PKR as a conserved neuroinflammatory mediator under viral, bacterial, & metabolic challenge: A role in Parkinsonian pathogenesis?

Matthew Aric Jefferson
Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/etd

Part of the Allergy and Immunology Commons, Immunology and Infectious Disease Commons, Medical Immunology Commons, and the Neuroscience and Neurobiology Commons

Recommended Citation
https://lib.dr.iastate.edu/etd/16823

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Graduate Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
PKR as a conserved neuroinflammatory mediator under viral, bacterial, & metabolic challenge: A role in Parkinsonian pathogenesis?

by

Matthew Aric Jefferson

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Neuroscience

Program of Study Committee:
Marian L. Kohut, Major Professor
    Rudy J. Valentine
    Anumantha Kanthasamy
    Donald Sakaguchi
    Jesse Hostetter

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University
Ames, Iowa
2018

Copyright © Matthew Aric Jefferson, 2018. All rights reserved.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>LIST OF FIGURES</th>
<th>v</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>viii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>x</td>
</tr>
</tbody>
</table>

## CHAPTER 1. GENERAL INTRODUCTION ................................................................. 1
  - Organization of the Dissertation ................................................................. 1
  - Introduction to the Dissertation ................................................................. 2
  - Literature Review .............................................................................................. 3
  - The Burden of Neurodegenerative Disease ......................................................... 3
  - Parkinson’s Disease: What We Do and Do Not Know .......................................... 6
    - Clinical presentation ....................................................................................... 6
    - Motor symptoms ............................................................................................. 7
    - Neurobiology of motor deficits ....................................................................... 7
    - Non-motor symptoms ...................................................................................... 8
    - Parkinsonian classification ........................................................................... 9
    - Current diagnostic criteria ........................................................................... 11
  - PD risk factors .................................................................................................. 11
    - Environmental risk factors .......................................................................... 12
    - Host factor detriments ................................................................................ 14
  - Cellular deficits of PD ..................................................................................... 17
    - Mitochondrial dysfunction ............................................................................ 17
    - Oxidative stress ............................................................................................ 17
    - Autophagy ...................................................................................................... 18
    - Neuroinflammation ...................................................................................... 19
  - Peripheral Inflammation and CNS Infiltration .................................................. 21
    - Evidence for a neuro-immune axis ................................................................. 21
    - Cytokines as mediators of sickness behavior ................................................. 22
    - Sickness behavior and depression ................................................................ 23
    - Lessons from LPS studies ............................................................................ 25
    - BBB regulates neuro-immune communication .............................................. 26
    - Routes of neuro-immune communication ..................................................... 27
    - BBB disruption ............................................................................................. 28
    - Cytokine transport across intact BBB ........................................................... 28
    - BBB trafficking of immune cells ................................................................... 29
    - Microglia and astrocytes as inflammatory brain cells ................................... 30
    - Acute vs. chronic forms of neuroinflammation ............................................. 31
    - Summary ........................................................................................................ 33
  - Chronic Inflammation and Host Factor Detriments ........................................... 33
Discussion .................................................................................................................. 109
Figures and Tables .................................................................................................... 116
References .................................................................................................................. 125

CHAPTER 4. PKR AS A NEUROINFLAMMATORY MECHANISM IN DUAL MODEL OF DIET-INDUCED OBESITY AND ACUTE MPTP ............... 130
Abstract ....................................................................................................................... 130
Introduction .................................................................................................................. 131
Materials and Methods .............................................................................................. 133
Results ......................................................................................................................... 137
Discussion ................................................................................................................... 144
Figures and Tables .................................................................................................... 151
References .................................................................................................................. 161

CHAPTER 5. SUMMARY AND CONCLUSIONS ...................................................... 167

REFERENCES ............................................................................................................ 174

APPENDIX. SUPPLEMENTAL DATA ..................................................................... 198
Striatum Gene Expression Data .................................................................................. 199
Hippocampal Gene Expression Data .......................................................................... 200
Hippocampal Protein Expression Data ...................................................................... 201
LIST OF FIGURES

Figure 2.1 A single, systemic injection of LPS produces a pro-neuroinflammatory
gene signature in the hippocampus and striatum........................................ 78

Figure 2.2 Pharmacological inhibition of PKR with imoxin (IMX) reduces LPS-
induced neuroinflammation........................................................................ 79

Figure 2.3 LPS downregulates PKR phosphorylation (p-PKR) in the striatum, while
upregulating total PKR protein expression in the striatum and
hippocampus.................................................................................................. 80

Figure 2.4 LPS induces astrocyte activation that is significantly reduced by 0.5
mg/kg imoxin, while producing no changes to microglial activation......... 81

Figure 2.5 PKR inhibition reduced PKR protein activator (p-PACT) expression,
while LPS reduced protein expression for stereotypical PKR substrate
(p-eIF2-α). ........................................................................................................ 82

Figure 2.6 Neither LPS or Imoxin caused major change in cell proliferation markers... 83

Figure 2.7 Downstream signaling suggests an apoptotic function for PKR following
acute LPS challenge. ..................................................................................... 84

Figure 2.8 PKR signaling scheme........................................................................ 85

Figure 3.1 CNS PKR protein expression occurs by 15 DPI with Influenza
A/PR/8/34......................................................................................................... 116

Figure 3.2 Striatal and hippocampal apoptotic and inflammatory induction precedes
prototypical cell death, occurring in conjunction with astrocyte
activation, in BALB/c mice at 15 DPI with 0.5 HAU Influenza
A/PR/8/34 (n = 4) compared to control (n = 4). .............................................. 117

Figure 3.3 Neurons and microglia treated with influenza-conditioned media in vitro
exhibit increased cell death and inflammation.............................................. 118

Figure 3.4 Striatal and hippocampal inflammatory expression 3 DPI with combined
challenge of 0.1 HAU Influenza A/PR/8/34 and 18 /mg/kg MPTP......... 119

Figure 3.5 Striatal and hippocampal inflammatory expression 15 DPI with
combined challenge of 0.1 HAU Influenza A/PR/8/34 and 18 mg/kg
MPTP............................................................................................................ 120
Figure 3.6 Gene expression data for GFAP and IBA1 at 15 DPI with 0.1 HAU Influenza A/PR/8/34 and MPTP challenge. ......................................................... 121

Figure 4.1 Weight gain by High Fat Diet (HFD)............................................................... 153

Figure 4.2 Hippocampal PKR gene expression was induced by High Fat Diet (HFD), MPTP, and dual HFD+MPTP challenge, 1 day post-MPTP. ....... 154

Figure 4.3 Hippocampal PKR gene expression dissipates by 7 days post-MPTP. ...... 155

Figure 4.4 Hippocampal PKR expression is not likely mediated by astrocyte (GFAP) or microglial (IBA1) activation. ......................................................... 156

Figure 4.5 Dual HFD+MPTP induces inverse expression between phosphorylated PKR (p-PKR) and total PKR in the hippocampus, 1 day post-MPTP. ...... 157

Figure 4.6 MPTP treatment induced pro-inflammatory expression in the hippocampus, 1 day post-MPTP................................................................. 158
LIST OF TABLES

Table 2.1 LPS-induced weight loss and was not changed by imoxin................................. 86
Table 2.2 Primer sequence information for qPCR assays............................................. 87
Table 4.1 Hippocampal type I interferon gene expression profile after 12 weeks of diet-induced obesity (DIO)........................................................................ 151
Table 4.2 Striatal type I interferon gene expression profile after 12 weeks of diet-induced obesity (DIO)........................................................................ 152
ACKNOWLEDGMENTS

To begin, I would like to firstly thank my major professor, Marian L. Kohut, and my committee members, Rudy J. Valentine, Don Sakaguchi, Anumantha Kanthasamy, Jesse Hostetter, and Russell Morgan, for their time, efforts, and guidance throughout the course of my doctoral experience here at ISU. I am particularly thankful to Dr. Morgan, who I had the pleasure to earn my MS with. His continued support has meant the world to me. I am grateful for Dr. Kohut's contributions and have enjoyed serving as a member of her team; her kindness and generosity have been exemplary. This work was also not possible without the daily contributions of Dr. Valentine. As both a professional and personal mentor, his unwavering support was a frequent source of motivation.

In addition to the committee members, I would like to thank the leaders within the neuroscience graduate community: Dr. Sakaguchi, Dr. Heather Greenlee, Dr. Elizabeth Stegemoller, and Katie Blair. I have had the pleasure to contribute to numerous neuroscience service initiatives, which would not have been possible without their support. Dr. Stegemoller was instrumental in nurturing my passion for patient advocacy and support, for which I will always be grateful.

I would like to thank my fellow lab mates (both current and past) and wonderful friends for their contributions, both emotionally and scientifically: Jessica Alley who has been my sister, Hyeyoon Eo, Corey Summers, Kelly Fuller, and Carter Reed. My neuroscience peers have been exceptional team members to collaborate with and I will always value the experiences we have shared: Patricia Izbicki, Bhavika Patel, Emir Malovic, Lauren Laboissonniere, and Matthew Neal.
The department of Kinesiology has been an exceptional work environment. Their current faculty and administrative leadership have created a supportive atmosphere and their inclusion of interdepartmental graduate students is encouraging. In addition, I am appreciative of all of my interactions with staff and key figures that maintain the department.

Finally, I would like to thank my wonderful family and friends. Those who have provided an unyielding level of support throughout all of my endeavors include Gail and Ray Jefferson, whose love and support have been a constant my entire life. The proudest thing I could ever be is their son. My amazing wife Amanda Joy has supported me throughout my entire graduate journey, as well as her loving parents. Not only has my time here impacted her (both positively and negatively, at times), but she has kept me grounded and smiling at the worst of times. And of course, my countless friends who have never been anything but supportive of me. I wholeheartedly thank you all.
ABSTRACT

Neuroinflammation has emerged as a pathological component of neurodegenerative disease onset and progression, including Parkinson’s disease. Given the current absence of disease-modifying therapies for this progressive disorder, approaches have now begun to explore the inflammatory burden that detrimental host factors place on the brain and how they can drive disease development. Our lab has identified the novel expression of the double-stranded RNA-dependent protein kinase (PKR) within the rodent brain following i) metabolic inflammation (diet-induced obesity), ii) viral infection (non-neurotropic influenza), and iii) bacterial sepsis (lipopolysaccharide). PKR stereotypically functions as an antiviral kinase, but has been implicated as a signal transduction element in response to a number of cell stresses. This biological target has been proposed to be upstream of inflammatory signaling cascades that contribute to, and aggravate, Parkinsonian pathology. By studying CNS PKR expression in three host conditions known to perturb CNS homeostasis through pro-inflammatory insult of the brain, our lab has characterized the cellular and molecular level expression of PKR in animal models and attempted to determine this signal’s possible role in Parkinson’s disease pathogenesis. Our results suggest that PKR serves as a cell stress signal that may precede neurodegeneration and functions to promote apoptosis and inflammation. Studying PKR’s expression across a broad variety of host states may lead to the development of meaningful anti-inflammatory therapeutics that may be used against Parkinson’s disease and related conditions.
CHAPTER 1. GENERAL INTRODUCTION

Organization of the Dissertation

This dissertation is a collection of manuscripts that are in preparation for peer-reviewed publication. This dissertation is comprised of five chapters: a general introduction and literature review, three original research papers, and a final discussion that summarizes, synthesizes, and integrates this work as a whole. An acknowledgements section follows. References for each original chapter can be found at the end of their respective chapter. The references for the literature review and final discussion are found at the end of this dissertation. Figures for each chapter will be found at the end of that respective chapter and the numbering for figures will be specific to each respective chapter.

The Introduction to the Dissertation introduces the emergence of neuroinflammation in neurodegenerative disease and its potential to shape the future therapeutic landscape within neurology. An in-depth review of this information can be found in the literature review within chapter 1. This includes information on i) Parkinson’s disease, covering the clinical presentation down to the cellular deficits of pathophysiology, ii) peripheral inflammation and its infiltration of the central nervous system, followed by iii) the role of chronic inflammation in host factor detrims, and finally iv) how PKR may serve as a novel neuroinflammatory mechanism. Chapters 2, 3, and 4 discuss PKR’s role in neuroinflammation by investigating its resulting expression from LPS challenge, influenza infection, and diet-induced obesity, respectively. Additionally, PKR’s relevancy to neurodegeneration in these studies is assessed by studying whether these host conditions can predispose animals to greater Parkinsonian toxicity. Attention is brought to the downstream
cell signaling events that may govern a PKR-mediated stress response to neuroinflammatory insult. The original experimental data and results of these studies was collected by the author of this dissertation during the course of his doctoral work as a graduate student in the Interdepartmental Neuroscience Graduate Program, working in the Department of Kinesiology, under the supervision of his major professor Marian L. Kohut.

**Introduction to the Dissertation**

The pathological development of neurodegenerative diseases had long been thought to occur in conjunction with the clinical manifestation of symptoms. That is, symptoms would not arise in a patient unless there was a primary pathology acting on the brain and promoting dysfunction. However, dogma surrounding how these diseases develop and progress in the brain has steadily transformed to accept the notion that neurodegenerative diseases progressively occur decades prior to the onset symptoms. This has prompted research efforts to identify processes that occur early in the brain, which may lead to the development of diagnostics and therapeutic interventions that can modify the course of neurodegeneration, and not just treat symptoms.

As a relatively “young” field of research, neuroimmunology has impressively demonstrated how peripheral inflammation can promote dysfunction of the central nervous system (CNS) (Dantzer & Kelley, 1989, 2007). Early studies using potent immune stimuli to interrogate cross-talk between the peripheral and central immune systems showed that inflammation is a disease process that can promote neuronal dysfunction through the enhanced expression of pro-inflammatory cytokines and that these inflammatory signals are capable of disrupting behavioral and cognitive function (Capuron, Ravaud, & Dantzer, 2000; Cremona, Goujon, Kelley, Dantzer, & Parnet, 1998; Dantzer, 2001b; Konsman, Parnet, & Dantzer, 2002). As it became dubbed, neuroinflammation has emerged as a cellular process
that can not only drive the development of neurodegeneration in the brain, but dictate disease severity. Clinically in PD, this disease state progression is often best observed by examining non-motor symptoms (Chaudhuri & Schapira, 2009; Poewe et al., 2017), which are an under-appreciated component of this neurodegenerative disease.

As for what states produce neuroinflammation, a broad range of host conditions can produce inflammatory events that become broadcast to the CNS and disrupt homeostasis through the aberrant activation of microglia, the brain’s resident inflammatory effector cell. These cells are largely implicated as the primary effectors that promote and sustain neuroinflammation, and have emerged as therapeutic candidates in PD (Amor, Puentes, Baker, & van der Valk, 2010; Q. Wang, Liu, & Zhou, 2015). The challenge with this area of research is identifying neuroinflammatory events that reliably indicate CNS dysfunction and that are not dependent to a specific inflammatory context.

This work attempts to address these current gaps by studying a proposed stress signal in the brain, which is activated by a broad collection of both chronic and acute inflammatory insults, and determine its relevancy to Parkinson’s disease development. Overall, this research i) identifies a conserved neuroinflammatory-related event following various immune challenges, ii) highlights the importance of modifiable lifestyle factors and the necessity to maintain wellness through personal and preventative health measures, and iii) adds to the rapidly-growing body of literature implicating the immune system as an essential regulator of disease state susceptibility.

**Literature Review**

**The Burden of Neurodegenerative Disease**

Given their substantial burden on human health and disease, neurodegenerative diseases (NDDs) have become vilified in recent years. Dogmatically considered age-
dependent diseases, neurodegeneration is defined as the sporadic, progressive loss of neurons in the central nervous system (CNS) that leads to a subsequent loss of host function. Although the temporal and regional kinetics of their pathological hallmarks vary between individual diseases, neurodegeneration encompasses a spectrum of similar cellular and molecular dysfunction. These conserved, spectral hallmarks grossly consist of proteinopathy deposition that progressively causes neuronal cell death and is responsible for the clinically observable behavioral deficits associated with a given NDD (Amor et al., 2014; Erkkinen, Kim, & Geschwind, 2018; Perry, 2004; Walsh & Selkoe, 2016).

While the study of an individual NDD presents its own unique subset of challenges, multiple diseases are considered neurodegenerative, suggesting that therapeutic gains of one disease advance the therapeutic gain of another disease (Erkkinen et al., 2018). The most prevalent neurodegenerative disease in the U.S., Alzheimer’s disease (AD), currently affects 5.4 million adults (AA, 2016), with approximately 1 million Americans having Parkinson’s disease (PD) (PDF, 2017), fewer than 20,000 living with Amyotrophic lateral sclerosis (ALS) (May Clinic, 2018), and approximately 200,000 living with multiple sclerosis (MS) (Mayo Clinic, 2018). The clinical diagnosis, management, and treatment of any given NDD is further complicated by its umbrella of disease-specific subtypes. For example, AD can present as either Mild Cognitive Impairment or Vascular Dementia, while PD may clinically present as either Lewy Body Dementia or Multiple Systems Atrophy (Jankovic, 2008). Furthermore, symptoms of these NDD subtypes commonly overlap with one another, such as memory loss. Considering that neither AD or PD have quantitative, physiological diagnostic
diagnoses are an unfortunate, yet realistic aspect in the clinical management of these disorders (Splete, 2006)

The average age of diagnosis for AD and PD is approximately 60 years of age (Clark & Kodadek, 2016). By this point in time, the neurodegenerative cascades that pathologically define a NDD have been developing for years prior (Ascherio & Schwarzschild, 2016; Hirsch, Jette, Frolikis, Steeves, & Pringsheim, 2016). Observations such as these underscore the clinical need for reliable, physiological measures that are predictive of future NDD onset (i.e., biomarkers). However, the inherent nature of finding a biological target that can reliably predict a future disease state, at a time when no symptoms are present, is enormously difficult. Although individual NDDs vary by clinical symptoms and pathological signatures, there are shared features that underlie and connect these diseases along a spectrum. Identifying a biomarker for one NDD will insurmountably benefit that specific disease, but it will also provide insight on related spectral disease states. In other words, the therapeutic gains of one NDD disease, will likely produce gains in another.

This dissertation will specifically focus on Parkinson’s disease (PD), but with an understanding that the challenges and limitations associated with this individual disease are similar when applied to related NDDs. We will begin by defining and reviewing what is currently known about PD, how the disease is pathologically believed to manifest within the brain, the cellular and molecular deficits associated with these changes, and what host conditions may serve as risk factors. From this, we will bring attention to the role of neuroinflammation as a cellular event that precedes bona fide neurodegeneration and discuss how various host states have the capacity to exacerbate these inflammatory changes. Following this will be a discussion on a novel CNS therapeutic target identified by our lab,
RNA-dependent protein kinase (PKR), the characterization of its CNS expression, and its validity as a potential PD biomarker. It is with great hope that this basic work can contribute to and help shape what we know about PD, thereby reducing its malicious burden on human health.

**Parkinson’s Disease: What We Do and Do Not Know**

PD is characterized by the progressive loss of dopaminergic neurons within the substantia nigra. The premature death of these cells leads to striatal dopamine deficiency, which subsequently results in loss of voluntary motor function. This pathophysiology has dogmatically characterized PD as an age-dependent motor disorder for the past 200 years. However, the past 30 years have been illuminating for our basic understanding of how cell loss is accelerated in PD and the events that regulate these deterrents. To understand the challenges that face PD and what further knowledge is needed to reduce its burden on human health, we’ll take a top-down approach when discussing current knowledge. Beginning with a description of PD’s clinical presentation, we’ll discuss current risk factors, and then transition into the pathogenesis of PD in the brain and how those events account for disease symptoms.

**Clinical presentation**

In the U.S., the annual incidence of PD is estimated at 60,000 adults, with a prevalence rate of 1,000,000 adults living with PD. Although estimates vary based on study design, this translates to 21 cases per 100,000 (Savica, Grossardt, Bower, Ahlskog, & Rocca, 2013). Worldwide, prevalence rates of PD are estimated at 0.3% for the general population (Pringsheim, Jette, Frolkis, & Steeves, 2014), these rates exponentially increase with age. With the average age of diagnoses being 60 years old, it is not common for cases before 50 years old. Incidence rates substantially increase from 60-90 years of age. (Twelves, Perkins,
& Counsell, 2003). The disease is twice as common among men, compared to women (Hirsch et al., 2016; Pringsheim et al., 2014). As for ethnical differences, PD appears less common in African Americans and Asians within the U.S., but there are not any major differences among other groups (Van Den Eeden et al., 2003).

**Motor symptoms**

Although it is the 2nd most common neurodegenerative condition (Poewe et al., 2017), PD is the single most common movement disorder, having more cases than amyotrophic lateral sclerosis, multiple sclerosis, and muscular dystrophy combined ("Statistics on Parkinson's," 2017). As a movement disorder, PD is predominantly characterized by bradykinesia, with patients inevitably experiencing a reduction in the amplitude and frequency of movements. This reduced range of motion coincides with muscle rigidity, postural instability, gait freezing, a resting tremor, and a reduced ability to initiate planned motor movement (Poewe et al., 2017). Clinically, a PD diagnosis diverges into two primary subtypes: a tremor-dominant form that affects relatively younger patients, and a postural imbalance and gait disorder (PIGD) form that affects older patients. This classification becomes meaningful for patient prognosis because the tremor-dominant is associated with a progressive decline of motor function, while PIGD has a much more rapid decay of motor function (Selikhova et al., 2009).

**Neurobiology of motor deficits**

What largely accounts for these classic signs and symptoms are dopaminergic deficits to the thalamic-cortico-basal ganglia circuits. The basal ganglia are responsible for the control of goal-directed motor behavior. When dopaminergic neurons in the substantia nigra die, there is a stochastic reduction in dopaminergic transmission to the striatum, located in the forebrain. This loss of excitatory dopamine opposition results in a net inhibitory output
from the thalamus to the cortex, as a result of increased GABAergic output from basal ganglia neurons. The resulting hyper-inhibition of thalamic-cortical projections produces the hallmark motor symptoms of PD (bradykinesia, rigidity, and/or tremor), and whose appearance marks the early, clinical stage of PD (Poewe et al., 2017).

**Non-motor symptoms**

When Dr. James Parkinson first described PD in 1817 as “shaking palsy” and his initial characterization of the condition has largely remained true for the past 200 years. However, what he initially left out from his clinical assessment was the appearance of non-motor symptoms, claiming that PD occurs with, “…the senses and intellects being uninjured.” (Parkinson, 2002). More recent developments in the past 20 years have expanded our clinical outlook of PD to include non-motor symptoms, which are observed to antedate motor symptoms and can carry predictive validity for determining at-risk patients (Chaudhuri & Schapira, 2009; Postuma & Berg, 2016). In addition, the identification and management of these non-motor symptoms has a large potential to influence patient quality of life (Greenwell, Gray, van Wersch, van Schaik, & Walker, 2015). Encouraging studies that examine non-motor symptoms in at-risk populations will be beneficial to research communities who seek to identify and characterize preclinical biomarkers, as the presentation of motor deficits is too late of an end point for effective clinical management (Postuma & Berg, 2016).

The spectrum of non-motor PD features carries a high degree of heterogeneity. These can include, but are not limited to: REM sleep-behavior disorder, psychosis, dementia, depression, autonomic dysfunction (orthostatic hypertension, swallowing impairments, urogenital dysfunction, and constipation), and reduced olfactory discrimination (Chaudhuri & Schapira, 2009). As varied as they might be, one longitudinal study observed that non-
motor features are highly prevalent among PD patients. Although 74% of the original 136 participants died by the study endpoint, a 20-year study based in Sydney reported that dementia was present in 83% of its 20-year survivors, 48% experienced orthostatic hypertension, urinary incontinence in 71%, hallucinatory psychoses in 74%, and 48% experienced choking (M. A. Hely, Reid, Adena, Halliday, & Morris, 2008). While the decay of motor symptoms appears to asymptote in a finite period of time, it’s the progression of non-motor symptoms that become responsible for and dominate patient disability over time (Mariese A Hely, Morris, Reid, & Trafficante, 2005).

Although these symptoms are often under-recognized and under-reported, they highlight the evolution of our understanding of PD and underscore the importance of not dogmatically viewing PD as a pure motor disorder. Additionally, as these features appear prior to movement deficits and persist through disease advancement, they indirectly implicate broader pathological features. Identifying and characterizing what these features may be presents an advantageous approach to the future PD outlook and management.

**Parkinsonian classification**

Major changes in PD outlook have occurred in the manner that we clinically characterize this disease. PD is not simply an over-arching, all-encompassing neurodegenerative condition, but is purely one clinical subset that occurs under an umbrella of Parkinsonian Syndrome. Approximately 10% of confirmed PD diagnoses are misdiagnosed as ‘Parkinsonism’, a collection of Parkinsonian syndromes. These individual syndromes can often be difficult to define because they closely mimic true PD, which is predominantly idiopathic in origin, making them difficult to initially diagnose ("Parkinson's Disease vs Parkinsonism," 2017).
While subtypes do exist, the most common PD syndromes are: multiple systems atrophy (MSA), supranuclear palsy (PSP), Lewy Body Dementia (LBD), and corticobasal degeneration (CBD). The defining diagnostic features of MSA are progressive and severe autonomic dysfunction, which occurs early and dominates the clinical presentation. These symptoms involve a combination of urinary incontinence, constipation, orthostatic hypertension, and erectile dysfunction. Patients with PSP present almost synonymously as PD patients, but are often clinically distinguished when they exhibit resistance to levodopa therapy. The dominant PSP-subtype, referred to as Richardson syndrome, presents with a unique eye movement (a supranuclear vertical gaze palsy). In addition to levodopa resistance, this feature distinguishes PSP from classic PD. As for LBD, it often doesn’t present with a motor deficit, but is associated with progressive shifts in personality, decreased empathy towards others, and possibly visual psychosis. LBD may precede PD, or occur subsequently. CBD is one of the more challenging Parkinsonian syndromes to clinically diagnose, due to its extensively variable phenotype. This condition can present as any other PD syndrome (D. R. Williams & Litvan, 2013). Collectively, the variability in clinical presentation among Parkinsonian syndrome can produce error rates as high as 24%, even when diagnosed by movement disorder specialists (Hughes, Daniel, Kilford, & Lees, 1992).

This discussion is not to draw attention to the lapses in the clarity of diagnostic criteria for PD. Rather, it is to provide evidence that our outlook on what we thought PD was as a disease state has enormously expanded. As a scientific community devoted to reducing the burden that PD places on human health, it is important to consider these changes and
apply our ever-expanding knowledge to these contexts. In doing so, we can successfully gain new insight that may shape the future diagnosis and treatment of PD.

**Current diagnostic criteria**

Current diagnostic criteria revolve around two primary clinical features in PD patients: the presence of bradykinesia and the addition of either muscle rigidity, or a resting tremor (Tolosa, Wenning, & Poewe, 2006). With the average age of onset being near 60 years of age, the average duration from diagnosis to time of death is 14 years. With early onset PD occurring in the mid-40s, symptoms tend to progress much slower and the average time from diagnosis to death is 24 years (Selikhova et al., 2009). Bradykinesia can often be patient-reported as “slowing down” and is clinically assessed through repetitive finger tapping, hand opening, and or foot/toe tapping. Typically, these movements will diminish in amplitude and frequency within a 15 second. Early in disease progression, rigidity may manifest as pain, but is characterized by increased resistance in movements. Tremor is one of the stereotypical symptoms of PD. It increases in patients maintaining a fixed posture and can become particularly apparent in tasks that require deliberate mental concentration (D. R. Williams & Litvan, 2013). It is imperative to note that PD diagnosis is purely established from clinical observations and patient medical history; there are currently no physiological/biochemical assays, neuroimaging techniques, or genetic screening tests available.

**PD risk factors**

While genome-wide association studies (GWAS) studies have identified a number of polymorphisms that provide insight into the genetic basis of PD, these heritable, early-onset forms only comprise approximately 5-10% of cases (Poewe et al., 2017). For these cases, certain inheritance patterns have emerged. For example, point-mutations and duplications
that occur to the SNCA gene, an α-synuclein encoding gene, are associated with an autosomal dominant form of early PD (Soldner et al., 2016). Other mutations associated with autosomal dominant PD include LRRK2, which are associated with autophagy and mitochondrial impairments (Bose & Beal, 2016). Conversely, autosomal recessive forms are associated with mutations to the Park2 and Pink1 genes, which are associated with oxidative stress and have a role in mitophagy (Pickrell & Youle, 2015). While the study of these mutations has produced disease state insight, genetic testing is not a routine clinical practice and it does not currently appear to have an impact on the clinical management of PD.

Environmental and host risk factors are believed to have the greatest capacity to influence the risk of PD, although it is difficult to understand the causal impact that these individual risk factors may have on the overall course of PD progression.

**Environmental risk factors**

Although the environmental etiology of PD is richly multifactorial, it is important to identify and understand these individual factors to gain a global overview of its pathogenesis. Through this understanding, research communities may identify new cellular and molecular signatures that could be targeted for therapeutic intervention. The environmental risk factors identified as having some of the largest contributions to PD pathogenesis are air pollution, history of respiratory infections, heavy metal exposure, and pesticide exposure.

Although an arguably under-studied risk factor for NDD’s, air pollution represents a potent source of noxious environmental stimuli with an established capacity to perturb CNS tissue homeostasis (M. L. Block & Calderon-Garciduenas, 2009; Craig et al., 2008). The composition of particulate matter and level of exposure varies widely, making it a challenge for epidemiologists to understand the causal interaction between air pollution and CNS
detriments. However, a working definition of particulate matter is a mixture consisting of gases, organic compounds, and metals. The two primary fractions of particulate matter implicated in CNS disease, PM$_{2.5}$ and UFPM, are largely derived from metal processing facilities and mobile exhaust emissions, respectively (Craig et al., 2008).

Another understudied environmental risk factor for PD are respiratory viral infections, which is a relationship that has been recognized since the 1970s (Moore, 1977). The causative pathogen implicated are influenza viruses. Initial epidemiological evidence came from a high incidence of post-encephalitic Parkinson’s following the 1918 Spanish influenza pandemic (Kristensson, 2006) suggesting that peripheral immune challenges are capable of perturbing CNS homeostasis by producing aberrant, systemic inflammatory responses (J. A. Majde, 2010). While there are neurotropic strains of influenza that can directly infect CNS tissue, namely H5N1 avian influenza ((H. Jang et al., 2009), seasonal influenza are largely believed to be incapable of direct CNS infection and the subsequent production of clinical neuropathology. However, there have been reports of mouse-adapted human viruses (A/PR/8/34) that are capable of producing viral replication intermediates within the olfactory bulb of C57 mice (Jeannine A Majde et al., 2007). While the reports on the neurotropism of seasonal influenza viruses have been limited, it is largely agreed that mild seasonal infections in a given host produce sickness behaviors that are the result of glial cell activation by peripheral cytokines (Dantzer & Kelley, 2007; Konsman et al., 2002; J. A. Majde, 2010). The more these events occur over a lifetime, the higher the propensity for an overzealous immune response to produce CNS derangements.

Both epidemiological and mechanistic evidence has implicated chronic heavy metal exposure (iron, manganese, and mercury) as an additional Parkinson’s risk factor. While
metals have numerous homeostatic roles, its accumulation within the CNS promotes toxicity that breeds oxidative stress through mitochondrial dysfunction. These events augment additional intracellular dysfunction such as, ER stress, autophagy dysfunction, mis-folding of proteins, and apoptosis (P. Chen, Miah, & Aschner, 2016; Wright & Baccarelli, 2007; J. Zhang et al., 2013). Manganese primarily serves as a respiratory Parkinsonian toxicant from hazardous occupations, such as welding and mining (Kwakye, Paoliello, Mukhopadhyay, Bowman, & Aschner, 2015). While manganese toxicity produces a variety of cellular derangements, its mechanism of action related to neuronal death has been linked to aberrant proteolytic cleavage of protein kinase C-δ, (Latchoumycandane et al., 2005), which has relevancy for NF-κB inflammatory signaling (Gordon et al., 2016). Environmentally-derived copper, cadmium, and lead are additional metals implicated in PD pathogenesis and all similarly exert toxicity through oxidative stress mechanisms that are capable of exacerbating α-synuclein pathologies (P. Chen et al., 2016).

**Host factor detriments**

It has become increasingly more recognized that lifestyle factors have a large potential to influence one’s probability of crossing the threshold towards a Parkinson’s disease state phenotype. Understanding these various factors and their contribution to PD pathogenesis holds great promise for potential clinical interventions because these factors are largely modifiable.

Age remains the single greatest risk factor for PD (Poewe et al., 2017; Wyss-Corey, 2016). While there is no process that can delay or halt the process of aging, we have seen impressive increases in human life expectancy over the past century (Dong, Milholland, & Vijg, 2016). Although projections for whether lifespan will continue to increase are readily
contested (Brown, Albers, & Ritchie, 2017), a reasonable interpretation of this trend is that increased aging has and will continue to place a substantial burden on human health and disease. As for PD, age is the single greatest risk factor and that risk substantially increases beginning around 60 years of age (Driver, Logroscino, Gaziano, & Kurth, 2009; Reeve, Simcox, & Turnbull, 2014).

Obesity is a detrimental host condition linked with numerous chronic disease states and pathologically carries a slew of molecular and cellular dysfunction. Epidemiological evidence of obesity as a risk factor for PD has been mixed based on existing studies. There have been no significant relationships between Body Mass Index (BMI) as a measure of overall obesity, however, as a measure of central obesity greater waist circumference and waist-to-hip ratio were associated with greater risk in a population of non-smokers (H. Chen et al., 2004). Others meta-analyses have observed similar trends where there has been no difference between BMI and PD risk among overweight and obese groups (J. Chen et al., 2014). However, Type 2 Diabetes (T2D) has a clear risk association with PD (Santiago & Potashkin, 2013). Animal studies have provided robust evidence that obesity predisposes the brain to Parkinsonian insult and can result in a greater degree of dopaminergic degeneration (M. Bousquet et al., 2012; L. Wang et al., 2014). The pathological basis for this relationship has largely been associated with chronic, peripheral inflammatory events which will be explored later in this review.

Famed cases of Parkinsonism among professional athletes, such as Muhammed Ali, have highlighted the basic notion that head injury can potentiate a neurodegenerative cascade. This relationship has largely been linked to chronic traumatic encephalopathy (CTE), a neurodegenerative tauopathy that results from repetitive, mild, sub-concussive head
trauma (Brody, Benetatos, Bennett, Klemenhagen, & Mac Donald, 2015; Gardner & Yaffe, 2015). While predominantly associated with Alzheimer’s neuropathology, tau pathology is integral to Parkinsonian pathogenesis and it is believed to have a synergistic effect on α-synuclein aggregation and deposition (Clinton, Blurton-Jones, Myczek, Trojanowski, & LaFerla, 2010; Lei et al., 2010). Although the mechanism of how tau may drive disease state development in PD is unclear, the neuroinflammatory response resulting from head trauma is clearly recognized (Ramlackhansingh et al., 2011). These injuries have a potent capacity to injure neurons, disrupt blood-brain barrier integrity, and activate microglia that can persist for years after injury in humans (Gentleman et al., 2004).

While aging, diet, and injury have the capacity to potentiate neurodegenerative dysfunction, there is not direct causality between their occurrence and PD incidence. However, when these host factors are considered in combination, they have a more readily acknowledged ability to augment neurodegeneration. Moreover, there are a number of host factors that can provide neuroprotective benefits. For example, smoking reduces the likelihood of PD, with its therapeutic benefit largely attributed to stimulation of nicotinic receptors in nigrostriatal pathways (Quik, 2004). Moderate alcohol intake may also be associated with lower PD risk, although this relationship is stronger for reducing AD risk ((Campdelacreu, 2014; Wirdefeldt, Adami, Cole, Trichopoulos, & Mandel, 2011). Exercise is another valuable host strategy for reducing PD incidence and serves as an intervention strategy, due to its ability to stimulate neurotrophic factors that support the growth and survival of dopaminergic cells (Goodwin, Richards, Taylor, Taylor, & Campbell, 2008).
**Cellular deficits of PD**

Parkinson’s disease is neuropathologically defined by the progressive loss of dopaminergic neurons in the substantia nigra, intracellular proteinaceous inclusions consisting of α-synuclein, a stochastic decrease in striatal dopaminergic terminals, and aberrant behavior of neuroinflammatory cells (Obeso et al., 2010; Sudhakar R Subramaniam & Howard J Federoff, 2017). While the propagation and accumulation of α-synuclein in topographically-specific brain regions are considered the primary pathology responsible for the progressive death of dopaminergic cells, there are a myriad of homeostatic cell functions that become deranged and exacerbate these primary pathological events.

**Mitochondrial dysfunction**

Mitochondrial dysfunction is (rightfully so) one of the more well-recognized facets of disturbed cellular physiology in PD. Given their role in oxidative phosphorylation, mitochondria are a potent source of free-radical mediated oxidative stress, producing superoxide radicals as a byproduct of electron transfer. Four respiratory chain complexes (I-IV) comprise oxidative phosphorylation. It has been observed that an approximate 35% complex I deficiency occurs exclusively in the substantia nigra of PD patients (Mann et al., 1994) and that it does not even occur in related Parkinsonian syndromes (i.e., multiple systems atrophy) (Gu et al., 1997). Additionally, a number of genes encoding mitochondrial-associated proteins (DJ-1, Parkin, and PINK1) are associated with early-onset forms of PD when loss-of-function is observed (Canet-Avilés et al., 2004; Dodson & Guo, 2007).

**Oxidative stress**

Oxidative stress results from deranged mitochondrial function and contributes to the vicious cycle of PD pathogenesis. Autosomal recessive mutations, such as the antioxidant protein DJ-1, exacerbate degeneration of dopaminergic neurons in murine knockout models
(Di Nottia et al., 2017; Guzman et al., 2010), thereby highlighting the notion that oxidative stress contributes to neuronal death. When considering the oxidative burden these cells face in PD, one may naturally wonder why those neurons appear selectively vulnerable to stress. Anatomically, the cellular architecture of dopaminergic neurons in the substantia nigra is relatively large, with a high degree of arborization of its unmyelinated axon. These size and signaling requirements place a heavy energy demand on the cell, that when imbalanced, potentiate premature cell death (Bolam & Pissadaki, 2012; Pissadaki & Bolam, 2013).

Additionally, cytosolic metabolites of dopamine synthesis themselves become directly toxic and promote oxidative stress (Lotharius & Brundin, 2002; Mosharov et al., 2009). This state of increased oxidative stress in PD does not remain pathologically inclusive, as it promotes lysosomal depletion, functionally impairing the lysosomal-autophagy system (Dehay et al., 2010).

**Autophagy**

Proteolytic defense systems are an integral component of upholding intracellular homeostasis. These processes consist of both the ubiquitin-proteasome system (UPS) and the lysosomal autophagy system (LAS). The maintenance of these systems is particularly paramount in the context of neurodegeneration, as there is an age-related reduction in their function, which promotes intracellular accumulation of α-synuclein in neurons (Kaushik & Cuervo, 2015; Xilouri, Brekk, & Stefanis, 2013). LAS has been suggested as being a more efficient removal process of α-synuclein oligomers (Xilouri et al., 2013) and the accumulation of these oligomers has been shown to inhibit UPS (Emmanouilidou, Stefanis, & Vekrellis, 2010), thereby leading to macro autophagy inhibition (Winslow et al., 2010).

When dysfunctional, the relative contribution of each system to the pathogenic progression within a diseased neuron is unclear, as these processes are intimately associated
with one another. What is more clear is that the inhibition of either process promotes α-synuclein accumulation (Xilouri, Vogiatzi, Vekrellis, Park, & Stefanis, 2009) and the derangement of these processes is inherently linked with the disruption of other cellular processes believed to be central to cellular dysfunction in PD. For example, oxidative stress resulting from mitochondrial dysfunction has been shown to deplete lysosomes (Dehay et al., 2010). Together, the collective disruption of these proteolytic processes imposes a vicious cycle that impairs α-synuclein degradation and its subsequent accumulation within the cell.

**Neuroinflammation**

Neuroinflammation is yet another well-recognized facet of PD pathogenesis. Originally recognized as a secondary event to proteinopathies in neurodegenerative disease, recent developments have shifted that paradigm to support the notion that neuroinflammation occurs as a priming event that exacerbates the development of pathogenic processes. Historically, the field of neuroimmunology has largely failed to collectively and systematically define “neuroinflammation”. Despite the ambiguity of defining neuroinflammation, a well-recognized facet of this process has been the chronic activation of microglia, the brain’s resident macrophage. While the immune system is vital for repair and regeneration of damaged tissues, chronic activation of the innate immune system in neurodegenerative disease is representative of an unresolved state of injury. This unresolved state potentiates a negative feedback loop, creating aberrant microglial behavior, leading to chronic activation and subsequent production of inflammatory molecules called cytokines. Cytokines are small-protein, intracellular messengers that mediate a multitude of cell-cell signaling interactions involved in cell activation and proliferation, antibody production, and are an integral component of inflammation. Having both pro- and anti-inflammatory functions, these cytokines influence neuronal homeostasis, and have a potent capacity to
influence behavioral and cognitive functioning (C. Cunningham et al., 2009; Dantzer & Kelley, 2007; Perry & Teeling, 2013). This evidence suggests that immune system modulation could have therapeutic benefit in neurodegenerative diseases. Furthermore, epidemiological studies have shown reduced risk between long-term non-steroidal anti-inflammatory drug (NSAID) use and PD incidence (Gagne & Power, 2010).

As for the temporal kinetics of neuroinflammatory processes, microglia and astrocyte activation has been shown to occur between 24-72 hours (depending on inflammatory stimulus) (Hoogland, Houbolt, van Westerloo, van Gool, & van de Beek, 2015). This cell activation subsequently produces an inflammatory milieu within the brain parenchyma. Additionally, blood-derived immune cells (lymphocytes and monocyte-derived macrophages) are recruited to and infiltrate the CNS. These events augment the relatively delayed disruption of blood-brain barrier (BBB) tight junction regulation, leading to increased BBB permeability (Obermeier, Daneman, & Ransohoff, 2013; Ransohoff, 2016a, 2016b). Collectively, these events disrupt neuronal homeostasis and promote cell death processes that are relevant to neurodegenerative disease (Hirsch et al., 2016; Perry, 2004; Perry, Cunningham, & Holmes, 2007).

The influence of the peripheral immune system on CNS function has been a relatively new development, with its bi-directional relationship only being appreciated within the past 10-15 years. Although there is intense research interest in this relationship, there are many basic questions that remain unanswered before viable drug targets could begin to emerge. Therefore, identifying, characterizing, and understanding the functions of various cell signaling pathways is an essential effort in the battle to develop disease-modifying therapies for the treatment of neurodegenerative diseases. The scope of this dissertation is focused on
the role of neuroinflammation and whether a specific cell-signaling pathway has a role in Parkinsonian pathogenesis. Therefore, the subsequent sections of this literature review will be dedicated to understanding the current state of innate immune system activation and the role it plays in Parkinson’s disease and related disease states.

**Peripheral Inflammation and CNS Infiltration**

The functional significance of the immune system can be most appreciated when one considers the vitality of an organism’s capacity to survey and appropriately respond to foreign, potentially lethal antigens. The detection and subsequent clearance of these stimuli, while not compromising self-tissue, is vital for host survival and serves an essential evolutionary role. This function is maintained by the innate immune system, as opposed to the adaptive immune system which responsible for conferring immunological memory.

Innate immunity is operationally defined by inflammation, an immune response in which vascular changes deliver effector cells (both innate and adaptive cell types) to a site of host injury or illness (Parkin & Cohen, 2001).

**Evidence for a neuro-immune axis**

Central evidence for a relationship between the immune and central nervous systems has been through “sickness behavior”, a phenomena in which systemic inflammation produces behavioral and cognitive derangements (Dantzer & Kelley, 2007; Konsman et al., 2002). Best observed during periods of infection, this behavior is characterized by lethargy, reduced locomotor activity, reduced appetite, malaise, and fatigue. It’s largely supported that these collective behaviors exist as an adaptive response to limit host energy consumption and/or allow for increased energy consumption required for an immune reponse (Hart, 1988; Kluger, 1991). Importantly, the clinical presentation of these symptoms is non-specific, as is the inherent nature of the innate immune system; an eclectic array of clinical conditions is
capable of inducing an array of sickness-like behaviors. Historically, this rivaled the notion that the immune system operated autonomously of other physiological systems. For if the immune system did influence host behavior to pathogens, it could not do so without a neuro-immune feedback loop (Dantzer & Kelley, 1989; Kelley, Dantzer, Mormede, Salmon, & Aynaud, 1985). This idea only recently gained widespread acceptance by neuroscientists and immunologists with Kipnis’ discovery of lymphatic vessels within the CNS (Louveau et al., 2015). So, if a neuro-immune axis does exist, what are the central mediators of this communication?

**Cytokines as mediators of sickness behavior**

Cytokines are inflammatory molecules produced by immune cells and tissue that are responsible for orchestrating a slew of cell-to-cell communications. With regards to their role in the neuro-immune axis, cytokines in the brain were believed to be an artifact of a sickness response to immune challenge (Dantzer & Kelley, 1989). Until interleukin (IL)-1, or “lymphocyte activating factor” as it had originally been designated, was cloned in 1984 (Auron et al., 1984) there had not been direct evidence that a communicative surrogate even existed. However, when both purified and recombinant IL-1 was systemically administered to rodents, it led to activation of the hypothalamic-pituitary-adrenal (HPA) axis by stimulating production of corticotropin-releasing factor in the paraventricular nucleus (Berkenbosch, Van Oers, Del Rey, Tilders, & Besedovsky, 1987; Besedovsky, Del Rey, Sorkin, & Dinarello, 1986).

Early theories emerged that attempted to explain the effects of cytokine-induced sickness behavior. Hart postulated that sickness behavior occurred as a by-product of a fever, leading to an adaptive conservation of energy (Hart, 1988). However, it was later established that IL-1 can induce sickness behavior, independent of fever (Kent et al., 1992).
Others attempted to rectify cytokine-induced sickness behavior as an evolutionary attempt to cope with pain during inflammation (Maier, Wiertelak, Martin, & Watkins, 1993). It has since been asserted that cytokine-induced sickness behavior exists as a host motivational state to maintain relative homeostasis; sickness behavior dominates an organism’s behavior so that organism can efficiently cope with and clear an infection. Whatever those actions are, they are relative to that host, and will inherently vary (Bolles & Fanselow, 1980; Dantzer, 2001a; Konsman et al., 2002).

**Sickness behavior and depression**

This basic research concept received merit in the late 1980s/early 1990s when recombinant IL-2 and interferon-α were developed and clinically used as adjunctive therapies in cancer and hepatitis C patients (Dantzer, O'Connor, Freund, Johnson, & Kelley, 2008). Shortly after beginning immunotherapy, health care providers observed potent increases in major depressive disorders among patients (Denicoff et al., 1987; Renault et al., 1987). While these neuropsychiatric changes were initially believed to be a mere side effect of immunotherapy, it became established that they were caused by chronic cytokine administration and produced clinically distinct depressive phenotypes (Capuron et al., 2002; Capuron et al., 2000; Dantzer et al., 2008).

Clinical proponents began to emerge that implicated immune system dysfunction as the cause of depression, dubbing the macrophage theory of depression (Maes, 1993). This theory postulated that the acute, pro-inflammatory response mounted by the innate immune system had the capacity to induce depression. This observation that depression occurred under chronic cytokine administration was important evidence for the legitimacy of a neuro-immune axis. It was understood that an acute immune response would adaptively produce a milieu of pro-inflammatory factors that would act on the brain and influence behavior (i.e.,
sickness behavior). Conversely, depression represents a maladaptive immune response that occurred when an innate immune response was particularly vigorous, or its duration was prolonged (Dantzer et al., 2008). Indeed, this concept has been validated through clinical observations that major depressive disorder (MDD) patients have elevated levels of circulating Tumor necrosis factor (TNF)-α and IL-6 (Dowlati et al., 2010). Additionally, MDD patients often have a high degree of comorbidity with systemic inflammatory conditions, including diabetes, cancer, and cardiovascular disease (Iwata, Ota, & Duman, 2013). Collectively, chronic inflammatory diseases are seen as both a predisposing factor for depression, and a disease state that can exacerbate a depressive phenotype (Wohleb, Franklin, Iwata, & Duman, 2016).

The available repertoire of clinical inflammatory biomarkers is largely limited to IL-6, TNF-α, and to a lesser degree IL-1β (Dowlati et al., 2010). Although elevated levels of these circulating cytokines can consistently be observed in MDD patients, there is often a difference in pathophysiology among clinical subtypes of MDD, making it a challenge to characterize neuro-immune dysfunction based on disease phenotype (Gold, 2015). An additional facet that complicates the modeling, and subsequent study, of this relationship is the behavioral difference between sickness-like and depressive-like behavior. While sickness-like behaviors are associated with rapid, intense expression of pro-inflammatory cytokines, this expression is transient and represents an adaptive immune response. Conversely, stress-induced inflammatory changes associated with depressive-like behaviors are less pronounced and represent a chronic, maladaptive immune profile (Wohleb et al., 2016). An important and well-accepted paradigm for studying neuro-immune interactions is
through peripheral immune challenge with lipopolysaccharide (LPS), a bacterial endotoxin that potently stimulates a toll-like receptor (TLR) – 4 mediated immune response.

**Lessons from LPS studies**

The use of LPS in mechanistic neuro-immune studies has produced multiple seminal findings. First, systemic administration of LPS in rodents produces robust pro-inflammatory gene and protein expression in the brain, primarily characterized by increased TNF-α, IL-1β, and IL-6 (Breder et al., 1994; Gatti & Bartfai, 1993; Quan, Stern, Whiteside, & Herkenham, 1999; van Dam, Brouns, Louisse, & Berkenbosch, 1992). This observation came nearly 30 years after Neal Miller administered endotoxin to rats and studied motivational behavior changes. These animals were subjected to a forced wheel running paradigm, a volitional action negatively affected by sickness, and classically conditioned to press a lever for periods of rest. Instead of conserving energy during a period of sickness, animals increased their lever pressing to earn more rest (N. E. Miller, 1964). Secondly, this same degree of sickness behavior can be induced by either central or peripheral administration of recombinant TNF-α or IL-1β. These behavioral alterations present as reduced psychomotor activity, decreased reward-seeking, social withdrawal, anhedonia, and even reduced amplitude of diurnal rhythms (Dantzer, 2001a). Thirdly, these behavioral deficits can be abrogated following central administration of anti-inflammatory factors, such as IL-10 and IGF-I ((Bluthé et al., 1999; Dantzer, Gheusi, Johnson, & Kelley, 1999). Importantly, these changes clinically translate to humans. When administered to healthy humans at a sub-septic dose (0.8 ng/kg), LPS has the capacity to negatively affect memory independent of sickness, and these changes inversely correlate circulating TNF-α (Reichenberg et al., 2001).
**BBB regulates neuro-immune communication**

Given the observation that a peripheral immune challenge negatively influences behavior and that increased pro-inflammatory cytokine expression mediates this effect, one can reasonably begin to question how that inflammatory cascade is propagated into the CNS. The blood-brain barrier (BBB) is an important interface that mediates these central-peripheral immune interactions. Convention has taught generations of neuroscientists that the BBB is absolute in its restrictive abilities, thereby making the brain an immunologically “privileged” organ. However, that convention has been challenged over recent years and we have come to understand that progressive loss of BBB integrity is integral to neuroinflammatory and subsequent neurodegenerative pathology (Lange, Storkebaum, De Almodóvar, Dewerchin, & Carmeliet, 2016; Obermeier et al., 2013).

Endothelial cells (ECs) constitute the anatomical foundation of the BBB. Salient features of this brain vasculature include intracellular tight junctions, that combined with minimal fenestrations, strictly regulate transcytosis between the blood and brain (Abbott, Rönnbäck, & Hansson, 2006). These features insulate the parenchyma from the blood, enabling the BBB to tightly regulate influx and efflux of various factors and nutrients. Beyond EC regulation of the BBB, pericytes sheath neural blood vessels and contribute to BBB integrity through vessel stabilization, vaso-regulation, and blood flow (Winkler, Bell, & Zlokovic, 2011). The next layer of protection is provided by astrocytes, whose end-feet processes completely encircle the vessel. Astrocytes are believed to contribute to BBB integrity via maintaining tight junctions, in addition to producing phospholipid transporter molecules (i.e., APOE) (Gee & Keller, 2005). In conjunction with an interplay between neurons and microglia, these BBB components form the neurovascular unit (NVU). In
relation to neurodegeneration, NVU breakdown has increasingly become recognized as a contributor to disease progression (Zlokovic, 2008).

**Routes of neuro-immune communication**

In addition to the rich cellular interplay within the neurovascular unit, systemic inflammation has four well-recognized routes of communicating with the CNS, either through neural or humoral modalities (Konsman et al., 2002; Perry, 2004). Although each of these routes can independently propagate peripheral inflammation into the CNS, they are very likely redundant.

Neural routes of inflammatory transmission are largely delivered via efferent and afferent vagus sensory nerves. This can serve as a direct central-to-peripheral route from the gastrointestinal tract and spleen, when systemic inflammatory challenges are administered to the peritoneal cavity (Goehler et al., 1999). Humoral routes of transmission are a particularly important consideration for studying neuro-immune interactions because they rely on signals from blood-derived products to propagate inflammation into the CNS. These interactions form the basis of neuro-immune interactions and will be discussed according to: BBB disruption, active cytokine transport across an intact BBB, and immune cell trafficking.

Historically, the notion that the brain is “immune privileged” has led to a perception that the BBB is static and impermeable to insult. The basis of this early theory was rooted in the differentiated vascular anatomy of brain capillaries. Within the vessels of the BBB, there is a distinct lack of intracellular fenestrations, endothelial cells sealed by tight junctions, and a subsequent reduction in pinocytosis (Reese & Karnovsky, 1967). These studies arose from the observation that dyes injected into the bloodstream, such as Evan’s blue, did not stain the brain because they were bound by albumin in the blood and were prevented from barrier entry (Davson, 2012).
**BBB disruption**

The BBB, as a component of the neurovascular unit, is the quintessential gatekeeper between the CNS and the peripheral immune system. When the homeostatic balance of the BBB is lost, this system crosses the threshold into a disease state phenotype. Therefore, disruption of the BBB is an important pathophysiological event, known to underlie numerous neurologic diseases, including neurodegenerative disease (Varatharaj & Galea, 2017). BBB disruption is intimately linked with systemic inflammation, as exemplified by LPS studies in the 1980s that demonstrated BBB disruption after systemic bacterial challenge in rats (Wispelwey, Lesse, Hansen, & Scheld, 1988). Follow-up studies confirmed that this disruption was cytokine-induced, largely mediated by TNF-α (Banks & Erickson, 2010).

What remains to be determined about the role of BBB disruption in disease is the function and temporal kinetics of this disruption during disease progression. This disruption may represent a terminal breakdown and inability of the BBB to regulate self-repair, as a result of septic insult. Or, it may be that this disruption is not an end-stage process, but simply the by-product of physiologic cytokine-signaling, induced by chronic inflammatory host states (Banks, 2015; Obermeier et al., 2013; Varatharaj & Galea, 2017; Zlokovic, 2008).

Regardless of unanswered questions regarding the function of BBB disruption, it is undeniable that cytokine-mediated BBB disruption has major implications for a wide array of disease states.

**Cytokine transport across intact BBB**

Early evidence for the active transport of cytokines across the BBB came from IL-1 studies. Peripherally administered IL-1 induces sickness behavior, but when an IL-1 neutralizing antibody is concomitantly administered to the brain, these behavioral detriments can be mitigated (Cremona et al., 1998; Pugh, Fleshner, Watkins, Maier, & Rudy, 2001).
This effect is not limited to IL-1, as LPS-induced TNF can be transported across the BBB where it can stimulate microglia to subsequently release TNF stores, which promote neuronal apoptosis (Qin et al., 2007). To date, the recognized cytokine transport and signal propagation mechanisms into the CNS include 1) indirect entry via vagal nerve stimulation (Watkins, Maier, & Goehler, 1995), 2) the secretion of cytokines from blood-to-brain trafficked monocytes (Persidsky et al., 1997), 3) stimulated cytokine release from BBB endothelial cells (Verma, Nakaoke, Dohgu, & Banks, 2006), 4) uptake at circumventricular organs (Maness, Kastin, & Banks, 1998), and finally 5) receptor-mediated direct passage into the CNS (Banks, Farr, La Scola, & Morley, 2001).

**BBB trafficking of immune cells**

Uptake of immune cells across the BBB (i.e., diapedesis) and the subsequent increase in BBB permeability is linked to numerous neurologic and neurodegenerative diseases, including AD and PD (Obermeier et al., 2013). The stereotypical neuroinflammatory disease in the CNS is multiple sclerosis (MS), which is pathologically characterized by T cell and macrophage extravasation, and serves as a prime model for studying immune cell infiltration. Increased T cell infiltration is believed to increase BBB permeability, allowing entry of other pro-inflammatory leukocytes that promote a feed-forward loop of inflammatory pathogenesis (Lassmann, 2008). Regardless of the cells that do enter the CNS, the very recruitment of activated inflammatory immune cells produces a pro-inflammatory milieu within the neurovascular unit. This leads to increased localized oxidative stress, alterations to matrix metalloproteases, and increased cytokine expression that synergistically drives this vicious cycle forward by disrupting tight junction regulation within the BBB (Larochelle, Alvarez, & Prat, 2011).
**Microglia and astrocytes as inflammatory brain cells**

Deemed the brain’s “resident macrophage”, microglia are the primary inflammatory effector cells of the brain. However, developmentally unique from bone marrow-derived macrophages of the peripheral immune system, microglia originate from primitive yolk sac macrophages and colonize the embryonic murine CNS about E 9.5-10.5. Microglia are maintained through local proliferation; they are not replaced from circulating bone-marrow-derived elements, indicating that they have very low cell turnover (DiSabato, Quan, & Godbout, 2016; Ransohoff, 2016b). What we have recently come to understand and appreciate about microglia is that they have enormous capacity to respond to changes in their local environment; single cells have extraordinary heterogeneity in their cell stress response profiles, the transcriptomic profiles appear highly consistent across disease states in both human and animal models, and it is generally well-accepted that microglia activity dictates disease state progression in the brain (Glass, Saijo, Winner, Marchetto, & Gage, 2010; Gosselin et al., 2017; Keren-Shaul et al., 2017). A major limitation of studying microglia and their role in various neuroinflammatory states is that there is no reliable method for isolating, purifying, and characterizing the function of these cells (Korzhevskii & Kirik, 2016). Ionized calcium-binding adaptor molecule 1 (IBA1) is a very widely-used marker for microglial “activation” (Walker & Lue, 2015). This protein localizes with cytoskeletal actin bundles and is functionally involved in membrane ruffling and phagocytosis (D. Ito et al., 1998). While a plethora of published studies characterize microglial activation through IBA1 gene and protein expression, immunohistochemical methods that allow for the examination of physical alterations to microglia remain the gold standard. However, pro-inflammatory cytokine-producing microglia do not always exhibit morphological changes (Jeong, Ji, Min, & Joe, 2013; Norden, Trojanowski, Villanueva, Navarro, & Godbout, 2016; Walker & Lue, 2015).
Only within the past two years has there been the identification of a resting marker for microglial populations (i.e., TMEM119) (M. L. Bennett et al., 2016). Any experimental method to characterize “microglial activation” should be heavily critiqued. As for microglia’s (arguably) pathogenic role in neuroinflammation, they are considered the principle cytokine-producing cell and are frequently implicated in the production of pro-inflammatory TNF-α, IL-6, and IL-1β (Perry, Nicoll, & Holmes, 2010; H. A. Silverman et al., 2015). The increased expression of these three pro-inflammatory cytokines have regularly been used to phenotypically define a neuroinflammatory signature.

Astrocytes also have a role in neuroinflammation. As the brain’s most abundant glial cell type, astrocytes maintain BBB integrity, regulate immune cell trafficking, glutamate uptake, potassium uptake, and support metabolic activity of neurons (Farina, Aloisi, & Meinl, 2007). In animal models, the inactivation of NF-κB specifically in astrocytes, has been shown to reduce inflammatory burden in a number of injury and disease models (Colombo & Farina, 2016). Although these responses are context-dependent, astrocytes have also been shown to be the principle chemokine-producing cell in response to neurological insult, which augments T-cell mediated adaptive immune responses (Farina et al., 2007; Summers, Kangwantas, Nguyen, Kielty, & Pinteaux, 2010). It has also been shown that LPS-induced neuroinflammation leads to a rapid microglial response, followed by delayed activation of astrocytes via up-regulation of glial fibrillary acidic protein (GFAP) (Norden et al., 2016). This suggests that microglial activation augments astrocyte activation in instances of systemic inflammation.

**Acute vs. chronic forms of neuroinflammation**

A variety of cellular stimuli have the capacity to produce acute or chronic neuroinflammation. The point at which a neuroinflammatory event (acute or chronic)
because maladaptive, compared to protective, is frequently unclear. LPS models are a nice context for studying acute neuroinflammation, while diet-induced obesity serves as a strong model for studying chronic neuroinflammation. In each context, the functional immune response and inflammatory pathways involved will inherently vary.

As an acute neuroinflammatory challenge, LPS challenge is expected to elicit microglial activation that is reparative. Ultimately, this response functions to identify and remove toxic, aversive stimuli. Initially, this response remains local within tissue and can be characterized by a relatively high magnitude of pro-inflammatory cytokine production, often characterized by a robust induction of TNF-α production (Qin et al., 2007; Skelly, Hennessy, Dansereau, & Cunningham, 2013). If left unresolved over time, this will create a vicious cycle of local inflammation (Michelle L Block, Zecca, & Hong, 2007). Acute neuroinflammation will often produce some degree of parenchymal damage, cell death, and debris, followed by microglial and astrocyte activation, and recruitment of peripheral immune cells to the CNS to aid the inflammation resolution (Schwartz & Baruch, 2014). Microglial “activation” is often identified as the initial immune-reaction event followed acute neuroinflammatory insult (Shechter, London, & Schwartz, 2013).

Conversely, chronic neuroinflammation resulting from diet-induced obesity can be characterized by a relatively lesser magnitude of pro-inflammatory production. Obesity produces a form of low-grade systemic neuroinflammation that will progressively precipitate neurodegeneration, that is frequently characterized by modestly increased levels of IL-1β (Johnson, 2015; Perry, 2004; Perry et al., 2010). Chronic microglial activation and its subsequent neuroinflammatory phenotype becomes maladaptive to neuronal health. Additionally, the unresolved nature of chronic neuroinflammation requires constant, low-
grade CNS recruitment of peripheral immune cells (monocytes and lymphocytes) that promotes increased permeability of the BBB via cell entry into the parenchyma, which subsequently promotes more damage (Schwartz & Baruch, 2014).

**Summary**

Each of these events provides impressive evidence that the brain is not so much “immune privileged”, but rather immune specialized. While cytokine signaling and cell trafficking within the neurovascular unit holds important homeostatic function, it is also an important pathogenic event to consider when studying neurological pathogenesis. As we know that peripherally-derived inflammatory signals have the capacity to influence the brain and behavior, the study of how these signals are mechanistically transferred into the CNS is imperative to shed further light on the role of immune regulation in chronic CNS disease states. Coupled with recent landmark discoveries, such as the existence of lymphatics within the CNS (Louveau et al., 2015), the field of neuroimmunology has been enabled to shed new light on immune system dysfunction in neurodegenerative disease. We are not only understanding how inflammatory signals are propagated to the brain and the behavioral detriments they produce, but how they create self-sustaining cascades that drive disease progression forward and potentiate additional pathogenic events.

**Chronic Inflammation and Host Factor Detriments**

Immunological dogma has long held that immune responses to foreign antigen are adaptive and they functionally exist to identify, initiate, and resolve an appropriate response to ensure host survival. The basis of this innate immune response begins with inflammation, a collective cellular and vascular response intended to deliver immune cells to a site of injury or illness. Classically, this response is associated with cardinal signs of inflammation: heat, pain, swelling, and redness (Serhan, Ward, & Gilroy, 2010). The kinetics of a mild
inflammatory immune response detail a fast-acting, rigorous burst of cell signaling events that when temporally acute, often remain localized to the immediate site of injury. However, this is not always case, as severe acute immune challenges have a large capacity to elicit systemic inflammation. Conversely, a chronic, unresolved inflammatory response to either endogenous or exogenous stimuli will become maladaptive to the host.

Systemic inflammation has become increasingly recognized as a pathogenic factor capable of driving progressive neurodegeneration (C. Cunningham et al., 2009; Perry, 2004; Perry & Teeling, 2013). The underlying basis for this theory is that the innate immune systemic is chronically activated, leading to sustained production of pro-inflammatory mediators (Michael T Heneka, Kummer, & Latz, 2014). This phenotype is predominantly maintained by microglia, CNS-resident mononuclear phagocytes (Kreutzberg, 1996), and will be discussed in greater detail below. This sustained inflammatory state primes microglia to mount an exaggerated immune response when a given host encounters additional immune challenges (i.e., respiratory infections) (Perry et al., 2007). In order to gain insight into the influence that systemic inflammation has on neurodegenerative pathogenesis, with particular emphasis on PD, we will discuss aging, obesity, and infection as they directly pertain to this body of work. However, there are a number of host conditions that promote neuroinflammation. Given that chronic neuroinflammation occurs early in the development of PD, these processes hold a high degree of therapeutic potential for developing effective diagnostic and therapeutic tools which may lead to disease-modifying approaches for treating these diseases.
Host risk factors for systemic inflammation

Aging

Aging remains the single greatest risk factor for neurodegenerative disease, including PD. A dizzying array of cellular and molecular changes accompanies senescence. Immunologically, elderly individuals exhibit a diminished immune response to challenge, compared to young. Often best exemplified by a weaker immune response to influenza vaccine and a subsequent increase in vulnerability to these environmental pathogens (Fleming & Elliot, 2005), these deficits in immune initiation and resolution are driven by both innate (Solana et al., 2012) and adaptive changes (Montecino-Rodriguez, Berent-Maoz, & Dorshkind, 2013). As for the capacity of these changes to influence normal behavior, the cognitive impairments produced by an inflammatory challenge are often sustained in the elderly and drive dementia-like changes (Craft et al., 2012).

An important distinction to make when examining age-related changes in the brain is to differentiate “normal” aging from pathological events associated with neurodegeneration. For example, microglia isolated from aged murine brains display greater basal up-regulation of immune activation markers (MHCII, CD11b, and IBA-1) when controlling for cell number (Frank et al., 2006). This implies that aged microglia are primed to produce an exaggerated phenotype against immune challenges, characterized by greater production of pro-inflammatory cytokines. This response correlates with long-lasting memory deficits (Barrientos, Frank, Watkins, & Maier, 2010). While this immune behavior is restricted to the context of host immune challenges (i.e., aged microglia do not produce exaggerated responses without immune challenge), the more that an aged host experiences immune challenges via inflammatory insults, the greater the likelihood that the brain will cross the threshold for a disease state phenotype. For PD, this aberrant neuroinflammatory phenotype
in the aged brain can synergistically augment the aggregation of primary pathological hallmarks, such as α-synuclein misfolding (L. Wang et al., 2014). While PD risk is associated with a number of environmental insults and host factor detriments, the following sections will focus i) on aging, obesity, and influenza infections, ii) how they lead to changes in the PD brain, and iii) how they can contribute to Parkinsonian pathogenesis.

**Obesity**

Initial animal studies in the early 1990s established a causative relationship between obesity and behavioral deficits. Rats fed a diet high in saturated fatty acids for 12 weeks displayed spatial and associative memory deficits (Greenwood & Winocur, 1990). In humans, obesity can negatively affect executive function, that becomes exacerbated with age (Gunstad et al., 2007). Additionally, mid-life obesity is a predictor of age-related mild cognitive impairment and serves as a common risk factor for neurodegenerative disease, including Parkinson’s disease (Freeman, Haley-Zitlin, Rosenberger, & Granholm, 2014; Xu et al., 2011).

In addition to insulin resistance, oxidative stress, and altered vascularization (Gregor & Hotamisligil, 2011; C. N. Lumeng & Saltiel, 2011), obesity is a detrimental host state known to produce neuroinflammation (Freeman et al., 2014; A. A. Miller & Spencer, 2014). This pro-inflammatory signature in the brain is characterized by reactive astrocytes and microglia that produce increased cytokine expression of TNF-α, IL-6, and IL-1β, which can underlie behavioral and cognitive disturbances (Guillemot-Legris & Muccioli, 2017; Hao, Dey, Yu, & Stranahan, 2016; E. B. Kang et al., 2016; Pistell et al., 2010).

As diet-induced obesity and chronic consumption of excess nutrients leads to the accumulation of visceral adipose (white adipose tissue), fat tissue itself becomes a hot-bed for the sustained production of inflammatory molecules. Acting in an endocrine-like fashion,
adipose cells secrete inflammatory cytokines and chemokines which promote the recruitment of peripheral inflammatory cells (Wellen & Hotamisligil, 2003). Animal studies of obesity have observed that 45-60% of cells in adipose tissue are F4/80+ macrophages, compared to 15% in lean controls (Weisberg et al., 2003). The phenotype of these adipose tissue macrophages is largely pro-inflammatory, characterized by greater expression of TNF-α and nitric oxide synthase 2 (iNOS) (Carey N Lumeng, Bodzin, & Saltiel, 2007). As a key inflammatory mediator, both genetic deletion of IL-1 receptors and systemic administration of IL-1β neutralizing antibody has been shown to reduce neuroinflammation (Denes et al., 2012). Attenuating these chronic, pro-inflammatory processes can rescue BBB integrity and reduced the pathogenic burden of host factor detriments and neuroinflammatory disease (Argaw, Gurfein, Zhang, Zameer, & John, 2009; Obermeier et al., 2013).

**Influenza**

Although relatively understudied, influenza infections have long been recognized to produce behavioral and cognitive deficits that are attributed to systemic inflammation (Dantzer, 2001b). Additionally, a direct relationship between Parkinsonism and influenza infection has been recognized for the past 40 years (Moore, 1977), and is historically exemplified by the 1918 H1N1 Spanish Flu pandemic. Following primary infection with the 1918 H1N1, a secondary disease state referred to as encephalitic lethargica (EL) could be observed for up to a decade later and was characterized by lethargy, ophthalmoplegia, and delirium (Henry, Smeyne, Jang, Miller, & Okun, 2010). Approximately 80% of these patients went on to develop post-encephalitic Parkinsonism (PEP), which included bradykinesia and a resting tremor (Cunha, 2004; Maurizi, 2010). The notion that influenza infection can promote Parkinsonism is further supported by the observation that antivirals, like amantadine, have
therapeutic benefit for the clinical management of PD (Pahwa et al., 2015; Sadasivan, Sharp, Schultz-Cherry, & Smeyne, 2017).

When the brain parenchyma is directly infected with strains of influenza that can cross the BBB, neuroinflammation and Parkinsonian pathology is aggravated. C57BL/6 mice intranasally inoculated with influenza A/Vietnam/1203/04 (H5N1), a neurotropic virus that invades the CNS, exhibited long-lasting microglial activation and aggregation of α-synuclein that promoted degeneration of dopaminergic neurons in the substantia nigra (H. Jang et al., 2009).

However, most influenza strains are non-neurotropic (G. F. Wang, Li, & Li, 2010) and are capable of producing long-lasting neurobehavioral deficits (Luyt et al., 2012). It has previously been demonstrated that influenza-induced systemic inflammation causes microglia to exhibit a neuroinflammatory phenotype than can compromise hippocampal-mediated behaviors through architectural changes to dendritic branching and synaptic spine density (Jurgens, Amancherla, & Johnson, 2012). This phenotype consisted increased expression of the pro-inflammatory cytokines TNF-α, IL-6, and IL-1β in the hippocampus of male BALB/c mice 7 days post-infection with influenza A/PR/8/34. Furthermore, influenza-induced microglial activation is not limited to specific strains of influenza. Intranasal infection with non-neurotropic influenza A/California/04/2009 (H1N1) has been shown to produce chronic microglial activation in both the substantia nigra and hippocampus beginning 21 days post-infection (DPI) and persisting through 90 DPI (Sadasivan, Zanin, O'Brien, Schultz-Cherry, & Smeyne, 2015). Combining influenza infections with other inflammatory insults can precipitate a neurodegenerative cascade. Dual challenge with influenza A/California/04/2009 and the Parkinsonian toxin 1-methyl, 4-phenyl, 1,2,3,6-
tetrahydropyridine (MPTP) in mice has been shown to have no additive effect on microglial activation, but produces substantial loss of dopaminergic neurons in the substantia nigra (Sadasivan et al., 2017).

**Double-Stranded RNA-Dependent Protein Kinase (PKR) in Cell Stress**

Double-stranded RNA-dependent protein kinase (PKR) is a serine/threonine kinase that characteristically serves an antiviral role, in response to dsRNA viral intermediates. Acting as a pattern recognition receptor (PRR) for the innate immune system, its activation leads to the phosphorylation of the α subunit of the eukaryotic initiation factor 2, thereby inhibiting viral translation and subsequent protein production within infected cells. This response ultimately induces apoptosis (Garcia et al., 2006). Upon binding dsRNA, free PKR undergoes a conformational change by dimerizing and exposing an activation loop which autophosphorylates, thereby activating PKR (Clemens, 1997). Once activated, PKR is able to phosphorylate a number of substrates involved in translation regulation, or propagate IκB/NF-kB-mediated signal transduction, based on cellular stimuli.

**PKR activation mechanism**

In response to either cellular, viral, or synthetic dsRNA, PKR will autophosphorylate and activate via dual 70 amino acid, dsRNA-binding motifs in the N-terminus (CLEMENS & ELIA, 1997). In resting conditions, PKR exists in a latent state. The autoinhibition of PKR is regulated by two dsRNA-binding domains (dsRBD) that block catalytic activity of its kinase domain (M. A. Garcia et al., 2006). Each monomer of the dsRBD consists of two α-β folds, joined by a 20 amino acid linker with a random coil conformation (Nanduri, Carpick, Yang, Williams, & Qin, 1998). Both dsRBDs have been shown to be required for optimal dsRNA binding (H. M. Chen, Wang, & D'Mello, 2008). This linker is what affords PKR the flexibility to structurally wrap around dsRNA and optimally allow protein-RNA interactions,
while also providing an explanation for which length of dsRNA is required to activate PKR (Manche, Green, Schmedt, & Mathews, 1992). Although PKR can be activated by dsRNA of at least 30 base pairs, dsRNA of 80 base pairs or longer will yield optimal activation, thereby representing a diverse window of dsRNA activators.

As an inhibitor of translation, PKR is intracellularly localized to ribosomes. Ribosomal proteins have been shown to mediate PKR via the dsRBDs. Ribosomal localization affords PKR the functional flexibility to respond to activating stimuli (i.e., dsRNA), locally inhibit translation and protein synthesis, all the while not affecting global mRNA translation (M. Garcia et al., 2006). Once active, PKR will autophosphorylate and dimerize, exposing its catalytic domains. Within the activation loop, the amino acid residues Thr446 and Thr451 have been shown to be required for autophosphorylation. Substitution of Thr451 leads to PKR inactivation. Of particular note, PKR exhibits a bell curve pattern of activation in which high concentrations of dsRNA can actually inhibit PKR (B. R. Williams, 1999). Conversely, this implies that a high level of PKR expression would be elicited by moderate levels of cell stress.

**Mechanism of PKR inhibition with imoxin**

The two commercially available small molecule inhibitors of PKR are 2-aminopurine (2-AP) and 8-(imidazol-4-ylmethylene)-6H-azolidino [5,4-g] benzothiazol-7-one. The latter was identified by Jammi et al. (2003) and is referred to as Imoxin, C16, and PKRi in the literature. While 2-AP does inhibit PKR kinase activity, it has been shown to inhibit other kinases (J. Huang & Schneider, 1990; Posti et al., 1999). The oxindole/imidazole inhibitor Imoxin inhibits PKR by serving as an ATP site-directed competitor which blocks PKR autophosphorylation, although pharmacokinetic data is largely absent from existing literature (Ingrand et al., 2007; Weintraub et al., 2016). The estimated IC50 for Imoxin, a metric that
graphically represents inhibition of a compound by plotting percentage of activity versus concentration, was 186 ± 20nM compared to the 2-AP with an IC50 of 10 mM (Jammi, Whitby, & Beal, 2003). In functional contrast, 2-AP inhibits PKR-independent kinases at higher (millimolar) concentrations (Y. Hu & Conway, 1993). In neurons in vitro and ex vivo, Imoxin has respectively been shown to modulate expression for Jun N-terminal kinases (JNKs), p38 MAP kinases, and others (H. M. Chen et al., 2008; Couturier et al., 2011). Nonetheless, PKR inhibition in vitro with Imoxin prevents stress-induced neuronal apoptosis in numerous experimental contexts and could serve as a neuroprotective strategy (Weintraub et al., 2016).

**Activators of PKR**

*dsRNA*

Whether originating from cellular, viral, or a synthetic source (poly:IC), double-stranded RNA (dsRNA) leads to PKR activation through displacing autoinhibitory interactions that allow for substrate recognition. Natural viral PKR activators occur during viral replication in an infected host cell. Although intermediates of both RNA and DNA viruses can activate PKR, RNA viruses produce dsRNA intermediates necessary for producing sister copies. No matter the source, dsRNA at high concentrations will lead to PKR inhibition. At high concentrations, 2 PKR monomers that would ordinarily dimerize and activate, will dissociate as they compete to bind different dsRNA. Just as high concentration of dsRNA inhibits PKR, minimal concentrations will elicit a greater magnitude of PKR expression (F. Zhang et al., 2001).

*Toll-like receptors (TLRs)*

The TLR family is a highly conserved innate immune mechanism for identifying antigen and the subsequent production of an adaptive immune response that produces
immunological memory and (ideally) confers lifelong protection against pathogenic antigen. From previous work where PKR-deficient mice were systemically challenged with the TLR4 agonist, lipopolysaccharide (LPS), they exhibited reduced serum expression of proinflammatory IL-6 and IL-12 (Goh & Williams, 2000). While TLR3 does not directly activate PKR, extracellular dsRNA can activate TLR3 (Z. Jiang et al., 2003).

**Cytokines**

As a member of the interferon (IFN) response pathway, PKR is induced by the cytokines IFN-α, IFN-β, and IFN-γ. Interferons were one of the earliest identified classes of cytokines whose stereotypical function is to interfere with viral replication (Pindel & Sadler, 2011). IFN-γ-induced PKR activation in vitro has been shown to activate downstream NF-κB through a Janus kinase-1 (JAK1) mechanism. From the same studies, there was no observed IFN-γ-mediated activation NF-κB in PKR-null fibroblasts (HeLa S3, 2fTGH, and NIH-3T3) (Deb, Haque, Mogensen, Silverman, & Williams, 2001). NF-κB is a transcription factor that regulates and controls the production of cytokines relevant for a cell survival response (Lawrence, 2009).

Additionally, PKR has shown to be activated following activation of the proinflammatory cytokines, tumor necrosis factor-alpha (TNF-α). Genetic deletion of PKR in vitro reduces the TNF-α-induced activation of NF-κB (Takada, Ichikawa, Pataer, Swisher, & Aggarwal, 2007). That is, PKR partially modulates TNF-NF-κB signaling, implying a signal transduction function, rather than a signal effector.

**Protein activator of PKR (PACT)**

Following stimulation by extracellular stresses, PKR can be activated through intracellular protein-protein interactions with PKR-associated activator (PACT; aka PKRA; aka Rax, mouse ortholog) (Peters, Dickerman, & Sen, 2009). Independent of dsRNA, PACT
has been shown to mediate cell responses to a multitude of stimuli, such as H2O2 and arsenic (Singh & Patel, 2012). Phosphorylation of PACT at Ser246 and/or Ser287 leads to a conformational change of inactive PKR within the dsRNA binding domain, thereby activating PKR in a mechanism distinct from activation by dsRNA, but one that produces similar cell stress responses (Li et al., 2006). This PACT-PKR relationship has previously been shown to mediate apoptotic cell stress responses (Peters, Li, & Sen, 2006).

**Mediators of PKR signaling**

**Eukaryotic initiation factor 2-α (eIF2-α)**

The most well-characterized target and natural substrate for activated PKR is the α subunit of eukaryotic initiation factor 2 (eIF2-α). While PKR is one of four kinases that phosphorylate eIF2-α and exert the similar action of general translation inhibition, PKR’s dsRNA-binding domain is a unique structural and functional feature of the kinase, allowing it to inhibit the translation of viral mRNA within infected cells (Balachandran et al., 2000). This action is attributed to PKR’s high substrate affinity for eIF2-α (M. A. Garcia et al., 2006). Once active, autophosphorylation of the Thr446 residue promotes recognition of substrate and further phosphorylation. Within the C lobe of the kinase domain lies the αG helix that promotes initial interaction between PKR and eIF2-α. This interaction promotes a conformational change that allows the Ser51 residue to phosphorylate and unfold, allowing eIF2-α to fully access PKR’s catalytic cleft (Dar, Dever, & Sicheri, 2005).

**Interferon regulatory factors (IRF) and signal transducers and activators of transcription (STAT)**

As integral components of the Type I Interferon pathway, interferon regulatory factors (IRF) and signal transducers and activators of transcription (STAT) are two groups of transcription factors known to contribute to PKR signaling. The induction and activation of
these factors is often restricted to instances of viral infection, as the stereotypical function of interferon signaling is to interfere with viral replication (Lazear et al., 2013). IRF-1 has been implicated as a mediator of PKR-induced apoptosis following IFN-γ or poly:IC treatment (Kumar et al., 1997). As for PKR’s regulation of STAT1 signaling, a signal transducing role has been proposed. PKR knockout has been shown to reduce STAT1 activation, although catalytically active PKR is not required (Ramana et al., 2000). This evidence highlights that PKR has both direct and indirect cell signaling functions.

**p53**

As a tumor suppressor gene, p53 is frequently implicated as an essential mediator of tumor growth in numerous forms of cancer (Greenblatt, Bennett, Hollstein, & Harris, 1994). In TNF-α-treated lymphoma cells, PKR activation has been shown to induce p53 (Yeung, Liu, & Lau, 1996). Alternatively, p53 induction resulting from genotoxic insult activates PKR, leading to translation inhibition and induction of apoptosis via the eukaryotic initiation factor 2-α (eIF2-α) pathway (Yoon, Lee, Lim, & Bae, 2009). In PKR deficient MEF cells, p53-mediated arrest of the cell cycle is disrupted, but not completely abolished (Cuddihy, Wong, Tam, Li, & Koromilas, 1999). Thus, PKR and p53 have a bi-directional relationship where PKR is an important contributor to cell cycle regulation following stress.

**Mitogen-activated protein kinases (MAPKs)**

Of mammalian responses to cell stress, MAPKs are integral players in responding to a variety of insulting stimuli (Arimoto, Fukuda, Imajoh-Ohmi, Saito, & Takekawa, 2008). Consisting of the ERK, JNK, and p38 families, which regulate activation of stress-response transcription factors (NF-κB, ATF, STAT), many of these pathways also overlap with PKR. The involvement of PKR in these MAPK responses does not depend on its kinase function. For example, catalytically inactive PKR contributes to TNF-α-induced p38 expression (Deb,
Zamanian-Daryoush, Xu, Kadereit, & Williams, 2001). PKR’s exact role and contribution to these signaling events is context-dependent, but occurs in response to stimuli ranging from thermal shock to inflammatory cytokines (M. Garcia et al., 2006).

**Activating transcription factors (ATF)**

Among additional transcription factors related to PKR signaling is Activating Transcription Factor 3 (ATF-3). ATF-3 has been shown to promote p53 activation following cell stress (Yan, Lu, Hai, & Boyd, 2005) and overexpression of ATF-3 increased caspase-3 mediated apoptosis (Syed et al., 2005). Although ATF-3 gene and protein expression are relatively low in both neurons and glial cells in the CNS, cell stress will promote its induction following a variety of stresses (Anderson, 2012). In particular, ATF-3 is induced following endoplasmic reticulum (ER) stress and its induction has been shown to be dependent on the eif2α (H.-Y. Jiang et al., 2004). Collectively, ATF-3 expression mediates cell stress responses and regulates cellular proliferation by acting through the eif2α, whose phosphorylation is maintained by PKR.

**Fas-associated death domain (FADD) and caspase-8**

Of key mediators involved in PKR-mediated stress responses and apoptosis, the fas-associated death domain (FADD) and the initiator caspase, caspase-8 are key signaling events. FADD is an intracellular adaptor protein that can consequently activate the initiator caspase, caspase-8, in response to extracellular stress signals (Balachandran et al., 1998). FADD-activated caspase-8 can go on to either directly activate the executioner caspase-3, or promote Bcl-2 activation that leads to mitochondrial release of cytochrome c (Xiao-Ming, 2000). In HeLa CCL-2 cells treated with staurosporine, an apoptosis-inducing bacterial alkaloid, PKR was shown to induce apoptosis through FADD/caspase-8 signaling. This activation was not dependent on PKR phosphorylation, nor eIF2-α phosphorylation (von
Additionally, the PKR/FADD apoptotic pathway has been shown to mediate Aβ-induced cell death in SH-SY5Y neurons (Couturier et al., 2010). Fas-FADD are not the only pathways involved in caspase-8/PKR-mediated apoptosis, but also involve TNF-TNFR1 interactions (Gil & Esteban, 2000).

**NF-κB**

The NF-κB signaling pathway is master transcriptional regulator involved in a broad range of neuroinflammation and inflammatory processes. In unstimulated cells, NF-κB is sequestered in the cytoplasm by the inhibitory IκBs. Upon detection of cell stress, the IκB dimer dissociates and promotes nuclear translocation that leads to the transcription of inflammatory genes (Liu, Zhang, Joo, & Sun, 2017). PKR-mediated activation of the NF-κB pathway occurs either from direct activation from FADD via TNFR1 or Fas-induced apoptosis (W.-H. Hu, Johnson, & Shu, 2000; Ranjan & Pathak, 2016), or through PKR itself (Zamanian-Daryoush, Mogensen, DiDonato, & Williams, 2000). Furthermore, PKR-mediated activation of NF-κB does not require catalytically active PKR (Bonnet, Weil, Dam, Hovanessian, & Meurs, 2000). Additional evidence for PKR’s role in inflammatory signaling comes from *in vitro* studies (discussed in greater detail below). When murine macrophages are treated with known NLRP3 agonists, pharmacological PKR inhibition with Imoxin significantly reduces production of caspase-1 and subsequent IL-1β (Lu et al., 2012). What can be unclear from studies investigating NF-κB and PKR is whether NF-κB is activating as an anti-apoptotic response to limit PKR-mediated apoptosis, or if it is activating apoptosis to promote cell death following inflammation.

**NLRP3 inflammasome**

Inflammasomes are multimeric signaling platforms for caspase-1 that have been shown to be required for the production of IL-1β and IL-18, two pro-inflammatory cytokines
implicated in a number of disease states (Bauernfeind et al., 2009; Dagenais, Skeldon, & Saleh, 2012; Leemans, Cassel, & Sutterwala, 2011). A report by Lu et al. (2012) had implicated a direct relationship between PKR and NLRP3. Peritoneal macrophages were isolated from PKR<sup>++</sup> and PKR<sup>−/−</sup> mice and stimulated with a variety of stress-inducing stimuli that serve as danger-associated molecular patterns (DAMPs) for recognition by the innate immune system: synthetic dsRNA (poly:IC), ATP, monosodium urate (MSU), adjuvant aluminum, lipopolysaccharide (LPS). Activation and expression of caspase-1 and IL-1β was diminished in response to ATP, MSU, and Alum in PKR knockout samples. Pharmacological inhibition of PKR in ATP- and MSU- treated macrophages had similar effects, indicating that the anti-inflammatory action of PKR inhibition is not limited to genetic deletion. Additionally, immunoprecipitation of stimulated mouse macrophages indicated that PKR uniquely associated with NLRP3 and no other members of the NLR-family (Lu et al., 2012). This seminal paper provided the first evidence suggesting that PKR was a novel authorizing factor for the production of NLRP3.

However, subsequent reports went on to imply this interaction with NLRP3 was independent of PKR’s kinase action (Hett et al., 2013), while others have observed that PKR activity does not modulate the inflammasome at all (He, Franchi, & Nunez, 2013), or that PKR actually suppresses inflammasome activity (Yim et al., 2016). Others have reported that inhibiting PKR autophosphorylation with the flavonoid luteolin actually increases NLRP3 activation in macrophages and microglia (Dabo et al., 2017). In the context of obesity, NLRP3 has been identified as a critical mediator that regulates metabolic inflammation (i.e., ‘metainflammation’). However, there was no difference in the IL-1β phenotype of nigericin or ATP-treated bone marrow-derived macrophages (BMDMs) from obese wildtype or PKR
knockout mice (Lancaster et al., 2016). These results imply that NLRP3 activity is not dependent on PKR.

**Evidence of CNS PKR Expression**

**Clinical relevancy of PKR to neurodegeneration**

The first neuropathological report that PKR was expressed in the human brain came from 2002 findings that phosphorylated-PKR and eIf2α was expressed in degenerating hippocampal and cortical neurons of Alzheimer’s disease patients (Chang RC, 2002). In addition to AD, phosphorylated PKR has been found in hippocampal neurons of PD and Huntington’s disease patients, suggesting that PKR expression is not limited to a single disease state, but could serve as a general marker of cell stress in neurodegeneration. Furthermore, these authors proposed that increased PKR expression could serve as a cell stress signal that precedes a bona fide disease state (Bando et al., 2005). Multiple groups have gone on to confirm that PKR expression localizes with degenerating neurons and it functions as a signaling transducing node in response to inflammation, autophagy, and apoptosis association with neurodegenerative disease progression (Hugon, Mouton-Liger, Dumurgier, & Paquet, 2017).

**CNS expression of PKR**

Animal studies have extended our understanding of PKR by showing that genetic deletion of PKR leads to attenuated neuroinflammation and that pharmacological inhibition can produce similar effects. Repeated systemic administration of LPS (1 mg/kg I.P., O111:B4, daily for 3 days) led to increased TNF-α and IL-6 cytokine release, IBA1 microglial activation, and increased production of Aβ in the hippocampus of C57BL/6 mice. None of these changes were observed in PKR knockout mice (Carret-Rebillat et al., 2015). Directly relevant to PKR signaling, no changes were detected to eIf2α phosphorylation.
Either signaling changes had dissipated by the 24 hour post-LPS timepoint, or it alternatively suggests that PKR activation does not act downstream on its canonical substrate. Alternative evidence for involvement of the elf2α pathway with PKR signaling comes from transgenic Alzheimer’s models (APP/PS1) and oxidative stress assays on immortalized human neurons (SH-SY5Y). This report found a positive correlation between phosphorylated PKR, phosphorylated elf2α, and the Aβ peptide-producing enzyme BACE1 (Mouton-Liger et al., 2012). Similar results have been observed in thiamine-deficiency models of neurodegeneration, once again highlighting PKR’s broad potential to indicate neurological cell stress in a variety of disease states (Mouton-Liger et al., 2015).

Given the observation that PKR is induced following a variety of neurodegenerative-related cell stresses, it is reasonable to postulate that the inhibition of PKR could alleviate disease state burden. Others have shown that PKR inhibition can attenuate peripheral inflammation in white adipose tissue, both in vivo and in vitro (Nakamura, Arduini, Baccaro, Furuhashi, & Hotamisligil, 2014; Nakamura et al., 2010). Two commercial PKR inhibitors are available, 2-aminopurine (2-AP) and imoxin (aka C16 & PKRi) (Jammi et al., 2003; Weintraub et al., 2016). Imoxin is an ATP-site-directed competitor that blocks PKR autophosphorylation. In vitro studies using Aβ-induced oxidative stress in SH-SY5Y neurons have shown that treatment with imoxin can prevent PKR phosphorylation and activation. Additionally, PKR inhibition with imoxin prevented caspase-3-mediated cell death in these studies (Page et al., 2006). In age-related animal studies of PKR expression, inhibition with imoxin has been shown to reduce PKR and elf2α expression, while not stimulating the pro-survival mTOR pathway (Ingrand et al., 2007). In an in vivo 3-nitropropionic acid (3-NP) model of Huntington’s disease, imoxin reduced neuronal apoptosis and modulated cell cycle
progression. 3-NP is a neurotoxin that selectively caused neuronal cell death. When administered at the time of 3-NP, imoxin reduced the extent of striatal neurodegeneration and improved behavioral deficits (H. M. Chen et al., 2008). Additionally, imoxin attenuated 3-NP-induced expression of cyclin-dependent kinases (CDK) and the pro-apoptotic ATF-3, while not modulating eIF2α expression. These results implied that PKR inhibition with imoxin is modifying stressed neurons’ attempt to re-enter the cell cycle, which could lead to apoptosis. The therapeutic potential of PKR inhibition is not just limited to neurons. Primary co-cultures of mouse neurons, microglia, and astrocytes treated with Aβ exhibited decreased expression of pro-inflammatory cytokines TNF-α and IL-1β, reduced NF-κB activation, and caspase-3 activation was abolished, following treatment with imoxin (Couturier et al., 2010).

**PKR as a mediator of neuroinflammation**

Outside of its stereotypical antiviral function, PKR has proven to be a stress-induced cell signal that uniquely has crossover function in inflammation, autophagy, and apoptosis (M. A. Garcia et al., 2006). Thus, PKR can uniquely orchestrate cell stress responses to a variety of stimuli. While PKR relationship with NLRP3 is debatable (He et al., 2013; Lu et al., 2012; Yim et al., 2016), catalytically inactive forms of PKR are capable of activating NF-κB and promoting TNF-α production, thereby negating the role of PKR/eIF2-α signaling (Bonnet et al., 2000). Activated by inflammatory cytokines, both bacterial and viral infection, genotoxic stress, and additional forms of cell stress, PKR goes on to mediate the expression of MAPKs, Nf-κB, IRFs, and ATF’s.

Stress-induced PKR expression in the brain has regularly been shown to co-localize with neurons (Hugon et al., 2017). This increased expression has been shown to occur as both cytoplasmic and/or nuclear fractions of PKR (Marchal et al., 2014; X. Wang, Fan, Wang, Luo, & Ke, 2007). Additionally, PKR has been shown to co-localize with neuronal
expression of phosphorylated tau (Bose et al., 2011). As for non-neuronal expression of PKR, LPS-induced PKR expression has been shown in both primary rat microglia and astrocytes (J. H. Lee et al., 2005; Ong et al., 2005). In animal models of Gaucher disease, a lysosomal storage disease caused by a mutation to glucocerebrosidase (GBA1), PKR has been shown to co-localize with GFAP-positive astrocytes and MAC2-positive microglia (Vitner et al., 2016). While highly expressed throughout all major brain structures (isocortex, olfactory bulb, hippocampal formation, cortical subplate, striatum, pallidum, thalamus, hypothalamus, midbrain and cerebellum), basal mRNA PKR is not differentially expressed throughout the brain (Lein et al., 2007). The pons and medulla have the lowest relative mRNA expression in the mouse brain.

The inherent challenge in studying inflammatory processes is that this complex crosstalk of cell signaling produces both adaptive and maladaptive responses, which are highly context-dependent. Ideally, our work and the work of others can posit PKR as a molecular entity that is capable of predicting disease state progression and responding to therapeutic intervention for Parkinson’s disease and other neurodegenerative conditions.
CHAPTER 2. LOW-GRADE, ACUTE IMMUNE CHALLENGE WITH LPS INDUCES HIPPOCAMPAL AND STRIATAL PKR EXPRESSION AND PHARMACOLOGICAL INHIBITION WITH IMOXIN IS NEGATIVELY REGULATED BY FADD APOPTOTIC SIGNALING

Matthew A. Jefferson¹,²,*, Rudy J. Valentine², & Marian L Kohut¹,²

A manuscript prepared for submission to Brain, Behavior, & Immunity

¹Interdepartmental Neuroscience Graduate Program, Iowa State University, Ames, IA
²Department of Kinesiology, Iowa State University, Ames, IA

* Author for correspondence (MAJ): 235 Forker Building, Department of Kinesiology, Iowa State University, Ames, IA 50011-4008. E-mail: mjefferson83J@gmail.edu. Telephone: (309) 781-7590. (515) 294-8009.

Abstract

Inflammation has readily become recognized as an early pathogenic event that can drive the progression and development of neurodegenerative disease. Peripheral inflammatory signals have been shown to produce chronic, aberrant activation of microglial cells, which compromises neuronal homeostasis, promotes astrocyte activation, and progressively contributes to the pathogenesis of neurodegenerative disease. Our lab had previously identified increased expression of RNA-dependent Protein Kinase (PKR) in murine brains of a chronic model of systemic inflammation and sought to understand its role in an acute model of systemic inflammation. Functionally, PKR has diverse antiviral, apoptotic, and inflammatory responses that can occur independently of one another. Collectively, we sought to examine hippocampal and striatal CNS PKR expression in male C57/BL6 mice brains following a systemic immune challenge with LPS, the neuroinflammatory phenotype that coincided with PKR expression, the concurrent microglial and astrocyte phenotype, the therapeutic potential of the PKR inhibitor imoxin (IMX) to attenuate acute LPS-mediated neuroinflammation, and the cell signaling pathway modulated by PKR inhibition. We
observed a considerable degree of overlap in inflammatory and apoptotic signaling events, with PKR and NLRP3 expression appearing to be negatively regulated by FADD/Caspase-8. Together, this work suggests that PKR does not function as an effector molecule in systemic inflammation, but rather acts as a node whose functions are dependent on the availability of players in the response network and the context of the inflammatory challenge.

**Introduction**

In an ever-aging population, neurodegenerative diseases have irrefutably become established as one of the greater public health burdens among modern society. Current therapeutic options for the treatment of Alzheimer’s (AD) and Parkinson’s disease (PD), the two most prevalent neurodegenerative diseases, do not modify disease progression (Erkkinen et al., 2018). Of the deranged cellular processes implicated in neurodegeneration, inflammation has readily become recognized as an early pathogenic event that can drive the progression and development of neurodegenerative disease (Perry et al., 2010).

While neuroscientific dogma had previously postulated the brain as an immunologically “privileged” organ, we now appreciate that the blood brain interface is highly dynamic and capable of propagating peripheral inflammatory signals into the CNS (Erickson, Nicolazzo, & Banks, 2018; Obermeier et al., 2013; Zlokovic, 2008). These signaling events have largely been shown to produce chronic, aberrant activation of microglial cells, the brain’s resident innate immune cell. Chronic microglial activation becomes a pathogenic event that compromises neuronal homeostasis, promotes astrocyte activation (Liddelow et al., 2017), and progressively contributes to the pathogenesis of neurodegenerative disease (Colm Cunningham, 2013).

PKR is constitutively expressed in mammalian cells and stereotypically functions as an anti-viral kinase, phosphorylating its canonical substrate eukaryotic translation initiation
factor-2α (eIF-2α), thereby inhibiting protein synthesis within virally-infected cells (Berry, Knutson, Lasky, Munemitsu, & Samuel, 1985; Clemens, 1997; M. A. Garcia et al., 2006). While dsRNA species produced during viral synthesis activate PKR via autophosphorylation, PACT (RAX mouse ortholog) is recognized as a cytosolic protein activator of PKR in response to a multitude of extracellular stresses (T. Ito, Yang, & May, 1999; Li et al., 2006). Several knockout studies have eloquently implicated PKR’s role in neurodegenerative pathogenesis (Carret-Rebillat et al., 2015; Hugon et al., 2017; Mouton-Liger et al., 2015). Additionally, its neuropathological expression occurs in human brain regions relevant to Alzheimer’s, Parkinson’s, and Huntingtin’s disease pathogeneses (Bando et al., 2005; Chang et al., 2002; A. L. Peel et al., 2001). Functionally, PKR has diverse antiviral, apoptotic, and inflammatory responses that can occur independently of one another. This collective response synergistically contributes to a cell stress response that could either be detrimental or protective. Pharmacological inhibition of PKR has been shown to produce a myriad of diverse biological effects, including PKR-independent changes (M. A. Garcia et al., 2006).

Inflammation is notoriously fickle, producing both adaptive and maladaptive responses that are highly context-dependent. This complex crosstalk of cell signaling has made it a challenge to identify molecular entities that are capable of predicting disease state progression and responding to therapeutic intervention. PKR has a well-accepted role in inflammatory signaling, across a variety of host cell types (R. Kang & Tang, 2012). Using LPS as a model to stimulate peripheral-to-CNS inflammation, we hypothesized that LPS-induced PKR activation would regulate neuroinflammation, and the pharmacologic inhibition of PKR with Imoxin (IMX), would attenuate this neuroinflammatory phenotype. Given the pathophysiological involvement of these regions in AD and PD, we characterized the kinetics
of PKR expression across the hippocampus and striatum in male C57/BL6 mice, the
neuroinflammatory phenotype that coincided with PKR expression, the therapeutic potential
of IMX to attenuate acute LPS-mediated neuroinflammation, and the involvement of cell
death signaling pathways modulated by PKR inhibition.

Materials and Methods

2.1 Study Design

This work consisted of two studies. Study #1 was an initial LPS dose response and
timeline study to define LPS-induced PKR expression in the brain. Male C57BL/6 mice were
randomly assigned to either: saline (n = 5), a single dose of 5 mg/kg LPS I.P. and brains
collected 3 hours later (n = 5), a single dose of 1 mg/kg LPS I.P. and collected 3 hours later
(n = 5), or a single dose of 1 mg/kg LPS I.P. and brains collected 24 hours later (n = 3-4).
Samples were processed for gene expression via qPCR to determine the dose at which
transcriptional PKR expression in the striatum and hippocampus was the greatest, for follow-
up studies using a PKR inhibitor.

From Study #1, the 1 mg/kg LPS I.P. dose at 24 hours post-LPS was selected and
used in Study #2. This dose of LPS was selected for the PKR inhibitor study because it
elicited the same magnitude of PKR gene expression in the brain, while producing a modest
pro-inflammatory profile that would not mask a potential drug effect in Study #2.

Study #2 utilized the same paradigm as the LPS dose response study, with the
exception that two doses of the PKR inhibitor imoxin were administered prior to LPS
challenge. The objective of this study was to test the role of PKR in modifying downstream
stress responses to LPS. Animals were randomly assigned to one of five groups: Saline (n =
7), Saline + 2 S.C. doses of 1 mg/kg IMX (n = 5), one I.P. injection of LPS at 1 mg/kg and
brains collected 24 hours later (n = 6), pretreated with two S.C. doses of 0.5 mg/kg IMX +
LPS ($n = 7$), or pretreated with two S.C. doses of 1.0 mg/kg IMX + LPS ($n = 7$). As a positive control, imoxin alone did not modulate gene expression for any of our markers of interest, compared to saline. While not graphically represented, these data were included in analyses.

Doses of imoxin were administered 24 hours pre-LPS (hour 0) and again at 2-hours pre-LPS (hour 22). 1 mg/kg O111:B4 LPS was administered at hour 24, with animals sacrificed 24 hours later (hour 48). Samples from Study #2 were processed for gene expression via qPCR and protein expression via Western Blotting.

2.2 Animals

Male C57BL/6 mice (Charles River) were maintained in group housing ($n = 5$/cage) and kept on a 12-hour reverse light cycle in ventilated Innovive racks (Innovive, San Diego), with ad libitum food and water access. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Iowa State University. In Study #1, animals were I.P. challenged with Lipopolysaccharide (LPS) at 6 weeks of age, sacrificed via asphyxiation ($\text{CO}_2$), brains removed and micro-dissected for the hippocampus and striatum, then snap-frozen in liquid nitrogen for subsequent processing. Study #2 utilized the same overall design, with the exception that mice were only given one dose of LPS, and two pre-LPS doses of PKR inhibitor were administered.

2.3 Treatment

2.3.1 LPS Challenge

In Study #1, animals were administered 2 different doses of O111:B4 LPS I.P. (Sigma-Aldrich, St. Louis; L4391) with 2 post-LPS collection times to survey the difference in neuroimmune phenotype across different dose intensity and challenge duration: 5 mg/kg
LPS at 3 hours post-LPS, 1 mg/kg LPS at 3 hours post-LPS, and 1 mg/kg LPS at 24 hours post-LPS. Control mice were administered saline intraperitoneally. LPS was dissolved in saline at room temperature and given at a volume of 8 µl/kg (~200 µl per 25 g mouse). Body weights were recorded pre- and post-LPS challenge. The percent of weight loss from challenge in Study #1 and Study #2 is available in Table 1.

2.3.2 Imoxin Administration

Using a separate cohort of mice for Study #2, PKR inhibitor studies were performed using Imidazolo-oxindole PKR inhibitor Imoxin (Calbiochem; #527450). Imoxin was prepared by dissolving the stock powder in 100% ethanol (soluble at 20 mM) and further diluting doses in saline. Mice received either 0.5 mg/kg or 1.0 mg/kg IMX S.C. at a volume of 4 µl/kg (~100 µl per 25 g mouse). 1 mg/kg LPS was administered at hour 24, with animals sacrificed 24 hours later (hour 48). Injection times were appropriately staggered to ensure an accurate tissue collection schedule.

2.4 Gene Expression

At the time of processing, samples were kept frozen on liquid nitrogen and RNA isolation was performed using an RNA binding-column method (Qiagen RNeasy Micro Plus Kit; #74034). Total RNA was isolated from one hemisphere and reverse transcribed to cDNA (Qiagen RT² First Stand Kit; #330404) to be used in SYBR green assays for relative gene expression (Qiagen RT² SYBR Green Fluor qPCR Mastermix; 330513). qPCR was performed on a Bio-Rad MyIQ PCR system for 40 cycles of amplification, with a subsequent melt curve to validate the purity of amplified product. The primer sequences used are available in Table 2. Relative difference of gene expression between groups was analyzed
using the Pfaffl Method (Pfaffl, 2001), where threshold cycle (Ct) values were normalized to both β-actin and GAPDH, and expressed relative to control groups.

2.5 Protein Expression

Protein lysates from one hemisphere of each animal were homogenized with a motorized pestle in 150 µl of custom Lysis Buffer A containing: 30 mM NaHEPES, 5 mM EGTA, 3 mM EDTA, 20 mM KCL, 32% glycerol, phosphatase inhibitor cocktail 3 (Sigma-Aldrich; P0044), and HALT protease inhibitor cocktail (Thermo Fisher; 78425). Homogenates were centrifuged for 15 minutes at 15,000 g (4°C), and supernatant collected. Total protein was quantified using the bicinchoninic acid method (BCA; Pierce Biotechnology, Inc.; #23227) with bovine serum albumin standards. Western blot protein samples (10-30 µg) were run on 8-16% Criterion TGX Precast Gels (Bio-Rad) at 150V for 1.5 hours, transferred to polyvinylidene fluoride membranes (PVDF; EMD Millipore), and blocked in Tris-buffered saline (TBS; pH 8.0) containing 0.05% Tween-20 and 5% non-fat dry milk (LabScientific; #M0841) for 1 hour at room temperature. Membranes were incubated overnight at 4°C with primary antibody at the manufacturer recommended dilution. Primary antibody solutions were prepared in 1x TBST, 0.05% Na Azide, and 3% BSA and markers included: NLRP3 (Adipogen; cryo-2), p-PKR (Thr 451; EMB Millipore; #07-886), p-eIF2α (Ser 51; CST; #3398) and β-actin (CST; #4970). Primary mouse monoclonal antibodies from Santa Cruz Biotechnology included: PKR (sc-6282), p-PACT (sc-53524), PACT (sc-377103), CDK2 (sc-6248), ATF3 (sc-518032), FADD (sc-271748), Caspase-8 (sc-81656), NF-κB p65 (sc-8008), and RIP1K (sc-133102). Cut membranes were washed with 1x TBST (0.1% Tween), probed at room temperature for 1 hour with respective secondary antibody, treated with SuperSignal ELISA Femto Maximum Sensitivity Substrate (Thermo; #970740), and images
captured on a Bio-Rad Universal Hood II gel imager. Densitometry was performed using ImageJ software (NIH), normalizing all values to β-actin, and expressed relative to control groups. Cropped Western Blot images show group representative samples run from the same gel, with corresponding housekeeping control.

2.6 Analysis

Data were analyzed in GraphPad Prism (version 6 for Windows) using one-way analysis of variance (ANOVA) on both gene and protein expression values. Post-hoc multiple comparisons were performed using Sidak correction, with an α-level of 0.05. Figures were generated in GraphPad, reporting data as mean fold change with error propagated as 95% Confidence Intervals. Gene data were represented on a Log2 scale, with protein data on a linear scale. Significance above bar denotes difference from control, with between group differences denoted by line. **** \( p < 0.0001 \), *** \( p < 0.001 \), ** \( p < 0.01 \), * \( p < 0.05 \) by 1-way ANOVA. Additional images were generated using PowerPoint 2016 (Microsoft).

Results

3.1 LPS challenge produced a stereotypical neuroinflammatory phenotype

To assess the capacity for systemic inflammation to induce CNS PKR expression, we administered two doses of LPS (5 mg/kg & 1 mg/kg), with 2 post-LPS collection timepoints (3 hours & 24 hours), to the cohort of animals used in Study #1. To characterize the pro-inflamatory phenotype of our model, we used qPCR to describe the gene expression patterns of IL-6, TNF-α, and IL-1β. These are three well-validated pro-inflammatory markers of neuroinflammation (Harold A Silverman et al., 2014; Skelly et al., 2013).

A one-way ANOVA between Control, 5 mg/kg LPS at 3 hr Post-LPS, 1 mg/kg LPS at 3 hr Post-LPS, and 1 mg/kg LPS at 24 hr Post-LPS revealed that striatal IL-1β (F (3, 14) = 40.26, \( p < 0.0001 \)), TNF-α (F (3, 14) = 39.57, \( p < 0.0001 \)), and IL-6 mRNA (F (3, 14) =
39.57, \( p < 0.0001 \) were significantly upregulated from control in all groups receiving LPS (Figure 1A). Additionally, hippocampal mRNA was significantly up-regulated for IL-1\( \beta \) (F (3, 13) = 9.869, \( p = 0.0012 \)), TNF-\( \alpha \) (F (3, 13) = 56.38, \( p < 0.0001 \)), and IL-6 (F (3, 13) = 89.34, \( p < 0.0001 \)) compared to control for all three LPS groups. The only non-significant change was that 1 mg/kg LPS did not induce IL-6 mRNA expression at 24 hours post-LPS.

5 mg/kg LPS at 3 hours Post-LPS produced the greatest magnitude of change compared to Control for IL-1\( \beta \), TNF-\( \alpha \), and IL-6 across all LPS groups in both the striatum (181-fold, 187-fold, & 64-fold, respectively) and hippocampus (3-fold, 47-fold, & 211-fold, respectively). Across the three pro-inflammatory genes surveyed, IL-1\( \beta \) appeared to exhibit a greater degree of IL-1\( \beta \) induction in the striatum over the hippocampus.

In addition to cytokine expression, we examined markers pertaining to NLRP3 inflammasome gene induction. NLRP3 gene expression was significantly up-regulated from control in the striatum (F (3, 14) = 14.43, \( p = 0.0001 \)). Post hoc comparisons revealed that this was primarily driven from a difference between controls and 5 mg/kg LPS dose at 3 hours Post-LPS (\( M = 10.22, SD = 1.48 \)), \( t(14) = 6.042, p = 0.0002 \) (Figure 1B). Hippocampal NLRP3 mRNA expression exhibited the same pattern of up-regulation, with NLRP3 significantly different in 5 mg/kg LPS dose at 3 hours Post-LPS, compared to Control (\( M = 9.43, SD = 1.089 \)), \( t(13) = 7.142, p < 0.0001 \). Post hoc comparisons revealed that the NLRP3 adaptor molecule ASC was significantly up-regulated in the 1 mg/kg LPS dose at 24 hours Post-LPS in both the striatum (\( M = 2.57, SD = 0.42 \)), \( t(14) = 5.01, p = 0.0011 \)) and hippocampus (\( M = 2.33, SD = 0.67 \)), \( t(13) = 3.578, p = 0.0201 \), compared to controls (Figure 1B).
As PKR was our mechanism of interest, we sought to determine whether its expression would vary based upon LPS dose and if its level of expression changed over time. Striatal PKR mRNA was significantly upregulated in all 3 LPS groups: 5 mg/kg at 3-hours Post-LPS ($M = 1.80, SD = 0.195$), $t(14) = 5.320, p = 0.0006$, 1 mg/kg at 3-hours Post-LPS ($M = 1.712, SD = 0.222$), $t(14) = 4.889, p = 0.0014$, and 1 mg/kg at 24-hours Post-LPS ($M = 1.83, SD = 0.368$), $t(14) = 5.174, p < 0.0008$. There was no significant difference in mean fold change between treatment groups in the striatum. Hippocampal PKR mRNA was significantly upregulated in all 3 treatment groups: 5 mg/kg at 3-hours Post-LPS ($M = 2.79, SD = 0.211$), $t(13) = 6.119, p = 0.0001$, 1 mg/kg at 3-hours Post-LPS ($M = 2.65, SD = 0.226$), $t(13) = 5.799, p = 0.0002$, and 1 mg/kg at 24-hours Post-LPS ($M = 1.80, SD = 0.532$), $t(13) = 3.058, p = 0.0272$. There was no significant difference in mean fold change between treatment groups in the hippocampus (Figure 1C).

3.2 PKR inhibition changed neuroinflammatory phenotype and attenuated NLRP3 inflammasome expression

Following the LPS dose study (Study #1), the 1 mg/kg LPS dose at 24 hours Post-LPS was selected and used in Study #2. Two doses of PKR inhibitor imoxin (0.5 mg/kg or 1.0 mg/kg) were administered 2 and 24 hours prior to LPS.

A one-way ANOVA between Control, 1 mg/kg LPS, 0.5 mg/kg IMX + LPS, and 1.0 mg/kg IMX + LPS revealed that gene expression for striatal IL-1β ($F(4, 27) = 157.8, p < 0.0001$) and TNF-α ($F(4, 26) = 90.24, p < 0.0001$) were significantly upregulated. Hippocampal mRNA was also significantly upregulated for IL-1β ($F(4, 27) = 15.53, p < 0.0001$) and TNF-α ($F(4, 27) = 12.35, p < 0.0001$). As observed for the 1 mg/kg LPS dose at
24 hours in Study #1, IL-6 gene expression in the striatum and hippocampus did not significantly change (Figure 2A).

Striatal NLRP3 gene expression was significantly up-regulated by LPS ($M = 3.04$, $SD = 0.559$), $t(27) = 6.614$, $p < 0.0001$. Although attenuated, striatal NLRP3 gene expression was also significantly up-regulated with 0.5 mg/kg IMX + LPS ($M = 2.27$, $SD = 0.289$), $t(27) = 4.326$, $p = 0.0019$, as well as 1.0 mg/kg IMX + LPS ($M = 2.00$, $SD = 0.826$), $t(27) = 3.392$, $p < 0.0213$. Compared to LPS alone, 1.0 mg/kg IMX + LPS significantly decreased NLRP3 gene expression in the striatum ($t(27) = 3.355$, $p = 0.0234$). 1 mg/kg LPS at 24 hours led to significant gene up-regulation of NLRP3 in the hippocampus ($M = 2.34$, $SD = 0.377$), $t(27) = 6.461$, $p < 0.0001$. Hippocampal NLRP3 expression was significantly increased with 0.5 mg/kg IMX + LPS ($M = 1.64$, $SD = 0.244$), $t(27) = 6.461$, $p < 0.0001$, compared to control, but not significantly different from LPS alone. Additionally, 1.0 mg/kg IMX significantly increased hippocampal NLRP3 gene expression to a comparable magnitude of LPS alone ($M = 2.18$, $SD = 0.469$), $t(27) = 6.461$, $p < 0.0001$. ASC gene expression was not significantly upregulated in the striatum or hippocampus by LPS alone, however, hippocampal ASC was significantly upregulated from Control in the 0.5 mg/kg IMX + LPS ($M = 1.53$, $SD = 0.333$), $t(27) = 3.044$, $p = 0.0455$ and 1.0 mg/kg IMX + LPS ($M = 1.63$, $SD = 0.235$), $t(27) = 3.468$, $p = 0.0157$ groups (Figure 2A).

Gene data mirrored protein analysis (Figure 2B), which found striatal NLRP3 protein to be significantly up-regulated by LPS ($M = 2.15$, $SD = 0.773$), $t(20) = 3.047$, $p = 0.0376$, as well as 1.0 mg/kg IMX + LPS ($M = 2.44$, $SD = 0.632$), $t(20) = 3.836$, $p = 0.0062$. Hippocampal NLRP3 protein expression was significantly up-regulated by LPS ($M = 2.43$, $SD = 1.256$), $t(19) = 3.025$, $p = 0.041$. As seen in the striatum, hippocampal NLPR3
expression was also significantly increased in the 1 mg/kg IMX + LPS group ($M = 2.41$, $SD = 0.930$), $t(19) = 3.136$, $p = 0.0322$. In both regions, NLRP3 protein expression in 0.5 mg/kg IMX + LPS was not significantly different from control.

Consistent with results from Study #1, striatal PKR gene expression was significantly upregulated by LPS ($M = 2.46$, $SD = 0.491$), $t(20) = 7.657$, $p < 0.0001$. Although significantly increased by 0.5 mg/kg IMX + LPS ($M = 1.91$, $SD = 0.370$), $t(20) = 4.931$, $p = 0.0004$ and also 1.0 mg/kg IMX + LPS ($M = 2.11$, $SD = 0.273$), $t(20) = 6.052$, $p < 0.0001$, striatal PKR gene expression appeared to be decreased by 0.5 mg/kg IMX in a similar pattern to pro-inflammatory cytokine and inflammasome expression (Figure 2A). Just as with the striatum, hippocampal PKR gene expression was significantly upregulated by LPS ($M = 2.48$, $SD = 0.271$), $t(27) = 5.946$, $p < 0.0001$, 0.5 mg/kg IMX + LPS ($M = 1.68$, $SD = 0.407$), $t(27) = 3.525$, $p = 0.0137$, and 1.0 mg/kg IMX + LPS ($M = 2.22$, $SD = 0.235$), $t(27) = 5.416$, $p < 0.0001$, although hippocampal PKR expression was decreased by 0.5 mg/kg IMX.

3.3 The effects of PKR inhibition with imoxin may not produce changes that are dependent on its kinase activity

Striatal PKR protein expression was significantly increased with LPS ($M = 1.62$, $SD = 0.419$), $t(20) = 3.374$, $p = 0.018$ and 1.0 mg/kg IMX + LPS ($M = 1.82$, $SD = 0.385$), $t(20) = 4.487$, $p = 0.0014$. As for protein expression of phosphorylated PKR (p-PKR; Thr451) in the striatum, LPS significantly decreased p-PKR ($M = 0.413$, $SD = 0.103$), $t(20) = 5.815$, $p < 0.0001$, as did 0.5 mg/kg IMX + LPS ($M = 519$, $SD = 0.123$), $t(20) = 4.762$, $p = 0.0007$, and 1.0 mg/kg IMX + LPS ($M = 0.635$, $SD = 0.181$), $t(20) = 3.616$, $p = 0.0103$.

Conversely, pairwise comparisons revealed that hippocampal PKR protein was significantly increased by LPS ($M = 1.66$, $SD = 0.474$), $t(20) = 4.129$, $p = 0.0031$. 
Hippocampal p-PKR protein expression was only significantly different between 0.5 mg/kg IMX and 1.0 mg/kg IMX under LPS ($t(20) = 4.946, p = 0.0005$). Collectively, PKR protein expression was increased by LPS in both the striatum and hippocampus, while the 0.5 mg/kg dose of IMX appeared to attenuate striatal PKR expression. While LPS would have been expected to increase p-PKR, we observed a decrease in the striatum (Figure 3). The magnitude of p-PKR protein expression did not vary when data were analyzed as p-PKR alone, compared to p-PKR/total PKR.

3.4 LPS-induced PKR gene expression coincided with time-dependent astrocyte activation, not microglial activation.

In Study #1, striatal GFAP gene expression was significantly upregulated with 5 mg/kg LPS at 3-hours Post-LPS group ($M = 1.52, SD = 0.297$), $t(14) = 3.129, p = 0.0435$ and 1 mg/kg LPS at 24-hours Post-LPS group ($M = 3.24, SD = 0.368$), $t(14) = 8.340, p < 0.0001$. Given the significant up-regulation of GFAP in the 1 mg/kg LPS at 24-hours Post-LPS group compared to the 3-hour post-LPS timepoint using 1 mg/kg LPS ($t(14) = 8.566, p < 0.0001$), it is reasonable to conclude that the magnitude of GFAP gene expression is temporally-dependent on longer time points. While the hippocampus did up-regulate GFAP gene expression 1 mg/kg LPS at 24-hours Post-LPS group ($M = 2.09, SD = 0.414$), $t(13) = 3.653, p = 0.0174$, the magnitude of its expression was not as great as in the striatum (Figure 4A). Under our paradigm, we saw no significant change in IBA1 gene expression in either the hippocampus or striatum.

In Study #2, LPS significantly upregulated striatal GFAP gene expression ($M = 5.77, SD = 0.716$), $t(27) = 15.73, p < 0.0001$, 0.5 mg/kg IMX + LPS ($M = 3.52, SD = 0.333$), $t(27) = 8.66, p < 0.0001$, and 1.0 mg/kg IMX + LPS ($M = 5.56, SD = 0.723$), $t(27) = 15.65, p <$
Furthermore, pairwise comparisons revealed that 0.5 mg/kg IMX +LPS significantly decreased striatal GFAP expression from LPS alone \((t(27) = 7.411, p < 0.0001)\) and 1.0 mg/kg IMX + LPS \((t(27) = 14.43, p < 0.0001)\). Similar to Study #1, the only significant change we observed for IBA1 gene expression was upregulation in the striatum with 0.5 mg/kg IMX + LPS \((M = 2.17, SD = 0.392), t(27) = 3.589, p = 0.0129\). However, this difference is likely attributed to a smaller standard deviation. We observed no significant change for IBA1 gene expression in the hippocampus (Figure 4B).

3.5 PKR inhibition changes protein expression of PKR activator and canonical downstream substrate

Western blotting revealed a significant decrease of phosphorylated PACT (p-PACT) in the striatum with 1 mg/kg IMX + LPS, \((M = 0.648, SD = 0.086), t(20) = 3.743, p = 0.0077\). Hippocampal p-PACT was significantly decreased with 0.5 mg/kg IMX + LPS, \((M = 0.529 SD = 0.152), t(19) = 4.038, p = 0.0042\) and with 1.0 mg/kg IMX + LPS, \((M = 0.305, SD = 0.117), t(19) = 5.966, p < 0.0001\). Furthermore, 1.0 mg/kg IMX + LPS significantly decreased p-PACT, when compared to LPS alone \((t(27) = 4.779, p = 0.0008)\) (Figure 5A). These data suggests that while treatment with imoxin under LPS challenge reduces expression for PKR and other inflammatory mediators, this PKR inhibitor appears to decrease the better-characterized protein activator of PKR (M. A. Garcia et al., 2006).

As PKR’s cognate substrate, we examined whether LPS induced a change in phosphorylated eIF-2α (p-eIF-2α) and if imoxin was capable of modulating its expression. While previous reports have shown that repeated injections of systemic LPS do not modulate p-eIF-2α (Carret-Rebillat et al., 2015), we observed a significant decrease in striatal p-eIF-2α protein with a single dose of LPS \((M = 0.538, SD = 0.129), t(19) = 5.973, p < 0.0001\) (Figure
5B). Striatal p-eIF-2α was significantly decreased with 0.5 mg/kg IMX + LPS ($M = 0.589$, $SD = 0.115$), $t(19) = 5.211$, $p = 0.0003$ and 1.0 mg/kg IMX + LPS ($M = 0.703$, $SD = 0.120$), $t(19) = 3.493$, $p = 0.0145$.

Hippocampal p-eIF-2α protein expression was significantly decreased by LPS alone ($M = 0.597$, $SD = 0.051$), $t(19) = 5.973$, $p < 0.0001$ and with 0.5 mg/kg IMX + LPS ($M = 0.6942$, $SD = 0.054$), $t(19) = 5.973$, $p < 0.0001$, but was restored to baseline by 1.0 mg/kg IMX + LPS. This recovery in LPS-induced p-eIF-2α by imoxin was significantly different from LPS alone in both 0.5 mg/kg IMX + LPS ($t(20) = 6.396$, $p < 0.0001$) and 1.0 mg/kg IMX + LPS ($t(20) = 4.735$, $p = 0.0008$) (Figure 5B). These results suggest that inhibition of PKR in LPS-induced neuroinflammation can restore phosphorylation of eIF-2α in the hippocampus.

3.6 PKR inhibition modulated downstream effectors related to cell proliferation

Striatal ATF3 protein expression was not significantly different in any of the 3 treatment groups alone, compared to control. However, ATF3 was significantly increased between LPS and 1.0 mg/kg IMX + LPS ($t(20) = 4.067$, $p = 0.0036$), and between 0.5 mg/kg IMX + LPS and 1.0 mg/kg IMX + LPS ($t(20) = 3.178$, $p = 0.0280$). Similar to the striatum, hippocampal ATF3 protein expression was significantly different between LPS and 1.0 mg/kg IMX + LPS ($t(20) = 3.571$, $p = 0.011$) (Figure 6A). These results suggest that 1.0 mg/kg IMX can increased striatal and hippocampal ATF3 from LPS alone, and that this dose is more effective than 0.5 mg/kg IMX.

There were no significant differences in hippocampal CDK2 protein expression, however LPS alone led to a significant decrease of CDK2 in the striatum ($M = 0.858$, $SD = 0.037$), $t(20) = 3.117$, $p = 0.0321$ (Figure 6B). While we only observed modest LPS-induced
changes to cell proliferation events via ATF-3 and CDK2, others have reported that cyclin-dependent kinases more strongly mediate this relationship in an acute model rodent model of Huntington’s disease (H. M. Chen et al., 2008).

3.7 Administration of IMX may function through upregulating apoptotic markers early in the downstream signaling pathway, while downregulating others

LPS did not significantly change FADD or procaspase-8 protein expression in the striatum, but 0.5 mg/kg IMX + LPS did significantly increase both FADD ($M = 1.76, SD = 0.336$), $t(15) = 5.089, p = 0.0008$ and procaspase-8 ($M = 2.35, SD = 0.490$), $t(16) = 6.795, p < 0.0001$. This increase to striatal FADD with 0.5 mg/kg IMX + LPS was significantly different from LPS alone ($t(15) = 3.836, p = 0.0097$) and from 1.0 mg/kg IMX + LPS ($t(15) = 3.334, p = 0.0269$). Similarly, the increase to striatal procaspase-8 with 0.5 mg/kg IMX + LPS was significantly different from LPS alone ($t(16) = 5.519, p = 0.0003$) and from 1.0 mg/kg IMX + LPS ($t(16) = 3.501, p = 0.0176$). Striatal procaspase-8 was also significantly increased from control with 1.0 mg/kg IMX + LPS ($M = 1.95, SD = 0.174$), $t(16) = 4.777, p = 0.0012$. There were no changes to hippocampal FADD expression, but hippocampal procaspase-8 was significantly decreased with LPS ($M = 0.772, SD = 0.096$), $t(20) = 5.47, p = 0.0001$, with 0.5 mg/kg IMX + LPS ($M = 0.806, SD = 0.053$), $t(20) = 4.672, p = 0.0009$, and with 1.0 mg/kg IMX + LPS ($M = 0.768, SD = 0.044$), $t(20) = 5.572, p = 0.0001$.

LPS significantly decreased RIP1K in the striatum ($M = 0.694, SD = 0.213$), $t(20) = 3.777, p = 0.0071$, along with 0.5 mg/kg IMX + LPS ($M = 0.595, SD = 0.075$), $t(20) = 4.993, p = 0.0004$ and 1.0 mg/kg IMX + LPS ($M = 0.519, SD = 0.063$), $t(20) = 5.931, p < 0.0001$.

The hippocampus displayed an opposite RIP1K expression pattern. RIPK1 protein expression was significantly increased with 1.0 mg/kg IMX + LPS ($M = 1.319, SD = 0.137$),
t(23) = 3.991, \( p = 0.0035 \). This increase was also significantly different from LPS alone \( (t(23) = 6.06, p < 0.0001) \) and from 0.5 mg/kg IMX + LPS \( (t(23) = 3.90, p = 0.0043) \). In the striatum we observed an LPS-induced decrease in RIPK1 protein expression that was not changed by imoxin, while 1.0 mg/kg IMX + LPS increased hippocampal RIPK1, compared to control, LPS alone, and 0.5 mg IMX + LPS.

RIP1 kinase has previously been shown to serve as a regulator of cell-death which authorizes NF-\( \kappa \)B (Kawai & Akira, 2006). LPS significantly decreased striatal p65 protein expression \( (M = 0.731, SD = 0.118), t(20) = 3.705, p = 0.0084, \) as well as 0.5 mg/kg IMX + LPS \( (M = 0.756, SD = 0.147), t(20) = 3.354, p = 0.0188, \) although to a lesser extent. The hippocampus did not reveal any p65 changes.

**Discussion**

Our studies assessed CNS PKR expression following an acute systemic immune challenge and observed a severe degree of overlap in inflammatory and apoptotic signaling pathways. The is the first evidence of CNS PKR expression following a single, low-grade LPS challenge, in addition to showing that PKR is not dose- or duration-dependent, and that PKR’s function may not depend on its kinase action. Others have reported CNS PKR expression following repeated LPS (Carret-Rebillat et al., 2015) and subcutaneous boluses of cultured *E. coli* (Poon et al., 2015). Transcriptionally, this activation occurs by at least 3 hours post-challenge and remains elevated through 24 hours, suggesting that PKR is not an acute phase stress signal in the brain. Post-mortem observations of increased p-PKR and PKR in the brain of neurodegenerative patients supports the notion that this signal is enduring throughout disease progression (Bando et al., 2005; Chang et al., 2002; A. L. Peel et al., 2001). Furthermore, the relative magnitude of PKR gene expression was not largely
different between the hippocampus and striatum, suggesting that neither region has a greater proclivity over one another to express LPS-induced PKR. This could be meaningful if PKR became a therapeutic molecule of interest, because its expression may be relatively global and drug action would not be anatomically restricted to one region of interest.

Despite a highly selective substrate-recognition mechanism, PKR activates in response to a multitude of cellular stresses across various disease states. Pharmacological inhibition with the PKR inhibitor imoxin (aka C16 and PKRi) has previously been shown to protect against tunicamycin-induced apoptosis in SH-SY5Y neurons (Shimazawa & Hara, 2006). Additionally, pretreatment of SH-SY5Y neuronal cells with imoxin in an in vitro model of Alzheimer’s disease ameliorated Aβ42-induced increases in PKR phosphorylation (Thr451) (Couturier et al., 2010). While this ATP binding site-directed inhibitor blocks autophosphorylation of PKR (Jammi et al., 2003), it has also been shown to promiscuously produce a number of PKR-independent effects (Nakamura et al., 2014; Naz et al., 2015; Weintraub et al., 2016).

4.1 LPS decreased PKR phosphorylation in the striatum recovered with Imoxin

Challenge with LPS is known to influence a myriad of synergistic cell signaling processes related to proliferation, cell death (apoptotic and necrotic), and inflammation (C. Cunningham, Wilcockson, Campion, Lunnon, & Perry, 2005; Godbout et al., 2005; Hoogland et al., 2015; Qin et al., 2007). As an inflammatory insult, LPS was hypothesized to increase p-PKR, and subsequently total PKR protein expression. We observed increased total PKR with decreased p-PKR/PKR in the striatum. Imoxin did appear to recover this LPS-induced decrease in p-PKR/PKR, although non-significantly. In an effort to clarify these mixed findings, we performed protein analysis on excess tissue from samples taken in Study
#1 (cerebellum). We hypothesized that p-PKR, while downregulated in the striatum at 24 hours post-LPS with 1.0 mg/kg LPS, would be upregulated with 1.0 mg/kg LPS at 3 hours post-LPS in the cerebellum. Following a one-way ANOVA between control, 1 mg/kg LPS at 3 hours, and 1 mg/kg LPS at 24 hours, we observed that p-PKR/PKR was significantly increased with 1.0 mg/kg LPS at 3 hours post-LPS ($M = 1.36$, $SD = 0.117$), $t(11) = 2.835$, $p = 0.0479$. There were no LPS-induced changes in total PKR expression in the cerebellum (Supplementary Figure 1). We can conclude that PKR phosphorylation (Thr451) increases early and decreases by 24 hours, implying that our post-LPS collection time used in Study #2 did not accurately capture the kinetics of PKR phosphorylation.

4.2 LPS-induced decrease of eIF2-α phosphorylation in the striatum and hippocampus was recovered with 1.0 mg/kg IMX in striatum

Phosphorylation of PKR has been shown to directly correspond to the phosphorylation of eukaryotic translation initiation factor 2 (p-eIF2-α) and subsequent regulation of apoptosis in AD cell models (Chang et al., 2002), AD rodent models (A. Peel, 2003), and human AD lymphocytes (Paccalin et al., 2006). Furthermore, the inhibition of the PKR/ eIF2-α pathway in neonatal rat brains has been shown to reduce apoptosis, while not stimulating the mTOR survival pathway (Ingrand et al., 2007). Conversely, inhibiting eIF2-α phosphorylation does not fully protect cells from TNF-α-induced apoptosis (S. P. Srivastava, Kumar, & Kaufman, 1998). We observed LPS-induced decreases in p-eIF2-α in the striatum and hippocampus, with imoxin appearing to recover this decrease. Similarly, treatment of primary rat cortical neurons with either apoptotic-inducing homocysteic acid or camptothecin has been shown to decrease p-eIF2-α, with imoxin treatment recovering its expression (H. M. Chen et al., 2008).
4.3 LPS did not significantly change PACT phosphorylation in the striatum or hippocampus, but treatment with 1.0 mg/kg IMX significantly reduced PACT phosphorylation.

As the primary non-viral protein activator of PKR, PACT activation has been deemed necessary for PKR activation in non-viral forms of cell stress (Li et al., 2006; Singh, Fowlkes, Handy, Patel, & Patel, 2009). Phosphorylation of PACT (p-PACT) has previously been shown to directly correspond with p-eIF2-α activation (T. Ito et al., 1999; Marchal et al., 2014), although we observed LPS+imoxin-induced decreases of p-PACT with concurrent striatal and hippocampal increases in p-eIF2-α expression. While LPS did not significantly change p-PACT expression, the combination of imoxin with LPS led to a decrease in its protein expression within the striatum and hippocampus. Based on our protein analysis, we saw no changes in striatal or hippocampal total PACT levels following LPS. This finding is in line with previous reports that stress in human and mouse cells does not cause total PACT/RAX levels to vary, but that stressful stimuli lead to the phosphorylation of PACT (R. L. Bennett, Blalock, & May, 2004). Knowing that PKR signaling exhibits diverse responses under various conditions of host stress (M. A. Garcia et al., 2006), we can simply posit that the therapeutic action of imoxin likely influences cell signaling that is dependent and independent of direct PKR signaling following a single dose of LPS.

4.4 LPS significantly reduced striatal CK2 expression, but there were no other LPS-induced changes to cell proliferation activity.

There were largely no changes to ATF-3 or CDK2 protein expression, except the novel observation that acute LPS decreased striatal CDK2 expression. This observation is consistent with in vitro and in vivo reports that CDK2 is downregulated by LPS in models of...
diabetic nephropathy (Saurus et al., 2016). In an acute 3-nitropropionic acid (3-NP) model of Huntingtin’s disease, which causes selective death to striatal neurons, PKR inhibition with imoxin has been shown to be neuroprotective by acting through cyclin-dependent kinase 2 (CDK2) (H. M. Chen et al., 2008). These authors also conclude that IMX inhibits expression of pro-apoptotic activating transcription factor 3 (ATF-3) in primary neuronal cultures and in vivo, which they conclude functions as an apoptosis-regulated protein that contributes to cell cycle activation during stress. ATF-3 upregulation is associated with a host of cell stresses (Hai, Wolfgang, Marsee, Allen, & Sivaprasad, 1999), is selectively induced by PKR (Guerra, López-Fernández, García, Zaballos, & Esteban, 2006) and has been implicated as a key signaling event in the eIF-2 cell stress response (H.-Y. Jiang et al., 2004).

4.5 FADD and Caspase-8 apoptotic signaling increased with 0.5 mg/kg IMX + LPS

PKR has been shown to be required for interferon-induced receptor interacting kinase 1 (RIP1K) necrosis, which is negatively regulated by FADD and caspase-8 (Thapa et al., 2013). Following cell surface binding of the FAS and tumor necrosis factor receptor-1 (TNFR-1) death receptors, Fas-associated death domain (FADD) activation leads to a pro-apoptotic cascade (Chinnaiyan, O'Rourke, Tewari, & Dixit, 1995) that recruits caspase-8 and activates downstream effector caspases (e.g., caspase-3) (Kreuz et al., 2004). Overexpression of FADD in vitro has been shown to protect SH-SY5Y neurons from TNF- and Aβ-induced apoptosis (Cantarella et al., 2003). PKR inhibition with imoxin in Aβ-treated neurons has been shown to reduce p-PKR/FADD nuclear translocation and to protect against capase-8 and -3 induction (Couturier et al., 2010). PKR localizes to both the cytoplasm and nucleus (Jeffrey et al., 1995). Although poorly understood, nuclear accumulation of PKR may be indicative of neuronal stress (Couturier et al., 2010). Moreover, LPS challenge of bone
marrow-derived macrophages (BMDMs) has been shown to activate the NLRP3 inflammasome and that FADD and caspase-8 are upstream mediators of this expression (Gurung et al., 2014). NLRP3 activation has also been shown to be regulated by upstream RIP1K (Tao et al., 2018; Zhou et al., 2017). These findings suggest that in our studies, FADD/Caspase-8 may be a signaling platform through which we observed LPS-induced increased in PKR and NLRP3 expression. The increases in striatal FADD and Caspase-8 protein expression with 0.5 mg/kg IMX + LPS may arguably account for the observed decreases in striatal PKR and NLRP3 with this treatment. Additionally, this relationship has previously been observed to not be dependent on PKR phosphorylation (von Roretz & Gallouzi, 2010).

4.6 Striatal RIP1K and NF-κB p65 were significantly reduced with LPS and PKR inhibition did not recover these decreases.

FADD-mediated apoptosis has also been shown be regulated by RIP1K in adaptive lymphocytes and to mediate NF-κB activation (H. Zhang et al., 2011). In models of interferon-induced cell death, PKR-RIP1K signaling has been shown to be regulated by FADD (Thapa et al., 2013). RIP1K has recently been identified as a critical mediator of promoting a disease-associated microglial (DAM) phenotype in an APP/PS1 model of Alzheimer’s disease (Ofengeim et al., 2017) and has been shown to be critical for TNF-α-induced apoptosis in vitro and in vivo (Meng et al., 2018). As systemic LPS has been consistently observed to produce aberrant microglial activation (C. Cunningham et al., 2005; Godbout et al., 2005; Hoogland et al., 2015; Qin et al., 2007), increased apoptosis driven by increased RIP1K expression is expected with these changes. However, we did not observe a gene signature of microglial activation and observed an LPS-induced decrease in striatal and
hippocampal RIP1K. This RIP1K decrease could function to inhibit cell death (Ma, Temkin, Liu, & Pope, 2005). These events are also influenced by NF-κB, which has been shown to be protective against cell death following interferon-induced RIP1K activation (Ma et al., 2005). In our studies, LPS decreased NF-κB p65 expression in the striatum, with IMX appearing to restore that decrease. Furthermore, catalytically inactive forms of PKR alone are capable of activating NF-κB, thereby mitigating consideration of PKR/eIF2-α signaling in this experimental context (Bonnet et al., 2000).

4.7 LPS did not induce microglial activation in the striatum or hippocampus, but LPS did induce astrocyte activation, which was significantly reduced with 0.5 mg/kg imoxin.

Microglia are believed to be the primary harbingers of cytokine production under neuroinflammatory contexts (Colm Cunningham, 2013; DiSabato et al., 2016; Jeong et al., 2013). They are also indicated as the primary neuro-effectors of NLRP3 (Gustin et al., 2015), although astrocytes are indeed capable of NLRP3 production (Song, Pei, Yao, Wu, & Shang, 2017). Initial reports had implicated PKR and its kinase function to be a key regulator in inflammasome activation (Lu et al., 2012). Subsequent reports went on to dispute this role was independent of PKR’s kinase action (Hett et al., 2013), while others have observed that PKR activity does not modulate the inflammasome at all (He et al., 2013), or that PKR actually suppresses inflammasome activity (Yim et al., 2016). We observed significantly increased gene and protein expression of PKR in the hippocampus and striatum in both the LPS dose study (Study #1) and PKR inhibitor study (Study #2). This expression coincided with significant upregulation of gene and protein NLRP3, as well as, significant gene induction of TNF-α and IL-1β. Conversely, IL-6 exhibited a transient, acute phase response that appeared to dissipate by 24 hours with 1 mg/kg LPS. From our inhibitor study, we
observed concurrent LPS-induction of PKR and NLRP3, both of which were attenuated by 0.5 mg/kg imoxin. This PKR and NLRP3 decrease with 0.5 mg/kg IMX + LPS may be negatively regulated by FADD/Caspase-8, which to our knowledge, would be a novel finding in acute LPS models of neuroinflammation. Our studies did not explore the functional implications of this observation and it could merely be coincidental. Others have reported that inhibiting PKR autophosphorylation with the flavonoid luteolin actually increases NLRP3 activation in macrophages and microglia (Dabo et al., 2017).

Although, previous reports have shown amelioration of IBA1 microglial activity in PKR KO mice (Carret-Rebillat et al., 2015), we observed an absence of IBA1 gene expression in both the striatum and hippocampus, under all LPS doses. Lack of IBA1 gene expression may likely be a by-product of our 3 and/or 24-hour post-LPS collection. Mixed reports in mice have cited IBA1 gene down-regulation at 4 hours post-LPS (single dose; 8 mg/kg IP) (Hoogland et al., 2015; Harold A Silverman et al., 2014) and increased IBA1 protein expression via immunohistochemistry at 3 hours post-LPS (single dose; 5 mg/kg IP) (Qin et al., 2007). However, the phenotype of pro-inflammatory cytokine expression observed in our studies has been suggested to precede IBA1 changes following LPS in mice (Norden et al., 2016). Therefore, microglia would very reasonably be expected to mediate our observed LPS-induced neuroinflammation, but we did not capture their involvement at our timepoint.

Alternatively, peak GFAP astrocyte expression has appeared to indicate the resolution of microglial activation (Norden et al., 2016). We observed robust GFAP gene induction in our model and it responded to PKR inhibition, similar as PKR, NLRP3, and IL-1β. Thus, the induction of our pro-inflammatory signature could very well be microglial-derived and our
observed astrocyte activation could be secondary. Conversely, reactive astrocytes are induced by nitric oxide (Brahmachari, Fung, & Pahan, 2006) and PKR has been implicated as an essential component of this mechanism (He et al., 2013). Given our observation of robust GFAP gene induction and that glial expression of receptors for IFN-γ are most abundant on astrocytes (Hashioka, Klegeris, Schwab, Yu, & McGeer, 2010), we hypothesized that the pro-inflammatory cytokine IFN-γ promoted astrocyte activation and subsequent PKR expression. However, we observed no difference in IFN-γ protein expression in either the hippocampus or striatum (data not shown). Overall, the exact contribution of microglia and astrocytes to our observed neuroinflammatory signature is unclear, due to temporal limitations. We can conclude that PKR expression is not dependent on timing or LPS dose and that it appears to coincide with astrocyte activation.

While characterizing differential CNS PKR expression and its concurrent inflammatory phenotype was a primary objective of this study, one limitation of the current study is that our immunophenotyping of astrocytes and microglia was limited solely to gene expression. These cells have only recently become identified for their enormous capacity for disease-state heterogeneity (Gosselin et al., 2017; Keren-Shaul et al., 2017; Mathys et al., 2017; Norden et al., 2016; Ransohoff, 2016b). As cytokines mediate sickness behavior (Dantzer, 2001a; Dantzer et al., 2008), it would also be helpful to include behavioral measurements to assess the extent that cytokines may mediate CNS PKR expression and whether imoxin can recover LPS-induced behavioral detriments. Finally, the current study utilized a well-established model of immune challenge to interrogate peripheral-CNS communication. Future studies should expand upon PKR’s exact role in individual disease states by examining its expression in specific neurodegenerative disease models.
4.8 Conclusion

These data add to the increasingly staggering body of literature implicating a role for systemic inflammation in neurological disease, which is shown to precede the clinical manifestation of neurodegeneration. The present study provides *in vivo* evidence that CNS RNA-dependent protein kinase (PKR) expression is induced following a single, systemic administration of LPS and appears to function outside if its canonical anti-viral role (Berry et al., 1985; Clemens, 1997). Although further studies are needed to clarify the therapeutic potential of PKR inhibition in individual neurodegenerative disease models, we believe PKR could be used an indicator of disturbed CNS homeostasis following systemic inflammation and that inhibition of PKR could likely produce therapeutic benefit by modulating apoptotic pathways. Collectively, this work suggests that PKR does not function as an effector molecule in systemic inflammation, but rather acts as a node whose functions are dependent on the availability of players in the response network and the context of the inflammatory challenge.
Figure 2.1 A single, systemic injection of LPS produces a pro-neuroinflammatory gene signature in the hippocampus and striatum.

Animals received single I.P. injections of either saline, 5 mg/kg LPS and tissue collected 3 hours later, 1 mg/kg LPS and tissue collected 3 hours later, or 1 mg/kg LPS and tissue collected 24 hours later. (A) Gene induction for IL-1β, TNF-α, IL-6 was greatest with 5 mg/kg LPS. (B) NLRP3 inflammasome gene expression was significantly increased with 5 mg/kg LPS at 3 hours post-LPS. ASC gene expression was opposite of NLRP3, with significant up-regulation with 1 mg/kg LPS at 24 hours post-LPS. (C) Striatal and hippocampal PKR gene expression was significantly upregulated by LPS in all three treatment groups and the magnitude of its expression did not significantly vary between groups. Data represented as mean fold change ± 95% CI on a Log2 scale. Significance above bar denotes difference from control, with between group differences denoted by line. ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05 by 1-way ANOVA (n = 3-5 per group).
Figure 2.2 Pharmacological inhibition of PKR with imoxin (IMX) reduces LPS-induced neuroinflammation.

(A) 1 mg/kg LPS at 24 hours post-LPS reproduced the neuroinflammatory phenotype observed in Study #1 and pre-LPS treatment with 2 S.C. doses of 0.5 mg/kg IMX + LPS appeared to reduce gene induction the greatest for IL-1β, TNF-α, NLRP3, and PKR. (B) LPS-induced NLRP3 protein expression was attenuated with 0.5 mg/kg IMX. Data represented as mean fold change ± 95% CI on a Log2 scale, or linear scale for protein data. Cropped Western Blot images show group representative samples run from the same gel, with corresponding housekeeping control. Significance above bar denotes difference from control, with between group differences denoted by line. ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05 by 1-way ANOVA. (n = 6-7 per group)
Figure 2.3 LPS downregulates PKR phosphorylation (p-PKR) in the striatum, while upregulating total PKR protein expression in the striatum and hippocampus.

(A) p-PKR is downregulated in the striatum and the hippocampus had no significant differences from control. Total PKR protein expression was upregulated by LPS in both the striatum and hippocampus. Data represented as mean fold change ± 95% CI on a linear scale. Cropped Western Blot images show group representative samples run from the same gel, with corresponding housekeeping control. Significance above bar denotes difference from control, with between group differences denoted by line. ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05 by 1-way ANOVA. (n = 6-7 per group)
Figure 2.4 LPS induces astrocyte activation that is significantly reduced by 0.5 mg/kg imoxin, while producing no changes to microglial activation.

(A) In Study #1, GFAP gene induction was greatest with 1 mg/kg LPS at 24 hours post-LPS in the striatum and hippocampus. There were no significant differences in IBA1 gene expression. (B) In Study #2, striatal GFAP expression was significantly decreased by 0.5 mg/kg imoxin + LPS, while still remaining significantly elevated from control. Hippocampal GFAP gene expression was significantly increased from control in all treatment groups. Consistent with Study #1, LPS did not induce IBA1 gene expression. Data represented as mean fold change ± 95% CI on a Log2 scale. Cropped Western Blot images show group representative samples run from the same gel, with corresponding housekeeping control. Significance above bar denotes difference from control, with between group differences denoted by line. ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05 by 1-way ANOVA. (n = 4-7 per group)
Figure 2.5. PKR inhibition reduced PKR protein activator (p-PACT) expression, while LPS reduced protein expression for stereotypical PKR substrate (p-eIF2-α).

(A) PACT phosphorylation was significantly decreased with 1.0 mg/kg imoxin + LPS in the striatum and hippocampus, although not by LPS alone. (B) LPS significantly down-regulated phosphorylation of eukaryotic translation initiation factor 2 (p-eIF2-α). Pre-treatment with imoxin recovered this LPS-induced decrease and these differences were significant in the hippocampus. Data represented as mean fold change ± 95% CI on a linear scale. Cropped Western Blot images show group representative samples run from the same gel, with corresponding housekeeping control. Significance above bar denotes difference from control, with between group differences denoted by line. ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05 by 1-way ANOVA. (n = 6-7 per group)
Figure 2.6 Neither LPS or Imoxin caused major change in cell proliferation markers.

(A) 1.0 mg/kg imoxin + LPS significantly increased ATF3 from LPS alone in the striatum and hippocampus.
(B) LPS significantly decreased striatal CDK2 protein expression. Data represented as mean fold change ± 95% CI on a linear scale. Cropped Western Blot images show group representative samples run from the same gel, with corresponding housekeeping control. Significance above bar denotes difference from control, with between group differences denoted by line. ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05 by 1-way ANOVA. (n = 6-7 per group)
Figure 2.7 Downstream signaling suggests an apoptotic function for PKR following acute LPS challenge.

(A) LPS alone did not change FADD or Procaspase-8 protein expression in the striatum, but both were significantly increased with 0.5 mg/kg IMX + LPS. Hippocampal procaspase-8 was significantly decreased from control in all groups. (B) LPS significantly decreased striatal RIP1K expression and imoxin did not change this. Hippocampal RIP1K expression was significantly increased with 1.0 mg/kg imoxin +LPS, compared to LPS alone. Protein expression of proinflammatory NF-κB p65 was decreased with LPS in the striatum, with no changes occurring in the hippocampus. Data represented as mean fold change ± 95% CI on a linear scale. Cropped Western Blot images show group representative samples run from the same gel, with corresponding housekeeping control. Significance above bar denotes difference from control, with between group differences denoted by line. ****p < 0.0001, *** p < 0.001, ** p < 0.01, * p < 0.05 by 1-way ANOVA. (n = 6-7 per group)
The diverse response by PKR suggest that it signaling events are cell type-specific and context-dependent. A number of detrimental cell stresses have been shown to induce PKR expression, including inflammation, ER/oxidative stress, and dsRNA viral intermediates (Guerra et al., 2006; Marchal et al., 2014). Noxious cellular stimuli produced by these events leads to surface receptor binding that triggers a signaling cascade that can either activate PKR directly, or via phosphorylation of the PKR protein activator PACT (R. L. Bennett et al., 2004). PACT has high activation potential, given that a multitude of factors can lead to it phosphorylation, including IL-3 deprivation, H$_2$O$_2$, and TNF-$\alpha$, among others (Gilbert, Duance, & Mason, 2002; T. Ito et al., 1999). Once activated, PKR has been shown to regulate translation through numerous regulatory pathways, including IRF, STAT, p53, MAPKs, ATF3, and NF-$\kappa$B (M. A. Garcia et al., 2006). Identified as being largely localized to the cytoplasm, p-PKR fragments have been shown to accumulate in the nucleus following tunicamycin-induced apoptosis (Onuki et al., 2004). From our studies, we observed LPS-induced protein changes relating to cell growth and proliferation (ATF-3 and CDK2), and inflammation (RIP1K and p65). Moreover, we observed a negative relationship between FADD and Procaspase-8 with PKR in the striatum; inhibiting PKR with 0.5 mg/kg imoxin decreased PKR, while increasing FADD. PKR associates with RIP1K, which has been shown to be negatively regulated by FADD (Thapa et al., 2013). Therefore, FADD may negatively PKR and could represent a novel effect of imoxin. Neither of these pathways would be expected to function autonomously, but synergistically contribute to apoptosis. Furthermore, we observed changes to most of these proteins following PKR inhibition with imoxin, suggesting that the therapeutic potential of inhibiting PKR will likely exert PKR-dependent and PKR-independent effects on cell signaling. While we examined CNS PKR expression following a single, systemic injection of LPS, sustained inflammatory cell stress and subsequent PKR activation would be expected to promote pro-apoptotic processes and contribute to the neurodegenerative cascade associated with chronic neuroinflammation (C. Cunningham et al., 2009; Murray, Skelly, & Cunningham, 2011; Perry, 2004; Skelly et al., 2013).
Table 2.1 LPS-induced weight loss and was not changed by imoxin.

(A) The greatest amount of weight loss in the LPS dose study (Study #1) occurred in mice receiving 1 mg/kg LPS with collection 24 hours post-LPS. (B) The selected 1.0 mg/kg LPS dose reproducibly caused an equal amount of weight loss in the PKR inhibitor study (Study #2). Administration of imoxin itself did not produce weight loss. Data represented as mean fold change ± 95% CI (n = 3-5 per group).

<table>
<thead>
<tr>
<th>Group</th>
<th>Avg. Pre-LPS Weight (g)</th>
<th>Avg. Post-LPS Weight (g)</th>
<th>% Weight Loss of Original</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25.60 ± 1.24</td>
<td>25.18 ± 1.18</td>
<td>98.34</td>
</tr>
<tr>
<td>5 mg/kg LPS – 3 hr</td>
<td>26.87 ± 1.64</td>
<td>25.95 ± 1.30</td>
<td>96.45</td>
</tr>
<tr>
<td>1 mg/kg LPS – 3 hr</td>
<td>26.02 ± 1.52</td>
<td>24.93 ± 1.48</td>
<td>95.83</td>
</tr>
<tr>
<td>1 mg/kg LPS – 24 hr</td>
<td>27.31 ± 0.88</td>
<td>24.38 ± 1.17</td>
<td>89.26</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>Avg. Pre-LPS Weight (g)</th>
<th>Avg. Post-LPS Weight (g)</th>
<th>% Weight Loss of Original</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>22.98 ± 1.22</td>
<td>23.34 ± 1.35</td>
<td>101.53</td>
</tr>
<tr>
<td>Saline + 1.0 mg/kg IMX</td>
<td>21.91 ± 1.53</td>
<td>22.74 ± 1.38</td>
<td>103.78</td>
</tr>
<tr>
<td>LPS + Saline</td>
<td>24.00 ± 0.98</td>
<td>21.42 ± 1.28</td>
<td>89.24</td>
</tr>
<tr>
<td>LPS + 0.5 mg/kg IMX</td>
<td>21.89 ± 0.67</td>
<td>19.54 ± 0.57</td>
<td>89.26</td>
</tr>
<tr>
<td>LPS + 1.0 mg/kg IMX</td>
<td>22.04 ± 1.02</td>
<td>19.96 ± 0.67</td>
<td>90.53</td>
</tr>
</tbody>
</table>
Table 2.2 Primer sequence information for qPCR assays.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5’ → 3’)</th>
<th>Reverse (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>GCTTCAGGCAGGCAGTATC</td>
<td>AGGATGGGCTCTTCTTCTCAAAG</td>
</tr>
<tr>
<td>TNF-α</td>
<td>AGCCGATGGGTTGTACCTTG</td>
<td>ATAGCAAAATCGGCTGACGGT</td>
</tr>
<tr>
<td>IL-6</td>
<td>ACCGCTATGAAGTTCTCTCTC</td>
<td>CTCTGTGAAATGTCCTCCTCC</td>
</tr>
<tr>
<td>NLRP3</td>
<td>GCTCCAACCATTCTCTGACC</td>
<td>AAGTAAGGCCGGAATTCACC</td>
</tr>
<tr>
<td>ASC</td>
<td>AGGAGTGAGGAGGGAAGC</td>
<td>AGAAGACGCAGGAAGATGG</td>
</tr>
<tr>
<td>PKR</td>
<td>GATGGAATAATCCCAACAAGG</td>
<td>AGGCCCCAAAGCAAGATGTCC AG</td>
</tr>
<tr>
<td>GFAP</td>
<td>GGTTGAATCGCTGAGGAAG</td>
<td>CTGTGAGGTCTGGCTTTG</td>
</tr>
<tr>
<td>IBA1</td>
<td>CTTGAAGCGAAATGCCTGAGAA</td>
<td>GGCAGCTGGAGATGCTTTT</td>
</tr>
<tr>
<td>β-Actin</td>
<td>GTGACGTTGCATCCGTAAG</td>
<td>GCCGGACTCATCGTACTCC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>AGGTCGGTGTAACGGATTG</td>
<td>TGTAGACCATGTAATGAGGTCA</td>
</tr>
</tbody>
</table>
Supplementary Figure 2.1 Phosphorylated PKR (p-PKR) is significantly increased in the cerebellum, compared to control, at 3 hours Post-LPS.

From the initial LPS dose study, p-PKR is up-regulated at 3 hours post-challenge with 1 mg/kg LPS, with expression decreasing by 24 hours post-challenge with 1 mg/kg LPS. Data represented as mean fold change ± 95% CI on a linear scale. Significance above bar denotes difference from control. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ by 1-way ANOVA (n = 3-5 per group).
References


Huntington’s disease (HD) transcripts and is activated in HD tissue. *Human molecular genetics, 10*(15), 1531-1538.


CHAPTER 3. RESPIRATORY INFECTION WITH INFLUENZA A/PR/8/34 (H1N1) INDUCES LATENT STRIATAL AND HIPPOCAMPAL PKR EXPRESSION IN A MURINE MULTI-HIT MODEL OF NON-NEUROTROPIC INFLUENZA AND ACUTE MPTP

Matthew A. Jefferson1,2,*, Neeraj Singh3, Arthi Kanthasamy1,3, Rudy J. Valentine2, & Marian L Kohut1,2

A manuscript prepared for submission to npj Parkinson’s

1Interdepartmental Neuroscience Graduate Program, Iowa State University, Ames, IA
2Department of Kinesiology, Iowa State University, Ames, IA
3Department of Biomedical Sciences, Iowa State University, Ames, IA

* Author for correspondence (MAJ): 235 Forker Building, Department of Kinesiology, Iowa State University, Ames, IA 50011-4008. E-mail: mjefferson83J@gmail.edu. Telephone: (309) 781-7590. (515) 294-8009.

Abstract

Neuroinflammation resulting from various host and environmental factor detriments has emerged as a well-recognized component of Parkinsonian pathogenesis. Respiratory infection with non-neurotropic strains of influenza are capable of producing Parkinsonian sequelae, exemplified best by historic epidemiology of pandemic influenza outbreaks, and neuroinflammation has been implicated as the pathogenic mechanism mediating this relationship. However, the challenge of identifying neuroinflammatory preclinical drug candidates is understanding whether their cell signaling behavior is protective or pathogenic. Our lab has implicated the expression of double-stranded RNA-dependent protein kinase (PKR) as an intracellular danger-sensing mechanism that activates in response to influenza-induced neuroinflammation. To determine its potential relevancy to Parkinsonian pathogenesis, we used an intranasal mouse model of influenza infection to induce systemic inflammation, followed by an acute MPTP challenge (4x, 18 mg/kg i.p.). Under this multi-hit model of dual influenza + MPTP-mediated neurotoxicity, we anticipated synergistic
expression of CNS PKR. Although we demonstrated that influenza infection in the peripheral immune system can directly produce a proinflammatory microglial phenotype our evidence does not suggest that influenza-induced PKR expression in the brain is a direct a neuroinflammatory effector, but more likely a delayed signal transducer that promotes apoptotic and inflammatory signaling. Whether this PKR-mediated cell stress response is pro-survival or pro-death is unclear. Given that influenza infections result in 3-5 million global cases of severe illness annually, it is pertinent to identify novel mechanisms of how peripheral sickness may impact the brain and precipitate neurodegenerative cascades.

**Introduction**

Clinically described as a neurodegenerative movement disorder, Parkinson’s disease (PD) is pathologically defined by the progressive degeneration of dopaminergic neurons in the nigrostriatal pathway that produces bradykinesia, muscle rigidity, postural instability, gait freezing, and a resting tremor (Mosley, Hutter-Saunders, Stone, & Gendelman, 2012; Poewe et al., 2017). As for its etiology, we have come to recognize that the clinical manifestation of PD largely results from a combination of environmental neuroinflammatory insults that accumulate over the course of one’s lifetime and precipitously disrupt homeostasis within the central nervous system (CNS) (Amor et al., 2014; Chao, Wong, & Tan, 2014; Colm Cunningham, 2013).

Of implicated risk factors, influenza infections-alone have long been recognized to produce behavioral and cognitive deficits that are attributed to systemic inflammation (Dantzer, 2001b). Additionally, the relationship between Parkinsonism and influenza infection has been readily recognized for the past 40 years (Moore, 1977), epidemiologically exemplified by the 1918 H1N1 Spanish Flu pandemic. Following primary respiratory infection, a secondary disease state referred to as encephalitic lethargica (EL) was observed
for up to a decade later and was characterized by lethargy, ophthalmoplegia, and delirium (Henry et al., 2010). Approximately 80% of these patients went on to develop post-encephalitic Parkinsonism (PEP), which included bradykinesia and a resting tremor (Cunha, 2004; Maurizi, 2010). The notion that influenza infection can promote Parkinsonism is further supported by the observation that antivirals have therapeutic benefit for the clinical management of PD (Pahwa et al., 2015; Sadasivan et al., 2017).

Intracerebral administration of neurotropic strains of influenza A in animal models have previously shown preferential localization to the substantia nigra (Yamada, 1996), which could provide direct evidence of how influenza may produce a Parkinsonian sequelae. Additionally, C57BL/6 mice intranasally inoculated with influenza A/Vietnam/1203/04 (H5N1), a neurotropic virus that invades the CNS, produced long-lasting microglial activation and aggregation of α-synuclein that promoted degeneration of dopaminergic neurons in the substantia nigra (H. Jang et al., 2009). However, most influenza strains are non-neurotropic (G. F. Wang et al., 2010) and are still capable of producing long-lasting neurobehavioral deficits (Luyt et al., 2012), suggesting that the neuropathological effect of seasonal influenza on CNS function is through an indirect route.

Systemic inflammation has recently emerged as a primary pathological mechanism in which influenza infection can disrupt CNS homeostasis (C. Cunningham, Campion, Teeling, Felton, & Perry, 2007; Perry, 2004; Perry & Teeling, 2013). Infection with non-neurotropic influenza produces activation of the peripheral innate immune system, which leads to increased expression of the pro-inflammatory cytokines TNF-α, IL-6, and IL-1β in the hippocampus of male BALB/c mice 7 days post-infection with influenza A/PR/8/34 (Jurgens et al., 2012). This increased pro-inflammatory expression was met with increases in MHC-II
and IBA1 expression, implicating microglia as the primary harbingers of this neuroinflammatory response.

As it relates to Parkinsonian pathogenesis, intranasal infection of mice with non-neurotropic influenza A/California/04/2009 (H1N1) has been shown to produce chronic microglial activation in both the substantia nigra and hippocampus beginning 21 days post-infection (DPI) and persisting through 90 DPI (Sadasivan et al., 2015). In this study, influenza-induced neuroinflammation was not met with breakdown of the blood-brain barrier, or increased T cell extravasation; two events suggested to contribute to systemic inflammation in neurodegeneration (Lyck & Engelhardt, 2012; Obermeier et al., 2013). Dual challenge with influenza A/California/04/2009 30 days post-infection and the Parkinsonian toxin 1-methyl, 4-phenyl, 1,2,3,6-tetrahydropyridine (MPTP) in mice has been shown to have no additive effect on microglial activation, but produces substantial loss of dopaminergic neurons in the substantia nigra (Sadasivan et al., 2017), suggesting that multiple inflammatory challenges can have detrimental effects relevant to the development of Parkinsonism. However, a challenge in identifying preclinical inflammatory markers that reliably indicate PD onset is that these signals are highly context-dependent and often exhibit both adaptive and maladaptive functions (Clark & Kodadek, 2016; Postuma & Berg, 2016).

The current study investigated the *in vivo* and *in vitro* role of influenza-induced double-stranded RNA-dependent protein kinase (PKR) expression in brain and assessed its role in Parkinsonian pathogenesis. PKR is an antiviral kinase that has been shown to mediate cell stress responses to a variety of stimuli, both viral and non-viral (M. A. Garcia et al., 2006). We hypothesized that influenza would time-dependently induce PKR expression in the CNS and that upon dual challenge with influenza + MPTP, CNS PKR expression would
be additive. Following intranasal infection with influenza A/PR/8/34 for either 3- or 15-days post-infection, animals were acutely challenged with MPTP (4x I.P., 18 mg/kg) and the striatum and hippocampus were analyzed for PKR expression, the coinciding neuroinflammatory phenotype, and downstream cell signaling pathways that may mediate PKR’s expression.

**Materials and Methods**

2.1 Study Design

This study utilized *in vivo* and *in vitro* paradigms to model influenza infection. Three independent cohorts of mice were intranasally inoculated with 2 different influenza doses from the same batch of virus and brain tissue collected at either 3, 4, or 15 Days Post-Infection (DPI): i) male BALB/c mice infected with 0.5 HAU (256 HAU batch total) and samples collected 4 or 15 DPI (n = 3-4 per group), ii) male C57BL/6 infected with 0.1 HAU and samples collected 3 DPI (n = 3-4), and iii) male C57BL/6 infected with 0.1 HAU and samples collected 15 DPI (n = 3-6). The day of infection was counted as Day 0. Standard influenza modeling for our lab utilizes BALB/c strains (N. Huang et al., 2013) as they are deemed more susceptible to respiratory challenge (Bouvier & Lowen, 2010). However, a strain change to C57BL/6 mice was warranted for infection studies utilizing 1-methyl, 4-phenyl, 1,2,3,6-tetrahydropyridine (MPTP), as these animals are more susceptible to MPTP-toxicity (Sedelis et al., 2000). Respective tissues were processed for gene and protein expression.

In order to assess the direct effect of peripheral infection on dopaminergic neurons and microglia *in vitro*, conditioned media experiments were performed. RAW 264.7 macrophages were infected with influenza, and both cell lysates and conditioned supernatant were collected. Cell lysates were processed for protein expression and the supernatants were
used to treat N27 dopaminergic neurons, along with BV2 microglia. Gene expression and cell viability experiments were performed on microglia and neurons, respectively.

2.2 Animals and health monitoring

Male BALB/c and C57BL/6 mice (Charles River) were maintained in group housing (n = 5/cage) and maintained on a 12-hour reverse light cycle in ventilated Innovive racks (Innovive, San Diego), with *ad libitum* food (Harlan Teklad 2014) and water access. Prior to any treatment, mice were environmentally acclimated for at least 1 week. Six to eight-week-old mice were used in all studies. All studies were approved by the Institutional Animal Care and Use Committee of Iowa State University.

Following either influenza infection or MPTP challenge, animals were individually-housed and well-being was monitored twice daily. Body weight was recorded daily, with food and water monitored. In order to maintain wellness, mice too weak from infection were supplied with food and water combined in petri dishes for better access. Animals whose body weight loss exceeded 20% were removed from the study and sacrificed.

At the end of a study’s respective duration, animals were sacrificed via asphyxiation (CO₂), brains removed and micro-dissected for the hippocampus and striatum, then snap-frozen in liquid nitrogen for subsequent processing.

2.3 Treatment

2.3.1 Virus and immunizations

Influenza A/PR/8/34 H1N1 was grown in the amniotic-allantoic sac of embryonated eggs. For influenza virus used in all *in vivo* and *in vitro* studies, the hemagglutination unit (HAU) of stock virus was 256 HAU/0.05 ml. For all *in vivo* infections, mice were subjected
to light anesthesia with isoflurane (VAD Anesthetic Machine, Vetamac, Rossville, IN) and intranasally inoculated with 50 ul of chilled, serially-diluted virus prepared in saline.

2.3.2 MPTP Formulation

Following primary infection with influenza, animals were subjected to an acute MPTP paradigm to establish neuroinflammation and rapid dopamine degeneration. Mice received four intraperitoneal (I.P.) injections of 18 mg/kg MPTP-HCl dissolved in saline (free-base; Axon Medchem 1075) at 2-hour intervals. Animals were sacrificed either 1-day post-MPTP or 3 days post-MPTP.

2.4 Gene Expression

RNA isolation was performed on snap-frozen brain tissue using an RNA binding-column method (Qiagen RNeasy Micro Plus Kit; #74034). Total RNA was isolated from one hemisphere and reverse transcribed to cDNA (Qiagen RT2 First Stand Kit; #330404) to be used in SYBR green assays for relative gene expression (Qiagen RT2 SYBR Green Fluor qPCR Mastermix; 330513). Qualitative Polymerase Chain Reaction (qPCR) was performed on a Bio-Rad MyIQ PCR system for 40 cycles of amplification, with a subsequent melt curve to validate the purity of amplified product. The primer sequences used are available in Supplementary Table 1. Relative difference of gene expression between groups was analyzed using the Pfaffl Method (Pfaffl, 2001), where threshold cycle (Ct) values were normalized to both β-actin and GAPDH, and expressed relative to control groups.

2.5 Protein Expression

Protein lysates from one hemisphere of each animal were homogenized with a motorized pestle in 150 µl of custom Lysis Buffer A (30 mM NaHEPES, 5 mM EGTA, 3 mM EDTA, 20 mM KCL, 32% glycerol, phosphatase inhibitor cocktail 3 (Sigma-Aldrich;
Homogenates were centrifuged for 15 minutes at 15,000 g (4°C), and supernatant collected. Total protein was quantified using the bicinchoninic acid method (BCA; Pierce Biotechnology, Inc.; #23227) with bovine serum albumin standards. Western blot protein samples (10-30 μg) were run on 8-16% Criterion TGX Precast Gels (Bio-Rad) at 150V for 1.5 hours, transferred to polyvinylidene fluoride membranes (PVDF; EMD Millipore), and blocked in Tris-buffered saline (TBS; pH 8.0) containing 0.05% Tween-20 and 5% non-fat dry milk (LabScientific; #M0841) for 1 hour at room temperature. Membranes were incubated overnight at 4°C with primary antibody at the manufacturer recommended dilution. Primary antibody solutions were prepared in 1x TBST, 0.05% Na Azide, and 3% BSA and markers included: NLRP3 (Adipogen; cryo-2), p-PKR (Thr 451; EMB Millipore; #07-886), and β-actin (CST; #4970). Primary mouse monoclonal antibodies from Santa Cruz Biotechnology included: PKR (sc-6282), p-PACT (sc-53524), PACT (sc-377103), FADD (sc-271748), Caspase-8 (sc-81656), and NF-κB p65 (sc-8008). Cut membranes were washed with 1x TBST (0.1% Tween), probed at room temperature for 1 hour with respective secondary antibody, treated with SuperSignal ELISA Femto Maximum Sensitivity Substrate (Thermo; #970740), and images captured on a Bio-Rad Universal Hood II gel imager. Densitometry was performed using ImageJ software (NIH), normalizing all values to β-actin, and expressed relative to control groups. Cropped Western Blot images show group representative samples run from the same gel, with corresponding housekeeping control.

2.6 Cell culture and treatment paradigm

RAW 264.7 macrophages were grown in high glucose DMEM medium (Thermo, 11995-065) containing 10% FBS (GE Healthcare HyClone™, SH3008803HI), 100 U/ml
penicillin, and 100 µg/ml streptomycin at 37°C. For *in vitro* infection, cells were plated in a 6-well plate at a density of 500,000 cells/well and incubated overnight for 12-24 hours until ~80% confluence was achieved. Prior to infection, growth media was aspirated, and cells washed twice with pre-warmed 1X PBS. Cells were infected with 10 HAU Influenza A/PR/8/34 for 2 hours in 500 µl serum-free medium (DMEM + 1% Pen/Strep + 1 µg/ml TPCK-Trypsin), with plates rocked every 15 minutes. Following infection, virus-containing media was aspirated, monolayers washed once with 1X PBS, and 2 ml of serum-free medium (DMEM + 1% Pen/Strep + 1 µg/ml TPCK-Trypsin + 0.3% BSA) was applied. Plates were incubated for 6 hours, conditioned media collected, cells washed once with 1x PBS, and lysed. Homogenized lysates were analyzed for p-PKR, PKR, and NLRP3 protein expression using Western blotting. Conditioned media was used to treat BV2 microglia for 6 hours, with cell lysates subsequently processed for IL-18, IL-1β, NLRP3, and PKR gene expression using qPCR.

2.8 MTS Cell Viability Assay

Influenza-conditioned medium from RAW 264.7 macrophages was additionally used to treat N27 rat dopaminergic neurons in a cell viability assay. This immortalized cell line are mesencephalic dopaminergic neurons that can be differentiated to produce tyrosine hydroxylase and dopamine (Adams et al., 1996). Undifferentiated cells were grown in RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine, 50 units penicillin, and 50 µg streptomycin. N27 cells were seeded in a 96-well plate at 2E4 cells/well and treated in 100 µl serum-free RPMI with either: control media, Influenza A/PR/8/34-conditioned media for 24 hours, 0.5 µM rotenone for 24 hours (Sigma, St. Louis, MO), or 0.5 µM rotenone for 16 hours + A/PR/8/34 for 8 hours. Rotenone is a well-characterized complex I inhibitor used to
model mitochondrial toxicity in PD models (Greenamyre, Cannon, Drolet, & Mastroberardino, 2010). Cell viability was measured using the CellTiter 96 Aqueous One Solution Cell Proliferation Kit (Promega). Following treatment, cells were incubated with MTS dye and incubated for one hour at 37°C. Post-incubation, spectrophotometry measured absorbance of the cell viability indicator, formazan, at 490 nm.

2.9 Analysis

Data were analyzed in GraphPad Prism (version 6 for Windows) using a Student’s T test for studies comparing infection to control, and a one-way analysis of variance (ANOVA) on both gene and protein expression values for combined influenza and MPTP studies. Post-hoc multiple comparisons were performed using Sidak correction, with an α-level of 0.05. Figures were generated in GraphPad, reporting data as mean fold change with error propagated as 95% Confidence Intervals. Gene data were represented on a Log2 scale, with protein data on a linear scale. Significance above bar denotes difference from control, with between group differences denoted by line. ****p < 0.0001, *** p < 0.001, ** p < 0.01, * p < 0.05 by 1-way ANOVA.

Results

3.1 Peripheral infection with Influenza A/PR/8/34 produces delayed PKR expression in the hippocampus and striatum

Initial influenza studies infected male BALB/c mice with 0.5 HAU A/PR/8/34 and collected samples 4- and 15-days post-infection (DPI). A student’s t test between saline and infected mice revealed no significant difference in total PKR protein expression at 4 DPI, in either the striatum or hippocampus. However, PKR protein expression was significantly up-regulated from control 15 DPI in the striatum (M = 6.75, SD = 1.08), t(6) = 10.36, p < .0001).
Additionally, PKR was significantly up-regulated in the hippocampus 15 DPI \( (M = 2.83, SD = 1.40), t(6) = 2.57, p = 0.0423 \) (Figure 1).

3.2 PKR expression 15 days post-infection (DPI) coincides with apoptotic and inflammatory signaling

As downstream targets of PKR, 15 DPI with 0.5 HAU A/PR/8/34 produced significant up-regulation of fas-associated death domain (FADD) in the striatum \( (M = 2.52, SD = 0.39), t(5) = 4.19, p = 0.0086 \) and the hippocampus \( (M = 3.94, SD = 0.81), t(5) = 6.86, p = 0.001 \). Additionally, NF-κB p65 protein expression was significantly increased in the striatum \( (M = 8.25, SD = 0.94), t(6) = 15.41, p < 0.0001 \) and the hippocampus \( (M = 2.95, SD = 0.95), t(6) = 4.09, p = 0.0064 \) of influenza-infected mice. A primary effector caspase of apoptosis, caspase-3, did not reveal any protein changes in either the striatum or hippocampus (Figure 2A).

Upstream of PKR activation, phosphorylated protein activator of PKR (p-PACT), was significantly increased in striatum \( (M = 1.64, SD = 0.31), t(6) = 4.18, p = 0.0058 \) of infected mice, but not the hippocampus. As for expression of effector neuroinflammatory glial cells that may mediate striatal and hippocampal PKR expression, glial fibrillary acidic protein (GFAP) protein was significantly up-regulated in the striatum \( (M = 4.77, SD = 0.82), t(5) = 8.64, p = 0.00034 \) and hippocampus \( (M = 4.33, SD = 0.44), t(5) = 14.81, p < 0.0001 \) 15 DPI with influenza (Figure 2B).

3.3 Infection of peripheral macrophages in vitro directly produces neuroinflammatory expression and compromises neuronal integrity relevant to Parkinsonian pathology.

Conditioned media experiments from influenza-infected macrophages were used to treat immortalized BV2 microglial cells. Student’s t-tests revealed that influenza treatment
led to significant gene induction for IL-1β ($M = 7.05, SD = 1.29$), $t(9) = 10.35, p < 0.0001$), NLRP3 ($M = 2.65, SD = 0.52$), $t(9) = 6.91, p < 0.0001$), and PKR ($M = 2.42, SD = 0.58$), $t(10) = 5.92, p = 0.00015$) (Figure 3B).

The same infection-conditioned media was used to treat N27 dopaminergic neurons, and an MTS cell viability assay was performed to determine the direct effect of influenza, rotenone (ROT), or the combination of both influenza + ROT. A one-way ANOVA revealed a significant loss of viable neurons compared to control, resulting from Influenza A/PR/8/34 ($M = 86.05, SD = 2.031$), $t(20) = 7.005, p < 0.0001$), ROT ($M = 76.37, SD = 3.046$), $t(20) = 11.87, p < 0.0001$), and A/PR/8/34 + ROT ($M = 18.55, SD = 4.402$), $t(20) = 4.18, p < 0.0001$) (Figure 3A). Furthermore, pairwise comparisons revealed that the percentage of viable cells from ROT treatment was significantly lower than A/PR/8/34 treatment ($t(20) = 4.861, p = 0.0006$). The percentage of viable neurons from the combined treatment of A/PR/8/34 + ROT was significantly lower than ROT-alone ($t(20) = 6.686, p < 0.0001$) and A/PR/8/34-alone ($t(20) = 6.686, p < 0.0001$). Additive treatment led to the lowest percentage of viable dopaminergic neurons, compared to each treatment-alone.

Furthermore, protein analysis of influenza-infected macrophages did not reveal any significant differences in p-PKR/PKR ($M = 1.06, SD = 0.14$), total PKR ($M = 1.02, SD = 0.15$), or NLRP3 ($M = 0.99, SD = 0.12$) expression. This suggests that our observed striatal and hippocampal PKR expression is not produced by peripheral immune cells, which then propagates into the CNS parenchyma, but rather a localized stress signal.

3.4 Dual Influenza 3 DPI + MPTP challenge produces limited PKR and NLRP3 protein changes
As an initial in vivo experiment to assess CNS influenza-induced PKR expression and its role in a multi-hit model of Parkinson’s disease (PD), we performed a pilot experiment to identify a sub-lethal dose of combined influenza + MPTP. Male C57Bl/6 infected with 0.1 HAU A/PR/8/34 were challenged with 18 mg/kg MPTP 3 DPI and samples were collected 24 and 72 hours post-MPTP. 72 hour post-MPTP data is shown in Supplementary Figure 1. While qPCR (24 hour post-MPTP) indicated no significant gene changes in MPTP or MPTP + A/PR/8/34 groups for PKR, NLRP3, or IL-1β expression (Figure 4B), MPTP-alone treatment significantly reduced p-PKR/PKR protein expression in the striatum ($M = 0.51$, $SD = 0.12$), $t(7) = 3.946$, $p = 0.0166$ and hippocampus ($M = 0.29$, $SD = 0.089$), $t(7) = 5.091$, $p = 0.0042$) from control (Figure 4A). NLRP3 protein expression was significantly reduced in hippocampus by MPTP ($M = 44.13$, $SD = 0.809$), $t(7) = 6.375$, $p = 0.0011$) and combined MPTP + A/PR8/34 ($M = 23.47$, $SD = 0.85$), $t(7) = 3.817$, $p = 0.0196$) (Figure 4A).

### 3.5 Dual Influenza 15 DPI + MPTP challenge changes PKR protein expression

As a follow-up to the pilot 3 DPI experiment, we infected male C57Bl/6 mice with 0.1 HAU A/PR/8/34 and 15 DPI, challenged with 18 mg/kg MPTP and collected samples 24 hours later for gene and protein analysis. qPCR did not reveal any significant differences for PKR, NLRP3, or IL-1β gene expression in the striatum or hippocampus resulting from treatment with either MPTP-alone, A/PR8/34-alone, or MPTP + A/PR/8/34. Striatal TNF-α gene expression was significantly increased by MPTP ($M = 0.29$, $SD = 0.089$), $t(11) = 8.361$, $p < 0.0001$) and by MPTP + A/PR/9/34 ($M = 0.29$, $SD = 0.089$), $t(11) = 7.768$, $p < 0.0001$). Pairwise comparisons revealed a significant difference between MPTP and A/PR/8/34-alone ($t(11) = 9.646$, $p < 0.0001$) and between A/PR/8/34-alone and MPTP + A/PR/8/34 ($t(11) = 8.865$, $p < 0.0001$). Hippocampal TNF-α gene expression was not significantly different from
control in any group, but pairwise comparisons revealed TNF-α was significantly lower in MPTP-alone, compared to MPTP + A/PR/8/34 \( (t(18) = 3.014, p = 0.0439) \) and significantly lower in A/PR/8/34-alone, compared to MPTP + A/PR/8/34 \( (t(18) = 3.454, p = 0.0169) \). Hippocampal IL-6 gene expression was significantly increased from control with MPTP + A/PR/9/34 \( (M = 30.20, SD = 1.28), t(20) = 55.60, p < 0.0001) \). Pairwise comparisons revealed this expression was also significantly increased from MPTP-alone \( (t(20) = 54.47, p < 0.0001) \) and A/PR/8/34-alone \( (t(20) = 56.09, p < 0.0001) \).

Striatal PKR protein expression was significantly down-regulated in MPTP + A/PR/8/34 treated mice \( (M = 0.639, SD = 0.03172), t(12) = 3.315, p = 0.0244) \) and this expression was significantly lower from MPTP-alone \( (t(12) = 4.937, p = 0.0021) \). Also, striatal PKR expression was significantly lower with A/PR/8/34-alone, compared to MPTP-alone \( (t(12) = 4.295, p = 0.0052) \). Hippocampal PKR expression was conversely up-regulated with MPTP + A/PR/9/34 compared to control \( (M = 2.176, SD = 0.61), t(8) = 3.659, p = 0.0378) \) and pairwise comparisons indicated this increase was significantly different between A/PR/8/34-alone and MPTP + A/PR/9/34 \( (t(8) = 3.505, p = 0.0394) \).

When analyzed as p-PKR/PKR, phosphorylated PKR (p-PKR) protein expression was significantly different in the striatum between A/PR/8/34-alone and MPTP + A/PR/8/34 \( (t(12) = 4.409, p = 0.0051) \). p-PKR was significantly decreased in the hippocampus of MPTP + A/PR/8/34 mice \( (M = 0.659, SD = 0.027), t(8) = 4.53, p = 0.0115) \) and this was significantly lower from MPTP-alone \( (t(8) = 4.026, p = 0.0189) \) (Figure 5A). The magnitude of p-PKR protein expression did not vary when data were analyzed as p-PKR alone, compared to p-PKR/total PKR.

3.6 Astrocyte and microglial glial induction appears to be MPTP-mediated
In an effort to assess potential involvement of astrocytes and microglia, qPCR was performed for GFAP and IBA1 gene expression, respectively. Striatal GFAP gene expression was only different between MPTP and A/PR/8/34-alone (t(11) = 3.364, p = 0.0312) and between A/PR/8/34-alone and MPTP + A/PR/8/34 (t(11) = 4.015, p = 0.0121). Hippocampal GFAP gene expression was significantly increased from control with MPTP-alone (M = 2.242, SD = 0.105), t(20) = 4.94, p = 0.0005), and MPTP + A/PR/8/34 (M = 3.581, SD = 0.262), t(20) = 10.27, p < 0.0001). Additionally, pairwise comparisons indicated that GFAP for MPTP-alone was significantly increased over A/PR/8/34-alone (t(20) = 4.264, p = 0.0023), that MPTP + A/PR/8/34 expression was greater than A/PR/8/34-alone (t(20) = 9.592, p < 0.0001), and finally that GFAP gene expression from MPTP + A/PR/8/34 was greater than MPTP-alone (t(20) = 5.328, p = 0.0002).

Combined treatment MPTP + A/PR/8/34 led to significant down-regulation of IBA1 gene expression in the striatum (M = 0.29, SD = 0.622), t(12) = 3.997, p = 0.0106) and this decrease was significantly difference from A/PR/8/34-alone (t(11) = 3.694, p = 0.0153). There were no observed changes to hippocampal IBA1 gene expression.

**Discussion**

Pandemic influenza outbreaks have long been observed to produce neurological deficits. Yet, the relationship between influenza infection and Parkinsonian-like symptoms remains relatively understudied. Despite historical (1918 Spanish flu) and modern day (Cali 09) epidemiological evidence, few studies have addressed the mechanisms that mediate this relationship.

Seminal studies by Jang et al (2009) provided evidence that direct CNS infection with a neurotropic strain of influenza can promote PD neuropathology by promoting long-lasting microglial activation and feed-forward loop of α-synuclein aggregation, the intracellular
proteinaceous inclusion that makes up Lewy bodies (H. Jang et al., 2009). Once activated, Jurgens et al (2012) demonstrated that influenza-induced systemic inflammation causes microglia to exhibit a neuroinflammatory phenotype than can compromise hippocampal-mediated behaviors through architectural changes to dendritic branching and synaptic spine density (Jurgens et al., 2012). Additionally, we know that systemic inflammatory insults with influenza can have synergistic effects, in combination with the Parkinsonian neurotoxin MPTP (Sadasivan et al., 2017). In addition to reactive microgliosis in the hippocampus and substantia nigra (Sadasivan et al., 2015), the additive effect of influenza and MPTP results in a significant loss of dopaminergic neurons (Sadasivan et al., 2017). These studies have provided foundational evidence that implicates systemic inflammation as a pathological process that can sensitize the brain to secondary immune challenges and produce Parkinsonism. What has not been addressed in these studies to date are neuroinflammatory mechanisms that may serve as therapeutic targets of interest.

This report provides the first evidence that peripheral infection with non-neurotropic influenza produces PKR expression in the CNS. As a canonical anti-viral protein in infected cells, PKR activation leads to the inhibition of protein synthesis as a host defense mechanism (M. A. Garcia et al., 2006; Marchal et al., 2014). In addition to this function, PKR is expressed by numerous inflammatory cell types (R. Kang & Tang, 2012), has been found post-mortem in brains of PD patients (Bando et al., 2005), and has been shown to activate in response to a diverse array of cellular stress in acute and chronic disease states (Hugon et al., 2017; B. R. Williams, 1999). A specific aim of our studies was to determine the mechanism of PKR activation in the brain following a systemic inflammatory challenge with influenza and assess its role in an MPTP model of dopaminergic neuron death.
In our models, in vivo PKR expression appeared to be both time and dose-dependent on influenza. We observed protein expression in both the striatum and hippocampus at 15 DPI in BALB/c mice infected with 0.5 HAU, but not at 4 DPI (Fig. 1). The expression of PKR protein does not appear to be mediated by its autophosphorylation (Thr451), as we observed MPTP-induced decreased to p-PKR expression 24 hours post-MPTP (Fig. 4) and an MPTP-induced increase to p-PKR 72 hours post-MPTP (Suppl. Fig. 1). Increases in PKR, and its downstream action, would be expected to positively correlate with PKR phosphorylation. At 15 DPI with 0.1 HAU A/PR/8/34 in C57BL/6 mice, we observed a significant increase in hippocampal PKR protein, while p-PKR was significantly decreased. However, the action of influenza-induced PKR expression in our model may not be dependent on its kinase activity, as this has previously been shown in models of pyroptosis (Hett et al., 2013). The seemingly disparate, latent expression of influenza-induced PKR protein at 15 DPI may suggest that PKR expression is not dependent on its activation through phosphorylation. This would suggest that PKR is acting as a signal transducing node that supports apoptotic cell response. Alternatively, if influenza-induced CNS PKR expression is functioning in an apoptotic capacity, perhaps its expression is dependent on cell death within the brain. However, we did not observe changes to caspase-3 mediated cell death in BALB/c mice infected with 0.5 HAU A/PR/8/34 15 DPI (Fig. 2A).

During the typical course of infection, peak viral expression in the lungs occurs by 3-4 DPI, maximal weight loss and sickness by 5-8 DPI, with body weight loss recovered by 12-15 DPI (Kohut, Sim, Yu, Yoon, & Loiacono, 2009; Warren et al., 2015). This served as rationale for selecting early (3-4 DPI) timepoints. Weight loss is considered a reliable metric of diseases progression in animal influenza models (Matsuoka, Lamirande, & Subbarao,
2009). We observed more global expression of CNS PKR (i.e., both striatum and hippocampus) when infection was severe enough to produce ~20% loss of body weight. In follow-up studies that incorporated MPTP-challenged groups, we had changes to both animal strain used and dose of influenza. While arguably more susceptible to influenza infection (Bouvier & Lowen, 2010; B. Srivastava et al., 2009), BALB/c mice are resistant to MPTP-challenge, which is attributed to a difference in endogenous levels of brain monoamine oxidase-B (MAO-B) (Meredith & Rademacher, 2011). MAO-B is the enzyme responsible for the catalytic conversion of MPTP to MPDP+, which is subsequently oxidized to the neurotoxin MPP+. Having established that influenza-induced PKR occurs 15 DPI, our dose of influenza used in dual immune challenge (influenza + MPTP) had to be moderate enough that it did not produce lethality by 15 DPI. Hence, we opted for a dose of influenza (0.1 HAU A/PR/8/34 in C57BL/6 mice) that produced a modest ~5% loss of body weight.

Evidence from the past decade has irrefutably highlighted inflammation as a primary driver of neuropathology. It is now generally well-accepted that neuroinflammation precedes neurodegeneration (C. Cunningham et al., 2009; C. Cunningham et al., 2005; Glass et al., 2010; Sparkman & Johnson, 2008; Q. Wang et al., 2015) and these inflammatory changes have been shown to occur prior to neuronal changes induced by influenza infection (Jurgens et al., 2012). All nucleated cell types have been shown to express PKR, including murine neurons (Alirezaei et al., 2007), astrocytes (Ong et al., 2005), and microglia (J. H. Lee et al., 2005). Following inflammatory challenge with influenza, or in combination with MPTP, we postulated that microglia were expressing PKR as an inflammatory mediator. Although our data did not explicitly phenotype glial cell populations, we did not observe substantial changes to IBA1 microglia gene expression (Fig. 6). As we observed an influenza-induced
increased in FADD apoptotic signaling, a plausible conclusion may be that neurons are the primary source of stress-induced PKR. Much of the literature examining CNS PKR localization implicates the source as being neuronal (Carret-Rebillat et al., 2015; H. M. Chen et al., 2008; Mouton-Liger et al., 2012; Mouton-Liger et al., 2015; X. Wang et al., 2007). However, it also possible that microglia themselves could be undergoing apoptosis as a regulatory mechanism to limit neuroinflammatory damage (White, McCombe, & Pender, 1998). Infection of mixed primary mouse astrocytes and microglia with either H1N1 (A/Shantou/169/06) or H5N1 (A/Chicken/Guangdong/1/05) has been shown to induce a proinflammatory response, in addition to apoptosis of these cytokine-producing cells (G. Wang et al., 2008). Furthermore, PKR is activated and expressed in both astrocytes and microglia in genetic models of Gaucher’s disease (Vitner et al., 2016). Future studies should utilize cell-specific techniques to localize the cellular source of PKR.

Functionally, PKR has been implicated in a multitude of stress responses resulting in antiviral responses, anti-proliferative actions, stress-induced apoptosis, and inflammation (Marchal et al., 2014). Fas-associated death domain (FADD) and caspase-8, which precedes executioner caspase-3, have previously been shown to be downstream effectors of PKR-mediated apoptosis (Balachandran et al., 2000; Couturier et al., 2010; Gurung et al., 2014; Thapa et al., 2013; von Roretz & Gallouzi, 2010). Following infection with 0.5 HAU A/PR/8/34 in BALB/c mice, we observed significant upregulation of FADD in both the striatum and hippocampus following infection. Additionally, we saw increases in NF-κB p65, of which FADD has previously shown to be an activator (W.-H. Hu et al., 2000). Overexpression of FADD in SH-SY5Y neurons has previously been shown to be protective against TNF- and Aβ-induced neurotoxicity in in vitro models of Alzheimer’s disease.
(Cantarella et al., 2003) and may represent a PKR-mediated target for therapeutic interventions.

What remains to be determined from studies of systemic inflammation and neurodegeneration are the context-specific mechanisms for how peripheral inflammatory signals can propagate in to the CNS. Three primary routes of CNS entry have been proposed; neuronal) afferent vagal nerves express inflammatory cytokine receptors that can be activated by systemic inflammation, direct) inflammatory cytokines and chemokines can propagate inflammatory signaling via immune cells in circumventricular organs (specialized regions of the BBB that do not tightly regulate CNS entry), and humoral) trans-epithelial signaling from cytokines in the blood to perivascular macrophages in cerebral endothelial layers which promote microglial activation (Perry & Teeling, 2013; Teeling & Asuni, 2017). From our in vitro studies, we did not observe influenza-induced PKR expression in peripheral macrophages, but the post-infection media produced proinflammatory gene expression in treated microglia. This suggested that a soluble factor in conditioned media was capable of activating PKR. Although stereotypically induced by dsRNA intermediates produced viral infection, PKR activation can occur in response to a number of extracellular signals (M. A. Garcia et al., 2006). As for what CNS PKR activation may mean for PD pathology, treatment of dopaminergic neurons with influenza-conditioned media produces cell loss. When combined with a secondary, PD-specific toxin (rotenone), this cell loss is additive. These changes mirror what has previously been shown with dual influenza + MPTP challenges; although there are no increases in microglial activation, there is a significantly greater degree of dopaminergic neuron loss (Sadasivan et al., 2017).
The inflammatory insults that accrue over the course of one’s lifetime can produce long-lasting changes that compromise neuronal function and precipitously contribute to neurodegenerative disease development. Previous work has fundamentally shown that influenza produces neuronal and inflammatory perturbations in the brain and that these changes promote a neurodegenerative cascade. Building off of that work, this study provides the first report of a novel neuroinflammatory marker that is induced by respiratory influenza infection and its resulting systemic inflammatory phenotype. Our study suggests that PKR functions as an inflammatory and apoptotic stress signal, and as opposed to acting as a terminal signal, PKR is most likely a transducing signal to a variety of downstream effectors. Future studies should continue to investigate influenza-induced neuroinflammatory signaling mechanisms and in particular, address how these signals are propagated from the periphery into the brain.
Figure 3.1 CNS PKR protein expression occurs by 15 DPI with Influenza A/PR/8/34.

Following brief anesthesia, male BALB/c mice were intranasally inoculated with either 1) Saline (n = 8) or 2) 0.5 HAU Influenza A/PR/8/34 (n = 8). Independent groups were monitored twice daily for either 4 days post-infection (DPI) or 15 DPI (n = 4/group). Data represented as mean fold change ± 95% CI on a linear scale. Cropped Western Blot images show group representative samples run from the same gel, with corresponding housekeeping control. Significance above bar denotes difference from control, with between group differences denoted by line. ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05 by 1-way ANOVA.
Figure 3.2 Striatal and hippocampal apoptotic and inflammatory induction precedes prototypical cell death, occurring in conjunction with astrocyte activation, in BALB/c mice at 15 DPI with 0.5 HAU Influenza A/PR/8/34 (n = 4) compared to control (n = 4).

A) FADD and NF-κB p65 protein expression are upregulated in the striatum and hippocampus 15 DPI. B) Protein activator of PKR (PACT) protein is upregulated by influenza in the striatum, with evidence of astrocyte activation. Data represented as mean fold change ± 95% CI on a linear scale. Cropped Western Blot images show group representative samples run from the same gel, with corresponding housekeeping control. Significance above bar denotes difference from control, with between group differences denoted by line. ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05 by 1-way ANOVA.
Figure 3.3 Neurons and microglia treated with influenza-conditioned media in vitro exhibit increased cell death and inflammation.

RAW 264.7 macrophages were infected with 10 HAU Influenza A/PR/8/34 for 6 hours. A) Conditioned supernatant was collected and used to treat N27 dopaminergic neurons in an MTS cell viability assay, expressed as a percentage of controls (n = 6). B) Influenza-conditioned supernatant was also used to treat BV2 microglia for 6 hours and subsequent qPCR assays were performed for IL-18, IL-1β, NLRP3, and PKR gene expression (n = 6). Microglia significantly up-regulated PKR and inflammasome-mediated gene expression. C) Western blotting on influenza-infected RAW 264.7 macrophages reveal no significant differences in PKR or NLRP3 protein expression (n = 3). Data represented as mean fold change ± 95% Confidence Interval on a Log2 scale. Significance above bar denotes difference from control, with between group differences denoted by line. ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05 by 1-way ANOVA.
Figure 3.4 Striatal and hippocampal inflammatory expression 3 DPI with combined challenge of 0.1 HAU Influenza A/PR/8/34 and 18 /mg/kg MPTP.

Male C57 mice were treated with either 1) saline (n = 4), 2) 4 separate doses of MPTP (I.P.) (n = 3), or dual challenge with intranasal 0.1 HAU Influenza A/PR/8/34 and MPTP (4x; I.P.) (n = 3). Influenza-treated mice were challenged with MPTP 3 DPI and sacrificed 24 hours later. A) Phosphorylated-PKR (pPKR) protein expression was significantly decreased by MPTP alone, with no changes to total PKR. B) PKR, NLRP3, and IL-1β gene expression revealed no transcriptional changes. Data represented as mean fold change ± 95% CI on a linear scale (protein) or log2 scale (gene). Cropped Western Blot images show group representative samples run from the same gel, with corresponding housekeeping control. Significance above bar denotes difference from control, with between group differences denoted by line. ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05 by 1-way ANOVA.
Figure 3.5 Striatal and hippocampal inflammatory expression 15 DPI with combined challenge of 0.1 HAU Influenza A/PR/8/34 and 18 mg/kg MPTP.

A separate cohort of male C57 mice were treated with either 1) saline (n = 6), 2) 4 separate doses of MPTP (I.P.) (n = 6), or dual challenge with intranasal 0.1 HAU Influenza A/PR/8/34 and MPTP (4x; I.P.) (n = 6). Influenza-treated mice were challenged with MPTP 15 DPI and sacrificed 24 hours later. A) Protein expression showed down-regulated of pPKR and total PKR, with no changes to NLRP3. B) Gene expression revealed no PKR or inflammasome changes, but provides evidence of pro-inflammatory cytokine induction. Data represented as mean fold change ± 95% CI on a linear scale (protein) or log2 scale (gene). Cropped Western Blot images show group representative samples run from the same gel, with corresponding housekeeping control. Significance above bar denotes difference from control, with between group differences denoted by line. ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05 by 1-way ANOVA.
Figure 3.6 Gene expression data for GFAP and IBA1 at 15 DPI with 0.1 HAU Influenza A/PR/8/34 and MPTP challenge.

A) Striatal and hippocampal GFAP gene induction appears to be largely mediated by MPTP challenge. B) Striatal IBA1 gene induction is significantly lower from controls in dual Influenza + MPTP mice. Data represented as mean fold change ± 95% Confidence Interval on a Log2 scale. Significance above bar denotes difference from control, with between group differences denoted by line. ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05 by 1-way ANOVA (n = 6 per group).
Supplemental Figure 3.1 Striatal and hippocampal PKR phosphorylation subsequently increases 72 hours post-MPTP.

Additionally, hippocampal NLRP3 protein expression dissipates in dual-treated mice at 72 hours post-MPTP, versus 24 hours post-MPTP. Male C57 mice were treated with either 1) saline (n = 4), 2) 4 separate doses of MPTP (I.P.) (n = 3), or dual challenge with intranasal 0.1 HAU Influenza A/PR/8/34 and MPTP (4x; I.P.) (n = 3). Influenza-treated mice were challenged with MPTP 3 DPI and sacrificed 72 hours later. Data represented as mean fold change ± 95% CI on a linear scale. Cropped Western Blot images show group representative samples run from the same gel, with corresponding housekeeping control. Significance above bar denotes difference from control, with between group differences denoted by line. ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05 by 1-way ANOVA.
Supplemental Figure 3.2 Dose and strain-dependent weight loss by Influenza A/PR/8/34 across three independent studies.

The initial study using 0.5 HAU A/PR/8/34 (256 total HAU batch) in BALB/c mice produced approximately 20% weight loss. Follow-up studies using C57/BL6 mice, to model dual MPTP+Flu, tested 0.1 HAU A/PR/8/34 (256 total HAU batch) and saw minor weight loss that did not coincide with CNS PKR expression. Additional dose studies infecting C57/BL6 with a more potent batch of 0.1 HAU A/PR/8/34 (1024 total HAU batch) did not see weight loss. CNS PKR expression induced by peripheral influenza infection appears dependent on observable sickness. Data represented as mean ± 95% CI (n = 3-8 per group).
Supplemental Table 3.1 Primer sequence information for qPCR assays.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5’ → 3’)</th>
<th>Reverse (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>GCTTCAGGCAGGCAGTATC</td>
<td>AGGATGGGCTCTTTCTTCTCAAG</td>
</tr>
<tr>
<td>TNF-α</td>
<td>AGCCGATGGGTTGTACCTTG</td>
<td>ATAGCAAAATCAGGTAGACGTT</td>
</tr>
<tr>
<td>IL-6</td>
<td>ACCGCTATGAGTCTCTCCTC</td>
<td>CTCTGTGAAGTCTCTCTCC</td>
</tr>
<tr>
<td>NLRP3</td>
<td>GCTCCAACCATTCTCTCAGCC</td>
<td>AAGTAAGGCAGAATTCACC</td>
</tr>
<tr>
<td>ASC</td>
<td>AGGAGTGCGAGGGAAAGC</td>
<td>AGAAGACGCAGGAGATGG</td>
</tr>
<tr>
<td>PKR</td>
<td>GATGGAAAATCCCCGAACAGGAG</td>
<td>AGGCCCAAGCAAGAGATGTCCAC</td>
</tr>
<tr>
<td>GFAP</td>
<td>GGTGAATCGCTGGAGGAG</td>
<td>CTGTGAAGGTCTGGCTTTG</td>
</tr>
<tr>
<td>IBA1</td>
<td>CTTGAAGCGAATGCTGGAGAA</td>
<td>GCCAGCTCGAGATAGCTTT</td>
</tr>
<tr>
<td>β-Actin</td>
<td>GTGACGTTGACATCCGTAAGA</td>
<td>GCCGACTCATCGTACTCC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>AGGCAGGTTGTAACCAAGTTC</td>
<td>TGTAGACCATGTAGTTGAGGTCA</td>
</tr>
</tbody>
</table>
References


Sadasivan, S., Sharp, B., Schultz-Cherry, S., & Smeyne, R. J. (2017). Synergistic effects of influenza and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) can be eliminated by the use of influenza therapeutics: experimental evidence for the multi-hit hypothesis. *NPJ Parkinsons Dis, 3*, 18. doi: 10.1038/s41531-017-0019-z


CHAPTER 4. PKR AS A NEUROINFLAMMATORY MECHANISM IN DUAL MODEL OF DIET-INDUCED OBESITY AND ACUTE MPTP

Matthew A. Jefferson¹,²,*, Rudy J. Valentine², Arthi Kanthsamy¹,³, & Marian L Kohut¹,²

A manuscript prepared for submission to PlosOne

¹Interdepartmental Neuroscience Graduate Program, Iowa State University, Ames, IA
²Department of Kinesiology, Iowa State University, Ames, IA
³Department of Biomedical Sciences, Iowa State University, Ames, IA

* Author for correspondence (MAJ): 235 Forker Building, Department of Kinesiology, Iowa State University, Ames, IA 50011-4008. E-mail: mjefferson83J@gmail.edu. Telephone: (309) 781-7590. (515) 294-8009.

Abstract

Neuroinflammation resulting from host factor detriments, such as obesity, has emerged as a well-recognized component of Parkinsonian pathogenesis. While it’s understood that low-grade, systemic inflammation from obesity is capable of perturbing CNS homeostasis, the characterization of these inflammatory pathways in the brain is enigmatic and highly context-dependent. Our lab has previously identified the expression of double-stranded RNA-dependent protein kinase (PKR) in murine brains fed a long-term high fat diet. PKR may potentially serve as a neuroinflammatory target for therapeutic intervention in obese populations at risk for Parkinson’s disease (PD). PKR activation has been suggested to serve as an intracellular danger-sensing mechanism and may precede NLRP3 inflammasome activation. To determine its clinical relevancy to Parkinsonian pathogenesis, we established a chronic model of diet-induced obesity in C57BL/6 mice over 14 weeks, followed by an acute MPTP challenge (5 mg/kg, i.p.). Brains were micro-dissected at 1 and 7 days post-MPTP challenge and surveyed for gene (qPCR) and protein (western blot) expression, revealing PKR up-regulation in the hippocampus at 1 day post-MPTP. Additionally, this expression...
appears regionally selective to the hippocampus, does not correspond to NLRP3 expression, and produces downstream apoptotic and inflammatory expression. This suggests PKR acts as a cell stress response in the obese brain following Parkinsonian insult. Given the current absence of disease-modifying therapies for PD, focusing on modifiable lifestyle factors such as obesity and their neuroinflammatory signature may yield meaningful preclinical insights into the progressive nature of this disease.

Introduction
Although clinically characterized by bradykinesia, muscle rigidity, postural instability, gait freezing, and a resting tremor (Poewe et al., 2017), the pathophysiology of Parkinson’s disease (PD) has been shown to develop decades prior to the onset of symptoms (Obeso et al., 2010; Postuma & Berg, 2016). While the primary pathology is defined by a progressive loss of dopaminergic neurons within the substantia nigra, intracellular proteinaceous inclusions composed of α-synuclein, and a stochastic decrease in striatal dopaminergic terminals (Obeso et al., 2010), there is also an observed increase in aberrant behavior of neuroinflammatory microglia and astrocytes (S. R. Subramaniam & H. J. Federoff, 2017). Moreover, this chronic neuroinflammatory phenotype underlies and drives the development of neurodegenerative cascades (C. Cunningham et al., 2009; Dantzer et al., 2008; Perry & Teeling, 2013).

Characteristically distinct from stereotypical inflammation, neuroinflammation is defined by i) central nervous system (CNS) recruitment and infiltration of blood-derived lymphocytes and monocyte-derived macrophages, ii) a decrease in the regulation of blood brain barrier (BBB) tight junction regulation, iii) morphological alterations to microglia and astrocytes, and iv) an accompanying expression of prolonged, low-grade inflammatory factors in the parenchyma that is induced by systemic inflammatory insults (Buckman et al.,...
In addition to a flurry of metabolic changes (Gregor & Hotamisligil, 2011; C. N. Lumeng & Saltiel, 2011), obesity is a detrimental host state known to produce neuroinflammation (Freeman et al., 2014; A. A. Miller & Spencer, 2014). This proinflammatory signature is characterized by reactive astrocytes and microglia that produce increased cytokine expression of TNF-α, IL-6, and IL-1β, which can underlie behavioral and cognitive disturbances (Guillemot-Legris & Muccioli, 2017; Hao et al., 2016; E. B. Kang et al., 2016; Pistell et al., 2010).

Considering the multifactorial etiology of PD and related neurodegenerative diseases, it is postulated that the obese brain is sensitive to additional inflammatory insults (Choi, Jang, Park, & Kang, 2005; Koprich, Reske-Nielsen, Mithal, & Isacson, 2008; Sulzer, 2007). In MPTP models of PD, obesity-induced systemic inflammation has previously been shown to synergistically promote loss of dopaminergic neurons, through enhanced decreases in tyrosine hydroxylase (TH) and elevated neuroinflammatory expression (M. Bousquet et al., 2012; Y. Jang et al., 2017; Paul et al., 2017; L. Wang et al., 2014). What is less understood are the precise mechanisms that mediate obesity-induced neuroinflammation and subsequently underlie neuronal dysfunction in PD.

The double-stranded RNA-dependent protein kinase (PKR) has historically served an antiviral function, as an innate immune response that inhibits translation of virus-infected cells (M. A. Garcia et al., 2006). In addition to reducing viral replication, PKR acts as a signal transducing node and has been shown to mediate apoptotic cell stress responses, antiviral host protection, and cell growth/differentiation behavior in cancer (Marchal et al., 2014). Increased expression of hippocampal phosphorylated PKR has been identified post-mortem in the brains of PD and Huntington’s disease patients (Bando et al., 2005). PKR has
also been shown to be elevated in circulating lymphocytes of Alzheimer’s disease patients and this increase negatively correlates with learning and memory scores (Paccalin et al., 2006). In rodent models, PKR knockout has been shown to ameliorate LPS-induced IBA1 microglial activation (Carret-Rebillat et al., 2015). Additionally, PKR was once implicated as a mediator of NLRP3 inflammasome expression (Lu et al., 2012), a multimeric protein complex required for the production of IL-1β-mediated inflammation (Latz, Xiao, & Stutz, 2013). The genetic and/or pharmacologic inhibition of PKR has been shown to attenuate neuroinflammatory phenotypes (H. M. Chen et al., 2008; Couturier et al., 2011; Ingrand et al., 2007; Mouton-Liger et al., 2012; Mouton-Liger et al., 2015).

Overall, we hypothesize that PKR is a novel mechanism linking obesity-induced neuroinflammation with Parkinsonian disease progression. Using a combined murine model of diet-induced obesity (DIO) and an acute challenge with the Parkinsonian neurotoxin, 1-methyl, 4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP), we examined striatal and hippocampal PKR expression in male C57BL/6 mouse brains fed a high fat diet (HFD) for 14 weeks, followed by MPTP challenge for 1 or 7 days. In the following studies, we sought to determine i) the impact of DIO-mediated neuroinflammation on CNS PKR and NLRP3 expression, ii) PKR’s role in prototypical neuroinflammation, iii) the capacity in which DIO makes the brain susceptible to MPTP challenge through PKR signaling, and iv) whether PKR contributes to PD neuropathology by serving a pro-apoptotic function.

Materials and Methods

2.1 Study Design

Two independent cohorts of male C57BL/6 mice were subjected to a diet-induced obesity (DIO) paradigm with either a high fat diet (HFD) or control diet (CD) (see 2.2). Cohort #1 was maintained on their respective diet for 12 weeks and grouped by either i)
Control Diet, or ii) High Fat Diet (n = 3-4 per group). In Cohort #2, mice were maintained on their respective diet for 14 weeks and grouped by: i) Control Diet (n = 3 -5), ii) High Fat Diet (n = 3 -5), iii) Control Diet + MPTP (n = 3 -5), and iv) High Fat Diet + MPTP (n = 3 -5). Following dietary intervention, animals were acutely challenged with MPTP to induce a transient Parkinsonism (see 2.3). Animals were sacrificed either 1 day or 7 days post-MPTP. Both 1 and 7 day post-MPTP groups included respective controls.

2.2 Animals, Treatment, and Well-being

Male C57BL/6 (Charles River) were kept in group housing (n = 5/cage) and maintained on a 12-hour reverse light cycle in ventilated Innovive racks (Innovive, San Diego), with ad libitum food (Envigo, 2014 Teklad; 13% kcal from fat) and water access. Animals assigned to a HFD were fed ad libitum for 12-14 weeks (Research Diets, Inc.; 60% kcal from fat). Prior to any treatment, mice were environmentally acclimated for at least 1 week. Six to eight-week-old mice were used in all studies. All studies were approved by the Institutional Animal Care and Use Committee of Iowa State University.

Body weight was recorded weekly during dietary treatment. Following MPTP challenge, animals were individually-housed and well-being was monitored twice daily for food and water intake. Once MPTP-challenged, pre- and post-MPTP body weights were recorded. In order to maintain wellness, mice too weak from infection were supplied with food and water combined in petri dishes for better access. Animals whose body weight loss exceeded 20% were removed from the study and sacrificed.

At the end of a study’s respective duration, animals were sacrificed via asphyxiation (CO₂), brains removed and micro-dissected for the hippocampus and striatum, then snap-frozen in liquid nitrogen for subsequent processing.
2.3 MPTP Formulation and Administration

Animals were subjected to an acute MPTP paradigm to establish neuroinflammation and rapid dopamine degeneration. Mice received four intraperitoneal (I.P.) injections of either 5 mg/kg MPTP-HCl dissolved in saline (free-base; Axon Medchem 1075) at 2-hour intervals, or saline-alone in non-MPTP groups. Animals were sacrificed either 1 day post-MPTP or 7 days post-MPTP. To account for dosing differences, MPTP administration was based on Body Surface Area (BSA), as opposed to body weight (M Bousquet et al., 2012; Cheung et al., 2009).

Of special note, the original study design intended to repeat the 12-week DIO paradigm used in the initial study. However, at the time of the original MPTP-administration with 18 mg/kg (4x I.P.; every 2 hours), unexpected animal death occurred to 100% HFD-treated mice. This death occurred either before all 4 injections could be administered, or within 12 hours of the final injection. No other animals from any group experienced lethality with MPTP. A follow-up pilot MPTP dose response study tested the lethality of 10 mg/kg MPTP (4x I.P.; every 2 hours) and 5 mg/kg MPTP (4x I.P.; every 2 hours) in HFD-treated mice (n = 3 per group). The 10 mg/kg MPTP dose was lethal in 2/3 mice tested, while no mortality was observed following the 5 mg/kg MPTP dose. Hence, this dose was used in the remainder of the study and accounts for our limited sample size.

2.4 Gene Expression

RNA isolation was performed on snap-frozen brain tissue using an RNA binding-column method (Qiagen RNeasy Micro Plus Kit; #74034). Total RNA was isolated from one hemisphere and reverse transcribed to cDNA (Qiagen RT2 First Stand Kit; #330404) to be used in SYBR green assays for relative gene expression (Qiagen RT2 SYBR Green Fluor
qPCR Mastermix; 330513). Qualitative Polymerase Chain Reaction (qPCR) was performed on a Bio-Rad MyIQ PCR system for 40 cycles of amplification, with a subsequent melt curve to validate the purity of amplified product. Gene data from the first cohort of obese mice was collected using RT² Profiler PCR Arrays for Type I Interferon Response (Qiagen; cat. No. 330231). Gene data from the second cohort of mice was collected with primers for: TNF-α, IL-6, IL-1β, NLRP3, ASC, PKR, IBA1, and GFAP. The primer sequences used are available in Supplementary Table 1. Relative difference of gene expression between groups was analyzed using the Pfaffl Method (Pfaffl, 2001), where threshold cycle (Ct) values were normalized to both β-actin and GAPDH, and expressed relative to control groups.

2.5 Protein Expression

Protein lysates from one hemisphere of each animal were homogenized with a motorized pestle in 150 µl of custom Lysis Buffer A (30 mM NaHEPES, 5 mM EGTA, 3 mM EDTA, 20 mM KCL, 32% glycerol, phosphatase inhibitor cocktail 3 (Sigma-Aldrich; P0044), and HALT protease inhibitor cocktail (Thermo Fisher; 78425)). Homogenates were centrifuged for 15 minutes at 15,000 g (4°C), and supernatant collected. Total protein was quantified using the bicinchoninic acid method (BCA; Pierce Biotechnology, Inc.; #23227) with bovine serum albumin standards. Western blot protein samples (10-30 µg) were run on 8-16% Criterion TGX Precast Gels (Bio-Rad) at 150V for 1.5 hours, transferred to polyvinylidene fluoride membranes (PVDF; EMD Millipore), and blocked in Tris-buffered saline (TBS; pH 8.0) containing 0.05% Tween-20 and 5% non-fat dry milk (LabScientific; #M0841) for 1 hour at room temperature. Membranes were incubated overnight at 4°C with primary antibody at the manufacturer recommended dilution. Primary antibody solutions were prepared in 1x TBST, 0.05% Na Azide, and 3% BSA and markers included: NLRP3
(Adipogen; cryo-2), p-PKR (Thr 451; EMB Millipore; #07-886), and β-actin (CST; #4970).

Primary mouse monoclonal antibodies from Santa Cruz Biotechnology included: PKR (sc-6282), p-PACT (sc-53524), PACT (sc-377103), FADD (sc-271748), Caspase-8 (sc-81656), and NF-κB p65 (sc-8008). Cut membranes were washed with 1x TBST (0.1% Tween), probed at room temperature for 1 hour with respective secondary antibody, treated with SuperSignal ELISA Femto Maximum Sensitivity Substrate (Thermo; #970740), and images captured on a Bio-Rad Universal Hood II gel imager. Densitometry was performed using ImageJ software (NIH), normalizing all values to β-actin, and expressed relative to control groups. Cropped Western Blot images show group representative samples run from the same gel, with corresponding housekeeping control.

2.6 Analysis

Data were analyzed in GraphPad Prism (version 6 for Windows) using a Student’s T test for studies comparing high fat diet to control diet, and a 2-way analysis of variance (ANOVA) on both gene and protein expression values for combined DIO and MPTP studies. Post-hoc multiple comparisons were performed using a Tukey test, with an α-level of 0.05, and reported as (mean fold change ± SD, p-value). Figures were generated in GraphPad, reporting data as mean fold change with error propagated as 95% Confidence Intervals. Gene data were represented on a Log2 scale, with protein data on a linear scale. Significance above bar denotes difference from control, with between group differences denoted by line. ****p < 0.0001, *** p < 0.001, ** p < 0.01, * p < 0.05 by 1-way ANOVA.

Results

3.1 Diet-induced obesity (DIO) modulates type I interferon gene expression in the hippocampus and striatum
To determine the effect that diet-induced obesity can have on the Type I Interferon gene expression profile in the murine brain, we performed a microarray analysis on the hippocampus and striatum of male C57BL/6 mice fed a high fat diet (60% kcal from fat) for 12 weeks. This duration of diet has previously been shown to produce a neuroinflammatory phenotype in the hippocampus (Hao et al., 2016). Of 84 different genes analyzed, the expression of only 2 genes in the hippocampus were significantly changed by DIO (Table 1). PKR (3-fold), also referred to as eukaryotic initiation factor 2-alpha kinase, and met proto-oncogene (19-fold) were significantly up-regulated.

The striatum appeared to exhibit a greater number of DIO-induced genes changes (Table 2). The following genes were significantly up-regulated following DIO: PKR (5.6-fold), IFIT3 (6.5-fold), IFITM1 (6.9-fold), NMI (8-fold), OAS1A (8.8-fold), PML (17-fold), PACT (37-fold), PSME2 (42-fold), SHFM1 (9-fold), and HSP90 (6-fold). As part of the interferon pathway, the general function of these genes pertains to the antiviral response of the innate immune system (Akira, Uematsu, & Takeuchi, 2006; Balachandran et al., 2000). As PKR was significantly up-regulated in both the hippocampus and striatum, a follow-up study was performed to prioritize the characterization of CNS PKR in a DIO + MPTP dual model of inflammatory challenge.

3.2 Hippocampal PKR gene expression is not synergistic under dual DIO + MPTP inflammatory challenge

After 14 weeks of DIO, male C57BL/6 mice were challenged with MPTP, and sacrificed 24 hours post-MPTP. At 1 day post-MPTP, the only significant gene changes in the striatum were for the pro-inflammatory cytokine TNF-α. A two-way ANOVA between Diet and MPTP treatment revealed a significant main effect for MPTP, $F(1, 12) = 69.03, p <$
Post-hoc comparisons using a Tukey test revealed that when compared to control diet-alone, striatal TNF-α was significantly increased by MPTP-alone treatment (9.7±0.73, \( p = 0.0006 \)), and by HFD + MPTP (10.72±1.57, \( p = 0.0009 \)). Multiple comparisons were also significant between HFD and MPTP-alone (\( p = 0.0002 \)) and between HFD and HFD + MPTP (\( p = 0.0002 \)). There were no significant main effects for Diet and/or MPTP for striatal gene expression of IL-6, IL-1β, NLRP3, ASC, or PKR.

Hippocampal gene expression for IL-1β was significantly increased from control diet in both the MPTP-alone and HFD+MPTP groups at 1 day post-MPTP. A two-way ANOVA between Diet and MPTP treatment revealed a significant main effect for MPTP, \( F(1, 9) = 57.18, p < 0.0001 \). When compared to control diet-alone, post-hoc comparisons using a Tukey test revealed a significant increase in IL-1β by MPTP-alone (3.71±0.31, \( p = 0.0173 \)) and with HFD + MPTP (6.21±0.28, \( p = 0.0022 \)). There was also a significant difference between HFD and MPTP-alone (\( p = 0.0021 \)) and between HFD and HFD + MPTP (\( p = 0.0003 \)).

Also, in the hippocampus at 1 day post-MPTP, there was a significant interaction between MPTP and Diet for PKR gene expression, \( F(1, 11) = 6.498, p = 0.027 \). Post-hoc comparisons using a Tukey test revealed that when compared to control diet-alone, PKR gene expression was significantly increased by HFD-alone (2.28±0.40, \( p = 0.0113 \)), MPTP-alone (2.48±0.25, \( p = 0.0059 \)), and with HFD + MPTP (2.71±0.21, \( p = 0.003 \)). The relative magnitude of gene expression did not vary between treatment groups, suggesting that the level of CNS PKR induction does vary between systemic inflammation from DIO, acute neurotoxicity from MPTP, or the combined inflammatory challenge of HFD + MPTP.
There were no hippocampal gene changes 1 day post-MPTP for TNF-α, IL-6, NLRP3, or ASC.

3.3 Hippocampal PKR gene expression is time-dependent, following MPTP

In addition to the 1 day post-MPTP timepoint, an additional cohort of animals were sacrificed 7 days post-MPTP and striatal and hippocampal samples were assessed for TNF-α, IL-6, IL-1β, NLRP3, ASC, and PKR. Neither HFD nor MPTP caused gene expression changes to any gene of interest. However, there was no detection for any of the assessed genes in the HFD + MPTP group at this timepoint. No follow-up experiments were performed to investigate this phenomenon, but a tempting conclusion would be that dual HFD + MPTP treatment lead to a decrease in viable, signal-producing cells.

At 7 days post-MPTP in the hippocampus, there were significant gene expression changes for IL-1β and NLRP3. Compared to control, a two-way ANOVA between Diet and MPTP treatment revealed that hippocampal IL-1β was significantly decreased by MPTP, $F(1, 11) = 35.05, p = 0.0001$. Post-hoc analysis revealed a significant difference between control diet and MPTP-alone ($0.41±0.98, p = 0.0227$), between control diet and HFD + MPTP ($0.30±0.26, p = 0.0023$), between HFD and MPTP-alone ($p = 0.0114$), and between HFD and HFD + MPTP ($p = 0.0021$).

Hippocampal NLRP3 gene expression at 7 days post-MPTP exhibited a similar pattern of down-regulation as IL-1β. MPTP treatment produced a significant main effect, $F(1, 12) = 42.02, p < 0.0001$. Post-hoc analysis indicated that group difference was significant between control diet and MPTP ($0.51±0.12, p = 0.0071$), control diet and HFD + MPTP ($0.46±0.33, p = 0.0023$), HFD and MPTP-alone ($p = 0.0040$), and HFD and HFD + MPTP ($p = 0.0013$).
There were no observed changes to hippocampal TNF-α, IL-6, or ASC gene expression 7 days post-MPTP. The only gene that exhibited changes at both the 1 and 7 day post-MPTP timepoints was IL-1β, increasing at 1 day with MPTP and decreasing by 7 days post-MPTP. Hippocampal gene expression of PKR 7-days post-MPTP had a significant main effect of MPTP, $F(1, 10) = 9.86, p = 0.011$. Post-hoc analysis with a Tukey test revealed this effect was mediated by a significant different between HFD and MPTP-alone ($p = 0.0394$). This attenuation of HFD-induced PKR signal could also be attributed to loss of PKR-expressing cells at this time point, similar to what was observed for gene expression in the striatum. However, protein analysis for caspase-3 in the hippocampus at 7 days post-MPTP did not reveal any significant differences in expression of this executioner caspase (data not shown).

3.4 No detectable expression of microglial activation

In an effort to survey the involvement of astrocytes and microglia in our models, we performed gene expression for GFAP and IBA1 at 1 and 7 days post-MPTP. The only significant changes in glial cell activation markers were to GFAP in the striatum at 1 day post-MPTP. A two-way ANOVA revealed that MPTP treatment had a significant main effect, $F(1, 12) = 58.48, p < 0.0001$. Post-hoc analysis with a Tukey test indicated GFAP gene expression was significantly increased by MPTP-alone (6.57±1.21, $p = 0.0023$), and by HFD + MPTP (10.71±0.50, $p = 0.0007$), when compared to control diet. Additionally, there was a significant difference between HFD and MPTP-alone ($p = 0.0009$), and between HFD and HFD + MPTP ($p = 0.0003$).

There was no significant gene induction for IBA1 in either the striatum or hippocampus, at either 1 or 7 days post-MPTP.
3.5 Expression of phosphorylated PKR is inverse of total

Having only observed hippocampal PKR gene induction at 1 day post-MPTP, our protein analysis using Western blotting only examined these tissues. For PKR changes, we observed a significant decrease to phosphorylated PKR, when taken as a ratio over total PKR changes. A two-way ANOVA revealed a significant main effect of MPTP treatment, $F(1, 12) = 17.8, p = 0.0012$. Post-hoc analysis with a Tukey test indicated that hippocampal p-PKR protein was significant decreased from control with dual HFD + MPTP treatment ($0.56 \pm 0.14, p = 0.0059$) and that this difference was also significant between HFD and HFD + MPTP ($p = 0.0056$) (Figure 5A). As for changes to total PKR, there were significant main effects for MPTP treatment, $F(1, 12) = 18.0, p = 0.0011$ and also for Diet, $F(1, 12) = 8.07, p = 0.0149$. However, there was no significant interaction. Post-hoc analysis indicated that MPTP produced a significant increase in PKR protein ($1.56 \pm 0.21, p = 0.0311$), and in HFD + MPTP animals ($1.81 \pm 0.28, p = 0.0017$), when compared to control (Figure 5A).

As for central PKR signaling events we observed a significant interaction to upstream protein activator of PKR (PACT) between Diet and MPTP, $F(1, 12) = 7.07, p = 0.021$. However, post-hoc analysis revealed no group differences. Also, two-way ANOVA revealed a significant main effect of MPTP, $F(1, 11) = 5.44, p = 0.0397$ for phosphorylated-eukaryotic initiation factor 2α (p-eIF2α) (Figure 5B). As with PACT, post-hoc analysis did not reveal any significant differences between groups (Figure 5B). This suggest that PKR’s downstream actions is likely mediated through other signaling pathways. The magnitude of p-PKR protein expression did not vary when data were analyzed as p-PKR alone, compared to p-PKR/total PKR.

3.6 Dual DIO + MPTP challenge produces synergistic changes to pro-inflammatory cytokine
Of expected inflammatory changes, diet-induced obesity was expected to produce a neuroinflammatory signature. Additionally, MPTP paradigms have previously been shown to produce a myriad of inflammatory changes, both systemically and centrally. A two-way ANOVA revealed a significant interaction between Diet and MPTP for hippocampal IL-1β protein, $F(1, 8) = 6.65, p = 0.0327$, with post-hoc analysis indicating a significant increase in IL-1β by MPTP-alone (2.97±0.64, $p = 0.0149$), compared to control.

There was a significant interaction between Diet and MPTP for hippocampal NLRP3 protein, $F(1, 12) = 18.0, p = 0.0012$. Post-hoc analysis indicated that NLRP3 was significantly decreased by HFD + MPTP ($p = 0.0026$), compared to HFD, and also between MPTP-alone and HFD + MPTP ($p = 0.0365$).

A two-way ANOVA of TNF-α protein expression indicated a significant interaction between Diet and MPTP, $F(1, 11) = 5.54, p = 0.0382$. Post-hoc analysis indicated this significant difference was between control diet and an increase with HFD + MPTP (3.0±0.71, $p = 0.0005$). Additional differences were between HFD and HFD + MPTP ($p = 0.0057$) and between MPTP-alone and HFD + MPTP ($p = 0.0004$) (Figure 6).

3.7 DIO increases hippocampal expression of proapoptotic and proinflammatory markers

As for hippocampal protein expression of PKR downstream effectors, Diet produced a significant difference in FADD protein expression, $F(1, 10) = 17.6, p = 0.0018$. Post-hoc analysis indicated that this apoptotic marker was significantly increased by HFD (2.07±0.44, $p = 0.0085$), compared to control, and significantly different between HFD and MPTP ($p = 0.0424$).

For caspase-8 expression, an initiator caspase that forms a death signaling complex with FADD, a two-way ANOVA produced a significant main effect of Diet and MPTP that
led to a significant interaction, $F(1, 11) = 14.4, p = 0.0030$. Post-hoc analyses indicated that caspase-8 was significantly increased by HFD + MPTP (2.68±0.60, $p = 0.0004$), compared to control diet. There was also a significant difference between HFD and HFD + MPTP ($p = 0.0045$), and between MPTP-alone and HFD + MPTP ($p = 0.0004$). Elevated expression of caspase-8 appeared to depend on dual HFD + MPTP challenge.

Downstream of FADD/Caspase-8 signaling, NF-κB p65 was significantly changed by both Diet and MPTP, yielding a significant interaction, $F(1, 12) = 6.29, p = 0.027$. Post-hoc analysis indicated that p65 was significantly increased from control diet by HFD-alone, (2.22±0.37, $p = 0.0009$). Additionally, there was a significant difference between HFD and MPTP-alone ($p = 0.0013$), and between HFD and HFD + MPTP ($p = 0.027$). These differences were mediated by the HFD-induced increase in p65 (Figure 7).

**Discussion**

The present study proposes a novel neuroinflammatory mechanism, double-stranded RNA-dependent protein kinase (PKR), that mediates the effect of diet-induced obesity (DIO) on the brain and the capacity in which DIO predisposes the brain to Parkinsonian insult. Collectively, this report is the first to show that DIO elicits PKR expression in the brain, PKR does not regulate NLRP3 in the brain following dual DIO + MPTP challenge, and that DIO-induced PKR activation mediates the expression of apoptotic FADD/caspase-8 in the brain.

**4.1 Diet-induced obesity (DIO) through a high fat diet elicits PKR gene activation**

In an initial study characterizing the gene expression profile of the Type I Interferon pathway in the brains of mice fed a HFD for 12 weeks, we observed increased expression of double-stranded RNA-dependent protein kinase (PKR) in the striatum and hippocampus. Reports to-date of PKR expression in the brain have come from observations that elevated phosphorylated PKR (p-PKR) can be found accumulating in neurons, circulating
lymphocytes, and cerebrospinal fluid of Alzheimer’s, Huntington’s, and Parkinson’s disease patients, as well as in animal disease models of neurodegeneration (Bando et al., 2005; Hugon et al., 2017; A. Peel, 2003). Under the context of beta-amyloid (Aβ)-mediated neurodegeneration, the primary neuropathology in Alzheimer’s disease, PKR has been implicated as a pro-apoptotic kinase that exacerbates neuronal death and accumulation of proteinopathy (Mouton-Liger et al., 2012; Mouton-Liger et al., 2015). As it relates to Parkinson’s disease, PKR has recently been shown to phosphorylate and promote α-synuclein pathology, the protein that comprises “Lewy bodies” (Reimer et al., 2018).

Although a pro-apoptotic function of PKR has been well-established, PKR has also been implicated as a key inflammatory mediator capable of producing innate immune responses to a variety of extracellular stressors (R. Kang & Tang, 2012; Marsollier, Ferré, & Foufelle, 2011).

4.2 PKR does not appear to contribute to DIO-induced NLRP3 CNS expression

In an effort to capture the mechanism through which inflammatory PKR expression is induced in the obese brain and how this signal is impacted by Parkinsonian insult, we examined gene and protein expression of the NLRP3 inflammasome. NLRP3 has been implicated as a key neuroinflammatory player in neurodegeneration (De Nardo & Latz, 2011; Gustin et al., 2015; M. T. Heneka et al., 2013; Jha et al., 2010; Song et al., 2017). We did not observe any gene changes to NLRP3 expression at 1 day post-MPTP in either the striatum or hippocampus. This timepoint is when we observed upregulation of hippocampal PKR by HFD, MPTP, and HFD + MPTP. We did however observe NLRP3 downregulation in the hippocampus at 7 days post-MPTP, following MPTP and HFD + MPTP. This expression was
driven by MPTP, which is consistent with previous reports (E. Lee et al., 2018), and did not appear to be influenced by DIO.

While PKR had previously been suggested to function as an upstream authorizing event prior to NLRP3 expression (Lu et al., 2012), our observations do not support this relationship. Others have supported this by observing that PKR does not modulate the inflammasome at all (He et al., 2013; Lancaster et al., 2016) or have suggested that PKR actually suppresses inflammasome activity (Yim et al., 2016). Additional evidence that challenges a PKR-NLRP3 relationship in the brain is that astrocytes do not express NLRP3 (Gustin et al., 2015), despite expressing PKR (Farina et al., 2007).

4.3 PKR expression does not appear dependent on prototypical neuroinflammation

We hypothesized that PKR induction would occur as a response to increased expression of pro-inflammatory TNF-α, IL-6, and IL-1β. As a triad of pro-inflammatory cytokines that are induced in the hippocampus following obesity (Guillemot-Legris & Muccioli, 2017), we expected their expression to coincide with PKR activation. The only cytokine up-regulated at the time of hippocampal PKR was interleukin-1β. PKR expression has previously been shown to occur independent of IL-1β in virus-infected epithelial cells, but to be dependent on TNF-α (Meusel, Kehoe, & Imani, 2002). This is further supported by the observation that PKR knockout in bone marrow-derived macrophages (BMDMs) of HFD-induced obese mice does not suppress IL-1β secretion (Lancaster et al., 2016). High fat feeding has been shown to produce an early transient spike in CNS TNF-α that recedes, but will return if HFD feeding is sustained (Spencer et al., 2017; Thaler et al., 2012; Waise et al., 2015). As for the peripheral source of this inflammatory signal, white adipose tissue exhibits increased production of TNF-α and IL-6. When treated with a PKR inhibitor in a genetic
model of obesity, this cytokine expression is reduced (Nakamura et al., 2014; Nakamura et al., 2010).

As for the CNS cell type mediating neuroinflammation and contributing to disease progression, astrocytes and microglia are implicated as the primary neuroinflammatory effectors (Teismann & Schulz, 2004). We hypothesized that PKR expression would be derived from activated microglia, yet we did not observe IBA1 gene expression in either the striatum or hippocampus. Ionized calcium-binding adaptor molecule 1 (IBA1) is a well-validated marker of microglial expression that corresponds to actin-mediated morphological changes of activation (Hoogland et al., 2015; Sasaki, Ohsawa, Kanazawa, Kohsaka, & Imai, 2001). Obesity-induced microglial activation, characterized by IBA1 expression (Hao et al., 2016), has regularly been implicated as a neuropathogenic event (Johnson, 2015; A. A. Miller & Spencer, 2014). Both astrocytes and microglia have been shown to express PKR in animal models of Gaucher’s disease, a form of Parkinsonism that occurs from glucocerebrosidase (GBA) mutation (Vitner et al., 2016). Based on previous work in human and animal tissues, neurons are the most likely source of PKR signal in our model (Carret-Rebillat et al., 2015; Hugon et al., 2017; Mouton-Liger et al., 2015). Even more so, hippocampal neurons in human PD brains have been shown to express PKR (Bando et al., 2005).

4.4 PKR expression is not additive under dual DIO + MPTP challenge

The pathogenic potential of inflammation, as a disease driver in neurodegeneration, is predicated on the basis that multiple inflammatory “hits” amass in an additive fashion and can disrupt cellular homeostasis (Colm Cunningham, 2013). As such, we hypothesized that CNS PKR expression would be greater is dual challenged mice (HFD + MPTP), compared to
HFD-alone, or MPTP-alone. While we observed significant PKR gene induction in the hippocampus by HFD-alone, MPTP-alone, and HFD + MPTP, there was no difference in the magnitude of its expression between groups. This transcriptional expression would suggest that PKR is functioning as a stress response node, as opposed to a terminal effector.

Hippocampal protein expression of PKR was significantly increased by MPTP and HFD + MPTP, which was mediated by MPTP and not obesity. Conversely, phosphorylated PKR protein was significantly down-regulated in the hippocampus with HFD + MPTP. Although HFD + MPTP does not induce a synergistic effect on PKR gene expression, it does for PKR protein expression. It would be expected that p-PKR and total PKR expression would positively correlate, as PKR phosphorylation has previously been shown to correspond to the phosphorylation of PKR’s canonical substrate in in vitro models of Alzheimer’s disease, eukaryotic initiation factor 2-α (eIF2-α) (Chang et al., 2002; M. A. Garcia et al., 2006). However, previous work by our lab has shown that PKR phosphorylation increases in the brain following systemic inflammation with LPS at 3 hours post-challenge, but dissipates by 24 hours post-challenge (Jefferson et al., in preparation). In the current studies, the observed decrease in hippocampal p-PKR could either be a limitation of our collection timepoint post-MPTP, or a compensatory decrease to cell stress that yields a total increase.

MPTP has a known capacity to induce neuroinflammation beyond the basal ganglia (Machado, Zoller, Attaai, & Spittau, 2016; Sriram et al., 2006), but we had to administer a dose much lower than what standard models utilize (5 mg/kg vs 15-20 mg/kg). This may have resulted in a reduced neuropathological insult. Additionally, our analyses are limited to smaller sample sizes, as we unexpectedly experienced attrition in our HFD + MPTP animals. In a neurodegenerative context, PKR-induced expression of p-eIF2-α has been shown to
regulate an apoptotic response (Ingrand et al., 2007; A. Peel, 2003). Our results do not support a PKR-eIF2-α pathway as a regulator of apoptosis in the brain following systemic inflammatory challenges with DIO and MPTP.

4.5 Increased pro-apoptotic FADD and inflammatory NF-κB are activated downstream of DIO-induced PKR expression

Chronic inflammatory states are known to precipitate neurodegenerative disease, including PD, and precede additional pathogenic processes (i.e., apoptosis) (Clark & Kodadek, 2016; Mosley et al., 2012; Sparkman & Johnson, 2008; Walker et al., 2015; Zitvogel, Kepp, & Kroemer, 2010). Uniquely having reported roles in both inflammation and apoptosis, we hypothesized that CNS PKR expression was serving a pro-apoptotic function following HFD + MPTP. We observed a HFD-mediated increase in fas-associated death domain (FADD) expression in the hippocampus, which has previously been implicated as a mechanism of PKR-mediated apoptosis (von Roretz & Gallouzi, 2010). FADD is an intracellular adaptor protein that can consequently activate the initiator caspase, caspase-8, in response to extracellular stress signals (Balachandran et al., 1998). FADD-activated caspase-8 can go on to either directly activate the executioner caspase-3, or promote Bcl-2 activation that leads to mitochondrial release of cytochrome c (Xiao-Ming, 2000). In HeLa CCL-2 epithelial cells treated with staurosporine, an apoptosis-inducing bacterial alkaloid, PKR was shown to induce apoptosis through FADD/caspase-8 signaling. This activation was not dependent on PKR phosphorylation, nor eIF2-α phosphorylation (von Roretz & Gallouzi, 2010). Additionally, the PKR/FADD apoptotic pathway has been shown to mediate Aβ-induced cell death in SH-SY5Y neurons (Couturier et al., 2010). Future studies should identify the extracellular mechanism responsible for activating PKR, as Fas-FADD are not
the only pathways involved in caspase-8/PKR-mediated apoptosis, but also involve TNF-TNFR1 interactions (Gil & Esteban, 2000).

Concurrently, we also observed a DIO-mediated increase in hippocampal NF-κB p65 protein expression. As DIO induces neuroinflammation, and inflammatory processes are mediated via NF-κB signaling, we expected to observe an increase in PKR-induced NF-κB expression. This activation of the NF-κB pathway occurred either from direct activation from FADD through TNFR1 or Fas-induced apoptosis (W.-H. Hu et al., 2000; Ranjan & Pathak, 2016), or through PKR itself (Zamanian-Daryoush et al., 2000). Furthermore, PKR-mediated activation of NF-κB does not require catalytically active PKR (Bonnet et al., 2000). What is unclear in our studies is whether NF-κB is activating as an anti-apoptotic response to limit PKR-mediated apoptosis, or is activating apoptosis to promote cell death following inflammation.

4.6 Conclusion

These data add to the rapidly accumulating literature that systemic inflammation, through detrimental host factors such as diet-induced obesity, can predispose the brain to additional environment insults. As one sustains multiple inflammatory “hits” over the course of their lifetime, this damage synergistically promotes neuronal dysfunction and progressively precipitates the development of neurodegenerative disease. Diet-induced obesity produces systemic inflammation, which following a Parkinsonian insult, elicits PKR activation in the brain that appears to serve an apoptotic function. As there are currently no disease-modifying therapies for PD, this work implicates PKR as a therapeutic target of interest to reduce neuronal cell death and also highlights the importance of modifiable lifestyle factors and their capacity to improve brain health.
Hippocampal type I interferon gene expression profile after 12 weeks of diet induced obesity (DIO).

Male C57BL6 mice were fed a 60% High Fat Diet (HFD) for 12 weeks, brains micro-dissected, and qPCR performed using Type I Interferon Arrays (Qiagen). Data excluded for genes of interest that did not have expression for all group samples. Data represented as mean fold change ± 95% confidence intervals, relative to control. ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05 by 1-way ANOVA. (n = 3-4 per group)
Table 4.2. Striatal type I interferon gene expression profile after 12 weeks of diet-induced obesity (DIO).

Male C57/BL6 mice were fed a 60% High Fat Diet (HFD) for 12 weeks, brains micro-dissected, and qPCR performed using Type I Interferon Arrays (Qiagen). Data excluded for genes of interest that did not have expression for all group samples. Data represented as mean fold change ± 95% confidence intervals, relative to control. ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05 by 1-way ANOVA. (n = 3-4 per group)

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>P value</th>
<th>Sig?</th>
<th>Control Diet</th>
<th>High Fat Diet</th>
<th>Difference</th>
<th>SE of difference</th>
<th>T ratio</th>
<th>df</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine deaminase, RNA-specific</td>
<td>0.658692</td>
<td></td>
<td>1</td>
<td>0.54507</td>
<td>0.45492</td>
<td>0.73307</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Bone marrow stromal cell antigen 2</td>
<td>0.905501</td>
<td></td>
<td>1</td>
<td>1.00794</td>
<td>-0.00794</td>
<td>1.32379</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Caspase 2</td>
<td>0.28616</td>
<td></td>
<td>1</td>
<td>4.30122</td>
<td>-3.30122</td>
<td>2.3465</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Caveolin 1, caveolin protein</td>
<td>0.60127</td>
<td></td>
<td>1</td>
<td>1.75493</td>
<td>-0.75492</td>
<td>1.34179</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Chemokine (C-X-C motif) ligand 1</td>
<td>0.995501</td>
<td></td>
<td>1</td>
<td>1.00794</td>
<td>-0.00794</td>
<td>1.32379</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Death-associated factor 2-alpha</td>
<td>0.215636</td>
<td></td>
<td>1</td>
<td>4.30122</td>
<td>-3.30122</td>
<td>2.3465</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Caveolin 1, caveolin protein</td>
<td>0.60127</td>
<td></td>
<td>1</td>
<td>1.75493</td>
<td>-0.75492</td>
<td>1.34179</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Chemokine (C-X-C motif) ligand 1</td>
<td>0.995501</td>
<td></td>
<td>1</td>
<td>1.00794</td>
<td>-0.00794</td>
<td>1.32379</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Death-associated factor 2-alpha</td>
<td>0.215636</td>
<td></td>
<td>1</td>
<td>4.30122</td>
<td>-3.30122</td>
<td>2.3465</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Caveolin 1, caveolin protein</td>
<td>0.60127</td>
<td></td>
<td>1</td>
<td>1.75493</td>
<td>-0.75492</td>
<td>1.34179</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Chemokine (C-X-C motif) ligand 1</td>
<td>0.995501</td>
<td></td>
<td>1</td>
<td>1.00794</td>
<td>-0.00794</td>
<td>1.32379</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Death-associated factor 2-alpha</td>
<td>0.215636</td>
<td></td>
<td>1</td>
<td>4.30122</td>
<td>-3.30122</td>
<td>2.3465</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

- **p < 0.05
- *p < 0.01
- **p < 0.01
- ***p < 0.001
- ****p < 0.0001
Figure 4.1 Weight gain by High Fat Diet (HFD).

Male C57/BL6 mice were ad-libitum fed a 60% HFD for A) 12 weeks in one study and for B) 14 weeks in a follow-up study. Mice fed a HFD gained approximately twice the amount of body weight as mice fed a standard control diet. Total weight gain was comparable between both obesity studies. Data shown as mean difference in body weight ± 95% confidence intervals. (n = 3-5 per group)
Figure 4.2 Hippocampal PKR gene expression was induced by High Fat Diet (HFD), MPTP, and dual HFD+MPTP challenge, 1 day post-MPTP.

Male C57/BL6 mice were fed a HFD for 14 weeks and then challenged with 5 mg/kg MPTP (4x I.P., every 2 hours) or saline. Brain samples were collected either 1 or 7 days post-MPTP. qPCR was performed on the striatum and hippocampus for A) pro-inflammatory cytokines, B) inflammasome-related, and C) PKR. The magnitude of PKR gene expression in the hippocampus was not additive under dual-DIO+MPTP challenge. Data represented as mean fold change ± 95% CI on a Log2 scale. Significance above bar denotes difference from control, with between group differences denoted by line. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ by 1-way ANOVA (n = 3-5 per group).
Figure 4.3 Hippocampal PKR gene expression dissipates by 7 days post-MPTP.

Male C57/BL6 mice were fed a High Fat Diet for 14 weeks and then challenged with 5 mg/kg MPTP (4x I.P., every 2 hours) or saline. Brain samples were collected either 1 or 7 days post-MPTP. qPCR was performed on the striatum and hippocampus for A) pro-inflammatory cytokines, B) inflammasome-related, and C) PKR. The only gene changes were to IL-1β and NLRP3 being downregulated by MPTP and HFD+MPTP, along with a difference in hippocampal PKR gene expression. Gene expression was not detectable (ND) in the striatum for HFD+MPTP samples. Data represented as mean fold change ± 95% CI on a Log2 scale. Significance above bar denotes difference from control, with between group differences denoted by line. ****p < 0.0001, *** p < 0.001, ** p < 0.01, * p < 0.05 by 1-way ANOVA (n = 3-5 per group).
Figure 4.4 Hippocampal PKR expression is not likely mediated by astrocyte (GFAP) or microglial (IBA1) activation.

Male C57/BL6 mice were fed a High Fat Diet for 14 weeks and then challenged with 5 mg/kg MPTP (4x I.P., every 2 hours) or saline. Brain samples were collected either 1 or 7 days post-MPTP. qPCR was performed on the striatum and hippocampus for A) glial-fibrillary acidic protein (GFAP), B) ionized calcium binding adaptor molecule 1 (IBA1). The only gene expression changes were MPTP-mediate GFAP upregulation in the striatum, day post-MPTP. Data represented as mean fold change ± 95% CI on a Log2 scale. Significance above bar denotes difference from control, with between group differences denoted by line. ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05 by 1-way ANOVA (n = 3-5 per group).
Following 14 weeks of diet-induced obesity with 60% kcal HFD, male C57/BL6 mice were acute challenged 5 mg/kg MPTP (4x I.P., every 2 hours) or saline. A) Protein analysis indicated a negative relationship between PKR and p-PKR (Thr451), following HFD+MPTP treatment. B) There were no changes to stereotypical PKR activators or downstream substrates. Data represented as mean fold change ± 95% CI on a linear scale. Cropped Western Blot images show group representative samples run from the same gel, with corresponding housekeeping control. Significance above bar denotes difference from control, with between group differences denoted by line. ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05 by 1-way ANOVA. (n = 3-5 per group)
Figure 4.6 MPTP treatment induced pro-inflammatory expression in the hippocampus, 1 day post-MPTP.

Protein analysis with western blotting indicated that modest MPTP (5 mg/kg, 4x I.P. every 2 hours) was sufficient to induce IL-1β expression, along with a significant increase in pro-TNF-α in the HFD+MPTP group. NLRP3 protein expression was significantly decreased with HFD+MPTP. Data represented as mean fold change ± 95% CI on a linear scale. Cropped Western Blot images show group representative samples run from the same gel, with corresponding housekeeping control. Significance above bar denotes difference from control, with between group differences denoted by line. ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05 by 1-way ANOVA. (n = 3-5 per group)
Western blotting in the hippocampus 1 day post-MPTP revealed that HFD alone increased apoptotic FADD and NF-κB p65 proinflammatory protein expression, while Pro-Caspase-8 expression was significantly increased from control with HFD+MPTP. Data represented as mean fold change ± 95% CI on a linear scale. Cropped Western Blot images show group representative samples run from the same gel, with corresponding housekeeping control. Significance above bar denotes difference from control, with between group differences denoted by line. ****p < 0.0001, *** p < 0.001, ** p < 0.01, * p < 0.05 by 1-way ANOVA. (n = 3-5 per group)
Supplemental Table 4.1. Primer sequence information for qPCR assays.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5' → 3')</th>
<th>Reverse (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>GCTTCAGGCAGGCAGTATC</td>
<td>AGGATGGGCTCTTTCTCTCAAG</td>
</tr>
<tr>
<td>TNF-α</td>
<td>AGCCGATGGGTTGTACCTTG</td>
<td>ATAGCAAAATCGGCTGAGGTT</td>
</tr>
<tr>
<td>IL-6</td>
<td>ACCGCTATGAAGTTCCTCTCT</td>
<td>CTCTGTGAAGTCTCTCTCTCC</td>
</tr>
<tr>
<td>NLRP3</td>
<td>GCTCCAACCATTCTCTGACC</td>
<td>AAGTAAGGCGGGAATTTCACC</td>
</tr>
<tr>
<td>ASC</td>
<td>AGGAGTGGAGGGGAAAAGC</td>
<td>AGAAGACGCAAGGAGATGG</td>
</tr>
<tr>
<td>PKR</td>
<td>GATGGAAAAATCCCGAACAAGGAG</td>
<td>AGGGCAAAAGCAAAGATGTCCAC</td>
</tr>
<tr>
<td>GFAP</td>
<td>GGTGGAATCGCTGGAGGAG</td>
<td>CTGTGAGGTCTGGCTTTGG</td>
</tr>
<tr>
<td>IBA1</td>
<td>CTTGAAGCGAATGCTGGAGAA</td>
<td>GGCAGCTGGAGATAGCTTT</td>
</tr>
<tr>
<td>β-Actin</td>
<td>GTGACGTTGACATCCGTAAGA</td>
<td>GCCGGACTCATCGTACTCC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>AGGTCGGTGTAACGGATTTG</td>
<td>TGTAGACCATGTTAGTGAGGTCA</td>
</tr>
</tbody>
</table>
References


CHAPTER 5. SUMMARY AND CONCLUSIONS

As introduced in the introduction to this dissertation, Parkinson’s disease (PD) is a neurodegenerative disorder that currently has no disease-modifying therapies. As such, the current course of treatment can only attempt to manage primary symptoms. Although clinically defined as a movement disorder, the non-motor symptoms of PD substantially compromise quality of life for patients. Pathologically, these symptoms predict disease state progression and suggest an underlying pathogenic mechanism, beyond the primary synucleinopathy. Neuroinflammation has recently been implicated as a form of chronic immune activation that can progressively promote a neurodegenerative cascade in the brain. Not only are these events capable of driving disease development in PD, but they can maintain a vicious cycle of neurodegeneration. This early pathogenic process holds a high degree of therapeutic potential because it can lead to the development of early diagnostic tools and drug candidates that can substantially alter the progression of disease.

However, the challenge associated with the study of neuroinflammation and neuroimmunology is that these processes have both adaptive and maladaptive functions. The goal of this work has been to examine the double-stranded RNA-dependent protein kinase (PKR) as a mechanism by which various host factor detriments promote neuroinflammation, that can subsequently contribute to Parkinsonian neurodegeneration. In doing so, it is our goal to highlight the signaling conservation in cell stress responses that can contribute to and precipitate PD development.

In Chapter II, our first aim was to characterize central nervous system (CNS) PKR neuroinflammatory gene expression following acute, systemic bacterial challenge with
lipopolysaccharide (LPS) to determine dose and temporal kinetics. The magnitude of hippocampal and striatal PKR gene expression did not vary between LPS dose intensity (5 mg/kg or 1 mg/kg I.P), nor duration of LPS challenge (3 or 24 hours). This was an unexpected finding. Inflammatory responses generally correspond to the magnitude of the stimuli, as evidenced by pro-inflammatory cytokine expression in these studies. TNF-α gene expression was greatest with 5 mg/kg LPS at 3 hours post-LPS, lower with 1 mg/kg LPS at 3 hours post-LPS, and lowest with 1 mg/kg LPS at 24 hours post-LPS. We hypothesized that PKR expression would behave in this manner, but the magnitude of its gene expression did not vary with the magnitude of LPS challenge. This suggests that PKR may not be acting as a terminal neuroinflammatory effector, but more so as a signal transducing node. Further supporting evidence for this claim is that there was no observable IBA1 gene induction, indicating an absence of microglial activation.

Our second aim was to determine the capacity in which a systemically administered PKR inhibitor (PKRi) can ameliorate an LPS-induced PKR inflammatory phenotype in the CNS. Using 1 mg/kg LPS at 24 hours post-LPS, we repeated our PKR and neuroinflammatory phenotype from the LPS dose and duration study. We also pre-treated LPS-challenged animals with the PKR inhibitor imoxin at two doses, 0.5 mg/kg or 1.0 mg/kg, that were administered 2 and 24 hours pre-LPS. By pre-treating with imoxin, we wanted to know if LPS-induced neuroinflammation would be attenuated because of PKR’s diminished activity. Imoxin was hypothesized to decrease this phenotype. The lower 0.5 mg/kg imoxin dose reduced neuroinflammation to a greater extent, but did not completely abolish LPS-induced neuroinflammation. The dose difference may be attributed to PKR activation kinetics. The observation that imoxin did not reduce LPS-induced
neuroinflammation to baseline suggests that there are non-PKR-mediated mechanisms contributing to inflammatory signal propagation in the brain. Thus, PKR does not appear to act as a sole regulator of neuroinflammation, but rather a signal transmission node.

Chapter III initially characterized murine CNS PKR expression following influenza challenge to determine the dose and temporal kinetics of this response. If PKR served as a mediator of influenza-induced neuroinflammation, we hypothesized that its induction would have occurred early (3-5 days post-infection) and occurred in conjunction with pro-inflammatory cytokine expression, as others have shown hippocampal neuroinflammation by 4 days post-infection (DPI) (Jurgens et al., 2012). From our initial influenza studies, we learned that infection resulting in substantial sickness led to delayed PKR induction in the striatum and hippocampus (15 DPI). This observation did not support our hypothesis. Follow-up studies were performed in vitro where immortalized macrophages were infected with influenza and the conditioned media was used to treat immortalized microglia. This treatment led to proinflammatory cytokine induction, providing direct evidence that infection of peripheral inflammatory immune cells can produce soluble factors which can activate microglia to produce a proinflammatory profile.

This conditioned media was also used to treat immortalized rat dopaminergic neurons in a cell viability assay, in which neurons were dual-treated with influenza-conditioned media and the Parkinsonian neurotoxin, rotenone. Cell loss was additive in influenza+rotenone treated samples, compared to influenza- and rotenone-alone conditions. This highlighted the synergistic potential of influenza-induced neuroinflammation and Parkinsonian-relevant stress. From in vivo studies to examine this multi-hit model of influenza and PD, we were unable to replicate the original magnitude of influenza-induced
PKR expression in the brain. However, we observed modest PKR protein changes and gained insight into the potential influence of strain differences. Returning to our original 15 DPI samples, we identified that PKR expression led to the induction of downstream apoptotic and inflammatory expression. These results suggest that PKR may serve as an influenza-induced stress response that promotes inflammation and apoptosis.

The objective of Chapter IV was to characterize CNS PKR expression in a murine model of diet-induced obesity and determine the extent to which it contributes to MPTP-induced Parkinsonian pathogenesis. After identifying striatal and hippocampal PKR gene expression from microarrays performed on the brains of mice fed a chronic high-fat diet, we performed a follow-up study using a mouse model of diet-induced obesity (DIO), in combination with MPTP. We hypothesized that PKR expression would be greatest in obesity + MPTP samples, compared to obesity-, or MPTP-alone. Only the hippocampus exhibited PKR expression in DIO and DIO + MPTP animals, but this expression was mediated by obesity and the magnitude of expression did not vary between these two groups. PKR signaling coincided with obesity-mediated induction of apoptotic and inflammatory signaling. Additionally, PKR expression did not appear dependent on astrocyte or microglial activation and is likely mediated by neurons, as a cell stress signal.

MPTP models of neurotoxicity have a well-characterized neuroinflammatory signature. From our use of MPTP in our diet-induced obesity studies, we saw up-regulation of PKR at 24 hours post-MPTP, which would support a potential role for PKR in MPTP-induced microglial activation. Following MPTP challenge, microglia activation generally occurs by 24 hours post-MPTP and can remain elevated through at least 7 days post-MPTP (Członkowska, Kohutnicka, Kurkowska-Jastrzębska, & Członkowski, 1996). MPTP-induced
astrocyte activation can occur between 3-4 days post-MPTP (Breidert et al., 2002), although the role of astrocytes in neuroinflammatory processes and Parkinsonian syndrome is much less understood. The chronic activation of microglia and subsequent astrocyte activation, combined with CNS infiltration by adaptive T lymphocytes, creates a vicious neuroinflammatory cycle that promote neuronal distress and neurodegeneration (Hirsch et al., 2016).

We have observed LPS- and MPTP-induced PKR expression following acute challenge (i.e., 24 hours post challenge) and we have also observed delayed PKR expression following influenza and diet-induced obesity (2 weeks and 12 weeks post challenge). Given that we were unable to observe evidence of stress-induced microglial activation in all three of our experimental conditions, operationally defined by gene expression of IBA1, we cannot reasonably infer that PKR expression is dependent on microglial activation. Although our LPS and influenza studies suggest that PKR expression may coincide with astrocyte activation, we did not observe similar patterns of GFAP and PKR expression in our obesity study. Given that MPTP-induced astrogliosis is a latent event (3-4 days post-MPTP), our observed upregulation of GFAP may not be indicative of bona fide morphological activation of astrocytes. Additionally, expression of the pro-inflammatory cytokines TNF-α and IL-1β occurs early following MPTP challenge (6-24 hours post-challenge), while IL-6 upregulation is often delayed (7 days post-challenge). As we had initial evidence (Lu et al., 2012) that implicated PKR upstream of NLRP3 and subsequent IL-1β production, we would have reasonably expected PKR expression to solely exist as an acute phase stress response (Machado 2016). However, we observed PKR expression at delayed time points, in which (relatively) acute phase cytokine production had dissipated. This does not support a direct
role for PKR as a direct neuroinflammatory regulator. What we can reasonably conclude is that CNS PKR expression can occur both early (LPS & MPTP) and delayed (Influenza & Obesity) following immune inflammatory challenge. Future studies should utilize cell-specific in vitro experimental designs to delineate the role of PKR in specific CNS cell types.

Together with existing literature implicating PKR as a neuron-derived stress response signal, we conclude that CNS PKR expression most likely localizes to neurons, occurs rapidly, and can remain elevated over the course of inflammatory challenge. Although entirely context-dependent, this early PKR expression likely functions as a pro-survival signal related to apoptotic signal transduction. We have no evidence of classic cell death to suggest that our observed CNS PKR expression is detrimental to cell health. Although sustained expression of PKR may lead to pro-apoptotic changes within the CNS, this early expression likely acts a compensatory response to stressful cell stimuli. Future studies should take care to temporally define PKR expression and any coinciding cell death that may occur, which would clarify if PKR functions as a terminal death-related cell signal.

Collectively across immune challenges with LPS, influenza, and diet-induced obesity, we have identified the novel expression of PKR, whose induction in these host contexts is outside of its non-canonical antiviral function. In addition to identifying PKR expression in brain regions relevant to Parkinsonian neurodegeneration, we have shown that its CNS induction across seemingly unrelated inflammatory disease states serves as a broad cell stress response. As PKR expression does not appear to correspond to the intensity of inflammatory stimuli, as do stereotypic pro-inflammatory mediators (i.e., cytokines), PKR likely acts as a signal transduction node that elicits downstream effectors to mount various cell responses. FADD/caspase-8-mediated apoptosis and NF-κB p65-mediated inflammation are two
downstream signals that likely mediate the mechanism of neuroinflammation-induced PKR expression. Important next steps in determining PKR’s candidacy as a neurotherapeutic target against PD will be i) to determine whether PKR’s expression is to promote cell survival via apoptotic pathways, and ii) if pharmacologically manipulating PKR expression will improve disease state outcomes, and iii) if these improvements can be substantial enough to modify the course of disease progression in the brain.
REFERENCES


Gilbert, S. J., Duance, V. C., & Mason, D. J. (2002). Tumour necrosis factor α up-regulates protein kinase R (PKR)-activating protein (PACT) and increases phosphorylation of PKR and eukaryotic initiation factor 2-α in articular chondrocytes: Portland Press Limited.


Sadasivan, S., Sharp, B., Schultz-Cherry, S., & Smeyne, R. J. (2017). Synergistic effects of influenza and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) can be eliminated by the use of influenza therapeutics: experimental evidence for the multi-hit hypothesis. *NPJ Parkinsons Dis, 3*, 18. doi: 10.1038/s41531-017-0019-z


White, C. A., McCombe, P. A., & Pender, M. P. (1998). Microglia are more susceptible than macrophages to apoptosis in the central nervous system in experimental autoimmune encephalomyelitis through a mechanism not involving Fas (CD95). International Immunology, 10(7), 935-941.


The purpose of this study was to examine the capacity for Imoxin (IMX), a PKR inhibitor that blocks kinase autophosphorylation, to ameliorate diet-induced obesity (DIO)-induced PKR activation and neuroinflammation, and minimize the severity of MPTP-induced neuronal damage. Male C57BL/6 mice were fed either a control diet (10.2% kcal fat, 58Y2) or a high-fat diet (61.2% kcal fat, 58Y1) for 14 weeks, collecting body weight weekly. At the start of week 12, began daily administration of the PKR inhibitor IMX (0.5 mg/kg SC or saline) for 14 days. At end of 14 weeks of feeding, we administered MPTP (4x 5 mg/kg IP at 2 hour intervals) or saline to respective groups and sacrificed 24 hours later. Brains were micro-dissected for the striatum and hippocampus. Groups included: Control Diet (CD) (n = 8), CD + MPTP (n = 8), High Fat Diet (n = 8), HFD + IMX (n = 8), HFD + MPTP (n = 8), and HFD + IMX + MPTP (n = 8). Samples were processed for gene and protein expression. qPCR was performed on striatum and hippocampus for: Actin and GAPDH housekeeping, PKR, NLRP3, ASC, TNF-α, IL-1β, IBA1, and GFAP. Western blotting was performed on hippocampal samples for: PKR, PACT, phosphor-eIF-2α, RIP1K, IKKi, NF-κB, TNF-α, NLRP3, and CDK2.
Striatum Gene Expression Data

- **PKR**
- **TNF-α**
- **IL-1β**
- **NLRP3**
- **ASC**
- **GFAP**
- **IBA1**
Hippocampal Gene Expression Data

PKR

TNF-α

IL-1β

NLRP3

ASC

GFAP

IBA1
Hippocampal Protein Expression Data

**PKR**

- Control Diet
- High Fat Diet
- HFD + IMX

**PACT**

- Control Diet
- High Fat Diet
- HFD + IMX

**p-eIF-2α**

- Control Diet
- High Fat Diet
- HFD + IMX

**RIP1K**

- Control Diet
- CD + MPTP
- High Fat Diet
- HFD + IMX
- HFD + MPTP
- HFD + IMX + MPTP

**IKKβ**

- Control Diet
- CD + MPTP
- High Fat Diet
- HFD + IMX
- HFD + MPTP
- HFD + IMX + MPTP

**NF-kB p65**

- Control Diet
- CD + MPTP
- High Fat Diet
- HFD + IMX
- HFD + MPTP
- HFD + IMX + MPTP

**PAC1**

- Control Diet
- CD + MPTP
- High Fat Diet
- HFD + IMX
- HFD + MPTP
- HFD + IMX + MPTP

**TNF-α**

- Control Diet
- CD + MPTP
- High Fat Diet
- HFD + IMX
- HFD + MPTP
- HFD + IMX + MPTP

**NLRP3**

- Control Diet
- CD + MPTP
- High Fat Diet
- HFD + IMX
- HFD + MPTP
- HFD + IMX + MPTP

**Cdk2**

- Control Diet
- CD + MPTP
- High Fat Diet
- HFD + IMX
- HFD + MPTP
- HFD + IMX + MPTP