreased affinity for the microtubules, allowing them to dissociate and increase their propensity to self-aggregate into pathogenic NFTs.[250,250,251] In a recent study, hyperphosphorylated Tau isolated from AD brain samples was injected into the hippocampus of human transgenic mice resulting in hyperphosphorylated Tau at Ser202/Thr205, Thr212, Ser214, Thr217, Ser262, and Ser422.[34]

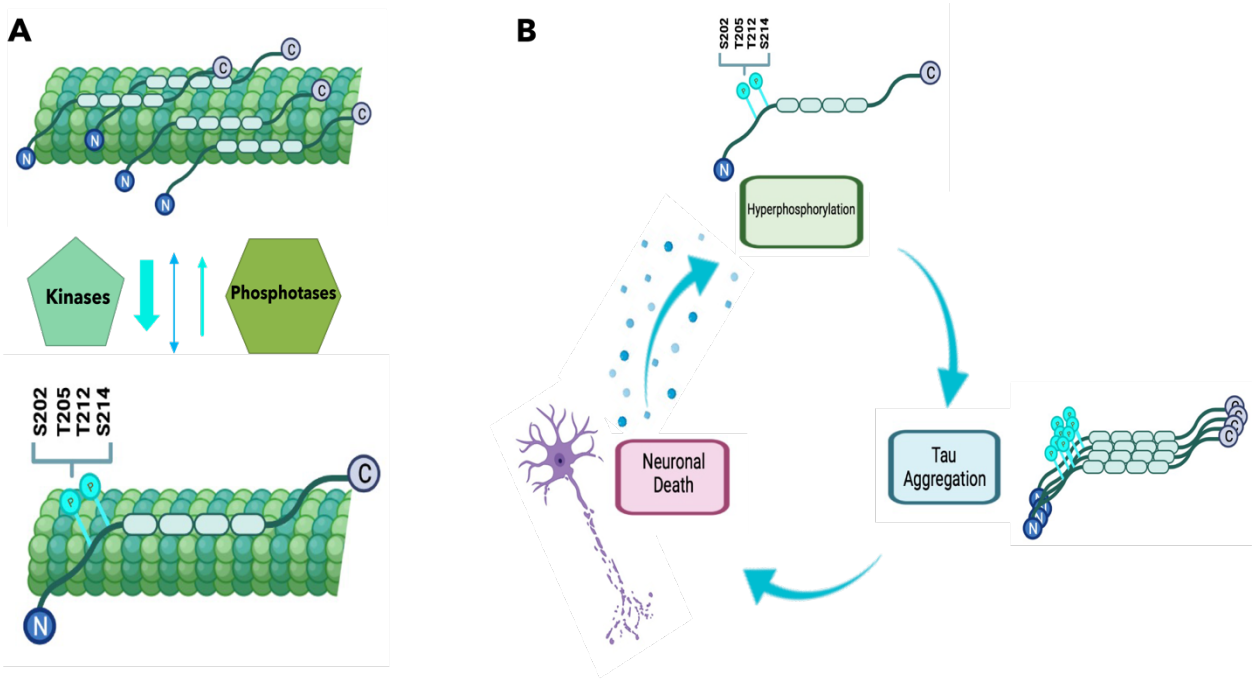

Figure 2: Tau phosphorylation regulates normal function in stabilizing microtubules and in a pathological aggregation. a, The balance of Tau phosphorylation is maintained by kinases and phosphatases, but can be disrupted by toxic factors, resulting in the hyperphosphorylation of Tau. **b,** Hyperphosphorylation of Tau contributes to the creation of Tau aggregates, NFTs, and ultimately neuronal death, further reacting in a cascade manner.

The mechanisms that disrupt this balance of phosphorylation remain elusive, however, it is thought that inflammatory cytokines as well as infectious agents may play a role in inciting neurodegenerative pathology.[241,252] We have summarized a microbial infections hypothesis linking numerous viral infections to increased Tau hyperphosphorylation as well as deposition of NFTs and damage in primary neurons.[252] Furthermore, activated immune signaling pathways were detected in some of these infection models as well as detection of enhanced pro-inflammatory cytokine secretion from CNS-resident immune cells including microglia, astrocytes, and monocytes.[253–257] These findings further support the notion infections may promote AD onset.[247,258–266] Further research into the role infectious agents may play in the accumulation of Tau aggregates is essential to gain a better understanding of the pathogenesis of NDDs as well as identifying potential therapeutic targets.

The focus of this thesis is to gain a comprehensive understanding of the effects of acute viral encephalitis on the brain, including the development of AD-related Tau pathology, with the use of a novel model of neurotropic viral infection. Previous research has focused on the interplay between microbial infection, Aβ accumulation and stimulation of inflammatory cytokines such as TNF-α, IL-6, and IL-1β.[257–260,267] However, not much is known about the relationship between neurotropic viral encephalitis, neuroinflammation, and Tau. In addition to its role in stimulating chronic inflammation, several researchers have suggested Aβ may protect against infection by acting as an antimicrobial peptide in a number of models through direct binding to the cell wall of the microbe or via insertion into the viral envelope.[262,265,268] A previous study suggested that Aβ acts as an antimicrobial agent in a transgenic AD mouse model (5XFAD) following bilateral hippocampal injection of $5\ x\ 10^6$ plaque forming units (PFU) HSV-1. Their results showed increased survival in transgenic mice compared to the wild type

(WT) littermates, and data suggested that Aβ is able to directly bind to HSV-1 and inhibit its replication.[262] Bourgade et al also demonstrated similar antimicrobial activity of Aβ against HSV-1 through direct binding, further suggesting that Aβ accumulates as an immune defense mechanism.[268] Furthermore, Aβ was shown to protect against *Salmonella enterica* serotype *typhimurium* (*S.* typhimurium) in AD mouse models and *Candida albicans* and *S. typhimurium* in nematode and cell culture models.[265] These data present Aβ as an antimicrobial peptide; however, whether the Tau protein, which forms intracellular aggregates similar in structure to Aβ aggregates, can also act as an antimicrobial peptide is unknown.

The goal of this study was to determine whether the Tau protein may act as an antiviral protein. For this we used a murine model of WNV neurotropic infection. WNV has long been studied for its ability to cause cognitive impairment. Previous studies utilized models of WNV infection with the strain isolated from New York in 1999 (WNV-NY99). However, use of this strain poses challenges for the study of neuronal effects of viral infections. Specifically, when WNV-NY99 is infected peripherally via footpad inoculation, the level of virus that crosses into the CNS is variable; however, when WNV-NY99 is inoculated i.c., 100% of mice die, thus limiting the ability to study recovery mechanisms.[269] To circumvent this, I utilized a subtype of WNV, Kunjin virus (KUNV). Endemic to Australia, KUNV is a naturally attenuated subtype of WNV, making it less virulent. Through use of this model, I studied whether neurotropic KUNV infection (1) contributes to Tau deposition and (2) whether Tau can act as an antimicrobial peptide against KUNV.

2.2: Materials and Methods

2.2.1: Tauopathy and Frontotemporal Dementia Mouse Model

Male transgenic mice containing the Tau P301S mutation and female non-carrier (NC) mice were purchased from Jackson Laboratory (PS19 line). The PS19 line was kept on a crossed background with C57BL/6 and C3H. Mice with the transgene are hemizygous (+/0) and express the P301S mutant form of human *MAPT* (1N/4R) under the direction of the mouse prion promoter. Expression of the transgene is 5-fold higher than the expression of the endogenous mouse MAPT protein. Male mice carrying the transgene are paired with non-carrier (NC) females for breeding. Both male and female mice of both NC and hemizygous genotypes were used for experiments, as noted.

2.2.2: Genotyping

Ear clips were taken from mice prior to 8 weeks of age and processed via polymerase chain reaction (PCR) to determine genotypes. Ear clips were run in ear digest (100 μL Direct PCR (Taik) (102-T, Viagen) + 1.5 μL Proteinase K 20 mg/mL (E192-5mL, VWR)) overnight in a thermocycler (55℃ for 12 hours, 85℃ for 45 minutes, 4℃ hold). They were then mixed with 0.1 μLP301S Tg Rev (5'-GGT ATT AGC CTA TGG GGG ACA C-3', IDT), 0.1 μL P301S Tg Fwd (5'-GGC ATC TCA GCA ATG TCT CC-3', IDT), 0.1 μL P301S Int Post CTRL Fwd (5'-CAA ATG TTG CTT GTC TGG TG-3', IDT), and 0.1 μL P301S Int Pos CTRL Rev (5'-GTC AGT CGA GTG CAC AGT TT-3', IDT) primers, 3.6 μL ddH2O, and 5 μL DreamTac Green PCR Master Mix (2x) (K1081, Thermo Scientific) and run in a thermocycler (1. 94℃ for 5 minutes, 2. 94℃ for 1 minute, 65℃ for 1 minute (-0.5℃ per cycle), 72℃ for 1 minute (step 2 is repeated for 10 cycles), 3. 94℃ for 1 minute, 60℃ for 1 minute, 72℃ for 1 minute (step 3 is

repeated for 28 cycles), 4. 72℃ for 10 minutes, 5. 4℃ infinite hold). Samples were run on a 1.5% Agarose gel with 5 μL Sybr Safe DNA gel stain (S33102, Fisher) for 30 minutes at 100 V and imaged using BioRad Image Lab.

2.2.2: C636 Cells, Vero Cells, and Kunjin Virus

2.2.2.1: Isolation of Kunjin Virus

Vero cells were grown and maintained in Dulbecco modified Eagle medium (DMEM) and supplemented with 5% fetal calf serum and antibiotic/antimycotic. Cells were infected with KUNV (Clone FLDSX) at a multiplicity of infection (MOI) of 0.5 and incubated at 37℃. Supernatant was collected at 40 hours after infection and centrifuged at 1300 x g for 10 minutes, then supernatants were pooled together in a sterile flask. To purify virus, 5 mL of cold 25% glycerol in Tris/NaCl/Ethylenediaminetetraacetic acid (EDTA) (TNE) was placed in the bottom of OptiSeal Polyallomer Tubes (NC9691210, Fisher) and overlayed with 25 mL of viral supernatant. The samples were placed in a SW32Ti Beckman Coulter rotor and ultracentrifuged at 30,000 rpm for 4 hours at 4℃. Virus remained in the pellet, which was resuspended in 1 mL TNE and aliquoted in single-use tubes for quantification by plaque assays.

2.2.2.2: Intracranial Infection with Kunjin Virus

Mice were heavily anesthetized under 5% isoflurane and injected with 10 μL KUNV (100 PFU) (prepared in 1% FBS in HBSS) in the third ventricle of the brain.

2.2.2.3: Plaque Assays

6 well tissue culture treated plates were plated with 2 mL of baby hamster kidney (BHK) cells at 600,000 cells/mL mixed with 450 mL EMEM (30-2003, ATCC) + 50 mL HI FBS (16140071, Gibco) and incubated overnight at 37℃. 10-fold dilutions were made from KUNV viral stock (0: stock virus, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) and 150 μL placed in each well and incubated at 37℃ for 1 hour. Wells were then overlayed with 3 mL of 2% SeaPlaque Agarose (BMA50100, Fisher Scientific) mixed with 2X MEM and allowed to solidify at room temperature for 1 hour and transferred to a 37℃ incubator for 3 days. Plates were then fixed with 10% Formaldehyde for 2 hours, after which plugs were removed and wells were stained with 1 mL Crystal Violet (10 g Crystal Violet (C581-100, Fisher), 200 mL of 100% Ethanol, and 800 mL of H_2O).

2.2.4.: Tau Aggregation or Phosphorylation Analysis

2.2.4.1: BCA Assay

Tissues were homogenized using a Bead Mill 24 Homogenizer (15-340-163, Fisher) at 4 m/s for 1 minute at 4℃. Homogenized tissue was centrifuged at 16,000 x g for 1 minute, and 25 μL of lysate supernatant was placed in a well of a 96-well plate, in addition to albumin standards made using the Pierce BCA Protein Assay Kit (23227, Thermo Scientific). A working reagent was created by adding 50 parts of reagent B to 1 part reagent A. 200 μL of working reagent was then added to each well containing lysate samples. The plate was then incubated at 37℃ for 30 minutes and read on a SpectraMax iD5 Multi-Mode Microplate Reader at 562 nm. Using the calculated values of the albumin standards, an equation to determine protein concentration of the lysates was created and implemented.

2.2.4.2: Sarkosyl Solubility Assay

Cortex samples from 8 wk-old KUNV-infected or mock-infected Tau P301S mice 30 dpi were homogenized using a Bead Mill 24 Homogenizer at 4 m/s for 1 minute. Homogenized tissue was centrifuged at 16,000 x g for 1 minute, and 100 μL of lysate supernatant was mixed with 300 μL of a Salt/Sucrose buffer (For a 50 mL volume: 0.8 M of NaCl, 1mM of Sodium orthovanadate (J60191, Alfa Aesar), 5 of Piece Protease Inhibitor Mini Tablets, EDTA-Free (A32955, Thermo Scientific), 10% Sucrose (J65148, Alfa Aesar), 30 mM of Na F 99% (A13019, Alfa Aesar), 10 mM Tris HCl pH 7.4) and spun at 16,000 x g for 20 minutes. The supernatant was pulled off and saved. 500 μL of Salt/Sucrose buffer was added to the sample pellet and spun at 16,000 x g for 20 minutes. The supernatant was pulled off and pooled with the first supernatant. 100 μL of 10% Sarkosyl (S3376, Teknova) was added for a final concentration of 1%. Samples were placed on a rocker to incubate at room temperature for 1 hour. Samples were placed in 1 mL Quick-Seal tubes (348184, Beckman Coulter) and sealed. Samples were placed in a Type 25 rotor (Beckman Coulter, 347261) and run at 25,000 rpm at 4℃ for 1 hour. Lysates, soluble Tau, were pulled off and stored at -20℃. Pellets, insoluble Tau, were reconstituted with 250 μL of 50 mM Tris/HCl, transferred to new microcentrifuge tubes, and stored at -20℃.

2.2.4.3: Western Blot

Cortex samples from 8 wk-old KUNV-infected or mock-infected Tau P301S mice 30 dpi were homogenized using a Bead Mill 24 Homogenizer at 4 m/s for 1 minute. Protein concentrations were equalized according to the BCA assay and brought up to 11.25 μL with sterile milliQ water. 3.75 μL of 4x Laemmli Buffer (1610747, BioRad) mixed with dithiothreitol

(DTT) was added to each sample. Samples were then boiled at 95℃ for 5 minutes and spun at 16,000 x g for 1 minute. 15 μL of each sample was loaded into Any kD Mini-PROTEAN TGX Precast Protein Gels (4569036, BioRad). Precision Plus Protein Kaleidoscope ladder (161-0375, BioRad) was used. Chamber was filled with 1X Running Buffer (10X Tris/Glycine/SDS (1610772, BioRad)) and run at 80 V for 30 minutes and 150 V for 1 hour. Blots were then transferred to PVDF membranes at 0.1 A overnight at 4℃ in 1X Transfer Buffer (10X Tris/Glycine (1610771, BioRad)). Membranes were then placed in 5 mL blocking buffer (5% NFDM (M17200-100, Research Products International) and 1X TBST (AAJ62938K2, Fisher Scientific + 10 mL Tween 20 (BP337-100, Fisher Bioreagents))) and incubated at room temperature for 1 hour. Membranes were then placed in 5 mL blocking buffer mixed with 5 μL of 0.2 mg/mL Phospho-Tau (Ser202, Thr205) Monoclonal Antibody (AT8) (MN1020, Invitrogen), 5 μL of 0.2 mg/mL Phospho-Tau (Thr212, Ser214) Monoclonal Antibody (AT100) (MN1060, Invitrogen), 5 μL of 5A6 1° Antibody Mouse IgG (DSHB) (0.2 ug/mL), 5 μL of 0.2 mg/mL Tau-5 Mouse Monoclonal Antibody (PIMA512808, Fisher Scientific), or GAPDH Mouse Antibody (4466S, Cell Signaling Technologies) and incubated at 4℃ overnight. Membranes were rinsed three times for ten-minute increments with 1X Tris Buffered Saline + Tween (TBST; 1X TBS + 10 mL Tween 20) and placed in 5 mL blocking buffer mixed with 5 μL of 0.2 mg/mL Anti-Mouse IgG HRP-Linked secondary antibody (7076S, Cell Signaling Technologies) and incubated at room temperature for 1 hour. Membranes were once again rinsed three times for ten-minute increments with 1X TBST and transferred to 1X TBS (AAJ62938K2, Fisher Scientific) for imaging. Membranes were placed in 1 mL western blotting detection reagent (RPN3243, GE Healthcare) for 1 minute and imaged using BioRad ChemiDoc Imaging System 27444.

2.2.4.4: Densitometry

Densitometry was performed on the western blots described above using Bio Rad Image Lab. Values were then normalized to GAPDH values using the following equation:

$$Target\ Mouse\ A\ x\ \frac{GAPDH\ mouse\ A}{Average\ GAPDH} = normalized\ Target\ Mouse\ A\ value.$$

2.2.4.5: Immunohistochemistry

2.2.4.5.1: Tissue Collection and Fixation

To collect tissues for immunohistochemistry (IHC), mice were anesthetized with isoflurane, and perfused in the apex of the heart with cold phosphate buffered saline (PBS; BP3994, Fisher Bioreagents). Brains were post-fixed overnight in 4% paraformaldehyde (PFA; 100496-496, VWR) in PBS and transferred into 30% sucrose solution the following day and changed twice more until sedimented. Once sedimented, brains were embedded in Tissue-Plus O.C.T. compound (23-730-571, Fisher Scientific) and kept at -80℃.

2.2.4.5.2: Tissue Sectioning

Tissue samples were transferred from -80℃ to -20℃ and allowed to acclimate for one hour. Sagittal sections (10 μm) were cut from a frozen block using a cryostat and plated on Superfrost Plus slides (12-550-15, Fisher Scientific). Slides were stored at -80℃.

2.2.4.5.3: Immunofluorescence Labeling

Sections were prepped with PBST (1X PBS + 10 mL Tween) and tissues circled with a PAP pen. Samples were then incubated in 50 μL of blocking buffer (500 μL of goat serum

(100241-872, VWR) + 9.5 mL PBST) for 1 hour. The primary antibody mix was prepared by adding 1 μL of 0.2 mg/mL Phospho-Tau (Ser202, Thr205) Monoclonal Antibody (AT8) (MN1020, Invitrogen) or 1 μL of 0.2 mg/mL Phospho-Tau (Thr212, Ser214) Monoclonal Antibody (AT100) (MN1060, Invitrogen) to 500 μL of blocking buffer and 0.5 μL of NeuN (D3S3I) rabbit monoclonal antibody (12943S, Cell Signaling Technologies; used for neuronal staining). 50 μL of the primary antibody mix was placed on each sample and incubated at 4℃ overnight. Samples were rinsed in PBST for ten minutes twice followed by PBS. The secondary antibody mix was prepared by adding 2 μL of 2 mg/mL Alexa Fluor 488 goat anti-mouse IgG (H+L) secondary antibody (A11001, Life Technologies Corporation) and 2 μL of 2 mg/mL Alexa Fluor 555 goat anti-rabbit IgG (H+L) secondary antibody (A21428, Life Technologies Corporation) to 800 μL of blocking buffer. 50 μL of secondary antibody mix was added to each sample and incubated at room temperature for 1 hour. Samples were washed in PBST for ten minutes followed by PBS. Nuclear staining was done with DAPI (D1306, Life Technologies Corporation) by adding 50 μL of diluted DAPI (1 μL of DAPI (1 μg/mL) + 1 mL PBS) to each sample and incubated for ten minutes at room temperature. Tissue slides were washed in PBST for ten minutes followed by PBS, dried carefully, and mounted with Prolong Gold (P36930, Life Technologies). Tissue slides were allowed to cure for at least 24 hours before analyzed using a Keyence BZ-X800 Series. Images were taken at 20X magnification, 3 images per tissue sample.

2.2.4.5.4: Immunofluorescent Analysis

Images described above were analyzed using FIJI Image J by transforming to 8-bit, overlaying a mask, and fluorescent signal quantified. Data was then graphed using GraphPad Prism and analyzed for statistical differences via unpaired t-tests.

2.3: Results

2.3.1: Kunjin Virus Productively Stimulates Tau Hyperphosphorylation in Eight-Week-Old Tau P301S Mouse Cortex Eighteen Days Post Infection

To study Tau hyperphosphorylation and aggregation, I used the PS19 mouse model (Tau P301S), as it allows for breeding of hemizygous carriers of the 1N4R human P301S mutation on the *MAPT* gene (Tau P310S +/0) as well as non-carrier (NC) littermates. The P310S mutation has been well characterized in human disease models of Frontotemporal Dementia and has been shown to cause Tau pathology similar to human AD patients, including Tau aggregates and NFTs by 6 months and hind-limb paralysis by 9 months, in mutant mice. Additionally, I used KUNV as an infection model to incite neuroinflammation. Previous data in the lab showed increased weight in 8 wk-old C57BL/6J mice infected i.c. with 1 or 10 PFU KUNV, whereas infection with 100 PFU resulted in decreased weight, indicating 100 PFU is a proper dose to use for i.c. inoculation (Fig 3). AD-related Tau pathology can be characterized by the hyperphosphorylation of Tau stimulating aggregation of Tau fibrils and NFTs. Therefore, the sites of hyperphosphorylation are of immense importance.

To determine whether infection causes phosphorylation at sites associated with aggregation, I examined levels of Tau phosphorylation following i.c. inoculation of KUNV 100 PFU into 8 wk-old, 11 wk-old and 15 wk-old Tau P301S +/0 mice. Compared to 8 wk-old mice, 11 and 15 wk-old mice revealed lower amounts of hyperphosphorylated Tau at sites Ser202/Thr205 and Thr212/Ser214 within samples of the cortex and spinal cord, at 18 dpi (Fig 4). This indicates Tau is phosphorylated more at these sites in an infected 8 wk-old model compared to infected 11 and 15 wk-old models, further establishing 8 wk-old mice as the optimal age of infection.

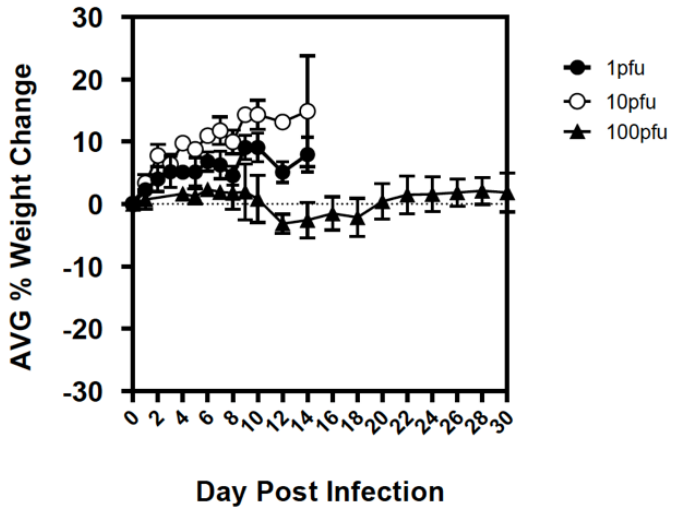

Figure 3. Decreased average weight change observed in 8 wk-old mice post i.c. KUNV (100 PFU) infection. Average weight change of 8 wk-old C57BL/6J mice following intracranial (i.c.) inoculation with KUNV at 1 PFU, 10 PFU, and 100 PFU doses. Results showed consistent weight loss as a measure of illnes following infection with 100 PFU, indicating optimal infectious dose.

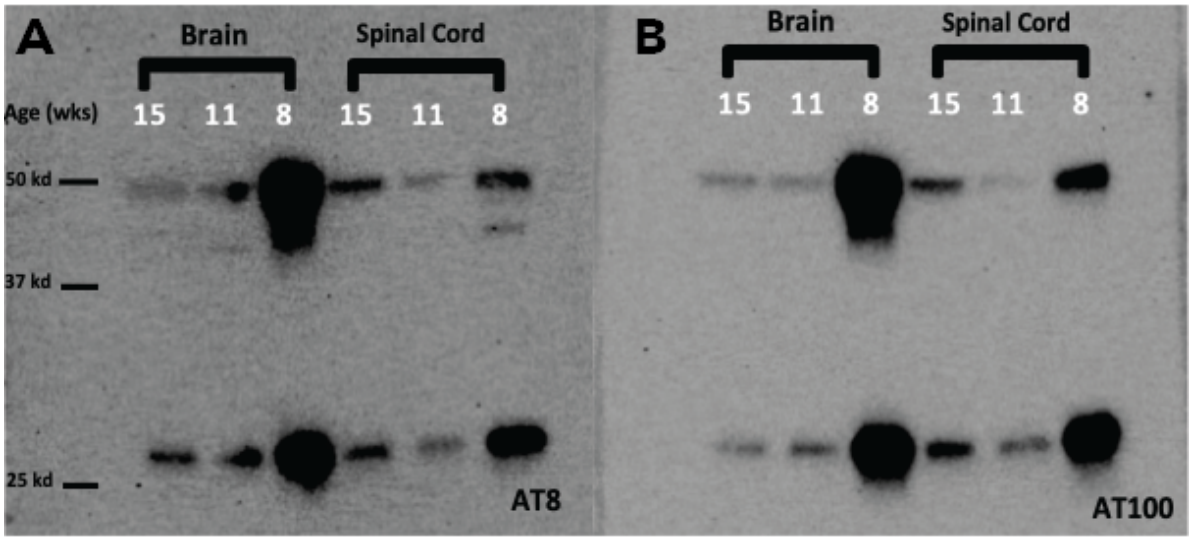

Figure 4. Increased Tau phosphorylation observed in KUNV-infected Tau P301S mice. a-b, 18 days post i.c. KUNV infection showed increased Tau hyperphosphorylation (**a**, site Ser202/Thr205, detected by AT8 antibody; **b**, Thr212/Ser214, detected by AT100 antibody) in the cortex and spinal cord of the 8 wk-old Tau P301S mice compared to the 11 and 15 wk-old models.

2.3.2: KUNV Infection Stimulates Tau Hyperphosphorylation in Tau P301S Mouse Cortex

To ascertain whether KUNV infection induces Tau pathology, 8 wk-old Tau P301S mice were infected i.c. with KUNV (100 PFU) or mock-infected and observed for 30 dpi. The cortex lysate from each sample was then analyzed via Western Blot Analysis and probed for Tau phosphorylation at sites Ser202/Thr205 and Thr212/Ser214 using the AT8 and AT100 antibodies, respectively (Fig 5A and 5B). Results show that Tau was phosphorylated at Ser202/Thr205 and Thr212/Ser214 sites in the KUNV-infected but not in the mock-infected Tau P301S mice. Additionally, the cortex lysate was probed for Total Tau, using the 5A6 antibody, and GAPDH (Fig 5C and 5D) which showed equal levels in both the KUNV-infected and mock-infected Tau P301S mice. Western blots were analyzed by densitometry and AT8, AT100, and Total Tau signals were normalized to GAPDH. Normalized values were analyzed on GraphPad Prism for statistical differences using unpaired t-tests. No statistical difference was observed in Total Tau levels (detected by 5A6) between the KUNV-infected and mock-infected Tau P301S mouse cortex ($p = 0.8113$); however, analysis revealed a significant difference ($p = 0.0325$) in the phosphorylation of sites Thr212/Ser214 (detected by AT100) with higher levels seen in the KUNV-infected mouse cortex. Additionally, there is a similar trend in the phosphorylation of sites Ser202/Thr205 (detected by AT8) with higher levels seen in the KUNV-infected mouse cortex, though it was on the cusp on being statistically significant ($p = 0.059$). Together these data show equal levels of Tau (detected by 5A6) and GAPDH between the KUNV-infected and mock-infected Tau P301S mice, but elevated Tau phosphorylation in the KUNV-infected Tau P301S mice, indicating KUNV incites increased Tau phosphorylation in the Tau P301S mice.

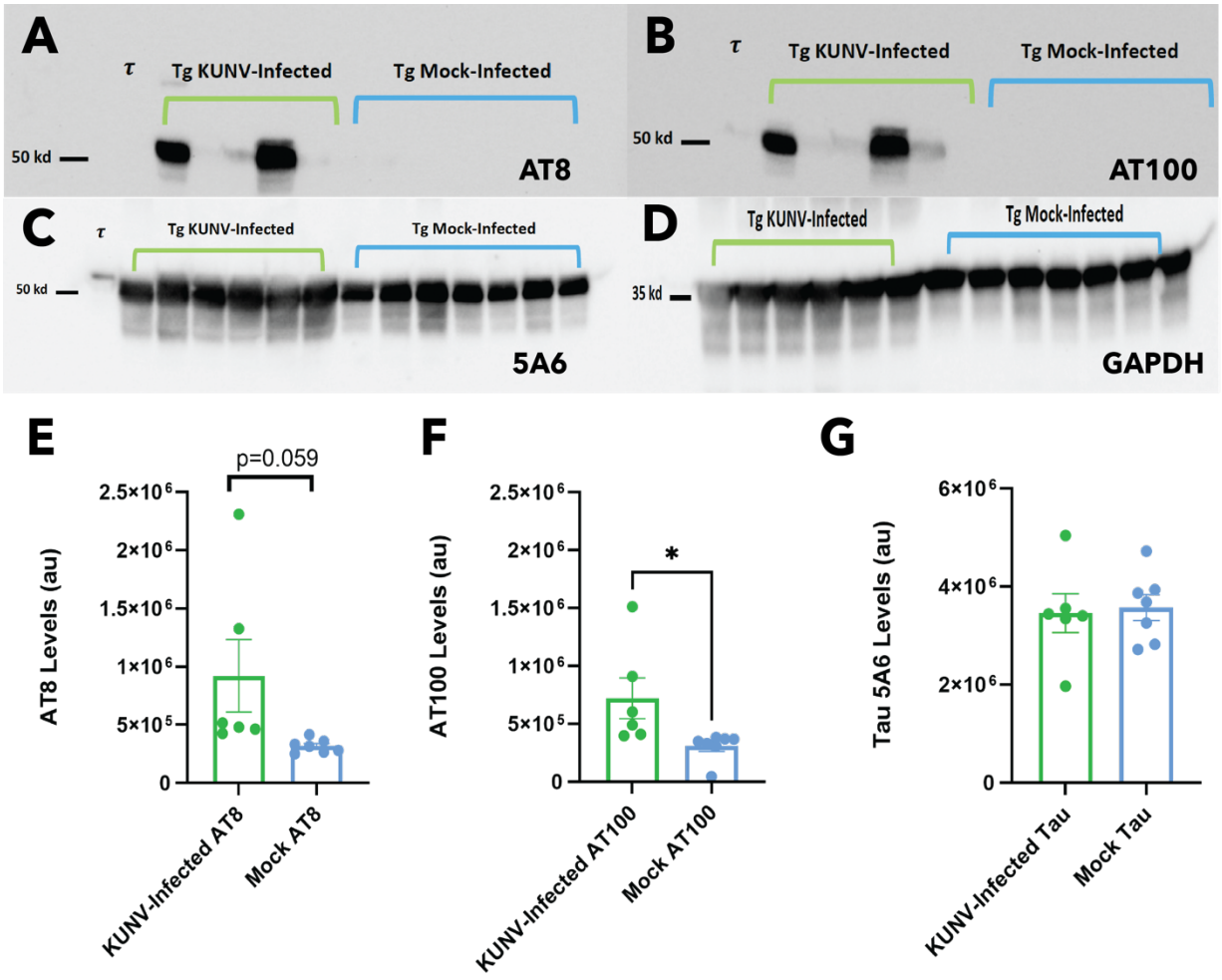

Figure 5. Elevated Tau phosphorylation in KUNV– versus mock-infected Tau P301S mouse cortex. a-b, 30 days post i.c. KUNV or mock infection of 8 wk-old Tau P301S mice showed significant Tau phosphorylation (a, site Ser202/Thr205, detected by AT8 antibody; b, Thr212/Ser214, detected by AT100 antibody) in the cortex of the KUNV-infected Tau P301S mice whereas mock-infected Tau P301S mice showed no detectable Tau phosphorylation. **c-d**, Equal levels of Total Tau, detected by 5A6 antibody, and GAPDH were shown in the KUNV-infected and mock-infected mouse cortex. **e-g**, Densitometry was performed on the AT8, AT100, and Total Tau blots, the values normalized to GAPDH and graphed on GraphPad Prism, revealing (e) p = 0.059,

2.3.3: KUNV Infection Stimulates Tau Hyperphosphorylation in Tau P301S Mice via Immunohistochemistry

To investigate where Tau hyperphosphorylation occurs within the brain, I performed immunohistochemistry on 8 wk-old KUNV-infected and mock-infected Tau P301S mouse brains at 30 dpi and probed for the Ser202/Thr205 and Thr212/Ser214 phosphorylation sites using the AT8 and AT100 antibodies, respectively. Phosphorylated signal coverage of the samples was analyzed using FIJI ImageJ. A significant increase was detected (Fig 6B) at the Thr212/Ser214 hyperphosphorylation sites (detected by AT100) in the KUNV-infected mice compared to the mock-infected ($p = 0.0299$). However, no statistical difference was detected (Fig 6A) at the Ser202/Thr205 hyperphosphorylation sites (detected by AT8) ($p = 0.2931$). Combined, these data suggest KUNV infection induces Tau hyperphosphorylation, with a more notable increase occurring at the Thr212/Ser214 sites.

2.3.4: KUNV Infection Induces Proteolytic Cleavage of Tau in Tau P301S Mouse Cortex

As shown in Fig 7, full length Tau is approximately 60 kDa; however, in my analyses, I noticed a second band consistently appearing at lower molecular weights in KUNV-infected samples (Fig 4A and 4B). To investigate the identity of this second band, I immunoblotted with the Tau 5 antibody, which binds further down the Tau protein at amino acids 210-230 (Fig 7) . Interestingly, increased Tau signal at various weights occurred (Fig 8), indicating that proteolytic cleavage of Tau may be occurring incited by KUNV.

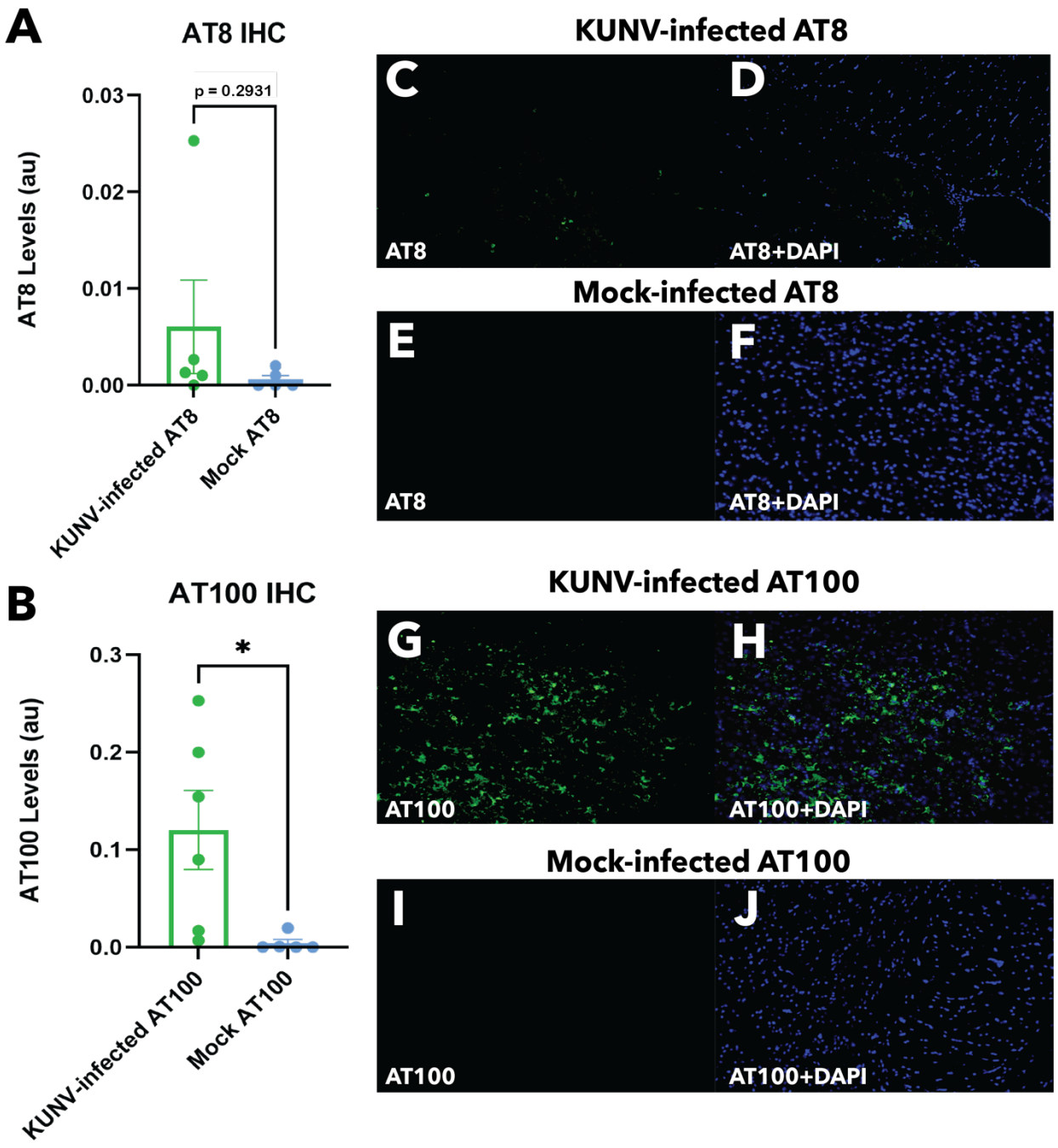

Figure 6: Elevated Tau phosphorylation at Thr212/Ser214 sites in KUNV-infected Tau P301S mice. a-b, Immunohistochemistry was performed on 30-day post KUNV-infected and mock-infected 8 wk-old Tau P301S mice. Tau phosphorylation signal coverage was assessed using FIJI Image J software and a statistical analysis was run via unpaired t-tests showing no statistical difference between the KUNV- and mock-infected Tau P301S mice at the Ser202/Thr205 sites (detected by AT8) (**a**, p=0.2931), but a significant difference at the Thr212/Ser214 sites (detected by AT100) (**b**, p=0.0299). **c-d**, Depicts AT8 (**c**) and AT8+DAPI (**d**) in KUNV-infected Tau P301S mice. **e-f**, Depicts AT8 (**e**) and AT8+DAPI (**f**) in mock-infected Tau P301S mice. **g-h**, Depicts AT100 (**g**) and AT100+DAPI (**h**) in KUNV-infected Tau P301S mice. **i-j**, Depicts AT100 (**i**) and AT100+DAPI (**j**) in mock-infected Tau P301S mice.

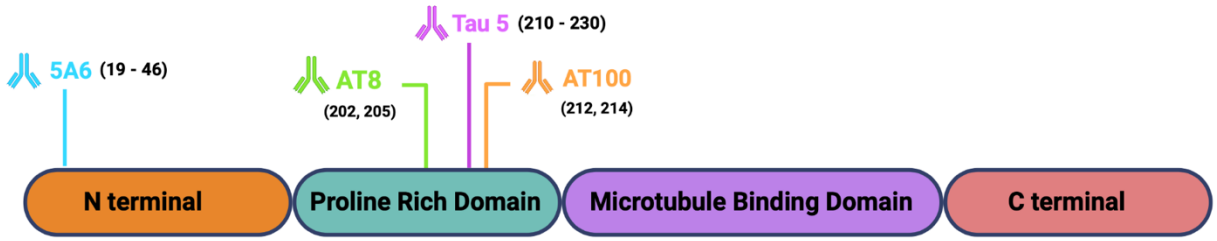

Figure 7. Tau protein structure with antibody epitopes. This study used four different antibodies in analyses, two phopho-Tau antibodies (AT8 detects at Ser202/Thr205; AT100 detects at Thr212/Ser214) and two non-phosphorylation-dependent Tau antibodies (5A6 detects at amino acids 19-46; Tau 5 detects at amino acids 210-230). The purpose of the Tau 5 antibody was to investigate the second band appearing a lower molecular weight on the 5A6 blots (seen in Fig 4).

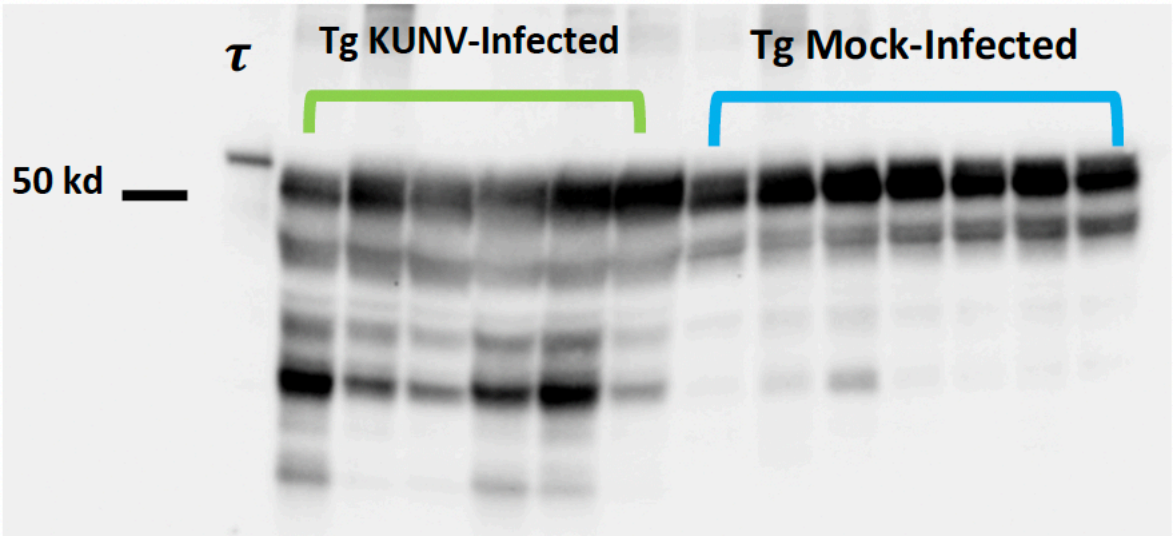

Figure 8. Increased Tau protein observed at various molecular weights in KUNV-infected Tau P301S mouse cortex. 30 days post i.c. inoculation 100 PFU KUNV of 8 wk-old Tau P301S mice revealed increased Tau signal, when probed for the Tau 5 antibody, at various weights compared to the mock-infected Tau P301S mice. This is suggestive of proteolytic cleavage of Tau occurring.

2.3.5: KUNV Infection Induces Tau Aggregation in Tau P301S Mouse Cortex

To determine whether KUNV-infection stimulates Tau aggregation, 8 wk-old Tau P301S mice were infected i.c. with KUNV (100 PFU) or mock-infected, then cortical tissue collected at 30 dpi. The cortex lysate from each sample was then processed with sarkosyl—an anionic detergent capable of extracting insoluble Tau from soluble Tau—and analyzed by western blot analysis with the Tau 5A6 antibody. Equalized protein concentrations were loaded, revealing elevated insoluble (aggregated) and soluble Tau levels in the KUNV-infected Tau P301S mouse cortex compared to the mock-infected Tau P301S mouse cortex (Fig 9A and 9B). Densitometry and statistical analysis via Dunnett's T3 multiple comparisons test showed significantly elevated levels of the soluble Tau fraction in the KUNV-infected Tau P301S cortex samples compared to the mock-infected Tau P301S cortex samples. Visually, Tau appeared to be elevated in the insoluble fraction of KUNV-infected versus mock-infected cortices as well, however, densitometry and statistical analysis showed no significant differences. Together, these results indicates that more Tau is present in the KUNV-infected Tau P301S mice, possibly in hyperphosphorylated form, increased Tau production, and possible proteolytic cleavage. Further experiments should be conducted to determine the form(s) of Tau present in the soluble fraction.

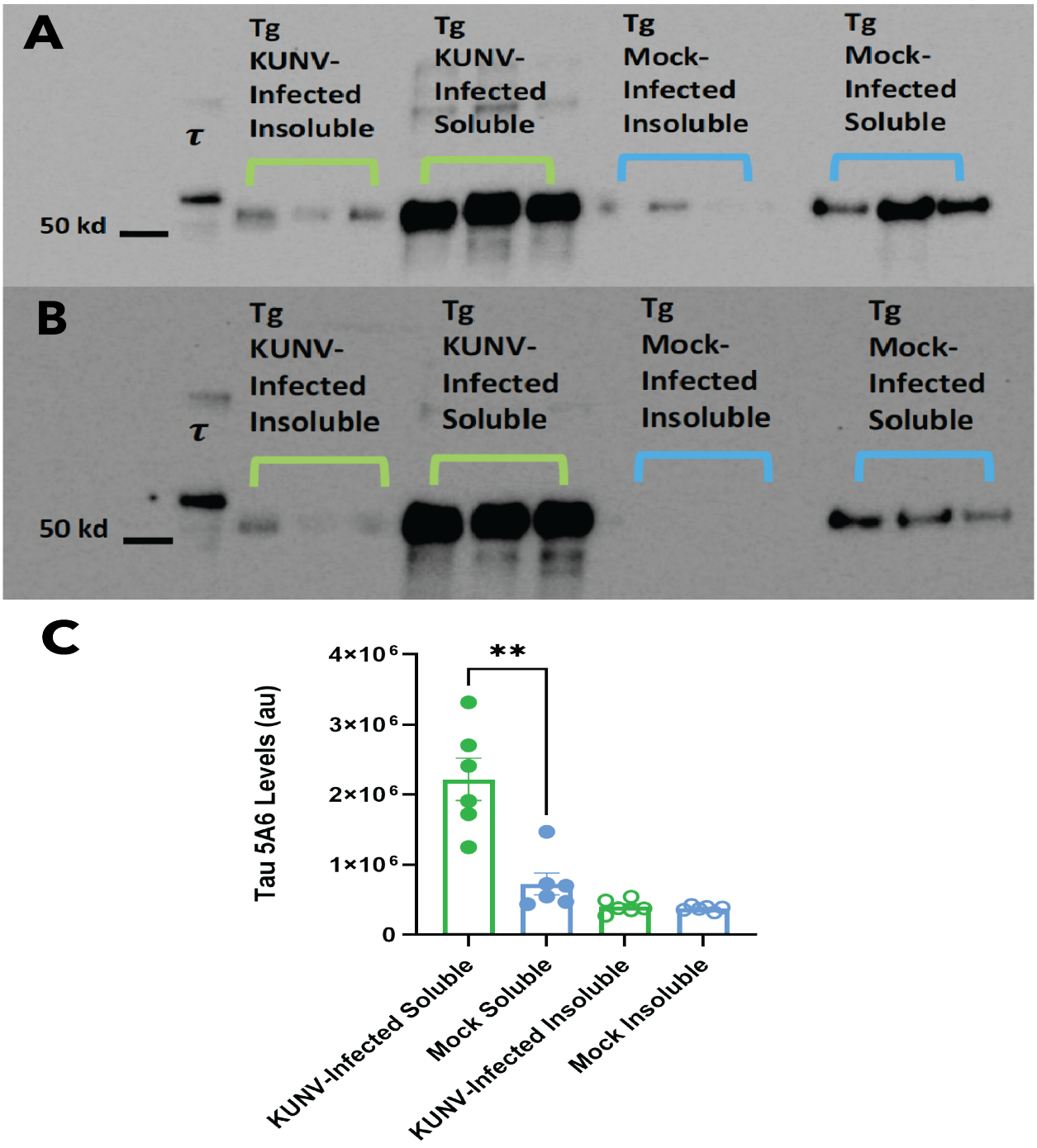

Figure 9. Elevated levels of soluble Tau in KUNV-infected Tau P301S mouse cortex. a-b, 30 days post i.c. inoculation with 100 PFU KUNV or mock-infected Tau P301S mouse cortex was treated with sarkosyl, then immunoblotted for 5A6, showing elevated levels of soluble Tau in KUNV-infected Tau P310S mice. **c**, Densitometry analysis was performed, and values normalized to the GAPDH values and graphed in GraphPad Prism, revealing p = 0.0063 for the soluble samples and p =0.7960 for the insoluble samples.

2.3.6: The Tau P301S Mutation Does Not Improve Survival Against KUNV Infection

Prior studies have suggested that Aβ may function as an antimicrobial peptide and improve survival of infected mice. To test this, I infected 8 wk-old Tau P301S and NC mice i.c. with KUNV (100 PFU) and assessed survival and weight loss out to 30 days. The recovery data was then analyzed with unpaired t-tests for statistical differences between the genotypes (Fig 10A), including sex specific differences (Fig 10B and Fig 10C), by using Graph Pad Prism Software. Analysis showed no statistical genotypic (Fig 10A-10C: $p=0.4339$, $p=0.8159$, $p=3879$) or sex-specific difference. However, there appears to be an interesting trend between the Tau P301S and NC males, which should be reanalyzed after more samples have been added to determine if a difference exists. In addition to monitoring for recovery, weight change in these mice was monitored out to 30 dpi as well. The average weight change per day of each genotype was calculated and graphed and a statistical analysis on all the mice from this experiment was done via a two-way ANOVA revealing a statistically significant difference between the two genotypes ($p<0.001$) (Fig 10D). Additionally, the average weight change per day of each genotype was then reanalyzed with only the mice that survived out to 30 dpi and assessed via a two-way ANOVA. Analysis revealed a significant statistical difference between the genotypes in the mice that recovered post KUNV infection ($p<0.001$) (Fig 10E). These data suggest Tau aggregation does not improve recovery from KUNV infection, but that the Tau P301S mice lose significantly more weight on average than NC mice post KUNV infection.

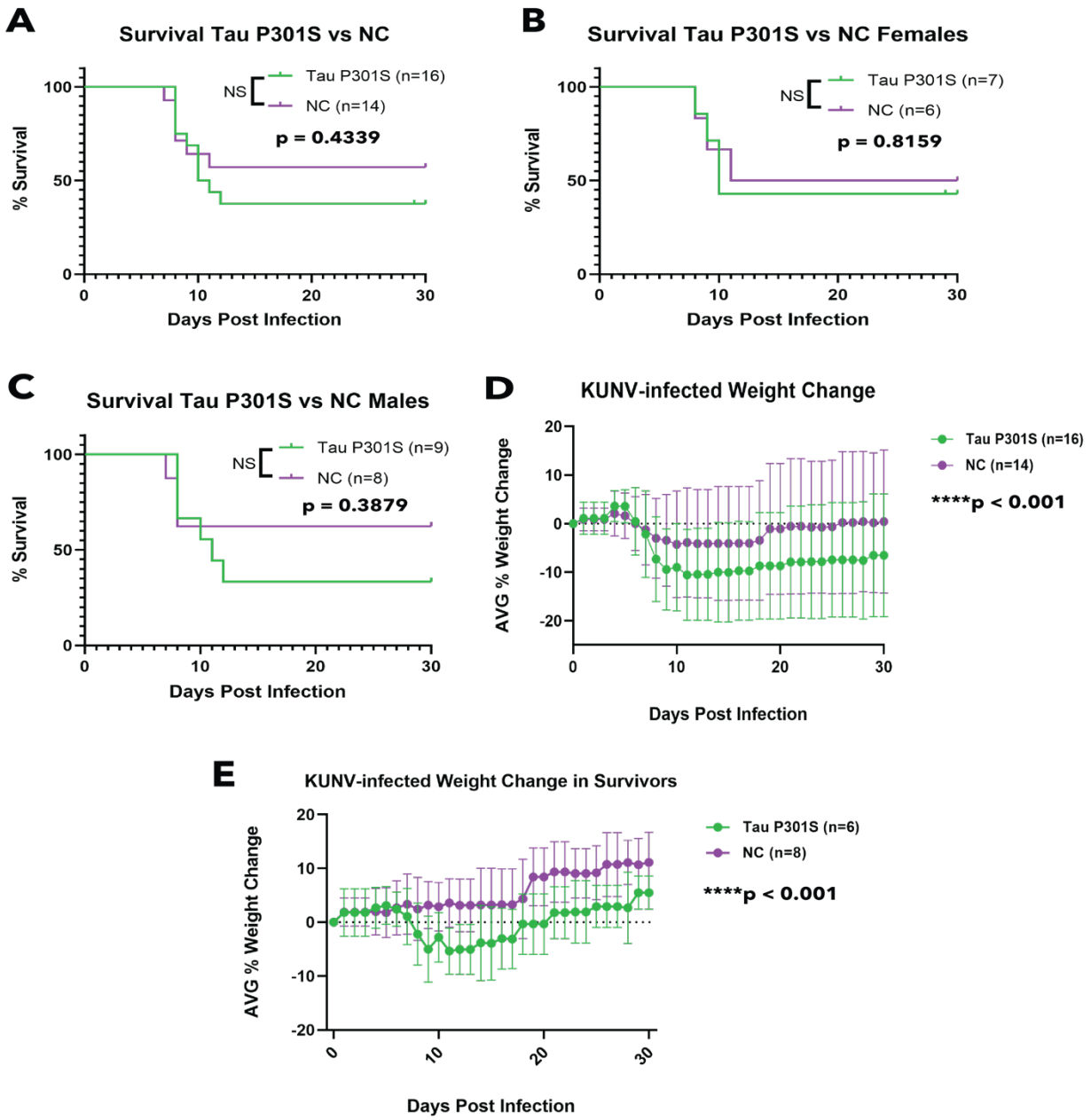

Figure 10. Survival and weight change between Tau P301S and NC 8 wk-old mice post i.c. KUNV infection. **a**, 8 wk-old Tau P301S and NC controls were infected i.c. with 100 PFU KUNV and monitored for recovery 30 dpi. **b**, Recovery data from 10a was reanalyzed using only the females from each genotype. **c**, Recovery data from 10a was reanalyzed using only the males from each genotype. **d**, Weight change was monitored for all mice (survived and those who died) shown in 10a and graphed as the average percentage weight loss for each day. **e**, Weight change for only the mice that survived to 30 dpi was graphed as the average percentage weight loss for each day. For **a-c**, statisical analyses were performed using Mantel-Cox test. For **d** and **e**, statistical analyses were perfomed by two-way ANOVA.

2.3.7: Decreased Viral Burden Observed in KUNV-Infected Tau P301S Mice Compared to Non-Carriers Six Days Post Infection

Recent findings showed Aβ acting as an antimicrobial peptide against HSV-1. To investigate whether Tau aggregates similarly impair viral replication of KUNV, I infected 8 wk-old Tau P301S and NC mice i.c. with KUNV (100 PFU) and harvested the olfactory bulb, brain stem, cerebellum, cortex, hippocampus, spinal cord, kidney, and spleen from samples at 3dpi and 6dpi. Viral load was assessed via plaque assays with baby hamster kidney (BHK) cells. Decreased viral levels were found in the cortical tissues of the 6-day post KUNV-infected Tau P301S mice compared to the KUNV-infected NC mice; however, it was found to not be statistically significant by an unpaired t-test ($p = 0.1619$) (Fig 11A). Although not significant, there does appear to be a trend that should be reexamined with more samples and possibly at a later time point (e.g., 8 or 9 dpi). This trend suggests that Tau aggregation may inhibit KUNV replication. Additionally, viral levels in the hippocampal tissues of the 6-day post KUNV-infected Tau P301S and NC mice were assessed via plaque assays. Data revealed no statistical difference between the two genotypes; however, there is a similar trend of lower viral levels in the Tau P301S mice (Fig 11B). Furthermore, it is important to note that higher viral levels were detected in the hippocampal tissue compared to the cortical tissue, likely due to inoculation occurring in the third ventricle of the brain. Together these data indicate there is no significant difference in viral levels at this time point between the Tau P301S and NC mice.

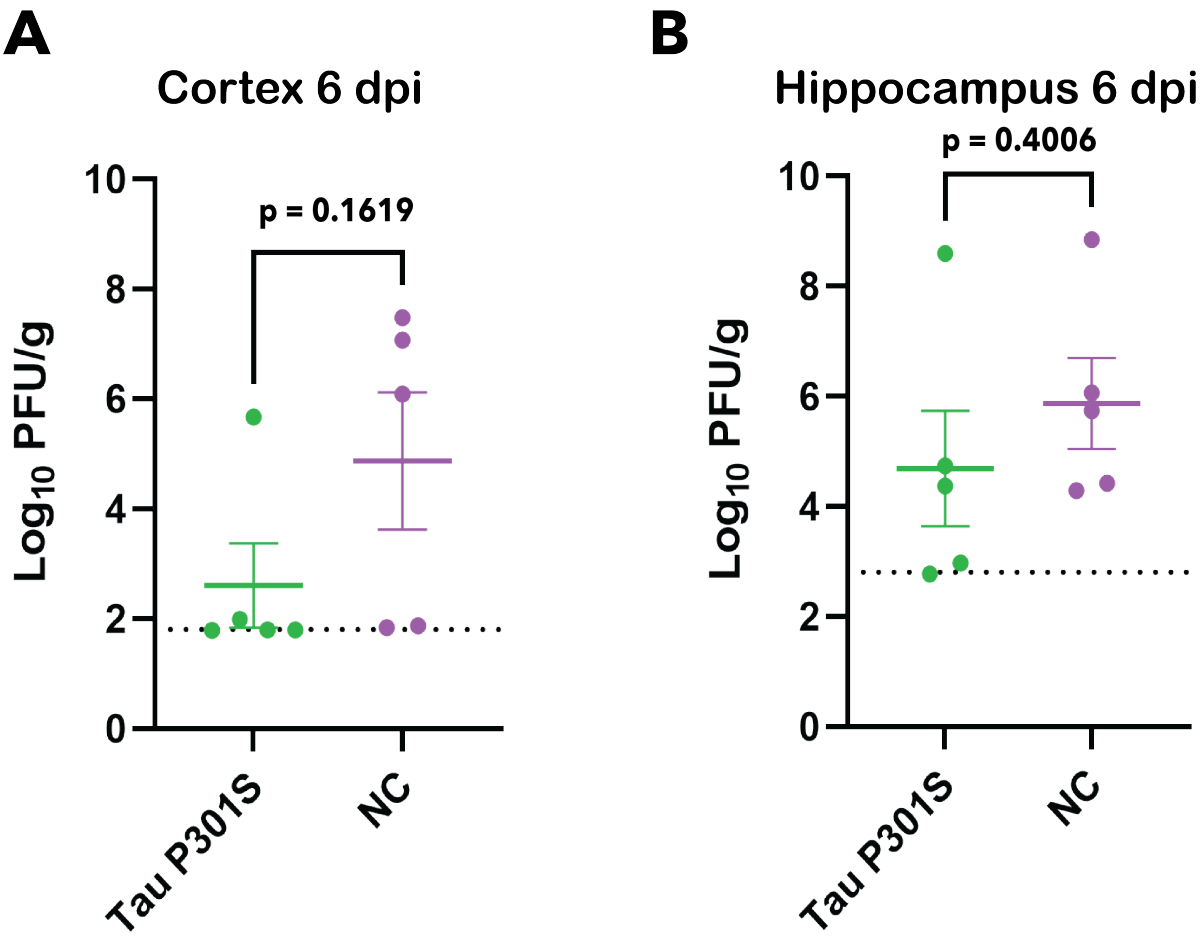

Figure 11. No statistical difference found in viral levels between 8 wk-old KUNV-infected Tau P301S and NC mouse cortex or hippocampus 6 days post infection. a-b, 8 wk-old Tau P301S and NC mice were inoculated i.c. with 100 PFU KUNV and tissues taken 6 dpi. Cortical (**a**) and hippocampal (**b**) tissues from 6-day post KUNV infected Tau P301S (n=5) and NC (n=5) mice were homogenized, and lysate assessed via plaque assays. Statistical analysis was run via unpaired t-tests showing no statistical difference between the two genotypes of KUNV levels 6dpi (**a**, p=0.1619; **b**, p=0.4006).

2.4: Discussion

It has previously been shown that neurotropic infections increase Aβ and Tau aggregates, hallmarks of AD, in mouse and cell culture models, suggesting infections are a risk factor for NDDs.[252] In this study, I focused on the relationship between KUNV and the intracellular protein Tau. The data presented here showed i.c. infection with KUNV productively increases Tau production and phosphorylation in a Tau P301S mouse model. There is significant evidence (Fig 5 and Fig 6) showing elevated phosphorylation occurring in the KUNV-infected Tau P301S mice at sites Thr212/Ser214 (detected by AT100) and a similar trend at sites Ser202/Thr205 (detected by AT8). These data correlate with previous data that showed increased phosphorylated Tau in the hippocampus of HIV-1-infected individuals compared to age-matched controls.[92] The mechanisms by which this phosphorylation occurs need to be further explored; however, routes such as CDK5 and GSK-3β activation in a cell culture model of Human T-cell leukemia virus type 1 (HTLV-1) infection have been identified.[107] Additionally, elevated levels of inflammatory cytokines such as IL-6, TNF (formerly known as TNF-α), and IL-1β have been identified alongside increased Tau phosphorylation in *Porphyromonas gingivalis* infection of mouse models, indicating they may play a role in the hyperphosphorylation of Tau.[264,270]

In addition to hyperphosphorylation of Tau, elevated levels of soluble Tau seen in the KUNV-infected Tau P301S mice (Fig 9) suggests an increase in Tau production and possible proteolytic cleavage (Fig 8). Though further examination is required to determine the forms of Tau present in this fraction, it has been noted that NFTs are primarily composed of hyperphosphorylated Tau as well as truncated forms of Tau.[271–273] In a HSV-1 infection of neurons and astrocytes, Tau cleavage was observed at D421 by caspase-3.[274] Additionally, Tau truncated at E391 was present in NFTs in brains of AD patients.[272] It is quite evident that

cleavage of Tau plays a large role in the deposition of NFTs, however, the mechanisms responsible for this toxic cleavage of Tau remain elusive.

Though the insoluble fractions (Fig 9) were not found to be statistically significant, this could be due to the length of time required for Tau aggregates to form. In transgenic rat models expressing truncated Tau (t151-391), sarkosyl insoluble Tau complexes within the brains were monitored at various time points, revealing a small signal of insoluble Tau in the 3-month-old model, with stronger signals occurring in the 9-month-old model, indicative of higher levels of aggregated Tau occurring in the 9-month-old model.[275] Therefore, this experiment should be repeated, and samples taken at later time points post infection (e.g., 45-90 dpi), a genotypic distinction of this fraction may become more notable. Formation of these aggregates may be done as an immune defense to perhaps trap and inhibit viral replication. Though, this will have to be examined further to elucidate whether direct viral binding is occurring.

Investigating the ability of Tau to act as an antimicrobial peptide yielded interesting results. Though not statistically significant, viral titer analysis revealed a consistent trend of decreased viral levels in the cortical and hippocampal tissues of KUNV-infected Tau P301S mice compared to KUNV-infected NC mice (Fig 11); whereas survival analysis (Fig 10A, 10B, and 10C) revealed there is no difference in survival between the two genotypes. Additionally, weight change analysis (Fig 10D and 10E) revealed a statistically significant difference between the two genotypes post KUNV infection, with the Tau P301S mice losing more weight than the NC mice. This suggests overexpression of Tau is exacerbating illness seen in these mice, either from an accumulation of Tau pathology, the immune signaling from affected Tau, or a mixture of both. Though not heavily researched, the inflammatory response during an infection model has shown increased levels of inflammatory cytokines (IL-6, IL-8, TNF, IL-1β) alongside

increases of Tau phosphorylation and aggregation, indicating that a cytokine cascade is occurring.[255–257,264,270] These data suggest Tau may be acting as an antimicrobial peptide to inhibit viral replication, however, the immune response generated by the infection and increased Tau pathology—cytokine storm and neuronal death—ultimately results in death of the Tau P301S mice.

Altogether these data indicate KUNV incites Tau deposition, suggesting it is a risk factor for AD. Additionally, data suggests Tau may be acting as an antimicrobial peptide to inhibit KUNV replication, though shown to not be statistically significant.

2.5: Conclusions and Future Directions

The data presented here yields strong indications that KUNV stimulates Tau aggregation and hyperphosphorylation in a Tau P301S mouse model. To further explore this notion, experiments should be performed *in vitro* with KUNV-infected and mock-infected primary cortical neurons from day 18 embryonic Tau P301S pups to ascertain whether KUNV similarly increases the rate at which these neurons develop tauopathies. Experimentation in this manner allows one to examine the direct effect of viral infection on neurons, in the absence of other CNS-resident cells such as microglia and astrocytes. An additional model system to explore are Induced Pluripotent Stem Cells (iPSCs) containing the same pathogenic *MAPT*-P301S mutation as the mouse model used above. The Karch lab has developed protocols to differentiate iPSCs out to cortical neurons.[276] Infection with KUNV of these iPSC-derived cortical neurons would allow for the impact of KUNV infection on Tau aggregation to be seen in a human cell model. Furthermore, this model similarly eliminates the presence of other CNS-resident cells in culture, allowing for a direct link between the primary cortical neurons and KUNV to be observed.

In addition to the assays described, an enzyme-linked immunosorbent assay (ELISA) should be performed on lysate from both *in vitro* models as well as an *in vivo* model to determine specific cytokine and chemokine involvement. Furthermore, the decreased trend in viral levels in the hippocampal and cortical tissues of the Tau P301S mice compared to the NC indicates Tau may be acting to inhibit viral replication. Investigation into specific cytokine involvement and levels present in KUNV-infected mice during peak viral infection (days 8-9) could elucidate whether the cause of death in the KUNV-infected Tau P301S mice is from the cytokine storm and Tau pathology incited by the virus.

Viral titer analysis of hippocampal and cortical tissues (Fig 11A and 11B) showed a

consistent trend of decreased viral levels in the KUNV-infected Tau P301S mice compared to the NC, though not statistically significant. The viral levels should be assessed in the other tissues (olfactory bulb, cerebellum, brains stem, spinal cord, and kidney) collected at this timepoint as well to see if this trend holds. Additionally, tissues should be taken at a later timepoint post infection.

To investigate whether Tau directly binds to KUNV, a RNAScope *in situ* hybridization assay should be performed and co-stained via IHC to visualizes if co-localization occurs between Tau aggregates and KUNV RNA. Additionally, binding can be explored through immunoprecipitation (IP) of tissue and neuronal samples by purification of Tau and analysis via quantitative polymerase chain reaction (qPCR) flow through. If Tau is found to co-localize with KUNV RNA, this would indicate direct binding, further suggesting Tau is capable of inhibiting KUNV replication.

In addition to exploring Tau's ability to inhibit KUNV replication *in vivo*, exploration *in vitro* with primary cortical neurons should be performed as well. As described above, experimentation in this manner would eliminate the presence of other CNS-resident cells, allowing for the relationship of Tau and KUNV to be observed.

Additional samples should be taken and added to the current data, however, future samples should also be assessed for Tau hyperphosphorylation and Total Tau via ELISA.

This study has determined that a relationship exists between Tau and KUNV. Through further exploration, immense knowledge may be gained regarding the role Tau plays in the presence of microbes, such as KUNV, as well as the ability of neurotropic infections to incite AD-related Tau pathology. A more thorough understanding into the mechanisms by which these events occur would not only add to the knowledge we possess of the risk factors that incite

NDDs, such as AD, but it would aid in the understanding of a hallmark, toxic lesion associated with AD and perhaps identify potential therapeutic targets to reduce Tau aggregates and NFTs as well.

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