UNIVERSITY OF CALIFORNIA SAN DIEGO

Astrocyte-Specific Removal of PHLPP2 Alters Activation of Cell Survival Pathways Following LPS-Induced Inflammation

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

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2021

The Thesis of Shirag Hrand Ohannesian is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California San Diego

2021

DEDICATION

In recognition of my family's continuous love and support, this project is wholeheartedly dedicated to my beloved parents and brother. Without the inspiration, drive, and support that you have given me, I might not be the person I am today.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

CNS	Central Nervous System
ROS	Reactive Oxygen Species
PHLPP	Pleckstrin Homology domain Leucine-rich repeat Protein Phosphatase
LPS	Lipopolysaccharide
BBB	Blood-Brain Barrier
GFAP	Glial Fibrillary Acidic Protein
SCN	Suprachiasmatic Nucleus
SCOP	Suprachiasmatic nucleus Circadian Oscillatory Protein
RA	Ras Association
PH	Pleckstrin Homology
LRR	Leucine-Rich Repeat
PP2C	Protein Phosphatase 2C
PI3K	Phosphatidylinositol 3-Kinase
TSC2	Tuberin Sclerosis Complex 2
mTOR	Mechanistic Target of Rapamycin
GSK-3	Glycogen Synthase Kinase-3
FoxO	Forkhead Box O
β-TrCP	β-Transducin repeat-Containing Protein
SCF	Skp1/Cullin 1/F-box
NF-κB	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
IKK	IκB Kinase
IL	Interleukin

TNF-α	Tumor Necrosis Factor-α
COX	Cyclooxygenase
TGF-β	Transforming Growth Factor-beta
MAPK	Mitogen-Activated Protein Kinase
ERK	Extracellular signal-Regulated Kinase
JNK	c-Jun N-terminal Kinase
Mst1	Mammalian Sterile 20-like Kinase 1
RTK	Receptor Tyrosine Kinase
WT	Wild-Type
KO	Knockout
HBSS	Hank's Balanced Salt Solution
PBS	Phosphate Buffered Solution
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal Bovine Serum
IP	Intraperitoneal
RIPA	Radioimmunoprecipitation Assay
BCA	Micro Bicinchoninic Acid
SDS	Sodium Dodecyl Sulfate
MOPS	3-(N-morpholino)propanesulfonic Acid
TA	Tris-Acetate
PVDF	Polyvinylidene Fluoride
TBS-T	Tris-Buffered Saline-Tween-20
BSA	Bovine Serum Albumin

GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
RT	Reverse-Transcription
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
Bcl-2	B-cell lymphoma-2
Bax	Bcl-2-associated X
Bak	Bcl-2-antagonist/killer
Bad	Bcl-2-associated agonist of cell death
HO-1	Heme Oxygenase 1
NOX4	Mouse NADPH Oxidase 4
Nqo1	Quinine Oxidoreductase 1
Nrf1	Nuclear respiratory factor 1
GPX3	Glutathione Peroxidase 3
SOD2	Superoxide Dismutase 2
ULK-1	Unc-51 Like autophagy activating Kinase 1
CCL2	Chemokine C-C motif Ligand 2
LC3	Light Chain 3
ELISA	Enzyme-Linked Immunosorbent Assay

LIST OF FIGURES

LIST OF SCHEMES

LIST OF TABLES

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ABSTRACT OF THE THESIS

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Neurodegeneration is the progressive damage and deterioration of neurons often as a result of Alzheimer's disease and Parkinson's disease (Dauer and Przedborski, 2003), and the role of astrocytes in this process is becoming recognized. Neurodegenerative diseases have a

positive correlation with other pro-inflammatory conditions such as diabetes mellitus, hypercholesterolemia, atherosclerosis, severe autism, and cardiovascular disease suggesting that chronic inflammation may play a role in the pathogenesis of neurodegenerative diseases (Exalto et al., 2012; Ledesma & Dotti, 2012; Emanuele et al., 2010). Astrocytes are crucial regulators of neuroinflammation. The extent of the inflammatory response is a delicate balance between cell survival and cell death. Astrocytic responses are context-dependent, and may aggravate inflammatory reactions and worsen tissue damage, or may suppress inflammation and increase tissue repair. Pleckstrin Homology domain and Leucine-rich repeat Protein Phosphatase (PHLPP) inhibits and terminates numerous important cell survival pathways through dephosphorylation of AGC Kinases such as Akt. Our laboratory has previously shown that removal of PHLPP1 in the brain is protective during ischemic damage (Chen et al., 2012). However, the role of PHLPP2 in the astrocytic response remains largely unknown. We used astrocyte-specific PHLPP2 knockout mice compared with global PHLPP2 knockout to study the role of PHLPP2 in astrocytes following a 4-hour lipopolysaccharide (LPS) treatment to induce inflammation. Changes in gene and protein expression of proteins involved in inflammation, NF-κB activation, apoptosis, mitogen-activated protein kinase stimulation, antioxidant, and autophagy levels were analyzed through Western blot and mRNA. We demonstrated that astrocyte-specific removal of PHLPP2 activates NF-κB, attenuates TNF-α and pro-apoptotic mediators, alters antioxidant activity, and increases activation of autophagy after 4 hours of LPS treatment. There seems to be more overall protection than harmful changes with astrocyte-specific removal of PHLPP2 in the pathways that we studied. However, we cannot conclude that those protective activations outweigh the harmful effects nor that removal of PHLPP2 in astrocytes will lead to better survival.

Introduction

Neurodegeneration is the progressive damage and deterioration of neurons often as a result of Alzheimer's and Parkinson's disease (Dauer and Przedborski, 2003), and the role of astrocytes in this process is becoming recognized. According to the National Institute of Neurological Disorders and Stroke, neurodegenerative diseases affect approximately 50 million Americans each year and expected to double by 2030 as the population ages, due to having a mid- to late-life onset (Harvard NeuroDiscovery Center). Neurodegenerative diseases have mild symptoms at first, such as weakened coordination or failure to recall minor details, but symptoms may worsen to as severe as the inability to function independently due to physical and mental disabilities, and ultimately become fatal.

Neurodegenerative diseases may be genetic or caused by a stroke and lead to the death of neurons. Neurodegenerative diseases have a positive correlation with other pro-inflammatory conditions such as diabetes mellitus, hypercholesterolemia, atherosclerosis, severe autism, and cardiovascular disease suggesting that chronic inflammation may play a role in developing neurodegenerative diseases (Exalto et al., 2012; Ledesma & Dotti, 2012; Emanuele et al., 2010). Neuroinflammation, defined as an inflammatory response in the central nervous system (CNS), is an important characteristic of neurodegeneration. (Wiedermann et al., 1999; Zhang et al., 2010). Neuroinflammation is a multifaceted response that involves glial activation, release of inflammatory signals such as cytokines, chemokines, and reactive oxygen species (ROS) (Milatovic et al., 2017). Pleckstrin homology domain leucine-rich repeat protein phosphatase (PHLPP) is a Ser/Thr phosphatase that has been shown to play a role in several diseases including diabetes, cancer, and cardiovascular disease (Grzechnik and Newton, 2016) but whether it alters neuroinflammation is largely unknown. Our goal of the study is to understand

the role of the PHLPP isoforms in the regulation of astrocytic responses to injury, specifically in the presence of the endotoxin lipopolysaccharide (LPS) to find potential therapeutic approaches in minimizing inflammation and neuronal degeneration.

Astrocytes

The CNS of the human body consists of two types of cells: neurons and glia. Neurons are the principal cells that carry out neuronal functions to process and transmit information. Neurons, once damaged or degenerated, cannot be repaired or regenerated in the CNS (Ransohoff, 2016). Glial cells are subclassified into microglia, astrocytes, and oligodendrocytes (Vallejo et al., 2010). Glial cells provide structural and physiological support and respond to injury, infection, or neurodegenerative disease to protect the neurons (Wood and Bunge, 1984). Astrocytes are the most abundant glial cell and make up about 50% of the human brain. They tile the entire brain and spinal cord and have numerous essential complex functions in a healthy CNS. Under normal conditions, astrocytes are essential for the regulation of the blood-brain barrier (BBB) and brain homeostasis (Schousboe and Westergaard, 1995; Wolburg and Risau, 1995). They support neurons by supplying metabolites and growth factors to aid in synaptic formation and plasticity, and regulating neurotransmitters and extracellular ion balance (Colombo and Farina, 2016; Schousboe and Westergaard, 1995).

Astrocytes are crucial regulators of the inflammatory response triggered by microglia and are necessary for neurite outgrowth and synaptic remodeling (Colombo and Farina, 2016; Ridet et al., 1997) and detect danger signals by responding to cytokines released by microglial activation. Microglial cells are resident immunocompetent and phagocytic cells that mediate immune responses by upregulating inflammatory signals (leading to inflammation) and scavenge cellular debris and dead neurons through phagocytosis during CNS insult (Kim and de Vellis,

2005). During injury in the brain, neurons and microglial cells produce pro-inflammatory cytokines and ROS that act as activators of astrocytes and lead to reactive astrogliosis, a hallmark of all types of CNS insults (Sharma et al., 2007; Rivieccio et al., 2005). Reactive astrogliosis is characterized by astrocytic hypertrophy, ROS production, and accumulation of glial fibrillary acidic protein (GFAP) (O'Callaghan, 1993; O'Callaghan et al., 2014). GFAP is the main intermediate filament protein in astrocytes. It is a highly regulated protein and has a structural role in addition to astrocytic functions such as: neuron-glia interactions, signal transduction, regulation of BBB, regeneration, synaptic plasticity, and scar formation (Bonni et al., 1997; Kahn et al., 1997; Middeldorp and Hol, 2011).

Reactive astrogliosis is a gradated continuum of molecular, functional, and cellular changes that are regulated by context-dependent signals to reduce ROS accumulation and tissue damage during inflammation (Sofroniew and Vinters, 2009). These changes range from reversible gene expression alterations and localized cell hypertrophy to scar formations in severe cases that lead to permanent tissue structure rearrangement and cell proliferation (Sofroniew and Vinters, 2009). As scar formation occurs in the brain, it confines inflammatory mediators released by physically sealing the lesion site and secretes chemical growth inhibitors to prevent the spread of cellular damage, microbial infections, and free radicals (Rolls et al., 2009; Fitch and Silver, 2008). Conversely, due to the secretion of chemical growth inhibitors and physical blockage, scar formation obstructs axon regeneration and impedes normal CNS function recovery in the long term (Fitch and Silver, 2001; Huang et al., 2014). Once astrogliosis is activated, astrocytes secrete further cytokines, chemokines, and growth factors (Rivieccio et al., 2005) that lead to activation of several downstream neuroprotective pathways including apoptosis (caspase/calpain activation), excitotoxicity (neuronal excitability), immune activation

(BBB permeability), and cytotoxicity (ROS production) (Colombo and Farina, 2016; Lull and Block, 2010; Yang et al., 2007). Understanding the pathways that alter the inflammatory response of astrocytes to injury is important for the protection of neurons. Therefore, they play a critical role in neuronal survival and recovery following brain injury (Chen and Swanson, 2003).

PHLPP

Structure

The PHLPP gene has two isoforms, PHLPP1 and PHLPP2. Furthermore, PHLPP1 consists of splice variants PHLPP1α (Gao et al., 2005) and PHLPP1β, also known as suprachiasmatic nucleus (SCN) circadian oscillatory protein (SCOP) due to its oscillatory expression in a circadian pattern in the SCN (Shimizu et al., 1999). PHLPP consists of an N-terminus Ras Association (RA, only present in PHLPP1β and PHLPP2), a Pleckstrin Homology (PH) domain, a Leucine-Rich Repeat (LRR) region, a Protein Phosphatase 2C (PP2C) domain, and a PDZ binding motif (Scheme 1) (Grzechnik and Newton, 2016). PHLPP1and PHLPP2 isoforms share about 50% overall amino acid identity. PHLPP1α protein consists of 1205 amino acids, PHLPP1β consists of 1717 amino acids, and PHLPP2 consists of 1323 amino acids (Gao et al., 2005; Brognard et al., 2007). The crucial difference between the two isoforms is an amino-terminal extension of about 14 kDa in PHLPP2 (shorter N-terminal in PHLPP1) and some variation in the PDZ-binding motif which is involved in the dephosphorylation of Akt (Gao et al., 2005, Brognard et al., 2007).

Akt Signaling and PHLPP Expression

PHLPP dephosphorylates several members of the AGC Kinase family to regulate cellular responses (Scheme 2). Akt is a member of the AGC family of kinases and is a pro-survival

serine/threonine kinase that is ubiquitously expressed in neurons, astrocytes, and many other cell types. Akt modulates the precise balance between cell growth and death. The typical pathway towards cell survival and proliferation includes the activation of its upstream kinase phosphatidylinositol 3-kinase (PI3K) (Altomare and Testa, 2005). PI3K is a lipid kinase that engages Akt to the plasma membrane where it is activated through the phosphorylation of the activation loop (Thr308) and the hydrophobic motif (Ser473), leading to cell growth, proliferation, and survival (Brazil and Hemmings, 2001). Akt consists of three isoforms: Akt1, Akt2, and Akt3 which have been shown in cancer to be regulated by different PHLPP isoforms (Grzechnik and Newton, 2016; Brognard et al., 2007). PHLPP is a negative regulator of the Serine 473 site in the hydrophobic motif of Akt without altering the Threonine 308 site. Both sites are necessary for complete activation by phosphorylation; therefore, the PHLPP isoforms play an important role in altering Akt activity during infection or injury (Scheme 2). Although both isoforms of PHLPP have been shown to regulate cell survival in cancer cells through dephosphorylation of Akt, PHLPP1 has been demonstrated to dephosphorylate Akt2 and Akt3 isoforms whereas PHLPP2 targeted Akt1 and Akt3 isoforms (Brognard et al., 2007). In physiological systems like the heart and brain, PHLPP1 can dephosphorylate all Akt isoforms equally (Moc et al., 2014; Chen et al., 2012) suggesting tissue specificity. However, unlike in cancer, removal of PHLPP2 in cardiomyocytes and astrocytes does not alter Akt activity (Moc et al., 2014; Chen et al., 2012). Our laboratory has demonstrated that removal of PHLPP1 increases Akt activity in astrocytes, leading to protection following ischemic injury. This protective effect of PHLPP1 removal during ischemic injury is identified in both astrocytes and neurons (Chen et al., 2012), suggesting a potential therapeutic approach against damage caused by strokes.

However, the role of both PHLPP1 and PHLPP2 in astrocyte signaling during inflammation is not well studied.

Akt is a pro-survival signaling pathway and plays a role in PHLPP expression. Akt phosphorylation activates p70S6K (also known as S6K1) through inactivation of tuberin sclerosis complex 2 (TSC2) and activation of mechanistic target of rapamycin **(**mTOR) (Gao et al., 2005). p70S6K is closely related to Akt in the AGC family and modulated by signaling inputs from growth factors, nutrients, and energy balance downstream of mTOR (Liu et al., 2011). Activated p70S6K leads to protein translation and promotes cell growth. Removal of PHLPP indirectly activates p70S6K through Akt activation. Activated p70S6K leads to expression of PHLPP, leading to a negative feedback loop with Akt (Scheme 2). PHLPP also directly inhibits p70S6K by dephosphorylating its hydrophobic motif (T389). p70S6K requires phosphorylation at the activation loop (T229) and the hydrophobic motif (T389) for full activation that leads to protein translation (Liu et al., 2011; Ma and Blenis, 2009). Thus, PHLPP can regulate the full activation of p70S6K directly and indirectly through altering Akt in cells to promote protein translation and cell growth. Akt also promotes cell survival by phosphorylating glycogen synthase kinase-3 (GSK-3) and the Forkhead Box O (FoxO) family (Jope and Johnson, 2004, Biggs et al., 1999). GSK-3 is believed to control glycogen metabolism and gene transcription (Welsh and Proud, 1993). PHLPP expression is modulated through multiple degradation pathways in Akt signaling. Firstly, PHLPP1 levels are regulated through proteasomal degradation. PHLPP1 is a proteolytic target of an E3 ligase, called β-transducin repeat-containing protein (β-TrCP) (Warfel, 2011; Li et al., 2009). PHLPP1 is phosphorylated by GSK-3β and that leads to the exposure of its phosphodegron motif to allow for the degradation of PHLPP through binding of β-TrCP (Li et al., 2009). Importantly, Akt is involved in a second

negative feedback loop with PHLPP1 through GSK-3β. Once Akt activity is increased, GSK-3β is inactivated by phosphorylation from Akt and PHLPP1 degradation is inhibited, thereby increasing expression and blocking Akt (Scheme 2). Studies in cancer cells have shown that PHLPP1α and PHLPP1β are phosphorylated by GSK-3β and initiate subsequent degradation during steady levels of Akt (Warfel, 2011). Secondly, elevated levels of Ca^{2+} in the brain lead to a rapid inhibition of PHLPP1 cellular levels through calpain proteins (Shimizu et al., 2007). Calpains are a class of proteases that modify the function of their substrates through protein cleavage and are calcium-dependent (Chan and Mattson, 1999; Baudry and Bi, 2016). Calcium influx activates calpain-1, leading to PHLPP1α and PHLPP1β degradation. Calpain-2 is phosphorylated momentarily after, and leads to the synthesis of PHLPP1β, resulting in a short period of PHLPP1 degradation (Baudry and Bi, 2016). In summary, activation of p70S6K during neuroinflammation could potentially aid in cell survival, and removal of PHLPP would lead to its activation and might be deemed protective against astrocytic and neuronal loss. Inhibition of GSK3 during neuroinflammation inhibits PHLPP degradation which in turn will dampen Akt signaling and lead to deleterious effects following injury. Thus, understanding the roles of PHLPP isoforms in those pathways during an inflammatory response is important for understanding the astrocytic response to neuroinflammation and cell survival following an injury.

NF-κB Activation

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a protein transcription factor (Salminen et al., 2008) that leads to nuclear transcription of cytokines and launches an innate immune response following infection or injury (Baltimore, 2009). NF-κB is a key regulator of neuroinflammation, astrogliosis, synaptic plasticity, neurodegeneration, and

neuronal survival in numerous mouse models such as brain injury and spinal cord injury (Kaltschmidt et al., 2005; Burda and Sofroniew, 2014; Brambilla et al., 2005; Raasch et al., 2011). NF-κB dimers are composed of p50 and p65 subunits that are inhibited under normal conditions by IκB proteins and are localized in the cytoplasm (Saggu et al., 2016). After CNS injury, NF-κB-dependent genes are highly expressed that lead to both protective and deleterious effects on cell death and neuronal damage (Brambilla et al., 2005). NF-κB activation is mediated by the removal of the inhibitory IκB proteins through the IκB kinase (IKK) complex, which phosphorylates IκB protein leading to its ubiquitination and degradation. NF-κB is free to translocate to the nucleus and activate gene expression (Noursadeghi et al., 2008; Huang and Hung, 2013; Shabab et al., 2016). NF-κB activation leads to secretion of pro-inflammatory cytokines, chemokines, and enzyme to initiate neuroinflammation such as Interleukin (IL)-1, IL-6, tumor necrosis factor-α (TNF-α), cyclooxygenase (COX)-1, and COX-2 (Shabab et al., 2017; Lull and Block, 2010; Park et al., 2011).

Interestingly, studies have shown that inhibition of NF-κB during brain injury alters astrocytic functions and correlates with protective effects. Transgenic inhibition of NF-κB in the mouse brain was found to be protective from white matter demyelination and axonal loss during injury through severely diminished reactive astrogliosis response, leading to significantly less neurodegeneration and cognitive impairment (Saggu et al., 2016), suggesting that NF-κB regulates the astrocytic response and might be deleterious in cases of severe astrogliosis response. Another study has found that activated transforming growth factor-beta (TGF-β) in astrocytes causes reduced neuroinflammation and cell damage through downregulation of NF-κB activity (Cekanaviciute et al., 2014). Bethea et al. have further studied the role of NF-κB activation in astrocytes to neuroinflammation by blocking nuclear translocation of NF-κB,

rendering it inactive (Bethea et al., 1998). Their finding suggests that inhibition of NF-κB signaling in astrocytes ameliorates neuroinflammation through reduced levels of chemokines and oxidative stress in the injured CNS, indicating that NF-κB regulates pro-inflammatory and oxidative stress pathways that are crucial during neuroinflammation and neurotoxicity. These findings together suggest that inhibition of NF-κB during brain injury might ameliorate survivability and attenuate neurodegeneration through alterations of astrocyte signaling and oxidative stress pathways. Thus, it is important to understand NF-κB signaling during neuroinflammation and whether removal of PHLPP would play a role in its functions.

In an attempt to find protein phosphatases that regulate NF-κB signaling, a study used RNAi screening and found that PHLPP acts as a positive regulator of NF-κB activity. In a siRNA-based screen with PHLPP knockdown, there was a drastic decrease in TNF-α induced NF-κB activation through decreased IκB levels, leading to decreased activation of Ser276 on the p65 subunit in HeLa cells (Wang et al., 2010). This finding suggests that PHLPP plays an important role in activating NF-κB, but the mechanism remains unknown. Akt has also been shown to activate NF-κB and lead to cell survival (Madrid et al., 2000), but the mechanisms are not well understood nor whether PHLPP and Akt are associated with activating NF-κB. Additionally, in human gliomas, PHLPP1 and PHLPP2 have been shown to inhibit NF-κB activity through inhibition of its upstream kinase IKKβ (Agarwal et al., 2014). NF-κB is strongly associated with tumorigenesis and IKK inhibitors have been shown to exert significant anti-tumor effects (Lee et al., 2007). The loss of both PHLPP isoform levels correspond with tumor severity, and overexpression of PHLPP1 was found to significantly inhibit inflammatory cytokines, indicating that PHLPP may act as a tumor suppressor as well as dampen the inflammatory response in human gliomas (Teng et al., 2016). These findings suggest that the

PHLPP isoforms may regulate NF-κB and inflammation but their role in astrogliosis and neuroinflammation are unknown. Further studies are needed to determine whether the isoforms have overlapping or divergent targets in the brain following injury.

Mitogen-Activated Protein Kinases

Mitogen-Activated Protein Kinases (MAPKs) are a family of serine/threonine protein kinases crucial for regulation of inflammatory processes and their activity is increased during glial activation (Velagapudi et al., 2014; Kaminska et al., 2009). LPS activates the MAPKs in the brain (Moriyama et al., 2006; Li et al., 2018). The family of MAPKs consists of three major signaling pathways, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 (Shen et al., 2005). Activation of ERK, JNK, and p38 leads to their translocation to the nucleus to transcriptionally modulate production of pro-inflammatory mediators including COX-2, TNF-α, and IL-6 (Velagapudi et al., 2014; Irving and Bamford 2002; Lim et al., 2018; Rao, 2001). *In-vivo* activation of JNK and p38 in the brain leads to cell death, whereas ERK signaling is involved in cell proliferation of astrocytes and memory consolidation in the hypothalamus (Shen et al., 2005; Song et al., 2012). In astrocytes, it has been shown that activation of ERK, JNK, and p38 signaling pathways directly activate NF-κB, and indirectly activate it through expression of cytokines IL-6 and TGF-β (Ma et al., 2010; Yi et al., 2014; Hamby et al., 2010).

PHLPP isoforms regulate MAPK levels in cancer cells. In colorectal cancer, PHLPP1 and PHLPP2 directly dephosphorylate and inhibit RAF1, which is upstream of ERK leading to negative regulation of MAPK signaling (Li et al., 2014). These findings suggest that PHLPP1β regulates ERK levels and plays a role in cell proliferation in cancer. In addition, it has been demonstrated that there is a positive feedback loop in which H-Ras oncogenically inhibits PHLPP1 expression in cancer cells and subsequently inhibits the p38 pathway and downstream

caspase-dependent cell death (Mason et al., 2016). Overall, these findings suggest that PHLPP mediates cell death through activation of the p38 pathway in cancer cells. Furthermore, PHLPP1 and PHLPP2 both dephosphorylate the transcription factor c-Jun, which is activated by JNK, to inhibit transcriptional activity in glioblastomas and head cell carcinomas, leading to decreased apoptosis and cell transformation (Zhu et al., 2014). Lastly, Qiao et al. has shown that both PHLPP1 and PHLPP2 dephosphorylate and activate the pro-apoptotic protein Mammalian Sterile 20-like kinase 1 (Mst1), leading to phosphorylation of JNK and p38 to induce apoptosis and inhibit cell proliferation in cancer cells (Qiao et al., 2010). In conclusion, PHLPP-mediated inhibition of MAPK signaling pathways in cancer cells occurs through direct inactivation of pathway components, including the dephosphorylation of K-RAS and RAF1, and through transcriptional silencing and downregulation of gene expression.

PHLPP regulation of MAPK has been studied in the brain. In rat and mouse brains, PHLPP1β has been shown to negatively regulate RAS-RAF-MEK-ERK cascade by directly interacting with K-RAS (Shimizu et al., 2003; Shimizu et al., 2007). It has also been demonstrated that ERK phosphorylation is increased following ischemic damage in pig brains (Aharon et al., 2004), whereas inhibition of ERK increases damage in the brain following a deep hypothermic circulatory arrest in pigs (Cho et al., 2004). These studies suggest that the ERK pathway signaling is involved in reducing cellular damage in the brain. In addition, there is a correlation between the activation of p38 and JNK pathways with increased cell death in regions of the brain with ischemic damage (Irving and Bamford, 2002). In support of this, it has been shown that administration of p38 inhibitors in the brain decreases apoptosis in the hippocampus and ameliorated negative behavioral outcomes in rats after ischemic injury (Sugino et al., 2000; Barone et al., 2001). These results suggest that inhibition of p38 and JNK expression might lead

to protective results during brain injury, however, the role of PHLPP in regulation of p38 and JNK during neuroinflammation is not well understood. Furthermore, our laboratory has demonstrated that removal of PHLPP1 globally in the mouse does not alter basal ERK signaling in the brain (Chen et al., 2012). However, a study used mouse models of controlled traumatic brain injury and observed neurologic outcomes in wild-type and PHLPP1 knockout mice (Jackson et al., 2018). They observed that PHLPP1 levels increased in wild-type mice, whereas PHLPP1 knockout mice had increased ERK (and Akt) activation. They also observed near normal memory function in PHLPP1 knockout samples (tested through the Morris water maze), whereas wild-type mice that were injured were significantly impaired. These results suggest that removal of PHLPP1 is protective during brain injury. This could potentially be through upregulation of ERK and Akt and activation of their downstream cell proliferation pathways. In neuroinflammation mouse models, it has also been shown that LPS induces increased expression of ERK 1/2, JNK, and p38 in dendritic cells (Lim et al., 2018) and primary astrocytes culture of neonatal rats (Liu et al., 2018). However, the role of both PHLPP isoforms in the brain on the MAPK pathway during reactive astrogliosis and neuroinflammation following brain injury is not well understood.

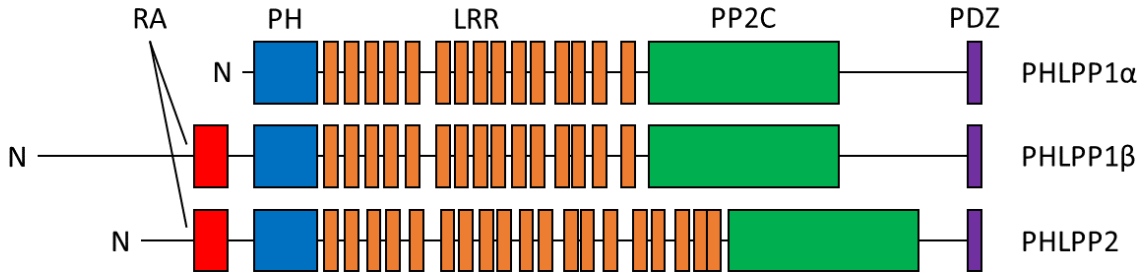
Neuroinflammation

Neuroinflammation plays a major role in the pathogenesis of neurodegenerative diseases and brain injury. The CNS is prone to diverse insults that evoke varying responses. Acute and localized damage lead to wound repair and tissue replacement, whereas diffuse and chronic insults lead to gradual damage across tissue (Burda and Sofroniew, 2014). Neuroinflammation occurs in the brain innately during CNS injury as a physiological response to protect tissue through several damage mechanisms (Russo and McGavern, 2016). Studies have shown the

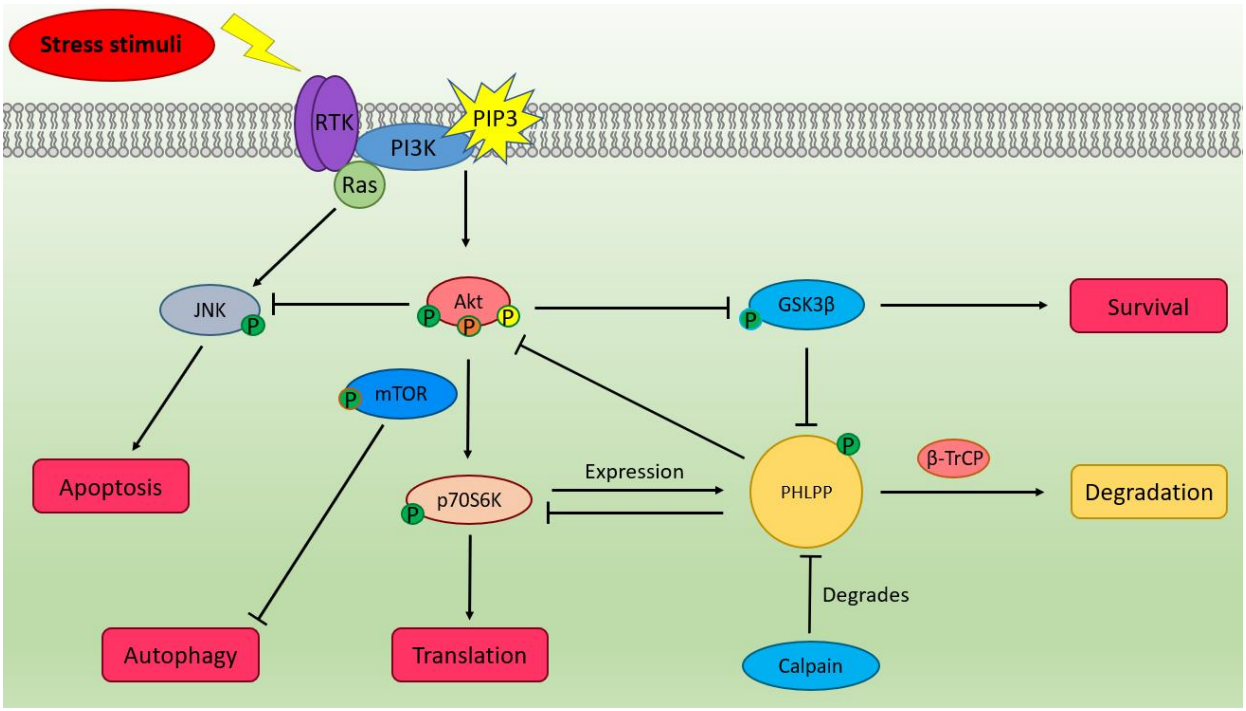
importance of the glial cell activation to injury, and the consequences of inflammation and wound healing responses on the severity of the overall permanent damage (Barral and Croibier, 2009). High levels of ROS occur during inflammation, mostly produced by neurons and microglia but also produced by astrocytes. Free radicals are highly reactive with cellular components that could lead to potential damage (Guyton et al., 1996; Lo et al., 1996; Sena and Chandel, 2012). ROS accumulation also has an immune defense function, as it acts as a switch for signaling cascade pathways and gene expression during inflammation and is essential for downstream pathways involved in protection of neurons such as preventing neuron excitotoxicity by inhibiting glutamate uptake/overactivation in neurons (Guyton et al., 1996; Sheng et al., 2013). As mentioned earlier, microglia are resident immunocompetent cells in the brain that constantly monitor their surroundings. They are the primary mediators of neuroinflammation following injury and respond to injury by upregulating inflammatory signals and ROS production (Kim and de Vellis, 2005). Astrocytes both respond to and secrete immunomodulatory signals, such as cytokines, inflammatory mediators, and chemokines (Barres 2008). During the inflammatory response, astrocytes experience an excessive number of stimuli simultaneously, activating a complex intracellular response. Although this inflammatory response to brain injury is critical, excessive glial activation leads to neurotoxicity and neuronal death (Russo and McGavern, 2016; Zhu et al., 2007). Thus, regulating the inflammatory response may potentially prevent further neuroinflammation and cell death, however, the mechanisms leading to deleterious response are currently not well understood (Yao et al., 2014; Qin et al., 2007).

LPS in the Brain

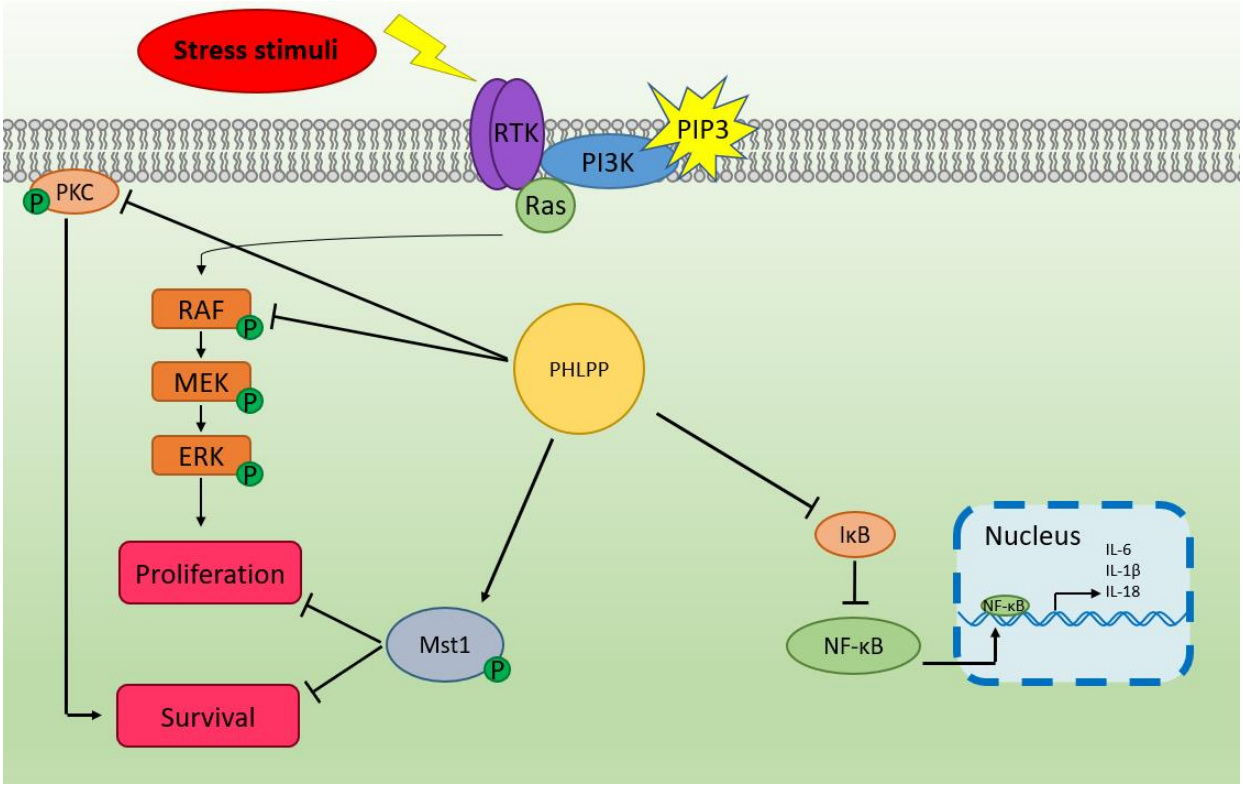
LPS is an endotoxin making up most of gram-negative bacteria's outer membrane wall. Endotoxins have been used as a classical model to induce neuroinflammation (Kang et al., 2019). Injecting high levels of LPS in the blood leads to systemic inflammation, brain microglial activation, and neuroinflammation by stimulating pro-inflammatory cytokines (Sandiego et al., 2015; Gabellec et al., 1995). Previous studies have demonstrated that LPS induced neuroinflammation leads to microglia and astrocyte activation and elevated COX-2, IL-6, IL-1β, TNF-α, MAPKs, and NF-κB expression (Fu et al., 2014; Rossol et al., 2011). These studies demonstrated progressive neuron damage and cell death (Gao et al., 2002). The microglia and astrocytic response to LPS not only leads to neuron damage but activation leads to further cell damage through neurotoxicity that does not fade away even though the stimulus is dissipated (Qin et al., 2007). Furthermore, a study found that LPS attenuated PHLPP1 expression in macrophages at both mRNA and protein levels, suggesting that PHLPP plays a role during an immune response (Alamuru et al., 2014; Alamuru et al., 2017). Understanding the roles of PHLPP in the astrocytic signaling pathways following an injury is necessary in order to potentially regulate the inflammatory response and minimize neuronal damage and cell death. In this study, we demonstrated that astrocyte-specific removal of PHLPP2 activates NF-κB, attenuates TNF-α and pro-apoptotic mediators, alters antioxidant activity, and increases activation of autophagy after 4 hours of LPS treatment.



Scheme 1: PHLPP Protein Structure. Both isozymes of PHLPP have similar structures including a Ras Association (RA) domain (only present in PHLPP1β and PHLPP2), a Pleckstrin Homology (PH) domain, a Leucine-Rich Repeat (LRR) region, a PP2C domain, and a PDZ binding motif.



Scheme 2: Akt Signaling and PHLPP Targets. PHLPP dephosphorylates Akt and p70S6K. PHLPP dephosphorylation inactivates Akt and p70S6K to suppresses cell proliferation and survival pathways. Akt activates p70S6K through mTOR, and mTOR acts as an inhibitor of autophagy. PHLPP is under a negative feedback loop with Akt through p70S6K. PHLPP is under a second negative feedback loop through removal of GSK-3β inhibition by high Akt levels; leading to PHLPP proteasome degradation through β-TrCP. Akt activation inhibits apoptosis through multiple pathways including inhibition of JNK.



Scheme 3: PHLPP Targets in Cancer. PHLPP dephosphorylates PKC, RAF, and Mst1 to suppress cell proliferation and survival pathways. PHLPP dephosphorylation activates Mst1 by removing the inhibitory phosphorylation. Mst1 then inhibits proliferation and survival. PHLPP also activates NF-κB by dephosphorylating IκB and removing its inhibition. NF-κB leads to nuclear translocation and pro-inflammatory cytokine release.

Materials and Methods

Animals

All animal procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of University of California, San Diego. Age-matched male and female wild-type (WT), PHLPP1 global knockout (KO), PHLPP2 global KO, and GFAP-PHLPP2 mice were used.

Astrocyte Isolation

Astrocytes were isolated from the brains of 1-3-day old WT, PHLPP1, PHLPP2, and GFAP-PHLPP2 KO mouse pups as previously described (Citro et al., 2007). Briefly, brains were isolated, and the meninges and cerebellum were removed. The remaining cerebral cortices were placed in cold Hank's Balanced Salt Solution (HBSS, Thermo Fisher Scientific) to avoid denaturation. To dissociate the brains, 2.5% trypsin and 4x pancreatin (2.5g/L in Phosphate Buffered Solution, PBS, Genesee Scientific) were added to HBSS at 37°C for 30 minutes. The cells were spun down and vigorously pipetted into a single-cell suspension and plated onto a T75 flask containing Dulbecco's Modified Eagle Medium (DMEM, Sigma Aldrich) with 10% fetal bovine serum (FBS, Thermo Fisher Scientific), glutamine (Thermo Fisher Scientific) and antibiotics (100 units/mL penicillin and100 µg/mL streptomycin, Genesee Scientific) were placed at 37°C in 5% CO_2 incubator. Media was repeatedly changed every 3-4 days until the culture was completely confluent. The flasks were shaken at 37°C on a rotary shaker at 240 rpm for 16-22 hours to remove oligodendrocytes, producing a culture of astrocytes with 95% purity. The media was removed and the flask rinsed twice with PBS to remove any remaining debris

from the astrocytes, then split 1:3 into new T75 flasks and incubated at 37°C in 5% CO_2 until grown to 90% confluency. The culture media was changed every 3-4 days. Once confluent, the cells were frozen for later experiments. For experimentation, the cells were thawed to T75 flasks containing 20mL of media and incubated at 37°C with 5% CO_2.

Cell Culture

Astrocytes were thawed to a T75 flask. Once the cells were confluent they were split at ratios of 1:3 for 6cm dishes or 1:6 in a 6-well plate and incubated overnight in media at 37°C in 5% CO_2. The following day, the cells are rinsed with PBS and serum-starved overnight by adding media without 10% FBS at 37°C in 5% CO_2.

LPS Stimulation

Following serum starvation, cells were stimulated with 1µg/mL LPS (Enzo) for 6 hours, 3 hours, and 0 hours (control) to induce inflammation. Cells were rinsed twice with cold PBS and harvested for isolation of protein or RNA.

***In-vivo* LPS Injection**

3-month-old GFAP-cre PHLPP2, PHLPP2 KO, and PHLPP1 KO male and female mice were used for acute inflammation studies. To induce inflammation *in-vivo*, LPS (1mg/kg) was administered by intraperitoneal (IP) injection for 4 hours. Control mice were injected with PBS. After 4 hours, brains were removed and snap-frozen in liquid nitrogen for protein and RNA isolation and analysis as described below.

Protein Isolation

Whole Brains

Brains were removed from WT, PHLPP1, PHLPP2, and GFAP-PHLPP2 KO mice at various ages (3-24 months) and snap-frozen in liquid nitrogen. Brains were pulverized with a mortar and pestle to powder while frozen and a portion of the tissue homogenized in 140 µL Western buffer (see Appendix). The homogenized extract was centrifuged at 14,000 rpm for 15 minutes at 4°C and the supernatant was removed for protein analysis.

Astrocytes

For cells in culture, following stimulation cells were harvested in radioimmunoprecipitation assay (RIPA) buffer (see Appendix). The lysed cells were placed in a water Sonicator for 5 minutes and centrifuged at 14,000 rpm for 15 minutes at 4°C. The supernatant was removed for protein analysis.

Protein Assay

For Western blot analysis, the protein concentration of the brain or cellular extract was determined using Micro Bicinchoninic Acid (BCA, Thermo Fisher Scientific) protein assay kit (see Appendix). For astrocytes, 10µg of each sample was mixed with H_2O and 1x final concentration of sodium dodecyl sulfate (SDS, see Appendix). For brain samples, 20µg was loaded (see Appendix). The protein samples were boiled at 95°C for 10 minutes and loaded onto an SDS-polyacrylamide gel (Invitrogen NuPage) alongside a protein ladder (BioRad and Genesee Scientific). Based on the proteins of interest being analyzed, 4-12%, 12%, or 8% Bis-Tris gels in 1x 3-(*N*-morpholino)propanesulfonic acid (MOPS, Spectrum Chemical) buffer or 3-8% gels in Tris-Acetate (TA, Thermo Fisher Scientific) buffer were used. Gels were run at 150

volts, for about 1.5 hours. Following electrophoresis, the proteins were transferred from the gel to polyvinylidene fluoride (PVDF, Millipore Sigma) membrane. The transfer took place in 1x Transfer Buffer (see Appendix) at 100 volts for exactly 2 hours. After the transfer was complete, the membrane was cut and blocked in 5% milk/0.1% Tris-buffered saline-Tween-20 (TBS-T) for 1 hour at room temperature on a rocker. Following blocking, the membranes were rinsed with 0.1 %TBS-T to remove the milk and the blots were probed overnight at 4°C on a rocker with primary antibodies in 5% Bovine Serum Albumin (BSA)/TBS-T (see Appendix for a list of antibodies used and their dilutions). The following day, primary antibodies were removed and membranes washed with 0.1 %TBS-T, 3 times for 7 minutes each on a rocker at room temperature. Following the last wash, secondary antibodies diluted in 5% milk/TBS-T were added based on the species that the primary antibody was raised in (see Appendix for dilutions and species). Secondary antibodies were kept on the membranes for 1 hour at room temperature on the rocker. Following binding, membranes were rinsed with 0.1 %TBS-T, 3 times for 7 minutes. Lastly, for visualization of the proteins, membranes were soaked in ProSignal Femto ECL reagent (Genesee Scientific) before being exposed on the myECL Imager (Thermo Fisher Scientific). AlphaView software was used to quantify band intensity and proteins were normalized to Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH).

RNA Isolation, cDNA synthesis, and qPCR

To analyze gene expression, RNA was purified and cDNA synthesized using reverse-transcription polymerase chain reaction (RT-PCR). Gene expression was analyzed using quantitative PCR (qPCR).

RNA Isolation

RNA was isolated from brains or astrocytes using 1 mL TRIzol reagent (Thermo Fisher Scientific) following the manufacturer's protocol. Each sample was lysed further by pipetting the entire volume up and down for 1 minute using a 1ml pipet. The TRIzol extract was frozen overnight. The following day, the extracts were incubated at room temperature for 5 minutes. To this 200µL of chloroform (Fisher Scientific) was added and incubated for 5 more minutes, while inverting to mix every 1-minute. Samples were centrifuged at 14,000 rpm for 15 minutes at 4°C, and the supernatant was removed and mixed with 500µL of isopropyl alcohol (Fisher Scientific). The samples were placed at -20°C overnight to recover more RNA. The following day, the samples were centrifuged at 14,000 rpm for 15 minutes at 4°C to collect the precipitated RNA pellet. The supernatant was discarded, and the pellet was rinsed with 70% ethanol and centrifuged at 10,000 rpm for 5 minutes at 4°C. The supernatant was removed and the pellet was left to air dry for 40 minutes. Once dry, the pellet was dissolved in 25µL of RNAse free sterile water (GE Life Sciences) and placed on a heat block at 55°C for 10 minutes, pipetting up and down for 30 seconds to completely dissolve the pellet. The concentration of the isolated RNA was determined using the BioTek Gen5 spectrophotometer (BioTek) and its data analysis software.

cDNA Synthesis

Based on the RNA concentration, 1µg of sample RNA was used to make cDNA with the Thermo Fisher Scientific's High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor following the manufacturer's protocol (see Appendix). Briefly, cDNA solution was prepared with 10X reverse transcription (RT) buffer, 25X deoxyribonucleotide triphosphate (dNTP), 10X RT primers, reverse transcriptase, RNAse inhibitor, and sterile water. The RNA

sample (1µg) was brought up to a total of 10µL volume with water and added to 10µL cDNA solution mix for a final volume of 20µL. Samples were run on an Eppendorf Mastercycler Nexus PCR Machine (see Appendix for RT-PCR protocol).

Quantitative Polymerase Chain Reaction (qPCR)

The newly synthesized 20µL of cDNA was diluted with 20µL of sterile water for a final volume of 40µL. The samples are further diluted 1:10 into new tubes. The final qPCR samples had 9µL of diluted cDNA, 1µL of 20x Qiagen probe, and 10µL of Genesee Scientific's qPCRBIO Probe Mix Lo-ROX (see Appendix for 20x primer list). All samples were run with GAPDH primer as control. The plate was run on a Thermo Fisher Scientific 7500 fast StepOne Real-Time PCR System. The resulting quantifications were analyzed using the comparative threshold cycle (C_t) method, normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein as described in Schmittgen and Livak, 2008.

Statistical Analysis

Results are presented in terms of averages ± standard error of the mean (SEM). Student's paired T-test was used to assess statistical significance (p values of <0.05 were deemed significant.

Results

Generation of global PHLPP2 knockout mice

Global PHLPP2 knockout mice were generated by removing exon 5 and 6 of the PHLPP2 gene. Floxed PHLPP2 mice for exon 5 and 6 were crossed with protamine-cre mice (Jackson Laboratories). Western blot analysis confirmed that removal of PHLPP2 protein in the brain of 3-month-old mice (Figure 1A) as well as other organs (data not shown). Removal of PHLPP2 did not alter the level of PHLPP1 in the brain.

Generation of astrocyte-specific PHLPP2 knockout mice

Astrocyte-specific PHLPP2 knockout mice were generated by crossing PHLPP2 floxed mice for exon 5 and 6 with GFAP-cre mice (Jackson Laboratories). To confirm removal of PHLPP2 in astrocytes only, Western blot of astrocytes from floxed PHLPP2 mice with and without GFAP cre were analyzed. PHLPP2 was removed from astrocytes (Figure 1B) without altering PHLPP1 levels (data not shown).

Removal of PHLPP2 accentuates LPS induced NF-κB activation in the brain

To determine whether removal of PHLPP2 alters inflammation, the activation of NF-κB in the brain was analyzed. Astrocyte-specific PHLPP2 KO mice and their controls were injected with LPS for 4 hours. Following LPS stimulation, we observed increased levels of p65 RNA in the brain following LPS treatment in both GFAP PHLPP2KO and WT samples (Figure 2A). There was no significant difference in p65 gene expression in WT and KO samples. To determine if NF-κB activity was altered, we examined the changes in phosphorylation of p65 and found that removal of PHLPP2 significantly increased NF-κB in the brain following LPS

treatment (Figure 2A). These findings suggest that removal of PHLPP2 may alter the inflammatory response during LPS-induced neuroinflammation.

Removal of PHLPP2 attenuates TNF-α gene expression in the brain following LPS stimulation

As we observed an increase in NF-κB activity, we investigated pro-inflammatory cytokine levels that may be altered through NF-κB alterations. LPS has been found to increase inflammatory cytokine expression in the brain (Rossol et al., 2011). We examined the change in inflammatory gene expression in the brain following 4 hours of LPS stimulation. We found in the brain that the gene expression of TNF-α was significantly attenuated with the removal of PHLPP2 in astrocytes (Figure 2B). We examined other cytokines (IL-1β, IL-6, COX-2, and chemokine C-C motif ligand 2 (CCL2)) activated with LPS treatment, however, there was no significant difference in activation in the brain between the KO and WT mice. This finding suggests that removal of PHLPP2 in astrocytes may be protective by attenuating the inflammatory response through weaker TNF-α activation during LPS-induced neuroinflammation and may potentially favor cell survival over cell death.

Removal of PHLPP2 attenuates activation of apoptotic mediators following LPS treatment

In the brain, LPS activates pro-inflammatory cytokine expression that can lead to apoptosis. To determine whether removal of PHLPP2 alters apoptotic mediators and therefore cell survivability, we examined the anti-apoptotic target B-cell lymphoma-2 (Bcl-2) (Luo et al., 1998) and the pro-apoptotic targets Bcl-2-associated X (Bax), Bcl-2-antagonist/killer (Bak), and Bcl-2-associated agonist of cell death (Bad) (Opferman, 2008; O'brien & Kirby, 2018). We found that following LPS stimulation, removal of PHLPP2 in astrocytes significantly decreased

pro-apoptotic proteins Bax and the phosphorylation of Bad (Figure 3) while there were no significant differences in Bcl-2 gene expression. This suggests that the removal of PHLPP2 may be protective and pro-survival through downregulation of apoptosis.

Activation of MAPKs was not detected at the 4-hour timepoint following LPS stimulation

Since we found alterations in apoptosis, we studied MAPKs levels which target downstream pro-apoptotic and pro-survival pathways (Shen et al., 2005; Song et al., 2012). MAPKs are a family of serine/threonine protein kinases crucial for regulation of inflammatory processes and their activity levels follow a cyclic pattern (Velagapudi et al., 2014; Kaminska et al., 2009). The three major pathways of MAPKs are ERK, JNK, and p38 (Shen et al., 2005) as discussed earlier. We observed protein expression of ERK, JNK, and p38 at 4 hours following LPS stimulation and we have found no activation of MAP kinases with the removal of PHLPP2 at the 4-hour timepoint.

Removal of PHLPP2 alters antioxidant activity following LPS stimulation in the brain

Neuroinflammation leads to excessive ROS production that induces oxidative stress and causes cellular damage (Milatovic et al., 2017). We looked at antioxidant RNA and protein levels to observe any changes with PHLPP2 removal. We observed that the gene and protein expression of Heme Oxygenase 1 (HO-1) was significantly attenuated with PHLPP2 removal, suggesting decreased oxidative stress (Figure 4A and 4B). We also found a significant increase of NOX4 gene and protein expression with PHLPP2 removal, suggesting protection (Figure 4A and 4B). Other antioxidants were analyzed; quinine oxidoreductase 1 (Nqo1), nuclear respiratory factor 1 (Nrf1), glutathione peroxidase 3(GPX3), and superoxide dismutase 2 (SOD2), however, there were no significant alterations with PHLPP2 removal (data not shown). These results

indicate that PHLPP2 removal may decrease oxidative stress through increased antioxidant activity.

Removal of PHLPP2 alters autophagy following LPS stimulation

Lastly, we looked at autophagy activation, an intracellular degradation system that removes harmful debris and damaged organelles (Eskelinen, 2005), for any alterations with the removal of PHLPP2. We observed increased activation of autophagy with the removal of PHLPP2 following LPS administration. We observed a drastic activation of light chain 3 (LC3)-II protein expression in LPS treated PHLPP2 KO samples (Figure 5). We also looked at phosphorylation of mTOR protein levels, which inhibits autophagy when activated, and did not see any changes with PHLPP2 removal. Activation of mTOR seems to be downregulated which could indicate activation of autophagy, however there was a lot of variation between the animals, therefore, it was difficult to see any statistically significant differences. We also analyzed the activation of Unc-51 like autophagy activating kinase (ULK) and p62 and did not find any differences between the two groups (data not shown). These findings suggest that removal of PHLPP2 may be protective through activation of autophagy.

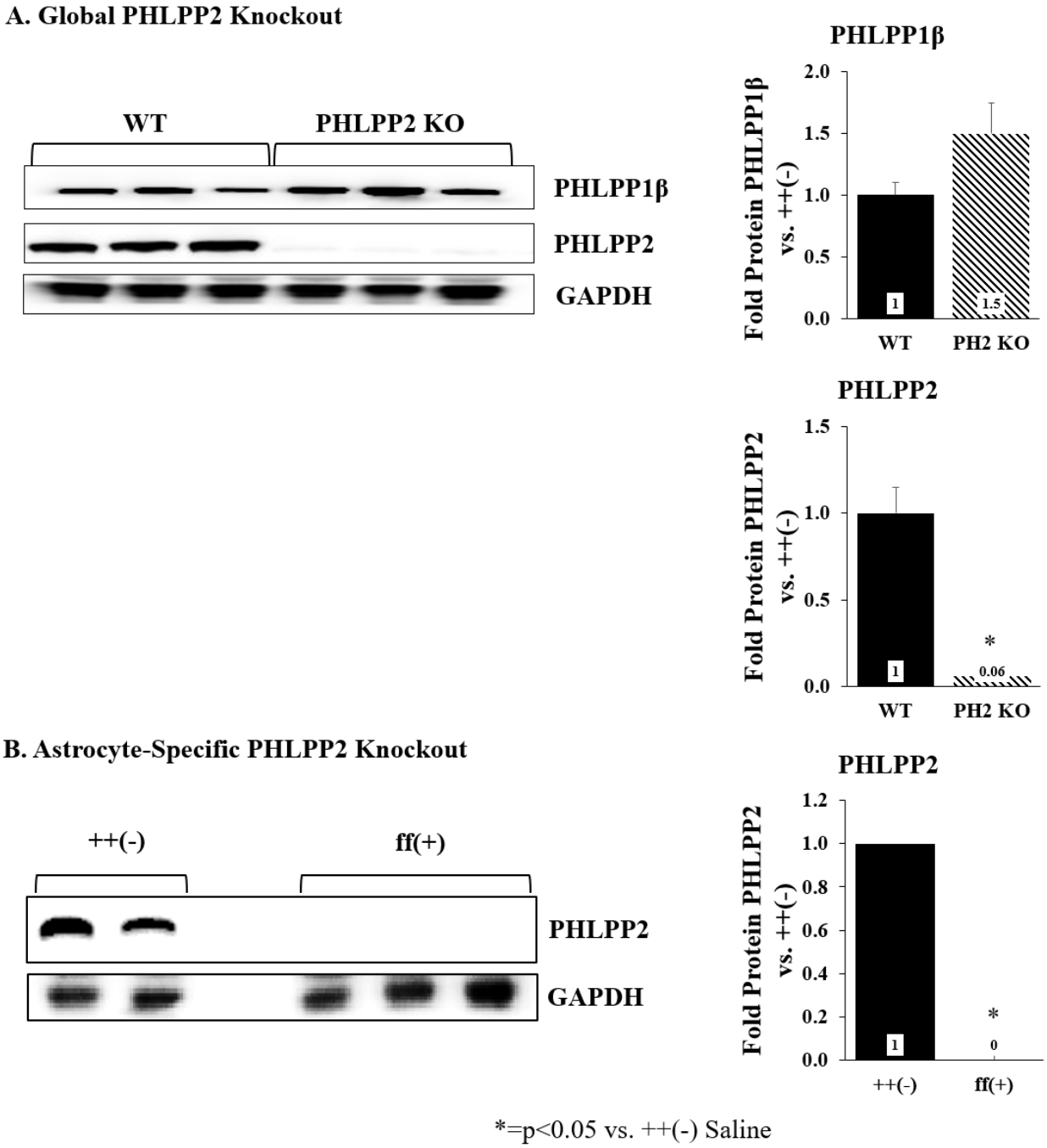


Figure 1: Generation of knockout mice. Western blot quantitation of **(A)** whole-brain extracts from 3-month-old global PHLPP2 knockout mice for PHLPP1β and PHLPP2 protein, and **(B)** isolated astrocytes from <3-day-old GFAP-Cre PHLPP2 knockout mice pups for PHLPP2 protein expression. (*p<0.05 vs. WT/++(-); (A) n=3; (B) n=2 for the ++(-); n=3 for ff (+).)

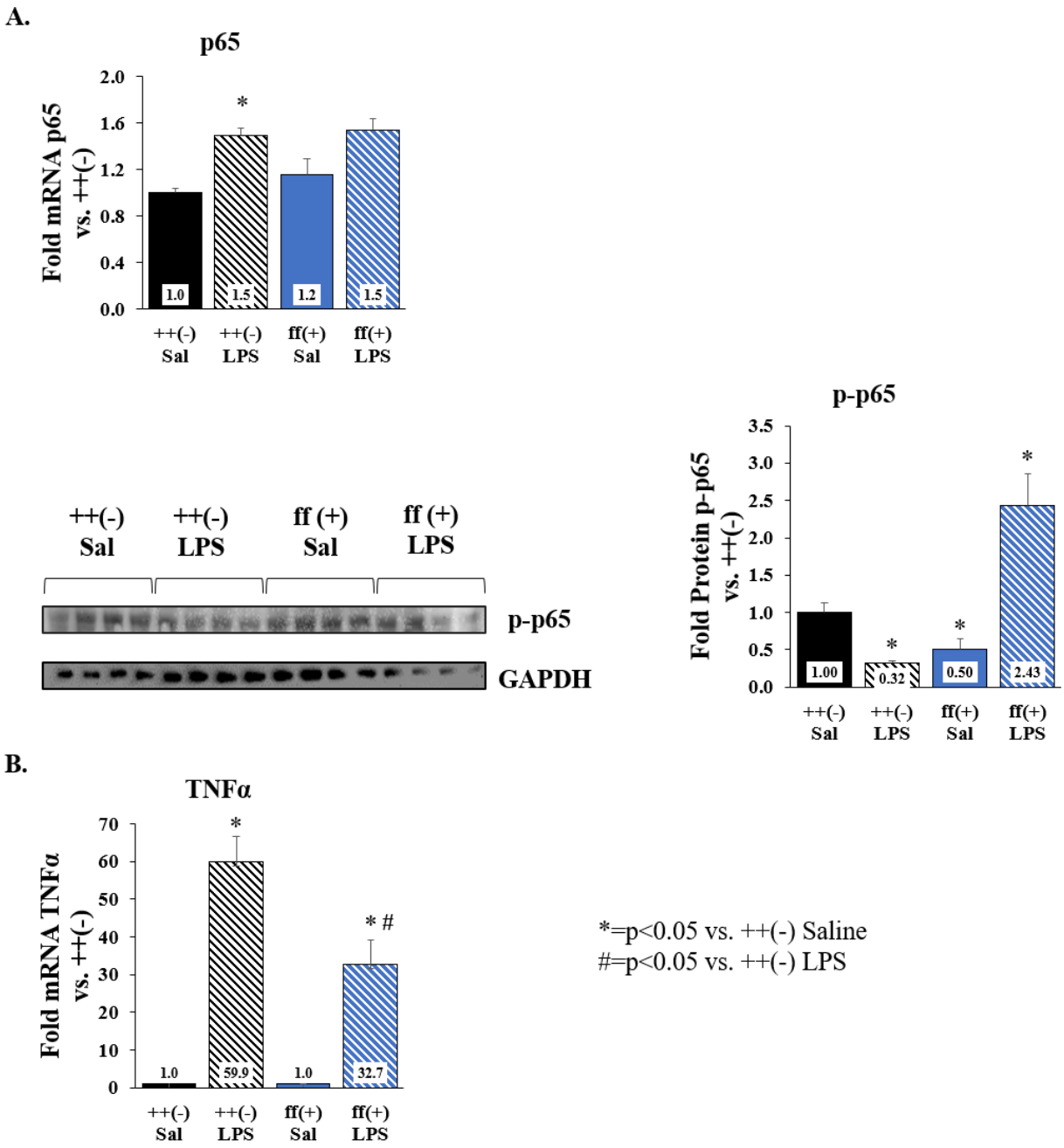


Figure 2: Removal of PHLPP2 increases NF-κB and attenuates TNF-α activation following LPS treatment. (A) RNA and Western blot quantitation of p65 expression from whole-brain extracts of 3-month-old GFAP-Cre PHLPP2 knockout mice. **(B)** RNA quantitation of TNF-α gene expression from whole-brain extracts of 3-month-old GFAP-Cre PHLPP2 knockout mice. (*p<0.05 vs. ++(-) Sal; #<0.05 vs. ++(-) LPS; RNA: n=3 for Sal, n=5 for LPS; Western blot: n=4.)

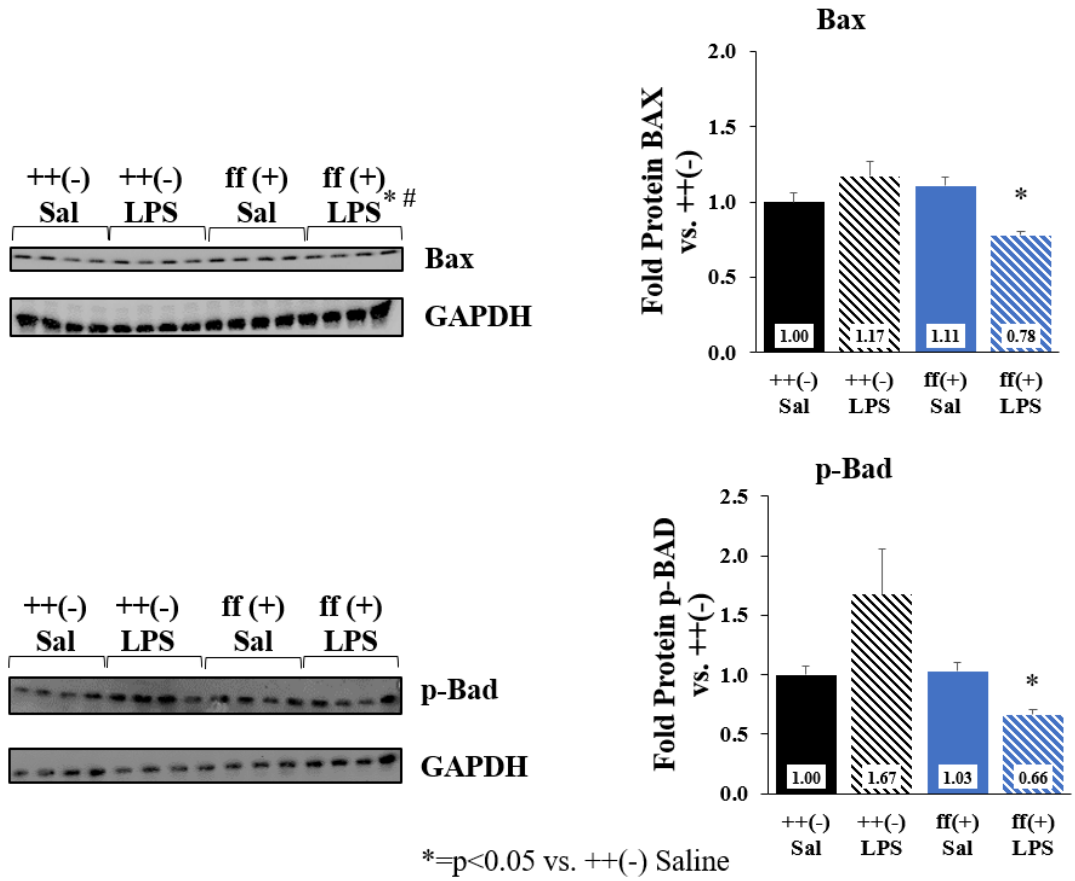


Figure 3: Removal of PHLPP2 attenuates activation of apoptotic mediators following LPS treatment. Western blot quantitation of Bax and p-Bad protein expression from whole-brain extracts of 3-month-old GFAP-Cre PHLPP2 knockout mice. (*p<0.05 vs. ++(-) Sal; n=4.)

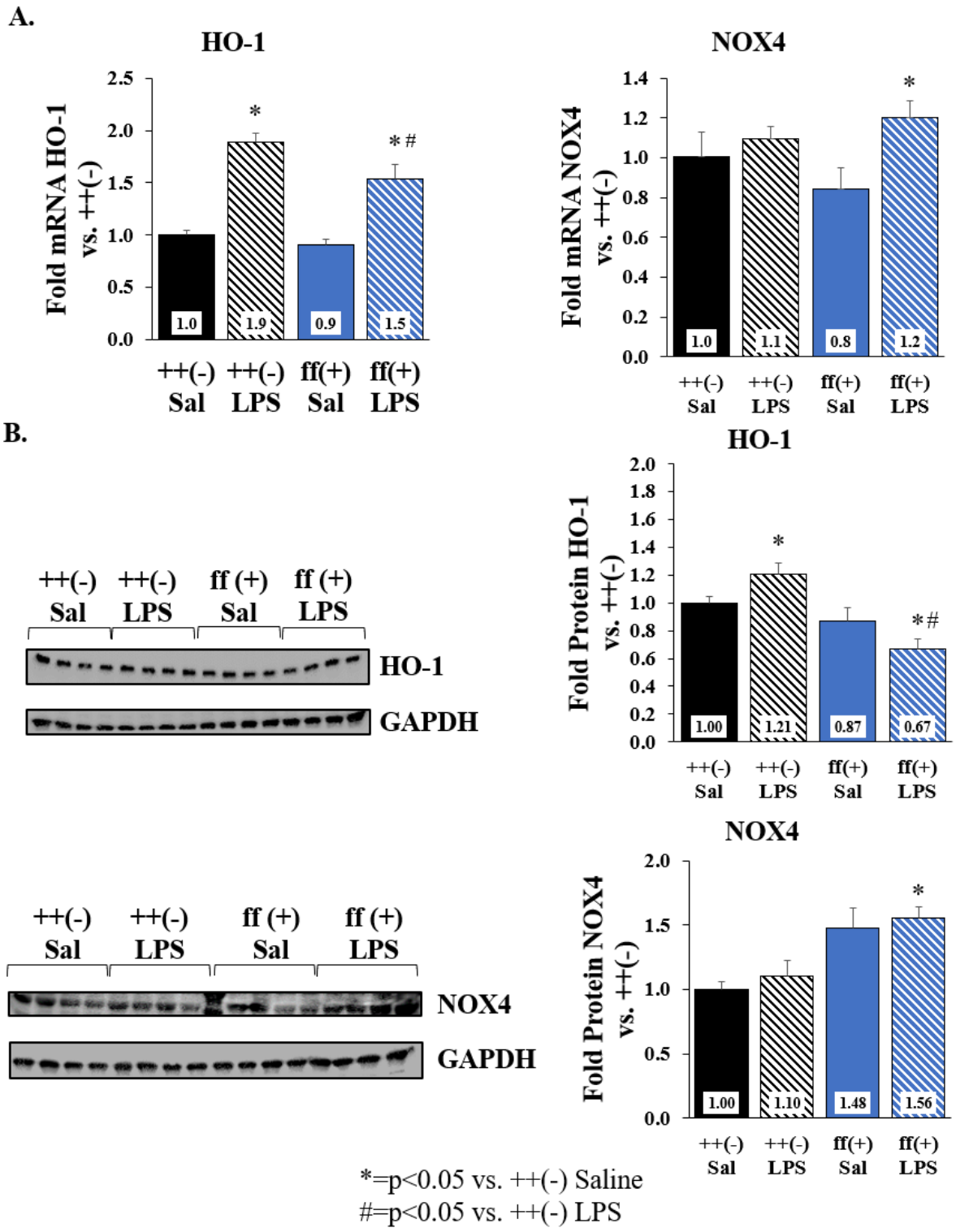


Figure 4: Removal of PHLPP2 alters antioxidant activity following LPS treatment. (A) RNA quantitation of HO-1 and NOX4 gene expression from whole-brain extracts of 3-month-old GFAP-Cre PHLPP2 knockout mice. **(B)** Western blot quantitation of HO-1 and NOX4 protein expression from whole-brain extracts of 3-month-old GFAP-Cre PHLPP2 knockout mice. (*p<0.05 vs. ++(-) Sal; #<0.05 vs. ++(-) LPS; (A) n=3 for Sal, n=5 for LPS; (B) n=4.)

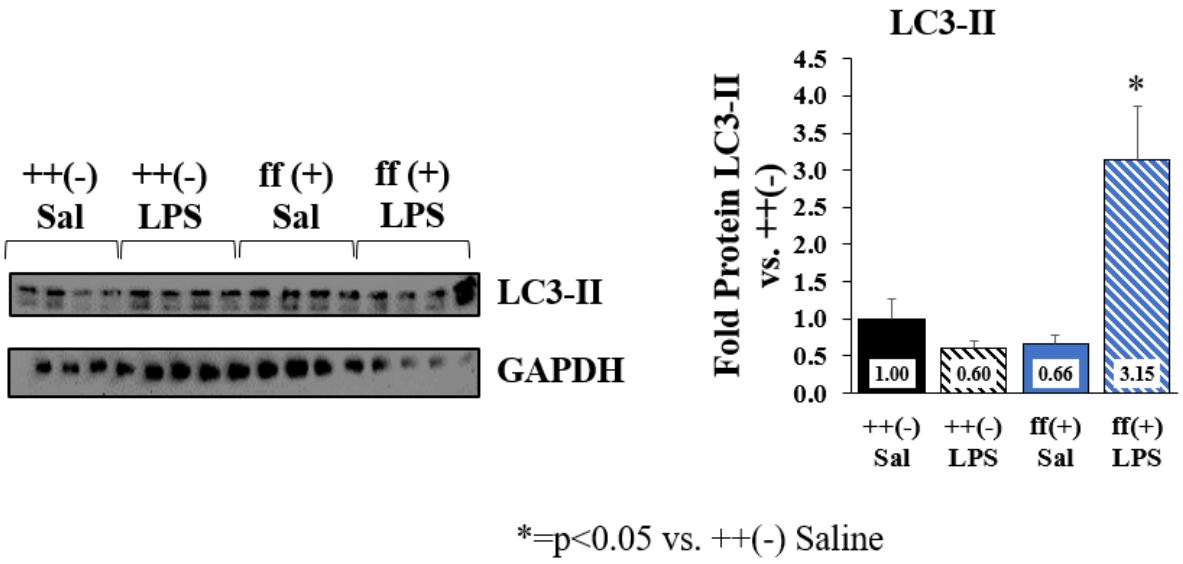


Figure 5: Removal of PHLPP2 increases activation of autophagy following LPS treatment. Western blot quantitation of LC3-II protein expression from whole-brain extracts of 3-month-old GFAP-Cre PHLPP2 knockout mice. (*p<0.05 vs. ++(-) Sal; n=4.)

Discussion

Neurodegenerative diseases affect approximately 50 million Americans each year. Neuroinflammation is a major hallmark of neurodegeneration. Astrocytes are crucial regulators of the inflammatory response in an injured brain and are necessary for neuronal survival. PHLPP1 and PHLPP2 are serine/threonine phosphatases that dephosphorylate several members of the AGC kinase family, including pro-survival kinase Akt (Gao et al. 2005; Grzechnik and Newton 2016). Our laboratory has demonstrated that removal of PHLPP1 increases Akt activity in astrocytes and the brain, leading to protection following ischemic injury, demonstrated through a stroke model in which mice had undergone a middle cerebral artery occlusion (Chen et al., 2012). However, unlike that found in cancer, removal of PHLPP2 was found to not alter Akt activity in cardiomyocytes and astrocytes in culture (Moc et al., 2014; Chen et al., 2012; Welch 2019). The role of PHLPP2 in astrocyte signaling during inflammation is not well studied. In this study, we have generated astrocyte-specific PHLPP2 knockout mice (3-months-old) and induced neuroinflammation through *in-vivo* LPS injection in efforts of understanding the role of PHLPP2 in astrocyte signaling during brain injury.

Activation of Akt leads to the activation of numerous downstream targets such as p70S6K and GSK3. In the present study, we found no changes in p-p70S6K and p-GSK3 protein levels between knockout samples and control following LPS treatment. These results suggest that removal of PHLPP2 in astrocytes may not alter Akt activation based on no difference in activation of two downstream targets, p70S6K and GSK3 levels. It is important to remember that PHLPP2 removal was astrocyte-specific, and these are expression analysis of whole-brain extracts. These results are consistent with a previous study that used global PHLPP2 knockout mice and analyzed the brain for changes in basal levels of Akt activity and found that removal of

PHLPP2 does not alter Akt (Welch, 2019). However, we looked at PHLPP2 knockout samples and saw no alterations in Akt activity. This suggests that PHLPP1 and PHLPP2 are not identical in signaling and may differing or even opposing roles in cell survival. To further confirm this finding, future studies may apply the same stroke model used in PHLPP1 knockout mice to PHLPP2 knockout mice.

Inflammation is activated LPS or through pro-inflammatory cytokines. LPS activates NF-κB signaling by removing the inhibitory IκB protein. This leads to nuclear translocation of the p65 subunit, activates transcription of cytokines such as TNF-α, IL-1β, IL-6, COX-2, and CCL2, and launches the immune response (Park et al., 2011; Shabab et al., 2017). Studies have shown that inhibition of NF-κB during brain injury alters astrocytic functions and correlates with protective effects. One study showed that transgenic inhibition of NF-κB in the mouse brain was found to be protective from white matter demyelination and axonal loss during injury through severely diminished reactive astrogliosis response (Saggu et al., 2016). Another study looked at the role of NF-κB activation in astrocytes to neuroinflammation by blocking nuclear translocation of NF-κB, rendering it inactive (Bethea et al., 1998), and found that inhibition of NF-κB during brain injury might ameliorate survivability and attenuate neurodegeneration through alterations of astrocyte signaling and oxidative stress pathways. Thus, it is important to understand NF-κB signaling during neuroinflammation and whether removal of PHLPP would play a role in its functions. Following LPS stimulation, we observed increased levels of gene expression of p65 in both KO and WT brain samples. In cancer, PHLPP2 removal has been found to increase NF-κB activity (Agarwal et al., 2014). However, to determine activity we analyzed the phosphorylation of p65 and found that removal of PHLPP2 significantly activates NF-κB, both basally and following LPS treatment when compared to WT controls. These

findings suggest that removal of PHLPP2 alters NF-κB activation and may alter the inflammatory response following LPS-induced neuroinflammation.

In the present study, we found that the gene expression of TNF-α was attenuated compared to WT with removal of PHLPP2 following stimulation in the brain. We analyzed other cytokines (IL-1β, IL-6, COX-2, and CCL2) and observed that they were activated by LPS, but there was no significant difference in activation between the two groups. A study looked at PHLPP siRNA-based knockdown and observed a drastic decrease in TNF-α induced NF-κB activation through decreased IκB levels, leading to decreased activation of Ser276 on the p65 subunit in HeLa cells (Wang et al., 2010). This finding suggests that PHLPP plays an important role in activating NF-κB, but the mechanism remains unknown. Although we observed a decrease in TNF-α activation, we did not see a decrease in NF-κB but rather a slight increase, as previously reported. Our finding suggests that removal of PHLPP2 in astrocytes may be protective by attenuating the inflammatory response through weaker TNF-α activation during LPS-induced neuroinflammation and may potentially favor cell survival over cell death.

LPS stimulation and pro-inflammatory cytokines also activate apoptosis. We examined the changes in pro-apoptotic and anti-apoptotic targets following LPS stimulation. Bax, Bak, and Bad are pro-apoptotic proteins that are activated with cellular stress and signal the release of pro-apoptogenic molecules to activate caspases and initiate apoptosis (Opferman, 2008; O'brien & Kirby, 2018). Anti-apoptotic targets include Bcl-2, which blocks the release of pro-apoptogenic molecules through Bax and Bak inhibition (Luo et al., 1998; O'brien & Kirby, 2018). We studied BAX, BAD, and BCL2 to examine any differences in cell death pathways. We found that PHLPP2 removal may attenuate apoptosis. We observed that protein expression of Bax and p-Bad were significantly decreased with removal of PHLPP2 following LPS stimulation. We also

looked at Bcl-2 gene expression and did not observe any changes. This suggests that the removal of PHLPP2 may be protective and pro-survival through the downregulation of apoptotic mediators.

MAPKs are a family of serine/threonine protein kinases crucial for regulation of inflammatory processes. MAPK activity is increased during glial activation and is known to have a cyclic pattern (Velagapudi et al., 2014; Kaminska et al., 2009). MAPKs are activated by LPS (Li et al., 2018). MAP kinases consist of three major pathways: ERK1/2, which is pro-survival and proliferation, and, JNK and p38, which signal cell death (Shen et al., 2005). MAPKs have been shown to directly activate NF-kB in astrocytes. In addition, ERK, JNK, and p38 MAP kinases translocate to the nucleus and transcriptionally modulate the production of pro-inflammatory cytokines including COX-2, TNF-α, and IL-6 (Ma et al., 2010; Yi et al., 2014; Hamby et al., 2010). PHLPP isoforms regulate MAPK levels in cancer cells. In colorectal cancer, PHLPP1 and PHLPP2 directly dephosphorylate and inhibit RAF1, which is upstream of ERK, leading to negative regulation of MAPK signaling (Li et al., 2014). In the brain, PHLPP1β has been shown to inhibit K-Ras, which is also an upstream activator of ERK (Shimizu et al., 2010). However, the role of PHLPP2 in MAPKs in the brain remains unknown. We observed gene expression and protein levels of ERK, JNK, and p38 at 4 hours following LPS stimulation and we have found no activation of MAP kinases with the removal of PHLPP2 at the 4-hour timepoint. Due to MAPK's cyclic activity in which their levels oscillate, we may have missed its activation period. We looked at 4 hours after LPS stimulation, which is only one timepoint and MAPKs were not active during that time. However, they might have been active earlier or later than the 4-hour timepoint. A future study would have to look at multiple timepoint in order to see activation of MAPKs.

Neuroinflammation leads to excessive ROS production that induces oxidative stress and causes cellular damage (Milatovic et al., 2017). Excessive ROS formation can induce oxidative stress, leading to cell damage that may ultimately cause cell death. Therefore, cells have an antioxidant system to scavenge excessive ROS levels (Sheng et al., 2013). We found that astrocyte-specific removal of PHLPP2 alters antioxidant activity following LPS treatment. We looked at antioxidant RNA and protein levels to observe any changes that PHLPP2 removal may have on antioxidant activity and we observed that gene and protein expressions of HO-1 were significantly attenuated in PHLPP2 KO LPS samples, indicating increased oxidative stress. We also found a significant increase of NOX4 activation in both gene expression and protein analysis, indicating protection. We analyzed other antioxidants, Nqo1, Nrf1, GPX3, and SOD2, and did not see any alterations with PHLPP2 removal. These results indicate that PHLPP2 removal causes alterations in antioxidant activity. However, we are unable to discern if these alterations are overall protective or harmful because they have opposing effects and further studies are required.

Autophagy is an intracellular degradation system that delivers cytoplasmic debris to the lysosome. It is pro-survival because it removes harmful debris and damaged organelles (Eskelinen, 2005). LPS-induced inflammation has been found to activate autophagy in the brain (Francois et al., 2014). During autophagy, autophagosomes engulf cytoplasmic components and lead to their degradation. LC3-I protein is conjugated to form LC3-II, which is recruited to autophagosomes. Increased levels of LC3-II have been a standard method of detecting activation of autophagy (Tanida et al., 2008). We observed that astrocyte-specific removal of PHLPP2 increases activation of autophagy following LPS treatment. We observed a drastic activation of LC3-II protein expression in LPS treated PHLPP2 KO samples compared with controls. We also

looked at the phosphorylation level of mTOR, which inhibits autophagy when activated, and we did not see any significant difference in activation with PHLPP2 removal when compared to control. Phosphorylation of mTOR seems to be downregulated which would indicate activation of autophagy but there was a lot of variation within the animals to see any statistically significant differences and further samples are necessary. Further, we analyzed p62, which is downregulated during autophagy (Moscat and Diaz-Meco, 2009), and phosphorylation level of ULK1 (Fan et al., 2015), which is upregulated during autophagy, and we did not observe any significant difference in activation with PHLPP2 removal when compared to control. These findings suggest that removal of PHLPP2 is protective through activation of autophagy and may lower the extent of cellular damage caused by the inflammatory response.

In conclusion, there seems to be more overall protection than harmful changes with astrocyte-specific removal of PHLPP2 in the pathways that we looked at. However, we cannot conclude that those protective activations outweigh the harmful effects nor that removal of PHLPP2 in astrocytes will lead to better survival.

Future Directions

Our current study looked at the effects of astrocyte-specific PHLPP2 removal in whole-brain expression following a 4-hour LPS treatment. Our study gave us a snapshot of what happens during inflammation with removal of PHLPP2 in astrocytes. Future studies may look at global PHLPP2 knockouts and compare those results with astrocyte-specific knockouts to form a more complete picture of astrocyte signaling. In addition, future studies may also look at different timepoints of LPS treatment to see patterns of activity, especially for MAPKs which have cyclic activity.

Furthermore, additional analysis techniques may be necessary. Such techniques include enzyme-linked immunosorbent assay (ELISA) analysis of cytokines in blood samples, cell death assay, and analyze ROS levels in knockout vs. control samples. Immunohistochemistry techniques would also render useful to look at protein expression in tissue. Lastly, new animal models that use overexpression of PHLPP2 levels in astrocytes and the whole brain to confirm trends observed in knockout samples.

Appendix

Table 1: 0.5 M Ethylenediaminetetraacetic acid (EDTA)

Reagent	Source	FW or Stock Concentration	Quantity	Final Concentration
EDTA	Aldrich Chemical Company	372.24	93 g	0.5 M
Sodium hydroxide	Thermo Fisher Scientific	40	10 g	0.5 M

Add the above components to 300 mL of water. Stir and bring the pH to 8. Bring up total volume to 500 mL by the addition of water.

Table 2: 100 mM Phenylmethylsulfonyl fluoride (PMSF)

Reagent	Source	FW or Stock Concentration	Quantity	Final Concentration
Phenylmethylsulfonyl fluoride	Calbiochem	174.2	1.4 g	100 mM
Isopropanol	Thermo Fisher Scientific	60.1	80 mL	N/A

Mix the above components together for a total volume of 10mL.

Table 3: RIPA Buffer

Reagent	Source	FW or Stock Concentration	Quantity	Final Concentration
Sodium chloride	Thermo Fisher Scientific	5 M	15 mL	150 mM
Tris (pH = 7.4)	Thermo Fisher Scientific	1 M	25 mL	50 mM
Nonnidet-P40 Substitute	USB Corporation	603	5 mL	1%
Sodium deoxycholate	Alfa Aesar	432.58	5 g	23 mM
SDS	Hoefer	10%	5 mL	0.1%
EDTA	See Table 1	0.5 M	2 mL	2 mM
Sodium Fluoride	Sigma Aldrich	1 M	25 mL	50 mM

Mix the above components and bring up the total volume to 500 mL by the addition of water.

Table 4: RIPA Mix

Reagent	Source	FW or Stock Concentration	Quantity	Final Concentration
RIPA Buffer	See Table 3	15.4%	5 mL	15.1%
Para-Nitrophenylphosphate	Sigma Aldrich	1 M	5 L	1 mM
Sodium orthovanadate (pH=10)	Thermo Fisher Scientific	500 mM	5 µL	0.5 mM
Leupeptin	Thermo Fisher Scientific	10 mg/mL	5 µL	10 µg/mL
Aprotinin	Sigma Aldrich	200x	33 µL	1x
PMSF	See Table 2	100 mM	50 µL	1mM

Thaw out and vortex sodium orthovanadate and PMSF to completely dissolve pellet before use. Add all the components and vortex to mix.

Table 5: Western buffer

Reagent	Source	FW or Stock Concentration	Quantity	Final Concentration
Sodium phosphate (pH=7)	Sigma Aldrich	0.5 M	20 mL	20 mM
Sodium chloride	Thermo Fisher Scientific	5 M	15 mL	150 mM
Magnesium chloride	Acros Organics	1 M	1 mL	2 mM
Nonnidet P40	Sigma Aldrich	100%	0.5 mL	0.1%
Glycerol	Thermo Fisher Scientific	100%	50 mL	10%
Okadaic acid	Sigma Aldrich	10 μM	0.5 mL	10 nM
Sodium fluoride	Sigma Aldrich	1 M	5 mL	10 mM
Sodium pyrophosphate	Sigma Aldrich	0.5 M	10 mL	10 mM
Dithiothreitol	Sigma Aldrich	1 M	0.5 mL	1 mM
Sodium orthovanadate	Sigma Aldrich	200 mM	0.0092 g	0.1 mM
Pepstatin	Sigma Aldrich	1 mg/mL	5 mL	10 μg/mL
Leupeptin	Sigma Aldrich	1 mg/mL	5 mL	10 μg/mL
Aprotinin	Sigma Aldrich	25 mg/mL	200 μL	10 μg/mL
N_{α}-Tosyl-L-lysine chloromethyl ketone hydrochloride	Sigma Aldrich	5 mg/mL	1 mL	10 μg/mL
L-1-Tosylamide-2-phenylethyl chloromethyl ketone	Sigma Aldrich	5 mg/mL	1 mL	10 μg/mL

Add the above components to 250 mL of water and stir. Bring up the total volume to 500 mL by the addition of more water and stir.

Table 6: 5x SDS Loading Dye

Reagent	Source	FW or Stock Concentration	Quantity	Final Concentration
Tris (pH=7)	Thermo Fisher Scientific	1 M	12 mL	0.25 M
Glycerol	Thermo Fisher Scientific	92.09	19.2 mL	40%
SDS	Hoefer	288.38	3.84 g	277 mM
β-Mercaptoethanol	Sigma Aldrich	78.13	9.6 mL	20%
Bromophenol blue	Thermo Fisher Scientific	669.96	0.048 g	0.0015 mM

Mix the above components and bring up the total volume to 48 mL by the addition of water.

Table 7: MOPS buffer

Reagent	Source	FW or Stock Concentration	Quantity	Final Concentration
MOPS	Spectrum Chemical	209.26	836.8 g	1 M
Tris-Base	Gentrox	121.14	484.8 g	1 M
SDS	Hoefer	288.38	80 g	69.4 mM
EDTA	Aldrich Chemical Company	372.24	24 g	16.1 mM

Dissolve the above components in 2 L of water. Bring up the total volume to 4 L to make 20x MOPS buffer. Dilute 1:20 in water to make 1x MOPS buffer.

Table 8: TA buffer

Reagent	Source	FW or Stock Concentration	Quantity	Final Concentration
TA buffer	Thermo Fisher Scientific	20x	50 mL	1x

Dilute 1:20 in water to make 1x TA buffer (950 mL for 50 mL stock TA buffer).

Table 9: 20x Transfer buffer

Reagent	Source	FW or Stock Concentration	Quantity	Final Concentration
Tris-base	Gentrox	121.14	145.6 g	0.24 M
Glycine	Gentrox	75.07	720 g	1.92 M

Mix the above components in 2 L of water and stir. Bring up the total volume to 5 L by the addition of water.

Table 10: 1x Transfer buffer

Reagent	Source	FW or Stock Concentration	Quantity	Final Concentration
20x Transfer buffer	See Table 9	20x	50 mL	1x
Methanol	Thermo Fisher Scientific	32.04 g/mol	200 mL	20%

Mix the above components and bring up the total volume to 1 L by the addition of water.

Table 11: 10x Tris Hydrochloride (TBS)

Reagent	Source	FW or Stock Concentration	Quantity	Final Concentration
TBS	Thermo Fisher Scientific	157.6	63 g	0.1 M
Sodium chloride	Thermo Fisher Scientific	58.44 g/mol	70.2 g	0.3 M

Mix the above components in 2 L of water. Bring pH to 7.5. Bring up the volume to 4 L by the addition of water.

Table 12: 0.1% TBS-T

Reagent	Source	FW or Stock Concentration	Quantity	Final Concentration
10x TBS	See Table 11	10x	2 L	1x
Tween-20	Chem-Impex International	1227.54	20 mL	0.1%

Mix the above components and bring up the total volume to 20 L by the addition of water.

Table 13: Western Blot Primary Antibody List

Antigen	Source/Catalog #	Dilution	Host
COX-2	CST D5H5	1:1000	Rabbit
IL-1β	CST 3A6	1:1000	Mouse
P-ERK 1/2 (p-p44/42 MAPK)	CST 9101S	1:1000	Rabbit
GAPDH	CST 2118S Proteintech 10494	1:1000 1:40000	Rabbit Rabbit
GFAP	CST 3670S	1:1000	Mouse
P-GSK3α/β	CST 9331S	1:1000	Rabbit
P-JNK (P-SAPK)	CST 4668S	1:1000	Rabbit
LC3B (D11) XP	CST 3868S	1:1000	Rabbit
P-mTOR (Ser2448)	CST 5536T	1:1000	Rabbit
NF-κb p65	CST 8242P	1:1000	Rabbit
Bax	CST D2E11	1:1000	Rabbit
P-p38 MAPK (T180/Y182)	CST 4511S	1:1000	Rabbit
P-p70S6K	CST 9202S	1:1000	Rabbit
PHLPP1β	Bethyl #A300-660A	1:2000	Rabbit
PHLPP2	Bethyl #A300-661A	1:2000	Rabbit
p62	CST 5114S	1:1000	Rabbit
p-Bad	CST 40A9	1:1000	Rabbit
HO-1	CST D60G11	1:1000	Rabbit
NOX4	CST D21F6	1:1000	Rabbit
SOD2	CST D9V9C	1:1000	Rabbit
p-ULK-1	CST D1H4	1:1000	Rabbit

Table 14: Western Blot Secondary Antibody List

Antigen	Source/Catalog #	Dilution
Anti-Rabbit Immunoglobin G	Sigma Life Sciences/SLBV9141	1:8000
Anti-Mouse Immunoglobin G	Sigma Life Sciences/SLBV2305	1:2000

Table 15: cDNA Synthesis Mix

Reagent	Source	FW or Stock Concentration	Quantity	Final Concentration
10x RT Random Primers	Thermo Fisher Scientific	10x	2 µL	1x
dNTP Mix	Thermo Fisher Scientific	100mM	0.8 µL	4mM
RNAse Inhibitor	Thermo Fisher Scientific	20 units/µL	1 µL	1 unit/µl
MultiScribe Reverse Transcriptase	Thermo Fisher Scientific	50 units/µL	1 µL	2.5 units/µl
10x RT buffer	Thermo Fisher Scientific	10x	2 µL	1x

Add the above components with 3.2 µL of sterile water. Add to 1 µg of RNA (10 µL volume final) for a total reaction volume of 20 µL.

Table 16: RT-PCR Protocol

Time	Temperature
10:00	25°C
2:00:00	37°C
5:00	85°C
Hold	4°C

40 cycles.

Table 17: qPCR Primer List

Integrated DNA Technologies (IDT) PrimeTime qPCR Primer Assays

Gene Symbol	Assay ID
BAD	Mm.PT.58.41918051
BAX	Mm.PT.58.4012210
BCL2	Mm.PT.58.7362966
CCL2 (MCP1)	Mm.PT.58.42151692
COX-2	Mm.PT.58.9154407
GAPDH	Mm.PT.39a.1
SOD2	Mm.PT.58.14276358
GPX3	Mm.PT.58.29885432
HO-1	Mm.PT.58.8600055
IL-6	Mm.PT.58.10005566
IL-1β	Mm.PT.58.41616450
NOX-4	Mm.PT.58.8820983
NRF-1	Mm.PT.58.13216611
PHLPP1	Mm.PT.58.9003883
PHLPP2	Mm.PT.58.13356316
Nqo1	Mm.PT.58.10871473
p65	Mm.PT.58.29633634
TNF-α	Mm.PT.58.12575861

Table 18: qPCR Protocol

Stage	Time	Temperature
Holding	2:00	50°C
Holding	10:00	95°C
Cycling	0:15	95°C
Cycling	1:00	60°C

40 cycles.

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