RESTORATION OF MICROGLIAL TRANSPORTER EXPRESSION BY NRF2 ACTIVATION

By

RACHEL NICOLE RITZAU

A thesis submitted to

The School of Graduate Studies

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Master of Science

Graduate Program in Toxicology

Written under the direction of

Dr. Lauren Aleksunes

And approved by

_____

_____

_____

New Brunswick, New Jersey

OCTOBER, 2017

ABSTRACT OF THE THESIS

Restoration of Microglial Transporter Expression by Nrf2 Activation

By RACHEL NICOLE RITZAU

Thesis Director: Dr. Lauren Aleksunes

Activation of brain microglia can be both beneficial and detrimental during the pathogenesis of neuroinflammation. Healthy microglia will propagate short-term inflammation as a means of protecting the central nervous system (CNS) from ensuing injury. However, when this process becomes dysregulated, chronic neuroinflammation results, which further propagates injury to the neuro-environment. Consequently, chronic neuroinflammation results in neuronal death and loss of synapses, which will eventually culminate in neurodegeneration. The principal aim of this dissertation research was to determine whether adaptive changes in the expression of microglial efflux transporters during a neuro-inflammatory state could be attenuated by activation of the antioxidant Nrf2 pathway. The primary function of efflux transporters is to remove both endogenous and exogenous substrates from the cell. This efflux can prevent the accumulation of toxic substrates and ensure proper cell-cell communication. Our laboratory has

previously demonstrated that increasing concentrations of the bacterial endotoxin, lipopolysaccharide (LPS), in immortalized mouse BV-2 microglial cells decreases the expression and function of the multidrug resistance proteins 1a and 1b (Mdr1a/1b) and the breast cancer resistance protein (Bcrp) transporter while increasing the expression of several multidrug resistance-associated protein (Mrp) efflux transporters. These findings were confirmed in the present study using the same model of microglial activation. Further, studies were conducted to assess the ability of a Nrf2 activator, dimethyl fumarate (DMF, Tecfidera®), to mitigate the down-regulation of efflux transporters in mouse microglial cells. Dose- and time-course studies for LPS and DMF were performed in BV-2 cells. Activation of the Nrf2 pathway was assessed by quantifying Nrf2 binding to a prototypical antioxidant response element and the expression of the Nrf2 target genes, NAD(P)H quinone oxidoreductase 1 (Nqo1) and heme oxygenase-1 (Ho-1). Microglial cells were co-treated with both LPS and DMF for 12 and 24 hr and profiled for expression of cytokines, Nrf2 targets and efflux transporters using qPCR and western blotting. In general, the treatment of BV-2 cells with LPS decreased the expression of Mdr1a, Mdr1b, and Bcrp. LPS treatment also up-regulated the levels of Mrp1 and Mrp5 mRNAs. Co-treatment of DMF with LPS prevented mRNA changes in cytokines and efflux transporters and, for Bcrp, restored protein expression to that observed in vehicle-treated BV-2 cells. Together, this research establishes a connection between neuroinflammation, Nrf2 activation, and efflux transporter expression that may be exploited pharmacologically to restore microglial functions.

ACKNOWLEDGEMENTS

My time as a graduate student has forced me to overcome several professional and personal challenges that have helped my growth as both a scientist and person. During this journey, multiple people have had an impact on my growth including my mentor Dr. Lauren Aleksunes. I would like to thank her for providing much guidance and support during my graduate career. Also for aiding and encouraging me through my research projects. I am grateful for all I have learned under both her mentorship and as a member of the Aleksunes lab.

Additionally, I would like to thank my committee members Dr. Guo and Dr. Richardson for helping me to complete the final steps of my graduate career. Specifically, I thank Dr. Richardson and his laboratory for also playing a role in my growth as a scientist by first sparking my interest in microglial research during my short rotation in his lab.

Next I would like to acknowledge everyone in the Aleksunes lab for providing an interesting and friendly work environment. Thanking the other graduate students and Dr. Xia Wen for showing me around the lab and teaching me essential laboratory techniques. As well as answering all my questions and giving beneficial advice.

Lastly, I have to thank my friends and family for always being so supportive of me during my time as a student. Especially my dad and brother for being there through all my failures and accomplishments, making sure I always had support and could keep moving forward.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

ARE, Antioxidant response element; ABC, ATP binding cassette; Bach1, BTB and CNC homolog 1; BBB, Blood-brain barrier; Bcrp, Breast cancer resistance; CNS, Central nervous system; DA, Dopaminergic; DMF, Dimethyl fumarate; GSH, Glutathione; Ho-1, Heme oxygenase-1; iNOS, Inducible nitric oxide synthase; IL-1β, Interleukin 1 beta; Keap1, Kelch-like ECH-associated protein 1; LPS, Lipopolysaccharide; MMF, Monomethyl fumarate; Mrp, Multidrug resistance-associated protein; Mdr1 Multidrug resistance protein 1; Nqo1, NAD(P)H quinone oxidoreductase 1; Nrf2, Nuclear Factor-E2 related factor 2; P-gp, P-glycoprotein; SF, Sulforaphane; tBHQ, *tert*-butyl hydroxyquinone; TNFα, Tumor necrosis factor alpha

INTRODUCTION

Brain

The brain is a complex network of cellular structures that is essential for human functioning. The ability to remember important dates, make simple to life-changing decisions and comprehend difficult concepts is enabled by a functioning nervous system. Due to this complexity, scientists work to elucidate the biological mechanisms behind thought and consciousness. However, at the most basic level the nervous system operates as an information processor that collects and processes internal and external data, and enacts an appropriate response (Haines *et al.*, 2013).

Organization: The nervous system consists of the central (CNS) and peripheral nervous systems (PNS). The CNS contains the brain and spinal cord while the PNS comprises of all the nerves that connect the CNS to other peripheral structures. Connectors are referred to as the spinal and cranial nerves. Neural tracts act to innervate muscles and glandular epithelium as well as transmit sensory information (Haines*, et al.*, 2013). The PNS is further divided into two sections: the autonomic and somatic nervous systems. The somatic system coordinates voluntary movements, while the autonomic system regulates the involuntary functions such as respiration and heart rate (Powley, 2013; Swanson, 2013). In the somatic nervous system, sensory and motor neurons are connected to the spinal cord allowing for rapid communication between the brain and other body systems. Once the sensory information has been processed motor neurons

of the spinal cord transmit the neuronal orders to the corresponding muscles (Swanson, 2013). On the other hand, the autonomic nervous system is responsible for processes such as heart contractions, circulation of the blood, and respiratory and is divided into two separate divisions termed the sympathetic and parasympathetic nervous systems. In general, the sympathetic system is activated during “fight or flight” situations, while the parasympathetic system initiates during periods of “rest and digest” (Powley, 2013).

The individual parts of the CNS include the spinal cord, brain stem, cerebellum, thalamus and the cerebral hemispheres. As previously mentioned, the spinal cord connects the brain and PNS through sensory fibers and motor neurons. The spinal cord transmits sensory data from the body wall, extremities and gastrointestinal tract to the brain while then allocating motor impulses back to the consequent muscles. The brain stem consists of the medulla oblongata, pons and the midbrain. The medulla oblongata is closely associated with the autonomic nervous system because it contains essential connection points and nuclei important for the regulation of respiration, heart rate and other visceral occupations. While the pons functions as a neuronal connector between the cerebral cortex and cerebellum. Similarly, the midbrain largely acts as a connection point between the cerebral cortex and brain stem. The cerebellum is critical to the production of smooth, uninterrupted movements by serving as the command center for individual muscle groups involved in voluntary movement. Whereas the thalamus is the essential collection point for all sensory information transferred to the CNS. All sensory data except for olfaction pass through the thalamus where

the data are processed and distributed to the appropriate area of the cerebral cortex. Lastly, the two cerebral hemispheres consist of three key subsections. These regions include the outer-most layer the cerebral cortex, and the inner layers: the subcortical white matter and basal nuclei (Haines*, et al.*, 2013). The cerebral cortex consists of elevated areas called gyri and depressions called sulci. Collections of gyri and sulci are further divided into six lobes based upon their functions, which include the frontal, occipital, temporal, parietal, limbic and insular lobes (Haines and Mihailoff, 2013). The subcortical white matter contains networks of myelinated axons that rapidly communicate with the cerebral cortex. The third subsection, basal nuclei contains a collection of neuronal cell bodies that are involved in motor processes (Haines*, et al.*, 2013).

Cellular Structure: Neurons are the essential functional unit of the nervous system and are maintained by glial cells. The basic structure of a neuron contains one cell body that is connected to one axon and one dendrite. Axons and dendrites are both branched processes that enable communication with surrounding neurons and glia. Dendrites receive signals, while axons send out the responding transmissions. The points of communication between neurons are called synapses where the axon of the pre-synaptic neuron releases chemical messengers into the dendritic space of the post-synaptic neuron. The neuronal chemical messengers or neurotransmitters are packaged into synaptic vesicles and released out into the synapse. There are a wide range of neurotransmitters that are used in this signal transmission, some of which include glutamate, GABA, dopamine, serotonin and glycine. Whether the message received by the post-synaptic neuron is inhibitory

or excitatory is not decided by the neurotransmitter released, but by the specific receptors the chemical messengers bind too. The post-synaptic receptor is capable of regulating the signal type transmitted; therefore, neurons have immense flexibility in the number of communicative combinations they can send and receive (Bloom, 2013; Hof *et al.*, 2013).

The other major cellular component of the nervous system are glial cells that support and protect neurons. The three types of glial cells located in the CNS include oligodendrocytes, astrocytes and microglia. While the fourth type of glial cell, Schwann cells are located exclusively in the PNS. Oligodendrocytes and Schwann cells enable rapid signaling between neurons through the creation of myelin sheaths synthesized onto axons. Unlike oligodendrocytes, Schwann cells only synthesize one myelin node on an axon, but they also further protect these axons through the creation of a basal lamina sleeve covering the whole tract of this communicator. Because the PNS only contains one glial cell type, Schwann cells therefore must fulfill multiple critical functions to completely support and protect the neurons in their care. Their responsibilities include synthesizing myelin, responding to injuries, eliminating debris and producing growth factors. Whereas the PNS has one multifunctional glial cell to perform the above-mentioned functions, the CNS delegates these responsibilities between three cell types. During brain development, oligodendrocytes form spaced sections of myelin along neuronal axons by wrapping their plasma membrane spirally along each section. Having spaced nodes of myelin along the axons enables the electrical signal or action potential to transfer from one node to the other without exhausting essential

energy during its transmission. Astrocytes accomplish multiple roles in the CNS mainly involving homeostasis. These star-shaped cells interact with neurons, oligodendrocytes and blood vessels using their foot-like projections. The projections enable astrocytes to accomplish multiple functions in several areas. Some of these occupations include the generation of growth factors, response to injury, detoxification of neuroactive substances, as well as the regulation, metabolism and inactivation of neurotransmitters. Lastly microglia are the resident immune cells of the CNS. Therefore, microglia respond to both physical trauma and various pathological assaults to the brain (Hof*, et al.*, 2013).

Microglia

Overview: Microglia are the resident macrophages of the CNS and are thus charged with its defense. They protect the brain from bacterial and parasitic infections as well as aid in the repair of damaged neural tissues. Unlike the other cells of the CNS, microglia do not originate from the neuroectoderm, but from myeloid progenitor cells created in the yolk sac that subsequently migrate into the CNS during early embryonic development. Following colonization of the brain, microglia become isolated from the periphery of the body due to formation of the blood-brain barrier (BBB). Microglia develop into a specialized autonomous cell population separated from blood-derived monocytes. A larger number of microglia are located in the gray matter compared to white matter of the brain. These macrophages are also found more abundantly in the evolutionarily recent regions

of the CNS such as the cerebral cortex and hippocampus compared to the older regions including the cerebellum and brainstem (Trapp and Herrup, 2011). Like all macrophages, microglia adapt to their environment. During periods of homeostasis, microglia monitor surrounding parenchyma through the branching of processes. These projections examine the surrounding extracellular fluid for cellular distress signals (Naftel *et al.*, 2013). Branching surveillance also enables the microglia to detect and eliminate damaged or unnecessary components of the CNS. This function is critical during brain development, thus initiating removal of surplus neurons unable to form appropriate neuronal synapses. Not only do microglia remove dysfunctional neurons, but they also participate in the pruning of redundant synapses during synaptogenesis. A key function of microglia is to secrete cytokines, immune mediators and growth factors that initiate immune responses and provide nutritional support to neurons (Trapp and Herrup, 2011).

During periods of distress involving infection such as viral encephalitis, lead encephalopathy or neurosyphilis, the morphology of microglia transforms into long rod-shaped cells that migrate and surround the compromised neurons. These actions prevent the spread of infection, enabling microglia to either rehabilitate or eliminate the infected neurons (Trapp and Herrup, 2011). By comparison, during situations involving direct tissue damage, infection, ischemia and inflammation, microglia assume an ameboid-shape that permits them to proliferate, surround and phagocytize the injured cells (Naftel*, et al.*, 2013). Another neuroprotective property of microglia encompasses the monitoring and remodeling of impaired synapses (Kraft and Harry, 2011).

Classification: Microglia exhibit a high degree of plasticity in response to their environment leading to multiple different phenotypic states, each with different features. These phenotypic states originate from the multiple interactions between microglia and neurons, astrocytes, oligodendrocytes, neighboring macrophages and cells outside the CNS. The simplest method of categorizing microglia is using the classical macrophage defined M1/pro-inflammatory or M2/anti-inflammatory states. Pro-inflammatory markers include the cytokines tumor necrosis factor alpha (TNFα) and interleukin 1 beta (IL-1β), reactive oxygen species, and the enzyme inducible nitric oxide synthase (iNOS). By comparison, anti-inflammatory markers include transforming growth factor beta (TGF-β), mannose receptor (CD206), arginase-1, and various other growth factors. This classification system is hampered by the fact that pro-inflammatory microglia can also express anti-inflammatory markers and vice versa (Biber *et al.*, 2014). Therefore, unlike the traditional M1/M2 macrophage classification system, microglia demonstrate a further dynamic phenotype that sanctions the adaptability to their environment, and accommodation to each unique situation (Kabba *et al.*, 2017).

Neuroinflammation: The inflammatory response induced by microglia is essential for brain defense. However, if this process becomes unregulated and/or chronically stimulated, the surrounding neural environment may be adversely affected. Not surprisingly, microglia have been implicated in the pathogenesis of a number of neurodegenerative disorders including Parkinson's and Alzheimer's disease. During neurodegenerative conditions, microglia contribute to the underlying inflammatory state in the brain. Throughout acute inflammation,

microglia produce a number of pro-inflammatory and cytotoxic factors as well as assuming phagocytic properties to eliminate foreign pathogens, clear deleterious debris and induce an additional immune response. Once the biological threat is eliminated, microglia undertake an anti-inflammatory state and initiate repair processes to stimulate the rehabilitation of the surrounding neuro-environment. Research performed in Alzheimer's patients and in transgenic mouse models have revealed that microglia present in neurodegenerative states are less likely to acquire anti-inflammatory features. These macrophages also demonstrate minimal repair and nutritional supportive functions commonly observed in healthy microglia (Block *et al.*, 2007).

One of the first neurodegenerative diseases linked with microglial dysfunction was Alzheimer's disease. Alzheimer's disease results in the progressive impairment of memory and cognitive decline, which are directly correlated to the widespread neuronal damage in the temporal and parietal lobes of the cerebral cortex as well as the hippocampus and amygdala (Block*, et al.*, 2007). The molecular characteristics of Alzheimer's include the cerebral accumulation of amyloid-beta aggregates, the generation of neurofibrillary tangles and large amounts of neuronal death (Kabba*, et al.*, 2017). The key evidence implicating microglial involvement in Alzheimer's disease was the observation that microglial activation increases with illness progression. The proposed mechanism associated with the neurodegeneration originates from the microglial response to amyloid-beta. Microglia detect amyloid-beta plaques as toxic protein aggregates that require immediate removal. Therefore, these macrophages respond by

surrounding and attempting to phagocytose the amyloid-beta plaques, generating excessive amounts of neurotoxic factors in the process (Block*, et al.*, 2007). Initially, microglia are able to disassemble and phagocytose amyloid-beta as well as clear out dying neurons (Fan *et al.*, 2015). However, a constant state of activation over time negatively impacts the ability to phagocytose less amyloid-beta, resulting in the excessive production of neurotoxic compounds, further damaging the surrounding tissue and propagating enhanced inflammatory signaling (Block*, et al.*, 2007; Kabba*, et al.*, 2017).

Parkinson's disease is a second neurodegenerative disorder where microglia have been implicated in the underlying pathogenesis. This disease is characterized by the presence of alpha-synuclein containing Lewy bodies, the destruction of the nigrostriatal ascending dopaminergic (DA) neurons, and overall degeneration of the substantia nigra. In Parkinson's patients, the corresponding physical symptoms mainly present as various levels of motor dysfunction (Purisai *et al.*, 2007). DA neurons are more susceptible to inflammatory insult because of their limited antioxidant capacity and their co-habitation with a large population of microglia. One of the chief proposed microglial mechanism involved in Parkinson's disease primarily involves injured DA neurons releasing distress signals, which then induce pro-inflammatory responses in the surrounding microglia population (Block*, et al.*, 2007). By comparison, another mechanism involves neuro-toxicant induced stimulation of microglia subsequently leading to elevated levels of oxidative stress, which negatively affect the surrounding DA neurons (Purisai*, et al.*, 2007). Alpha-synuclein either released by damaged neurons or from Lewy

bodies have been shown to induce an inflammatory response in microglia (Alvarez-Erviti *et al.*, 2011; Fellner *et al.*, 2013; Qin *et al.*, 2016). Microglial activation results in the generation of neurotoxic factors that cause additional neuronal damage and necrosis, thus creating a self-sustained inflammatory cycle. This cycle prevents microglia from aiding injured DA neurons and resolving the inflammation.

Inclusively, these findings suggest that microglia, when over activated in the pro-inflammatory state, have a prominent role in neurodegeneration by amplifying neuronal damage through exposure to pathological stimuli and neurotoxins (Block*, et al.*, 2007). Therefore, without proper microglial function the brain suffers from chronic inflammation, uncontrolled injury response and a deficient ability to recuperate after each insult (Kabba*, et al.*, 2017).

ABC Transporters: MDR1

Overview: Multidrug resistance protein (MDR1) or P-glycoprotein is a part of the ATP binding cassette (ABC) protein family, which is characterized by three conserved peptide motifs that consist of the Walker A and B sections as well as the 'ABC signature' sequence found between the Walker sections. Members of this family are active membrane transporters that translocate a broad range of substrates from numerous compartments of the body (Varadi *et al.*, 2002; Walker *et al.*, 1982). The corresponding *ABCB1* gene consists of 29 exons and the protein has a molecular weight of around 170-kDa (Chen *et al.*, 1990). MDR1 is positioned

in the plasma membrane on the apical surface of polarized epithelial cells enabling them to eliminate compounds already in the cell or prevent them from accumulating in the cell. The primary *in vivo* function of this transporter was elucidated after much study and was best characterized in knockout mice lacking P-gp expression (Schinkel *et al.*, 1997; Schinkel *et al.*, 1995). Researchers found that the P-gp-null mice had a heightened sensitivity to ivermectin-induced toxicity compared to mice with wild-type (WT) P-gp expression. Without functional P-gp, the mice accumulated toxic amounts of ivermectin in their brains leading to neurotoxicity (Lankas *et al.*, 1997). Similar results were observed in a study conducted in Mdr1-null and WT mice exposed to the antiparasitic drug, emodepside. Mdr1-null mice demonstrated increased neurotoxicity when compared to the control mice (Elmshauser *et al.*, 2015). These data provided empiric evidence that P-gp is a critical component of the BBB and protects against neurotoxicity (Schinkel*, et al.*, 1997).

Structure: Unfortunately, the X-ray crystal structure for the human P-gp has yet to be determined and without this critical piece of the puzzle the complete molecular mechanism of this transporter is still unknown. However, P-gp X-ray crystal structures and models have been published from mouse, hamster ovary cells, and bacteria, which help to provide a basis for proposals and hypothesizes of this mechanism. From this data and other studies researchers have found that MDR1 is made up of two transmembrane domains [TMD] containing 12 transmembrane segments and two nucleotide-binding domains [NBD] where ATP binds powering the transport of substrates (Choudhuri and Klaassen, 2006;

Rosenberg *et al.*, 1997; Rosenberg *et al.*, 2005; Sharom, 2014). Studies have also detected the location of the P-gp-drug-binding pocket between the two TMD halves. This central cavity contains aromatic and hydrophobic residues that are implicated in the binding of substrates through differing molecular interactions (Chufan *et al.*, 2013; Sharom, 2014). Additionally, a study conducted using mouse P-gp protein observed that the open and close structure conformation induces surface alterations inside the binding pocket suggesting another possible mechanism involved in P-gp transporter substrate recognition and interaction (Esser *et al.*, 2017).

Substrates: P-gp transports a large spectrum of structurally and functionally diverse compounds, which makes the definition of structure-activity relationships rather difficult. Additionally, the fact that the specific mechanisms this transporter utilizes to recognize their substrates are still largely unknown augments the difficulty of this task (Ieiri *et al.*, 2004). Evidence suggests that this protein has multiple sub-binding sites located inside the binding pocket either used for transport or allosteric-induced modifications. Studies have found that the major substrate binding sites are located between/at areas 5 and 6 of TMD1 and areas 11 and 12 of TMD2. In general, MDR1 substrate preferences includes chemicals that are hydrophobic, organic cations, existing at physiological pH, including one or more aromatic rings or with a molecular weight greater than 400 kDa (Sharom, 2014; Shilling *et al.*, 2006; Wang *et al.*, 2003; Zhou, 2008). These criteria generate an extensive list of substrates that include chemotherapy drugs, antibacterials, antidepressants, antihistamines, steroids, immunosuppressants,

cardiac agents, calcium channel blockers and HIV protease inhibitors (Fromm *et al.*, 1999; Ieiri*, et al.*, 2004; Ito *et al.*, 1997; Pauli-Magnus *et al.*, 2000; Saeki *et al.*, 1993; Schuetz *et al.*, 1998; Sparreboom *et al.*, 1997; Ueda *et al.*, 1992). P-gp can also be induced and inhibited by a large number of chemicals as well. One of the main explanations behind P-gp's promiscuity is the presence of several sub-binding sites with differing molecular interactions enabling the transporter to accommodate many structurally diverse compounds (Martin *et al.*, 1997; Martin *et al.*, 2000a; Sharom, 2014).

Transport Mechanism: Because the exact transport mechanism for P-gp is undetermined two main transport models have been proposed: the hydrophobic vacuum cleaner and the flippase models. The fundamental transport mechanism that these two models build upon first involves, the substrate binding to the inward-facing conformation located on the cytosolic membrane side. Next, ATP hydrolysis induces a conformational change from inward-facing to outward-facing state from the extracellular side releasing the substrate (Sharom, 2014). The hydrophobic vacuum cleaner model purports that the transporter can extract compounds from the cytoplasmic leaflet of the plasma membrane into P-gp's binding pocket and out into the extracellular space. Thus, directing substrates right back out of the cell similar to a vacuum cleaner. Lastly, in the flippase model substrates interact with the transporter binding pocket while moving into the cytoplasmic leaflet. Once bound, P-gp flips the substrate into the outer leaflet permitting the substrate to diffuse through the membrane out into the extracellular space (Borst and Schinkel, 1997; Choudhuri and Klaassen, 2006; Higgins and Gottesman, 1992; Sharom,

2014). Studies have shown that substrate interaction with binding sites are able to increase the rate of ATP cleavage resulting in 'drug-stimulated ATPase activity', such that once the substrate binds it can stimulate the energy generator required for its own transport. Additionally it has been exhibited that one NBD binds ATP for this purpose, while the other binds and hydrolyses ATP after transport in order to undergo the conformational change needed to bring the protein to its previous transport-ready state (Martin *et al.*, 2000b; Sauna and Ambudkar, 2001; Sauna and Ambudkar, 2000; Varadi*, et al.*, 2002).

Locations: P-gp can be found highly expressed in cancer cells, however it is also naturally found in multiple normal tissues throughout the body. For instance, MDR1 can be located in the kidney, liver, intestines, pancreas, adrenal gland, placenta and the BBB as well as in the blood-testes barrier. The fact that this transporter can be found in the canalicular membrane of hepatocytes, the apical membrane of enterocytes, proximal tubular cells and endothelial cells of both blood barriers emphasizes its role in the excretion of potential toxic compounds (Chin *et al.*, 1989; Cordon-Cardo *et al.*, 1989; Fojo *et al.*, 1987; Thiebaut *et al.*, 1987). Additionally, P-gp is expressed in single cells including lymphocytes, microglia, astrocytes and hematopoietic stem cells (Choudhuri and Klaassen, 2006; Ishikawa *et al.*, 2004; Svirnovski *et al.*, 2009). Not only is this transporter expressed in the endothelial cells of the BBB, but it is also found in the choroid plexus epithelium where it prevents potential toxins from moving into the blood stream and out of the cerebrospinal fluid (Rao *et al.*, 1999). Overall P-gp

serves as a major blockade between various tissues and potentially damaging endogenous and exogenous compounds.

MDR1 and Inflammation: Multiple studies have found evidence of inflammation induced changes in Mdr1 expression and function. For example, male CD-1 mice treated with the endotoxin lipopolysaccharide (LPS) displayed diminished expression and function of hepatic P-gp 24 hr after treatment. In contrast, LPS treatment induced up-regulation in the expression and function of renal P-gp. These findings demonstrate inflammation-mediated P-gp regulation is tissue-dependent (Hartmann *et al.*, 2005). Similar findings have been found in various *in vitro* studies investigating P-gp activity at the BBB. There has been little investigation of the effect on P-gp expression and function in microglia exposed to inflammation. Our laboratory has found that LPS treatment of immortalized mouse BV-2 microglial cells reduces the expression and function of Mdr1 in a 24-hr period (Gibson *et al.*, 2012). Interestingly, researchers have also found that this transporter is down-regulated at the BBB in acute inflammatory situations, but up-regulated in more chronic inflammatory states. This is perfectly demonstrated by Mdr1's response to acute (1-6 hr) and long-term (24 to 48 hr) exposure to TNFα in primary cultures of porcine or rat brain capillary endothelial cells (Hartz *et al.*, 2006; von Wedel-Parlow *et al.*, 2009; Yu *et al.*, 2008). For example, an *in vitro* study using primary rat brain capillaries found that short exposure (1-3 hr) to TNFα greatly reduced P-gp-mediated transport without affecting protein expression. But with continuous TNFα exposure (6 hr) an up-regulation of both transporter activity and expression was observed (Bauer *et al.*, 2007). Another *in vitro* study

conducted in immortalized human brain capillary endothelial cells (hCMEC/D3), treated with TNFα for 72 hr observed a significant increase in P-gp mRNA and protein expression (Poller *et al.*, 2010). However, in general *in vivo* experiments investigating acute and chronic inflammatory reactions have demonstrated decreased expression and activity of P-gp at the BBB (Goralski *et al.*, 2003; Roberts and Goralski, 2008).

In neurodegenerative disorders such as Alzheimer's and Parkinson's disease, Mdr1 expression and function appear to be diminished. This transporter plays an important role in Alzheimer's disease due to its ability to efflux β-amyloid from the brain, thus decreasing the number of β-amyloid plaques formed (Cirrito *et al.*, 2005; Hartz *et al.*, 2010). This connection suggests that the reduced functionality of P-gp might have a role in the pathogenesis of this disorder. To further demonstrate this association, multiple studies have found that stimulating P-gp expression through known inducers like, rifampicin, caffeine, verapamil (Abuznait *et al.*, 2011), and St. John's wort (Brenn *et al.*, 2014) resulted in reduction of β-amyloid accumulation in cellular and mouse models of Alzheimer's disease (Abuznait*, et al.*, 2011; Brenn*, et al.*, 2014; Qosa *et al.*, 2012). However, it is unclear whether declines in P-gp expression are due to aging or the neuroinflammatory environment itself or a combination of both (Abuznait*, et al.*, 2011; Brenn*, et al.*, 2014; Deo *et al.*, 2014). In Parkinson's disease, the connection with P-gp is less established. One of the main hypothesizes behind the cause of this disease involves chronic over exposure to neurotoxic substances, resulting in enhanced neuronal damage and augmented neuroinflammation.

Because P-gp's primary function is to eliminate these neurotoxic compounds from the brain, it suggests that Mdr1 may be dysfunctional or reduced in Parkinson's patients allowing for the increased accumulation of harmful compounds (Kortekaas *et al.*, 2005; Lacher *et al.*, 2015; Roberts and Goralski, 2008).

ABC Transporters: BCRP

Structure: Another member of the ABC transporter family is the Breast Cancer Resistance Protein (BCRP) or the *ABCG2* gene, which consists of 663 amino acids and was originally simultaneously discovered in a breast cancer cell line (Doyle *et al.*, 1998) as well as the placenta (Blazquez *et al.*, 2012). Unlike MDR1 and the MRPs, BCRP is a half-transporter embedded in the plasma membrane. BCRP is also unique among other known half-transporters due to its localization in the plasma membrane; other half transporters instead traffic to the intracellular membranes of mitochondria, endoplasmic reticulum and peroxisomes (Rocchi *et al.*, 2000). The BCRP half-transporter has one (NBD) and (MSD) containing 6 transmembrane sections. In order to become functionally active, BCRP forms homodimers or homotetramers allowing for substrate translocation across the plasma membrane. To date, only *in vitro* studies have suggested that BCRP can form higher level complexes; little is known from *in vivo* tissues. Without a complete X-ray crystal structure of the human BCRP transporter, investigations into the structure of the half-transporter alone or in dimers/oligomers and in turn, the mechanism involved in efflux are hampered. Although, what has been

discovered so far is that Bcrp contains multiple drug binding sites, which demonstrate basal levels of ATPase activity that is lesser than the activity observed in P-gp (Ni *et al.*, 2010).

Substrates: BCRP can transport both hydrophobic and hydrophilic conjugated organic anions. A wide range of compounds are transported by BCRP including chemotherapeutic drugs, dietary flavonoids, porphyrins, estrogens and the carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). Other drugs translocated by BCRP include prazosin, glyburide, nitrofurantoin, cimetidine and statins (Ni*, et al.*, 2010).

Locations and Functions: The placenta contains the highest amounts of endogenous BCRP protein within the human body indicating that this efflux transporter most likely plays an important role in the maternal-fetal blood barrier (Xiao *et al.*, 2015). Supporting this premise, an *in vivo* pregnancy study in mice found that the gestational diabetes drug, glyburide, is a substrate for Bcrp in the placenta. The fetal accumulation of glyburide was elevated in Bcrp knockout pregnant mice compared to wild-type mice (Zhou *et al.*, 2008). Similar results in wild-type and Bcrp knockout pregnant mice have been observed for the antibiotic and BCRP substrate, nitrofurantoin (Zhang *et al.*, 2007). Other tissues within the body also express BCRP including the small intestine, colon, liver, breast and in the endothelium of veins and capillaries. In all cases, BCRP is located on the apical side of the plasma membrane of polarized cells (Maliepaard *et al.*, 2001).

Bcrp was also found in the BBB and brain. Specifically, Bcrp immunostaining has been detected in endothelial cells, neurons, astrocytes and

microglia of humans, rats and mice (Doyle and Ross, 2003; Lee *et al.*, 2007). Even though Bcrp has a widespread brain distribution, most studies in this area have been more focused on the expression and functional Bcrp at the BBB rather than in specific parts or cell types of the brain. Experiments performed on Bcrp knockout mice treated with Bcrp specific substrates such as dantrolene or genistein have shown increased brain accumulation utilizing either continuous infusion for 120-150 mins or oral administration of both compounds compared to wild-type mice (Enokizono *et al.*, 2008; Enokizono *et al.*, 2007). Therefore, these findings demonstrate that Bcrp/BCRP is involved in efflux transport at the BBB to some extent, but that substrates that overlap with P-gp/MDR1, may have redundant pathways for extrusion from the brain (Enokizono*, et al.*, 2008; Enokizono*, et al.*, 2007; Meyer zu Schwabedissen and Kroemer, 2011).

Not only does BCRP protect the body from exogenous toxins, it is also involved in the handling of endogenous molecules such as porphyrins. Bcrp-null mice that were fed a diet containing the chlorophyll catabolite phenophorbide a, which caused the mice to exhibit signs of both protoporphyria and diet-dependent phototoxicity (Jonker *et al.*, 2002). Under normal conditions, BCRP limits the number of plant-derived compounds absorbed from ingested foods, but in the absence of Bcrp function, increased levels of phenophorbide a are able to interfere with the balance of porphyrins in the body, resulting in increased skin photosensitivity (Jonker*, et al.*, 2002). Overall BCRP functions to defend tissues against endogenous toxins and xenobiotics and regulate cellular homeostasis for endogenous substrates (Ni*, et al.*, 2010).

Neuroinflammation: Overall, the existing literature on the role of Bcrp in neuroinflammation are minimal; studies have instead focused largely on Bcrp efflux at the BBB. Two *in vitro* studies have demonstrated that pro-inflammatory cytokines reduced Bcrp mRNA and protein expression in the BBB (Poller*, et al.*, 2010; von Wedel-Parlow*, et al.*, 2009). For example, in hCMEC/D3 cells, BCRP mRNA and protein levels as well as function determined by Mitoxantrone accumulation, were decreased with exposures to IL-1beta, IL-6, and TNF-alpha (Poller*, et al.*, 2010). Our laboratory previously demonstrated in the immortalized mouse microglia BV-2 cell line that stimulation of inflammation using the bacterial cell wall component lipopolysaccharide (LPS) caused reductions in both the expression and function of Bcrp using Hoechst 33342 as a substrate (Gibson*, et al.*, 2012). The above evidence suggests that direct and indirect neuroinflammation results in decreased Bcrp expression and function, suggesting that this half transporter may be altered in neurodegeneration conditions.

ABC Transporter: MRPs

Overview: The C subfamily of ATP-binding cassette (ABC) transporter superfamily consists of nine multidrug resistance-associated proteins (MRPs/ABCC). These transporters are ATP-dependent efflux pumps embedded in plasma membranes that transport a variety of different endogenous and xenobiotic anionic substrates (Keppler, 2011). Similar to other ABC transporters, the MRPs contain two NBDs and two MSDs that each consist of six

transmembrane sections (Cole and Deeley, 2006). The only MRPs significantly detected in human, mouse and rat brain tissue are MRP1, 4 and 5 (Dallas *et al.*, 2003; Dauchy *et al.*, 2008; Hirrlinger *et al.*, 2002; Nies *et al.*, 2004).

Mrp1: The most well-known and studied MRP transporter is the multidrug resistance-associated protein 1 (MRP1/ABCC1). The *ABCC1* gene encodes a 190 kDa membrane glycoprotein that was discovered in lung cancer cells demonstrating a multidrug resistance phenotype in the absence of P-gp (Krishnamachary and Center, 1993). MRP1 contains the usual four-domain core structure of ABC transporters, but also has an additional MSD formed by five transmembrane sections (Cole and Deeley, 2006). This transporter is found in many cell types and tissues, but the highest expression levels are located in the lung, testis, kidney, skeletal and cardiac muscles, as well as in the placenta and macrophages. MRP1 is also found in blood-tissue barriers such as the choroid plexus and BBB (Keppler, 2011). MRP1 substrates are usually amphiphilic organic anions, endogenous conjugated metabolites as well as glutathione (GSH)-, glucuronide- and sulfate-conjugated compounds of phase II xenobiotic metabolism. Some specific examples of MRP1 substrates include, cysteinyl leukotriene (LTC_4), prostaglandin GSH-conjugates, glutathione disulfide (GSSG), aflatoxin B_1, 4-nitroquinoline-1oxide and GSH conjugates of antineoplastic agents, herbicides and pesticides. This substrate list indicates that MRP1 plays an important role in the detoxification and elimination of both endogenous and xenobiotic substances, which if left to accumulate in tissues could lead to harmful effects (Cole and Deeley, 2006).

One unique characteristic of the MRP1 transporter is the protein's relationship with GSH. GSH by itself is not a suitable substrate for MRP1, but when this molecule is paired with certain xenobiotics such as, verapamil or dietary flavonoids, GSH can be readily transported by MRP1. For example, in a study performed by Rappa *et al*, wild-type cells treated with either etoposide or sodium arsenite for 1 hr caused an increase in GSH efflux from the cell, which correlated with a simultaneous decrease in the intracellular GSH concentrations (Rappa *et al.*, 1997). However, when the researchers used a MRP knockout cell line, no changes in GSH concentrations were observed. It was concluded that MRP co-transports GSH along with certain substrates (Rappa*, et al.*, 1997). GSH can also act as an enhancer of MRP1-directed transport when interacting with glucuronidated and sulfated conjugated compounds (Keppler, 2011). In a study investigating the efflux of the carcinogen aflatoxin B_1, it was demonstrated that the MRP transporter was responsible for the movement of this toxin and its conjugates from the cells. MRP was capable of transporting the original form of the aflatoxin and that this efflux was augmented by the presence of GSH (Loe *et al.*, 1997). Unfortunately, the exact mechanism behind MRP1-mediated transport and how it recognizes specific substrates is still unclear, due mostly to the lack of a mammalian 3D crystal structure of this transporter. Although researchers hypothesize that GSH binds to and induces a conformational change in the MRP1 protein; in turn, this could allow binding of co-substrates with higher affinity and enhanced efflux (Cole and Deeley, 2006). MRP1 demonstrates a cellular

protective function by the efflux of harmful organic anion conjugates from the cell and thus aiding in their detoxification and excretion from the body.

Mrp4: The multidrug resistance-associated protein 4 (MRP4/ABCC4) is located in the prostate, kidney, BBB, liver, pancreas and choroid plexus. This transporter has also been found in erythrocytes, astrocytes, adrenal glands and in dendritic cells (Keppler, 2011). Unlike other members of the MRP family, MRP4 exhibits variable subcellular localization depending upon the cell type. For example, MRP4 localizes to the apical side of renal proximal tubules and brain endothelial cells. However, MRP4 also traffics to the basolateral side of both the choroid plexus ependymal cells and the hepatocytes (Belinsky *et al.*, 2007; Leggas *et al.*, 2004). The substrates of MRP4 include nucleoside-based anticancer drugs, and antiviral nucleotide analogues, cyclic AMP and GMP as well as monoanionic bile acids. Similar to MRP1, MRP4 transports GSH conjugates and requires the presence of GSH in order to move certain substrates across cellular membranes (Belinsky*, et al.*, 2007; Keppler, 2011). A study performed in Mrp4 knockout mice treated with the antiviral nucleotide analogue 9'-(2'-phosphonylmethoxyethyl)-adenine (PMEA) illustrated the ability of the Mrp4 transporter to act as an endogenous cellular protector (Belinsky*, et al.*, 2007). Mrp4 knockout mice treated with PMEA exhibited pronounced toxicity in multiple tissues including the bone marrow, spleen, thymus, and gastrointestinal tract as well as increased PMEA brain concentrations compared with wild-type mice. It was concluded that MRP4 is an important efflux protein in a number of tissues including the brain (Belinsky*, et al.*, 2007).

Mrp5: The multidrug resistance-associated protein 5 (MRP5/ABCC5) has been found in the heart, placenta, brain and in smooth and skeletal muscles (Dallas *et al.*, 2006). MRP5 is found specifically in pyramidal neurons and glia cells of the brain as well as in the BBB (Hirrlinger*, et al.*, 2002). In most tissues this transporter appears to be located on the basolateral plasma membrane, but in the capillary endothelial cells of the brain Mrp5 resides on the apical side of the membrane (Jansen *et al.*, 2015). This protein transports multiple nucleoside analogs, glutamate conjugates (Jansen*, et al.*, 2015), glutathione conjugates, as well as cyclic GMP and AMP (Ritter *et al.*, 2005). A lack of evidence exists corresponding to the physiological role of Mrp5, since Mrp5 knockout mice appear to be healthy and fertile. Although according to one study, the brains of Mrp5 knockout mice retain higher amounts of glutamate conjugates and changed gene expression, suggesting that the Mrp5 knockout mice could display a lesser neurological phenotype that is not easily recognizable in rodents (Jansen*, et al.*, 2015).

Neuroinflammation: Our laboratory has demonstrated that neuroinflammation as modeled by treatment of BV-2 microglia with LPS results in an increase in Mrp1 and Mrp5 expression, but a possible decrease in transporter function as illustrated by greater retention of the substrate calcein AM in activated microglia compared to control cells. This finding seems contradictive due to the fact that increased protein expression should result in an increase in function as well. However, this may be due to a change in membrane trafficking, limited availability of glutathione or the increase in protein could be a compensatory

reaction to dysfunctional transporters (Gibson*, et al.*, 2012; Rudd *et al.*, 2011). Another study performed in Alzheimer's patients analyzing their hippocampal brain samples, and found that Mrp1 protein was slightly increased in Alzheimer's samples compared to control brain samples. Together, *in vitro* and human studies indicate that the Mrp transporters may be up-regulated in chronic cases of neuroinflammation as well (Dallas*, et al.*, 2006).

Nrf2

Overview: The Nuclear Factor-E2 related factor 2 (Nrf2) pathway is the cell's most prominent defense against oxidative stress. This pathway becomes activated in the presence of oxidative stressors, such as damaging endogenous substances, toxicants and radiation. Nrf2 contains six highly conserved regions named Neh1 to Neh6. In the first domain, the Cap'n'Collar (CNC) and basic-leucine zipper regions are found (Itoh *et al.*, 1999). Being able to handle oxidative and chemical stress is an essential survival tool utilized by all animal species. Basic Nrf2-like systems have been observed in the budding yeast, fruit fly, laboratory worm and in zebrafish. For example, the Yap family proteins of budding yeast are basic leucine zipper type transcription factors, which are activated with oxidative stress, translocate into the nucleus and up-regulate the transcription of antioxidant target genes that contain the antioxidant response element (ARE) in their promoters. The fruit fly displays an antioxidant stress system that is phylogenetically similar to the Nrf2 pathway in humans. Instead of Nrf2, flies

possess a Cap'n'collar C (CncC) protein that is stimulated during oxidative stress. Then CncC translocates to the nucleus and heterodimerizes with *Drosophila* small Maf proteins and induces the transcription of antioxidant proteins. This evidence demonstrates the possibility that the mammalian Keap1-Nrf2 pathway evolved from a common ancestral system needed for species survival (Fuse and Kobayashi, 2017).

Mechanistic Pathway: During basal cellular states, the Kelch-like ECH-associated protein 1 (KEAP1) binds to the Neh2 region of Nrf2 keeping the protein inactive and sequestered in the cytoplasm. Keap1 functions as a restraint by forming a homodimer and binding with actin filaments. This localization allows the Keap1 dimer to catch Nrf2 proteins heading for the nucleus and keep them tethered in the cytoplasm until either Keap1 is forced to release Nrf2 or aid in its degradation. Studies have shown that Keap1 binding to actin filaments and forming a dimer are essential for Keap1 to repress Nrf2 activity (Kang *et al.*, 2004; Zipper and Mulcahy, 2002).

Nrf2 is released from Keap1 in the presence of oxidative stressors, which are normally electrophilic in nature with the tendency to react with sulfhydryl groups. This observation suggests that the electrophiles react with specific cysteine residues in Keap1 which cause a structural modification to the Keap1-Nrf2 complex releasing Nrf2. The Keap1 cysteine residue C151 is involved in the release of Nrf2 from Keap1 after exposure to sulforaphane and *tert*-butyl hydroxyquinone (tBHQ). This was shown by substituting the cysteine residue at 151 to a serine residue, which completely blocked the release of Nrf2 by

sulforaphane or tBHQ (Zhang and Hannink, 2003). Another possible Nrf2 release mechanism involves the phosphorylation of the Nrf2 protein by a protein kinase. Similarly, to the above study, researchers mutated a serine at position 40 and substituted it for an alanine. Protein kinase C (PKC) phosphorylated Nrf2 at position S40, which increased its dissociation from Keap1. This dissociation was negated after the S40 residue was mutated to alanine, demonstrating a correlation between Nrf2 phosphorylation and release from Keap1 (Huang *et al.*, 2002).

In addition to sequestering Nrf2, Keap1 regulates its degradation and turnover by acting as a substrate adaptor, recruiting Nrf2 proteins for targeting to the ubiquitin proteasome system (UPS). Keap1 binds to the scaffolding protein Cullin 3 (Cul3), which forms a ligase complex with Rbx1 and ubiquitin tagged E2. Once Nedd8 conjugates onto the ligase the Nrf2-Cul3-Keap1-E3 complex is ready for ubiquitination. When cellular oxidative stress is triggered, this ligase complex is inhibited allowing Nrf2 to be released from Keap1 and translocate to the nucleus in order to initiate gene transcription (Kobayashi *et al.*, 2004; McMahon *et al.*, 2003; Villeneuve *et al.*, 2010; Zhang *et al.*, 2004).

In the nucleus, Nrf2 heterodimerizes with small Maf, Jun or another unknown factor, which enables the complex to bind and activate the transcription of Antioxidant Response Element (ARE)-containing genes (Itoh *et al.*, 1997; Itoh, *et al.*, 1999). This activation is enhanced when the Nrf2 heterodimer also interacts with CREB-binding protein (CBP) (Loboda *et al.*, 2016). Transcription of ARE genes leads to the production of a network of anti-oxidative stress enzymes, proteins and transporters. Specific examples include the detoxifying enzymes

NAD(P)H:quinone oxidoreductase 1 (NQO1) (Loboda*, et al.*, 2016) and heme oxygenase 1 (HO-1) (Alam *et al.*, 1999), the antioxidant proteins glutathione peroxidase and glutathione reductase, as well as various ubiquitination enzymes (Chan *et al.*, 2001; Itoh*, et al.*, 1999; Loboda*, et al.*, 2016). Xenobiotic transporters regulated by Nrf2 include the MRPs (Maher *et al.*, 2007). Collectively, a battery of Nrf2-ARE genes participate in the detoxification, degradation and excretion of damaging proteins and compounds (Loboda*, et al.*, 2016).

Nrf2 Target Genes: HO-1 catalyzes the rate-limiting step in heme degradation with the aid of the NADPH cytochrome P450 enzymes. This reaction produces equal amounts of iron ions, biliverdin and carbon monoxide (CO). Both biliverdin and CO act as antioxidants, while the iron ions produced by HO-1 lead to the induction of the chaperone ferritin (Alam*, et al.*, 1999; Loboda*, et al.*, 2016). By increasing intracellular levels of ferritin and thus decreasing the levels of free iron ions, HO-1 greatly inhibits the ability of iron to catalyze damaging reactions such as lipid peroxidation and the generation of hydroxyl radicals through the Fenton reaction (Loboda*, et al.*, 2016). Biliverdin is quickly transformed into another antioxidant bilirubin by biliverdin reductase (Alam*, et al.*, 1999). The third product of HO-1 CO, mainly modulates mitochondria functions, glucose metabolism and serves to potentiate cytoprotective signaling in the cell. In the brain, CO has been observed increasing the oxygen consumption rate, which leads to both a decrease in glucose consumption and lactate generation (Wegiel *et al.*, 2014).

NQO1 is another detoxification enzyme induced through the Nrf2 pathway. This FAD-dependent flavoprotein catalyzes the reduction of multiple harmful reactive compounds such as, quinones, quinoneimines, nitroaromatics and azo dyes. NQO1 participates in the reduction of these quinone electrophiles, which prevents them from reacting with other molecules and causing the depletion of sulfhydryl groups or generating even more reactive products. The resulting hydroquinone products of NQO1 can be further metabolized to glucuronide and sulfate conjugates enabling their rapid removal from the body. Additionally, this flavoprotein has been indicated in the reduction of both estrogen and dopamine quinone derivatives as well as acting as a scavenger of superoxide (Dinkova-Kostova and Talalay, 2010).

Nrf2 is not an essential factor for life, through the use of Nrf2-null mice, which were able to survive to adult-hood (Chan *et al.*, 1996). Nonetheless, Nrf2 knockout mice have a heightened sensitivity to various disease pathologies and chemical toxicities. Researchers observed that the Nrf2-null mice are more vulnerable to toxicity produced by acetaminophen, cigarette smoke, and pentachlorophenol (Chan*, et al.*, 2001; Enomoto *et al.*, 2001; Iizuka *et al.*, 2005; Rangasamy *et al.*, 2005; Umemura *et al.*, 2006) For example, Nrf2 knockout mice were treated with acetaminophen (APAP, 300 mg/kg) either died or exhibited increased signs of liver damage compared to wild-type mice and pointed to Nrf2 having an important role in protecting from APAP-induced toxicity (Enomoto*, et al.*, 2001). Another study performed in Nrf2 knockout rats treated with aflatoxin B_1 showed increased hepatotoxicity compared to wild-type rats. The Nrf2 knockout

rats were unable to detoxify the carcinogen evidenced by the decrease in detoxification enzymes and the elevated number of DNA adducts found in the rat livers (Taguchi *et al.*, 2016). Consistent with these findings, researchers have observed that increased activation of Nrf2 causes beneficial effects. For example, deletion of *Keap1* gene in hepatocytes enabled the activation of the Nrf2 pathway and protected hepatocytes against APAP toxicity (Okawa *et al.*, 2006). Another study in rats exposed to aflatoxin B_1 and an analogue of oleanolic acid (CDDO-Im) demonstrated the protective effects of inducing Nrf2 activity (Yates *et al.*, 2006). It was observed that using this Nrf2 activity inducer was able to inhibit both aflatoxin-induced DNA adduct production and liver tumorigenesis. CDDO-Im treatment was also able to enhance the expression of genes involved in the metabolism of aflatoxin, ensuring the quick excretion of this carcinogen (Yates*, et al.*, 2006). On the other hand, un-regulated over-activation of Nrf2 *in vivo* has been shown to result in rather damaging effects, which has been demonstrated in a study done on Keap1-deficient mice (Wakabayashi *et al.*, 2003). These mice died within three weeks after their birth due to severe hyperkeratosis in the esophagus and forestomach. This caused the constriction of their esophagus and cardia, leaving these mice unable to get the nourishment needed to survive. Through this observation, the researchers found that Nrf2 also induces genes used in the squamous cell epithelia response to mechanical stress. This study demonstrates how important regulating Nrf2 functions are and how essential Keap1 is to this process (Wakabayashi*, et al.*, 2003).

Nrf2 Activators: In recent years, more focus has been placed on investigating the therapeutic effects of Nrf2 activators in neurodegenerative diseases, due to the heavy involvement of oxidative stress in the pathogenesis of these disorders. A number of studies have shown that induction of Nrf2 in the brain provides neuroprotection and improves neurodegenerative states, whereas its inhibition enhances neurodegenerative markers. One of the most promising pharmacological Nrf2 activators already on the market is dimethyl fumarate (DMF, Tecfidera®), which was approved in 2013 by the U.S. Food and Drug Administration for the treatment of multiple sclerosis (MS). DMF is metabolized in the gastrointestinal tract by esterases resulting in the formation of its active metabolite, monomethyl fumarate (MMF) (Bomprezzi, 2015; Dibbert *et al.*, 2013).

DMF is a strong Nrf2 activator, suppressing oxidative stress and the resulting inflammation, as well as promoting neurological recovery in MS and in various other *in vivo* models of neurodegenerative disorders. The mechanism of action for this drug is not completely understood. However, research suggests that DMF not only activates the Nrf2 pathway, but is also involved with a number of other cellular processes including the P53 (Brennan *et al.*, 2017), NF-κB (Gillard *et al.*, 2015; Kastrati *et al.*, 2016), HCA2 (Parodi *et al.*, 2015) and the ERK1/2 (McGuire *et al.*, 2016) pathways. Yet, through the use of Nrf2 knockout mice, neurological benefits have been largely found to be Nrf2-dependent (Ahuja *et al.*, 2016). Research suggests that DMF activates the Nrf2 pathway indirectly by generating oxidative stress through the binding and depletion of cellular GSH. A study showed that this GSH reduction was restored after 24 hr, suggesting a Nrf2-

dependent role in its recovery. DMF also acts by disrupting the Keap1-Nrf2 structure, thus allowing increased Nrf2 translocation into the nucleus. As further evidence, DMF is a member of the α, β carboxylic acid unsaturated ester chemical family and therefore is able to react with cysteine residues found on both GSH and Keap1 (Brennan*, et al.*, 2017). A study conducted by Ahuja *et al.*, showed that DMF reacts with the cysteine residues on Keap1, thus inhibiting the ubiquitin-dependent proteasomal degradation of Nrf2, resulting in the activation of the Nrf2 pathway (Ahuja*, et al.*, 2016). A correlation between DMF treatment and nuclear export of the Nrf2 repressor Bach1 was observed (Ahuja*, et al.*, 2016). Specifically, in microglia DMF treatment demonstrates reduction in cytokine inflammatory markers resulting from Nrf2 activation (Wilms *et al.*, 2010).

Methods and Materials

Chemicals: Unless otherwise noted, chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Cell Culture: The immortalized mouse (C57BL/6) microglia BV-2 cell line was provided by Dr. Bin Liu (University of Florida) and used for all experiments. BV-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% glutamax (Invitrogen, Carlsbad CA). BV-2 cells were maintained at 37°C with 5% CO_2. For all treatments, 1% fetal bovine serum was used.

LPS Dose-Response Study: BV-2 cells were treated with LPS from *Salmonella abortus equi* (Enzo Life Sciences, Inc., Farmingdale NY, ALX-581-009-L001) for 6, 12 or 24 hr. LPS was dissolved in distilled water. At each time point, cells (1 x 10^6/well) were treated in triplicate using LPS concentrations of 0, 10, 50 and 100 ng/ml.

DMF Dose-Response Study: Dimethyl fumarate (DMF) was dissolved in DMSO (final concentration of DMSO was 0.1%) BV-2 cells (1 x 10^6/well) were treated with DMF at 0, 5, 10, and 20 μM for 12 and 24 hr.

LPS-DMF Co-treatment Study: BV-2 cells were seeded (1 x 10^6/well) in six-well plates and treated with DMSO vehicle (final concentration of 0.1%), LPS (100 ng/ml) alone, DMF (20 μM) alone or a combination of LPS (100 ng/mL) and DMF (20μM) for either 12 or 24 hr. For western blot analysis, BV-2 cells were treated for only 30 hr. Two different combinations were performed: pre-treatment with either LPS or DMF for 30 mins. After the pre-treatment, the media was replaced with fresh media containing both LPS (100 ng/ml) and DMF (20μM).

RNA Isolation and Real-Time Quantitative PCR: Cells were treated according to one of the three experimental design paradigms described above. For the LPS dose response study, total RNA was isolated using the RNeasy mini kit (Qiagen, Frederick, MD). For all other RNA isolations, RNAzol RT reagent was used to

collect total RNA. RNAzol RT reagent was added directly to BV-2 cells and the lysate was homogenized through up-down pipetting. Next, DNA and proteins were precipitated with 400 µl RNAse-free water and 12,000 x *g* for 15 mins centrifugation. Then, total RNA was precipitated out with isopropanol overnight and the RNA pellet was collected through centrifugation for 10 mins at 12,000 x *g*. Next, the pellet was washed twice with 75% ethanol and finally dissolved in 20 µl RNAse-free water. RNA concentrations and purity were determined using both the 260/280 and 260/230 ratios from the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). cDNA was produced with the High Capacity cDNA Synthesis kit (Applied Biosystems, Foster City, CA). RNA samples were diluted in RNAse-free water to reach a concentration of 1 µg RNA. Next reverse transcription master mix was added to the diluted RNA samples, after which the reverse transcription reaction was performed in a thermal cycler (MultiGene OptiMax, Labnet International, Inc., Edison, NJ), yielding single-stranded cDNA. For qPCR, specific mRNA primers (Integrated DNA Technologies, Inc., Coralville, IA) for each gene were added to 1 µg of cDNA. The resulting messenger RNA expression was evaluated through real-time qPCR, using the SYBR Green-based method (Applied Biosystems). Amplified products were detected in the 384-well plate format using the ViiA7 Real Time PCR machine (Life Technologies, Grand Island, NY). Delta delta Ct values were generated from converted Ct values adjusted to a reference gene (Gapdh) (Livak and Schmittgen, 2001). Specific forward and reverse primer sequences used are listed in Table 1.

Alamar Blue Assay: The Alamar Blue assay was performed to determine cell viability based upon the reduction potential of metabolically active cells (Life Technologies). BV-2 cells (6 x 10^3/well) were seeded into a black clear-bottom 96-well plate and incubated with increasing concentrations of DMF (0-1000 μM). After 24 hr, 100 μl AlamarBlue reagent (Invitrogen) was pipetted into each well and incubated for 3 hr at 37°C. Finally, the fluorescence values were quantified at 570 nm excitation and 585 nm emission wavelengths using a SpectraMax M3 microplate reader (Molecular Devices, Sunnyvale, CA).

Western Blot Analysis: BV-2 cells were seeded onto either 60-mm cell culture dishes (5 x 10^6/dish) or 6-well plates (1 x 10^6/well) and incubated overnight. Cells were treated according to the LPS-DMF co-treatment design. BV-2 cells were washed once with cold PBS and then treated with 2-5 ml of trypsin to induce detachment. The cell pellet was then collected by centrifugation at 500 x *g* for 5 mins and washed with cold PBS. Cells were centrifuged again and the supernatant was removed, allowing the cell pellet to be re-suspended in 100 μl cell lysis buffer containing 1% protease inhibitors. This cell lysate was then centrifuged for 15 mins at max speed and the protein-containing supernatant was collected in a new Eppendorf tube. Protein concentrations of lysates were determined using the Bicinchoninic acid (BCA) assay (Pierce Biotechnology, Rockford, IL). Western blot analysis of target proteins was performed using 40 μg of protein homogenate per well by SDS-polyacrylamide gel electrophoresis on NuPage 8% gels (Invitrogen). Proteins were transferred overnight to a polyvinylidene fluoride membrane.

Membranes were blocked using a 5% nonfat dry milk solution containing PBS with 0.5% Tween 20. Primary antibodies were diluted in a 2% nonfat dry milk solution either overnight at 4°C or for 2 hr at room temperature. Primary antibodies were probed by using species-appropriate secondary antibodies and then detected using the SuperSignal West Dura blotting reagents (Thermo Fisher Scientific). The primary antibodies were used at the following concentrations: 1:1000 for Mdr1a/b (C219; Novus Biologicals, Inc., Littleton, CO), 1:2000 for Mrp1 (MRPr1; Enzo Life Sciences, Inc.), 1:2000 for Mrp4 (M_4I-10; Enzo Life Sciences, Inc.), 1:500 for Mrp5 (M_5I-10; Abnova, Walnut, CA), 1:5000 for Bcrp (BXP-53; Enzo Life Sciences, Inc.), 1:10,000 for Nqo1 (Abcam, Cambridge, MA) and 1:2000 for Gapdh (Abcam). The detection and semi-quantification of protein bands was performed by a FluorChem imager (ProteinSimple, Santa Clara, CA).

Nrf2 ELISA: Nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Fisher Scientific). Nrf2 nuclear accumulation was determined using the TransAM Nrf2 kit (Active Motif, Carlsbad, CA) and the assay was performed following the manufacturer's instructions. Briefly, diluted nuclear extract samples were added to the eight-well strips and then the strips were left to agitate for 1 hr. The wells were washed and the appropriate antibody was added followed by a 1 hr incubation period and washing steps. Next, 100 μl developing solution containing 3,3',5,5'-tetramethylbenzidine (TMB) was added and the color change was monitored and stopped using sulfuric acid (1N)

after 14 mins. The absorbance was determined at 450 nm using a SpectraMax M3 microplate reader (Molecular Devices).

Statistical Analysis: All statistical analysis was performed using GraphPad Prism version 6 (GraphPad Software, Inc., La Jolla, CA). Statistical significance between mean values of treatment groups was determined using a one-way ANOVA with Newman-Keuls post-test. Data are presented as mean ± standard error of the mean (SEM), with $p<0.05$ deemed statistically significant.

Table 1. qPCR Primer Sequences		
Target	**Forward Primer (5'→3')**	**Reverse Primer (5'→3')**
TNFα	AGCCCACGTCGTAGCAAACCAC	AGGAGCACGTAGTCGGGGCA
IL-1β	GACCTTCCAGGATGAGGACA	AGCTCATATGGGTCCGACAG
IL-6	TCCATCCAGTTGCCTTCTTG	ATTGCCATTGCACAACTCTTTT
iNOS	GGCAGCCTGTGAGACCTTTG	TGAAGCGTTTCGGGATCTG
Gapdh	GTTCCTACCCCCAATGTGTC	GTTGAAGTCGCAGGAGACAA
Mdr1a/Abcb1a	TGCCCCACCAATTTGACACCCT	ATCCAGTGCGGCCTGAACCA
Mdr1b/Abcb1b	GTGTTAAAGGGGCGATGGGCG	AGGCTTGGCCAGACAACAGCTT
Mrp1/Abcc1	GCTGTGGTGGGCGCTGTCTA	CCCAGGCTCAGCCACAGGAA
Mrp4/Abcc4	CCAGACCCTCGTTGAAAGAC	TGAAGCCGATTCTCCCTTC
Mrp5/Abcc5	AGGGCAGCTTGTGCAGGTGG	TGCTGTTCCCGCTTCCTTGCT
Bcrp/Abcg2	GCGGAGGCAAGTCTTCGTTGC	TCTCTCACTGTCAGGGTGCCCA
Nqo1	GAGAGTGCTCGTAGCAGGAT	GCAGGATGCCACTCTGAATC
Ho-1	GGTGACAGAAGAGGCTAAGACCGC	GCAGTATCTTGCACCAGGCTAGCA

Results

Activation of Microglia Using LPS: BV-2 microglia were activated to stimulate an inflammatory reaction using LPS as evidenced by time-dependent increases in the mRNA expression of acute phase cytokines namely, IL-1β, IL-6, TNFα, and the cytokine-stimulated enzyme, iNOS (Figure 1). Both IL-1β and IL-6 showed significant mRNA level increases at all time points tested, with greatest induction (30- and 50-fold) at 6 and 12 hr after LPS treatment. By 24 hr, IL-1β and IL-6 mRNAs had decreased in LPS-treated groups, but were still higher than control levels. TNFα mRNA levels peaked at 6 hr after LPS and steadily declined at 12 hr with a return to control levels at 24 hr. iNOS mRNA levels began to increase in response to LPS at 6 hr and were highest (20-fold) at 12 hr, while displaying variable responses at 24 hr. Though up-regulation of cytokine and iNOS mRNA levels exhibited time-dependent responses, most gene changes were not dependent upon the concentration of LPS. At 24 hr, LPS-treated groups did exhibit persistently elevated IL-6, IL-1β and iNOS mRNAs at either 50 or 100 ng/ml with lesser changes at 10 ng/ml.

In parallel to changes in cytokine mRNA expression, up-regulation of cellular and oxidative stress responsive genes, Ho-1 and Nqo1, were evaluated in response to LPS treatment (Figure 2). In general, 50 ng/ml and 100 ng/ml LPS provoked the greatest elevation in mRNA expression for both gene markers. Ho-1 up-regulation was highest in LPS-treated groups at 6 and 12 hr, and then returned

to control levels at 24 hr. By comparison, Nqo1 mRNA expression was enhanced 2-fold in response to 10-100 ng/ml LPS at all time points.

Regulation of Efflux Transporters in Microglia Activated with LPS: In general, microglia activated with LPS exhibited decreases in the mRNA expression of Bcrp and Mdr1a/1b and increases in Mrp1 and 5 transporters (Figure 3). For Bcrp, LPS treatment resulted in a significant 50% decrease in mRNA levels only at 24hr at the two highest concentrations (50 and 100 ng/mL LPS). The Mdr1a and 1b transporters also exhibited decreases in mRNA expression at 12 and 24hr as well. Both Mdr1a and 1b mRNA expressions were reduced by about 50% when treated with 100 ng/mL LPS. Contrary to Bcrp and Mdr1, Mrp1 mRNA expression was significantly increased in BV-2 cells with increasing LPS concentrations at all time points. Following treatment with 50 ng/mL of LPS, Mrp1 mRNA expression was up-regulated up to 120% compared to control at 12 hr. Similarly, Mrp5 expression increased 80% at the earliest time point (6 hr) at all LPS concentrations. No significant mRNA changes were observed for Mrp4. No significant mRNA changes were observed for either Mrp2 or Mrp3 as well (data not shown). Further experiments excluded Mrp2 and 3, because a previous study confirmed the absence of Mrp2 and 3 protein expression in BV-2 cells (Gibson*, et al.*, 2012).

Pharmacological Activation of Nrf2 Signaling in Microglia Using Dimethyl Fumarate (DMF): Recognizing the up-regulation of Nrf2 target genes, Nqo1 and Ho-1, in BV-2 cells activated with LPS (Figure 2), the next set of studies aimed to determine whether augmentation of this response using a pharmacological Nrf2 activator could restore transporter expression in LPS-treated

BV-2 cells. Using the Alamar blue assay, it was demonstrated that DMF did not significantly impact cell viability at doses lower than 50 μM (Supplemental Figure 1). At 150 μM DMF cell viability was decreased by about 40%, and then by 90% at 500 μM DMF. Concentrations of DMF between 5 and 20 μM were selected for subsequent experiments. To confirm that Nrf2 is activated in BV-2 cells by DMF treatment, nuclear extracts from DMF-treated cells were analyzed for their enrichment and binding to prototypical ARE sequence. At DMF concentrations of 10 and 20 μM, nuclear Nrf2 enrichment and binding in BV-2 cells increased by 200% compared to the vehicle-treated control cells (Figure 4). Nuclear translocation of Nrf2 was achieved after 4 hr of DMF treatment. Next, the ability of DMF to up-regulate the Nrf2 target gene, Nqo1, was assessed. The mRNA expression of Nqo1 was significantly increased at all doses of DMF (5-20 μM) at both 12 and 24 hr (Figure 5). The magnitude of Nqo1 induction was greater at 12 hr of DMF treatment, with a 500% elevation in mRNA expression compared to 100% at 24 hr. By comparison, the other Nrf2 target gene, Ho-1 only exhibited slight up-regulation with DMF treatments at 12 and 24 hr (data not shown).

Regulation of Antioxidant, Inflammatory and Transporter Pathways in Activated Microglia Treated with Dimethyl Fumarate: Two approaches were used for the co-treatment of BV-2 cells with LPS and DMF: pre-exposure where DMF was added for 30 min prior to addition of LPS or post-exposure where addition of DMF was delayed for 30 min once LPS was added to BV-2 cells. Activation of the Nrf2 pathway activation with DMF treatment was confirmed through increased mRNA levels of both Nqo1 and Ho-1 (Figure 6A). Unexpectedly,

Ho-1 displayed minimal change with DMF treatment at either time point. Instead, LPS treatment induced a higher magnitude of Ho-1 expression (over 200%) at 24 hr, which was attenuated with pre- or post-treatment with DMF. Nqo1 was a more sensitive marker of Nrf2 activation in this experiment and exhibited greater mRNA induction at 12 hr compared to 24 hr with DMF alone. At 12 hr, DMF induced a 250% increase in Nqo1 mRNA levels compared to control. The DMF-LPS treatments also induced Nqo1 mRNA levels compared to the control and LPS groups by 230% and 170% respectively. Whereas at 24 hr, there were only minimal increases to Nqo1 mRNA levels by 100% with DMF treatment compared to controls, but significantly increased with LPS treatment (alone and in combination with DMF). DMF treatment, both alone and in combination with LPS, increased Nqo1 protein expression at 30 hr was increased by 1000% (Figure 6B). There was also a trend for induction of Nqo1 protein expression (200%) in BV-2 cells treated with LPS, although it was not statistically significant.

Subsequent analysis assessed the ability of DMF to alter the up-regulation of cytokines and nitrative enzymes by LPS treatment. Similar to Figure 1, LPS increased the expression of IL-6, IL-1β, TNFα and iNOS mRNAs in BV-2 cells at 12 and 24 hr. The treatment of BV-2 cells with DMF alone had no effect on these genes. The co-treatment of LPS and DMF (both pre- and post-treatment) reduced the expression of IL-6, IL-1β, TNFα at 12 hr, but not at 24 hr. This decrease was exhibited by an mRNA reduction of 500% for IL-6 and 160% for IL-1β. At 24 hr, the co-treatment of DMF and LPS did not alter the LPS-mediated induction of IL-6, IL-1β and TNFα mRNAs. However, iNOS expression decreased 330% with the co-

treatment groups compared to LPS alone at 24 hr. Only the DMF pre-treated combination group exhibited decreased iNOS mRNA levels at 12 hr.

Interestingly, DMF treatment was able to restore mRNA expression of efflux transporters back to control levels (Figures 8 and 9). Similar to Figure 3, LPS treatment decreased Bcrp (30%), Mdr1a (30%), and Mdr1b (50%) mRNA expression and increased Mrp1 expression (40%) with no change in Mrp4 or Mrp5. Treatment with DMF alone had little effect on transporter mRNA expression with an exception of Mrp1, which was modestly up-regulated (Figure 9). Co-treatment with LPS and DMF significantly restored Bcrp mRNA expression compared to the LPS group at 24 hr, raising these levels near to basal expression. Similar results were observed at 12 hr although not statistically significant. Likewise, Mdr1a and 1b decreased with LPS treatment, which was abrogated by DMF at both 12 and 24 hr. Co-treatment with LPS and DMF (pre-treatment) greatly enhanced Mdr1 mRNA levels by at least 50% compared to LPS alone at both 12 and 24 hr. This mRNA elevation also rose slightly above control. In general, co-treatment with LPS and DMF (both pre- and post-treatment) were capable of raising the Mdr1 expression levels at least back to control. By comparison, co-treatment of LPS and DMF did not return Mrp1 mRNA levels back to control, but instead led to further increases. At 12 hr, this mRNA elevation was significantly increased by 80% compared to the control. While at 24 hr, Mrp1 mRNA was only slightly induced. Against the LPS-induced Mrp1 mRNA levels, the DMF-LPS treatments caused a slight increase at 12 hr, but no change at 24 hr. No significant changes were observed for Mrp4 or Mrp5 with any treatment.

By 30 hr, LPS treatment significantly decreased the protein expression of Mdr1, Mrp4 and Bcrp by 46%, 53% and 30%, respectively (Figure 10). LPS did not alter protein levels of Mrp1 or Mrp5. DMF treatment alone had minimal effects on protein expression, except for Bcrp and Mrp4, which were significantly increased 20 to 40% compared to control BV-2 cells. Furthermore, for Bcrp and Mrp4 expression the combination DMF-LPS treatment produced a significant increase in expression compared to the LPS stimulated BV-2 cells by 21% and 55% respectively. While Mdr1 and Mrp1 displayed negligible changes in protein expression with the combination treatment. Unexpectedly, Mrp5 expression exhibited a 45% reduction under the co-treatment group.

Discussion

The primary aim of the present study was to characterize the effects of dimethyl fumarate, a Nrf2 activator, on the expression of efflux drug transporters Mdr1, Bcrp and Mrp1, 4 and 5 using a model of microglial activation. First, the presence of an LPS-induced inflammatory response was confirmed, and demonstrated by the increase in cytokine mRNA levels. Next, increasing LPS concentrations resulted in the down-regulation of all efflux drug transporter expressions, except for the Mrp transporters. These outcomes agree with what was previously reported by Gibson *et al*. using the same BV-2 cell line and model of activation, lending further confirmation to the differential effects inflammation can have on efflux transporter expression and function in microglia (Gibson*, et al.*, 2012). In general, results of the current study demonstrate Nqo1's ability to serve

as a suitable marker of the Nrf2 pathway in microglia. Through activation of Nrf2, the study revealed DMF's ability to first decrease the LPS-induced cytokine mRNA levels, and to restore efflux transporter expression, specifically for Bcrp, Mdr1a/1b and Mrp4, in microglia stimulated by LPS.

The ability of DMF to activate the Nrf2 pathway was largely observed through the elevated expression levels of Nqo1 and to some extent Ho-1 in microglia. Even though both genes are considered Nrf2 pathway markers, according to our study Nqo1 proved to be the more sensitive indicator of Nrf2 activation in microglial cells. Nqo1 displayed a greater magnitude of mRNA elevation through DMF treatment within 12 hr, but diminished by 24 hr. Consequentially, the Nqo1 protein expression at 30 hr showed strong induction with only treatments containing DMF, while Nqo1 protein was slightly induced by LPS at this time point. These findings suggest that DMF and LPS may stimulate Nqo1 protein production through different mechanisms in BV-2 cells. An earlier time point may display the up-regulation of Nqo1 protein with LPS treatment. Similar mRNA results were observed in male C57B1/6 mice 8 weeks of age, dosed orally with 10 μl/g DMF, researchers observed a peak in brain (cortex, cerebellum and hippocampus) Nqo1 mRNA expression levels between 6 and 12 hr. After administering a single oral dose of 100 mg/kg DMF to the Nrf2-null mice, Nqo1 up-regulation was determined to be exclusively Nrf2-dependent in the hippocampus (Brennan *et al.*, 2016). This finding provides evidence that Nqo1 regulation via Nrf2 depends upon the specific brain region and likely cell type involved.

Ho-1 is known to be regulated by a number of effectors and pathways other than or in conjunction with Nrf2 such as, the BTB and CNC homolog 1 (Bach1) repressor (Reichard *et al.*, 2007; Shan *et al.*, 2006), and the phosphatidylinositol 3-kinase (PI3K/Akt) (Martin *et al.*, 2004), p38 mitogen-activated protein kinase (p38 MAPK) (Chen *et al.*, 2013) and the heme degradation pathways (Foresti *et al.*, 2013). In the current study, Ho-1 was greatly enhanced with LPS treatment, but minimally with DMF. Ho-1 has also been shown to display a more acute expression response with Nrf2 activation. Thus, while not greatly induced at 12 or 24 hr, Ho-1 mRNA expression may have been stimulated to a higher degree at 6 hr instead. This suggestion is supported by work from Brennan and colleagues (Brennan*, et al.*, 2016). As aforementioned, male C57B1/6 mice treated orally with 10 μl/g DMF, displayed Ho-1 mRNA levels that peaked substantially between 2 and 8 hr in the kidney and to a lesser degree in the liver and jejunum. Additionally, Ho-1 expression appeared to be only partially Nrf2-dependent in the kidney, but completely dependent upon Nrf2 in the jejunum. This dependence was demonstrated utilizing Nrf2-null mice given a single dose of 100 kg/ml DMF orally. Nrf2-null mouse kidneys displayed lower Ho-1 induction compared to the enhanced levels observed in WT mice treated with DMF. However, this induction was abolished in the jejunum of the Nrf2-null mice. These data show that Ho-1 expression is differentially regulated and tissue-dependent (Brennan*, et al.*, 2016).

Another means of Ho-1 regulation is related to the transcription suppressor Bach1 (Reichard*, et al.*, 2007). Treatment of human keratinocytes with 25 μM arsenite resulted in different cellular (nuclear vs cytosolic) distributions of NRF2

and BACH1. Arsenite elevated levels of nuclear NRF2 3 hr after treatment, with negligible amounts present in the cytosol, indicating rapid nuclear translocation of NRF2. In contrast to NRF2, BACH1 displayed rapid nuclear export within the first 30 mins of arsenite treatment and by 3 hr resulted in maximal BACH1 cytosolic accumulation. Consistent with this time-line *HO-1* mRNA was shown to be quickly induced 1 hr after treatment, most likely due to the absence of DNA-bound BACH1 enabling the present nuclear Nrf2 to bind and then stimulate HO-1 expression. These results suggest that *HO-1* transcription requires BACH1 inactivation in parallel with NRF2 activation. To further prove this mechanism a chromatin immunoprecipitation (ChIP) analysis was performed and resulted in the discovery that BACH1 and NRF2 interact with the same two enhancer sites containing multiple ARE motifs in the *HO-1* promoter. After arsenite treatment, NRF2 binds to both of these sites; however, in normal cells without arsenite BACH1 is bound to them instead. In another experiment, it was observed that cells treated with the proteasome inhibitor MG132 stimulated extensive nuclear NRF2 accumulation, but no *HO-1* mRNA induction. However, when treated with hemin the human keratinocytes displayed increased BACH1 inactivation and nuclear export as well as substantial *HO-1* mRNA induction. Thus, BACH1 inactivation was found to be more critical in the induction of HO-1 expression, when compared to NRF2 activation. According to these results, Nrf2 is capable of mediating Ho-1 transcription without an oxidative stress trigger so long as Bach1 is already inactivated. This could also explain the presence of Ho-1 induction during normal cellular states (Reichard*, et al.*, 2007).

The fact that LPS alone can activate the Nrf2 pathway and yet demonstrate extensive pro-inflammatory effects when compared to the anti-inflammatory properties displayed through DMF Nrf2 activation indicates the presence of a more complex pathway system. The two systems most likely involved in the LPS response of the current study are the Nrf2 and NF-κB pathways. The Nrf2 pathway is implicated through the elevation of both Ho-1 and Nqo1 expression. While NF-κB is a known regulator of cytokine expression and thus the main pathway involved in the inflammatory effects induced through LPS (Rushworth *et al.*, 2008). Furthermore, there is evidence of cross-talk between these pathways demonstrated by their ability to inhibit each other (Li *et al.*, 2008). In the case of LPS, it appears that the NF-kB pathway may overwhelm Nrf2 and diminish its' anti-inflammatory effects over time. Another possibility is that LPS may be capable of initially activating the Nrf2 pathway, but is unable to sustain the anti-inflammatory effects over time.

Studies performed in both primary human monocytes and the human monocytic cell line (THP-1) demonstrated LPS's ability to induce NQO1 and HO-1 expression and thus activate Nrf2 (Rushworth*, et al.*, 2008). The NQO1 mRNA expression was greatly increased at 4 and 8 hr, but steadily decreased by 12 hr. Interestingly this study also showed that LPS induces Nrf2 not through the destabilization of the Keap1-Nrf2 complex, but by synthesizing the protein de novo (Rushworth*, et al.*, 2008). These results give evidence towards the proposal that LPS can initially activate Nrf2, but cannot sustain the Nrf2 response. For example, it is possible that the newly transcribed LPS-generated Nrf2 protein is more

unstable compared to the Nrf2 already apart of the Keap1 complex. This is due to the fact that LPS only generated more Nrf2 protein without disabling the proteasomal degradation mediated by Keap1, meaning that large numbers of the Nrf2 protein could be disassembled minutes after their creation (Reichard*, et al.*, 2007). Furthermore, the appearance of increased Nrf2 cytosolic concentrations may trigger a feedback loop in place to prevent Nrf2 over-activation.

The present study was able to demonstrate DMF's anti-inflammatory functions by completely mitigating the LPS-induced mRNA cytokine response at 12 hr, but was unable to at 24 hr. This may either indicate that DMF has a shorter activity window in *in vitro* settings or that LPS was able to induce longer-acting effects that became more evident with lessening DMF activity. This enhanced cytokine response may be mitigated with a second dose of DMF after 12 hr to determine whether this result is due to the shorter activity period of DMF or whether there is a secondary mechanism that activates after a certain time period that down-regulates Nrf2 pathway activation. The ability of Nrf2 activators such as, DMF (Peng *et al.*, 2016; Scannevin *et al.*, 2012; Wilms*, et al.*, 2010), sulforaphane (SF) (Brandenburg *et al.*, 2010; Townsend and Johnson, 2016), *tert*-butyl hydroquinone (tBHQ) (Koh *et al.*, 2009), licochalcone E (Kim *et al.*, 2012), lutein (Wu *et al.*, 2015) and melatonin (Shah *et al.*, 2017) to down-regulate inflammatory markers has been demonstrated in multiple studies. For example, a study conducted by Brandenburg *et al.*, observed that SF was capable of nullifying the LPS-stimulated inflammatory response in microglial cells (Brandenburg*, et al.*, 2010). Both primary rat microglia and BV-2 cells were treated with a co-treatment

of 1 μM SF and 100 ng/ml LPS (pre-treated 30 mins with SF) for a range of time points (0-24 hr). mRNA expression of the cytokines, IL-1β, IL-6 and TNFα were all increased with LPS treatment. However, the co-treatment with SF attenuated TNFα, IL-6 and IL-1β mRNA induction maximally at 6 hr for TNFα and 12 hr for both IL-6 and IL-1β (Brandenburg*, et al.*, 2010). Another study, using primary rat microglia treated with DMF, demonstrated similar anti-inflammatory results. Microglia were co-treated with 10 μM DMF and 10 ng/ml LPS (pre-treated 30 mins with DMF) for 6 to 24 hr. After 6 hr, the researchers observed significant decreases to mRNA levels of all inflammatory target genes (IL-1β, IL-6, iNOS and TNFα) tested compared to LPS alone (Wilms*, et al.*, 2010). Both of these studies also demonstrated the ability of their chosen activator to induce Nrf2 protein expression, indicating the function of the Nrf2 pathway in the down-regulation of microglial inflammatory responses (Brandenburg*, et al.*, 2010; Wilms*, et al.*, 2010).

Discrepancies were observed in DMF's ability to completely restore efflux transporter expression back to basal levels. Bcrp was the only transporter that showed complete restoration in both their mRNA and protein levels with DMF treatment. Additionally, this study showed that DMF treatment alone was capable of significantly enhancing Bcrp protein expression, but not mRNA expression. There are multiple explanations for this difference in DMF-induced Bcrp protein and mRNA expression. For instance, the optimal time point for the mRNA induction might have occurred at 6 hr or earlier. Another mechanism may involve DMF-inducing Bcrp protein stabilization, allowing for extended Bcrp transporter accumulation, with lessened or normal levels of mRNA transcription. DMF

concentrations were not expected to have much effect on Bcrp expression due to the fact that until now, a direct transcriptional connection between Bcrp and Nrf2 has not been established. The only somewhat relevant study found was performed in rat brain capillaries treated with SF (Wang *et al.*, 2014). When treated with 10 mg/kg SF, Bcrp protein expression was significantly enhanced along with the Nrf2 target gene Ho-1. In order to investigate whether Nrf2 and p53 activation was necessary for Bcrp expression, the researchers co-treated the brain capillaries with both SF and a p53 inhibitor, pifithrin. Bcrp expression was down-regulated with pifithrin co-treatment, demonstrating the involvement of p53 in Bcrp induction. Thus, the study concluded that Nrf2 and p53 activity were required for Bcrp transporter elevation at the BBB (Wang*, et al.*, 2014). Further studies will need to be performed to investigate the possible influence of the Nrf2 pathway on Bcrp expression and function in microglia.

LPS alone stimulated consistent reductions in both mRNA and protein expression of the Mdr1a and 1b transporters. Mdr1 mRNA, but not protein expression was restored with DMF co-treatments. In contrast to Bcrp, Mdr1 has been shown to be regulated by a number of different factors such as, prostaglandin E2 (Mesev *et al.*, 2017), NF-κB (Gibson*, et al.*, 2012; Thevenod *et al.*, 2000; Wang*, et al.*, 2014), Nrf2 (Wang*, et al.*, 2014), heat-shock transcription factor 1 (HSF1) (Vilaboa *et al.*, 2000), p53 (Johnson *et al.*, 2005; Wang*, et al.*, 2014), pregnane X receptor (PXR) (Jain *et al.*, 2014), which can explain the results observed in this study. In the only other study performed in LPS-stimulated microglia, investigating efflux transporter expression and function, researchers

found a relationship between NF-κB inhibition, Nrf2 activation and Mdr1a expression. Utilizing the same microglial activation model as in the current study, LPS-induced down-regulation of Mdr1a mRNA expression was attenuated with treatment from the NF-κB inhibitor, BAY 11-7082 (Gibson*, et al.*, 2012). Nrf2 pathway involvement was implicated through the induction of Nqo1 mRNA with BAY 11-7082 treatment alone. Overall these results suggest the involvement of both NF-κB and Nrf2 pathways in the restoration of Mdr1a mRNA expression (Gibson*, et al.*, 2012).

In the same study that connected Nrf2, p53 and Bcrp expression, Wang and colleagues also investigated P-gp expression and function at the rat and mouse BBB (Wang*, et al.*, 2014). Rat brain capillaries were treated with 10 mg/kg SF, resulting in the enhancement of P-gp protein expression. P-gp transport was significantly increased with SF and another Nrf2 activator, *t*BHQ treatment demonstrated in an *in situ* brain perfusion method, measuring the buildup of [^{3}H] verapamil in rat brains. To further prove the role of Nrf2 in altering P-gp expression and function, brain capillaries from C57BL/6 WT and Nrf2-null mice were used. While P-gp transport function was increased with either SF or *t*BHQ treatment in WT mice, no enhancement of transport activity was observed in the Nrf2-null mice, giving a strong indication for Nrf2 modulation of P-gp transport. The researchers also determined that the Nrf2 signaling worked in concert with p53, p38 MAPK and NF-κB pathways. This was discovered first by co-treating rat brain capillaries with SF and the p53 inhibitor, pifithrin, which resulted in the nullification of SF-induced enhancement of protein expression and transport activity. The same experiment

using brain capillaries was performed, except the co-treatment consisted of SF and a specific p38 MAPK inhibitor, SB203580, resulted in similar results. Next, WT and p53-null mice were treated with SF, resulting in increased P-gp transport activity in WT mice, but this increase was not observed in the p53-null mice. Furthermore, in brain capillaries, SF treatment was found to translocate Nrf2, p53 and NF-κB into the nucleus, utilizing an electrophoresis mobility shift assay. Inclusively, the study concluded that p53, p38 MAPK, and NF-κB activation were required for Nrf2 up-regulation of P-gp expression and function at the rodent BBB (Wang*, et al.*, 2014). From these two studies, it is evident that Mdr1 regulation involves multiple different regulatory elements creating a complex signaling system that varies depending upon cell-type and tissue. For instance, in mouse microglia, it appears that NF-κB inhibition and possible Nrf2 activation are involved in the up-regulation of Mdr1a expression in an inflammatory environment. While at the rodent BBB, multiple signaling pathways including NF-κB activation are required for Nrf2-mediated up-regulation of Mdr1 expression and function.

Contrary to this pattern, Mrp1 mRNA expression was significantly increased with LPS treatment, but no differences in expression differences were observed at the protein level in BV-2 cells. The DMF-LPS co-treatments displayed elevated Mrp1 mRNA levels at 12 hr that were reduced at 24 hr. Similar LPS-mediated up-regulation in Mrp1 expression and function was observed in the mouse macrophage cell line (RAW 264.7). Regrettably, the researchers did not perform further experiments to discover the possible mechanism behind this result, although they did implicate Nrf2 pathway involvement in the discussion (Silverstein

et al., 2010). From these results and studies found in the scientific literature, it is difficult to decide whether Mrp1 expression is Nrf2-dependent or not. Multiple studies have connected Mrp1 regulation with Nrf2 activation (Gum and Cho, 2013; Hayashi *et al.*, 2003; Ibbotson *et al.*, 2017; Udasin *et al.*, 2016; Wang *et al.*, 2017); however, some studies disagree (Aleksunes *et al.*, 2008; Ghanem *et al.*, 2015). In fact, the Mrp1 gene promoter contains 2 AREs first located between -66 and -57 base pairs, while the second site is located at -499 to -490 bp, implicating a role for Nrf2-mediated regulation of Mrp1 (Ji *et al.*, 2013; Udasin*, et al.*, 2016). But whether these AREs are available for Nrf2 binding or already bound like Bach1 in HO-1, reveals another factor in Mrp1 regulation, which could explain the discrepancies between studies. It is evident that like Mdr1, Mrp1 regulation differs depending on the cell-type observed and treatment used. For example, in primary and PAM212 mouse keratinocytes exposed to 3 μM SF, Mrp1 expression and function were up-regulated (Udasin*, et al.*, 2016). To determine whether this up-regulation was Nrf2-dependent, researchers used primary keratinocytes isolated from Nrf2-null mice treated with SF, which resulted in the elimination of the SF-induced up-regulation in Mrp1 expression and function (Udasin*, et al.*, 2016). Similar to Mrp1 mRNA expression, LPS induced elevated Mrp5 mRNA expression significantly at 6 hr, but with only slight induction at 12 and 24 hr. Mrp5 protein expression was also slightly induced with LPS, but was not statistically significant. Interestingly, it appears that DMF down-regulates Mrp5 protein expression and significantly enhances this decrease when combined with LPS. In the experiments performed by Gibson *et al*., BV-2 cells treated with LPS resulted in enhanced Mrp1

and 5 expression levels, which did indicate a possible role of Nrf2 in this transporter mRNA enhancement, thus in agreement with the results found in the present study (Gibson*, et al.*, 2012).

In the present study, Mrp4 mRNA levels showed no significant changes. However, at the Mrp4 protein level DMF stimulated significant increases in expression, while LPS induced significant protein reduction. Similarly, to Bcrp, the DMF-LPS co-treatment stimulated a significant recovery of protein expression compared to the LPS treatment. These results demonstrate that Mrp4 expression may be regulated by Nrf2 in microglia. Indeed, this relationship between Mrp4 and Nrf2 was confirmed in a study performed on the livers of Nrf2-null mice treated with Nrf2 activators. The stimulation of Mrp4 transporter expression was Nrf2-dependent and discovered through further study that Nrf2 is capable of directly binding to the ARE located on the Mrp4 gene (Maher*, et al.*, 2007; Okada *et al.*, 2008). Similarly, another study performed in C57BL/6J mice treated with a single dose of 400 mg/kg acetaminophen (APAP) exhibited enhanced brain Mrp4 expression levels (Ghanem*, et al.*, 2015). Previous studies in livers of Nrf2-null mice have indicated that toxic APAP doses can activate the Nrf2 pathway and that Mrp4 elevated expression levels were correlated with Nrf2 activation (Aleksunes*, et al.*, 2008). Therefore Nrf2-null mice were utilized to investigate whether the brain Mrp4 induction was Nrf2-dependent. Indeed, brains from Nrf2-null mice demonstrated significantly down-regulated Mrp4 expression compared to the WT mice. This study provides further evidence that Mrp4 expression is regulated

through the Nrf2 pathway consistently in two different organ systems (Ghanem*, et al.*, 2015).

Differences in target expression levels between time points may involve DMF possibly undergoing cellular metabolism producing its' primary metabolite MMF. Researchers have demonstrated functional differences between these two compounds (Ahuja*, et al.*, 2016; Gillard*, et al.*, 2015; Peng*, et al.*, 2016; Scannevin*, et al.*, 2012). For example, primary mouse microglia were treated with DMF, MMF or co-treated with either DMF or MMF and LPS + interferon-γ (IFN-γ) (Peng*, et al.*, 2016). Through microarray analysis, the study observed that DMF induced around 1800 genes in control cells and about 2700 genes in activated microglia. In contrast, MMF only induced around 200 genes and 16 genes in control and activated microglia respectively. This striking difference in gene induction, suggests that DMF influences a large number of cellular functions compared to MMF. Another key gene induction difference between DMF and MMF was the fact that only DMF altered genes involved with NF-κB, suggesting that DMF, not MMF is responsible for the reduction of pro-inflammatory markers observed with DMF treatment (Peng*, et al.*, 2016). Unfortunately, none of the studies have focused on efflux drug transporters, but on the effects to Nrf2 activation and cellular functional changes. The differences between the outcomes of DMF and MMF are critical in *in vivo* situations since experimental data has proven that most of the initial DMF dose is rapidly metabolized to MMF, which has been more detectable in blood and tissues compared to DMF (Dibbert*, et al.*, 2013). In fact, most research suggests that MMF is responsible for the anti-

inflammatory characteristics of DMF, due to the slight if barely detectable presence of DMF in the body. However, other studies have detected GSH-conjugated forms of DMF both in blood and brain of rats, suggesting that some form of parental DMF may still transfer into the CNS and achieve pharmacological effect (Peng*, et al.*, 2016).

Unfortunately, the current study could not discern whether the results observed were primarily due to DMF, MMF, a mixture of the two or from a DMF-conjugated form. In future studies, it would be prudent to use both DMF and its' primary metabolite to determine whether both chemical forms demonstrate similar effects on efflux transporter expression and function. Another two experimental parameters to consider would be to either conduct an assay assessing the esterase activity of BV-2 cells or determine the concentrations of DMF and MMF in the treatment media after 12 and 24 hr using an analytical procedure such as HPLC-MS. The esterase activity assay would be an indirect method of determining the possible presence of MMF, since DMF is metabolized through esterases. In comparison, using an analytical method would directly asses the concentrations of DMF and MMF over the treatment duration. Additionally, an indirect method could be utilized involving the substitution of fetal bovine serum (FBS) for heat-inactivated FBS in the treatment media. This heat-inactivation will denature any present esterases in the FBS eliminating the possible metabolism of DMF during the experiments which could prove or disprove whether the observed effects were mediated exclusively by DMF.

The future directions of this project would be to first determine the functional effects of DMF on efflux transporters. The expression results primarily for Bcrp and Mrp4 display DMF-induced restoration at the protein level which would usually translate over to a similar effect observed in transporter function. However, these proteins might be present, but dysfunctional in some way that could involve being improperly incorporated into the plasma membrane or be functionally inhibited by another mechanism. Therefore, it is essential that these study results are confirmed through functional transport assays to determine the continuation of the project. The next step would be to conduct similar experiments using primary mouse and possibly human microglia to assess whether DMF would have similar effects in a more biologically natural cell type compared to the immortalized BV-2 microglia cells. A mouse study would be most definitive to demonstrate DMF's restorative effects on efflux transporters in an *in vivo* neuroinflammatory situation. The mice would be dosed in a similar treatment regimen as utilized in the *in vitro* studies. To further understand the mechanisms involved in Nrf2-modulation of efflux transporters in activated microglia, Nrf2-null mice and cellular Nrf2 knockout methods should be utilized in the future.

The present study still raises many questions regarding inflammatory effects on microglial drug transporters and their purpose in the inflammatory response. Indeed, Bcrp, Mdr1 and even Mrp4 appear to be down-regulated with LPS treatment suggesting that activated microglia have reduced efflux transporter expression. However, Mrp1 and Mrp5 are seemingly up-regulated in this scenario. The most likely reason is because these two groups of transporters are regulated

through different pathways. Is it possible that there is a beneficial effect to having Bcrp and Mdr1 down-regulated during inflammatory conditions? Or is this a detrimental product of microglial over-activation? Another influential factor involves the difference between acute and chronic inflammation and the impact of these states on efflux transporters. Therefore, in the present study the microglial activation model used and its effects on efflux drug transporters may apply more to acute inflammatory response than the detrimental chronic inflammatory state. Consequently, the reduction to Bcrp, Mdr1 and Mrp4 may in fact be beneficial, because acute inflammatory reactions are most commonly utilized as a microglial protective mechanism allowing for the elimination of foreign pathogens. Therefore, another *in vitro* study must be performed using an activated microglia model that more closely resembles chronic inflammatory conditions, instead of LPS to investigate the possible differential effects that acute vs chronic inflammation has on microglial efflux drug transporters. This is critical for furthering the understanding of microglial functions during chronic inflammatory states and thus neurodegeneration. Additionally, through better understanding novel drug targets and treatment options can be discovered to mitigate and one day cure neurodegenerative disease.

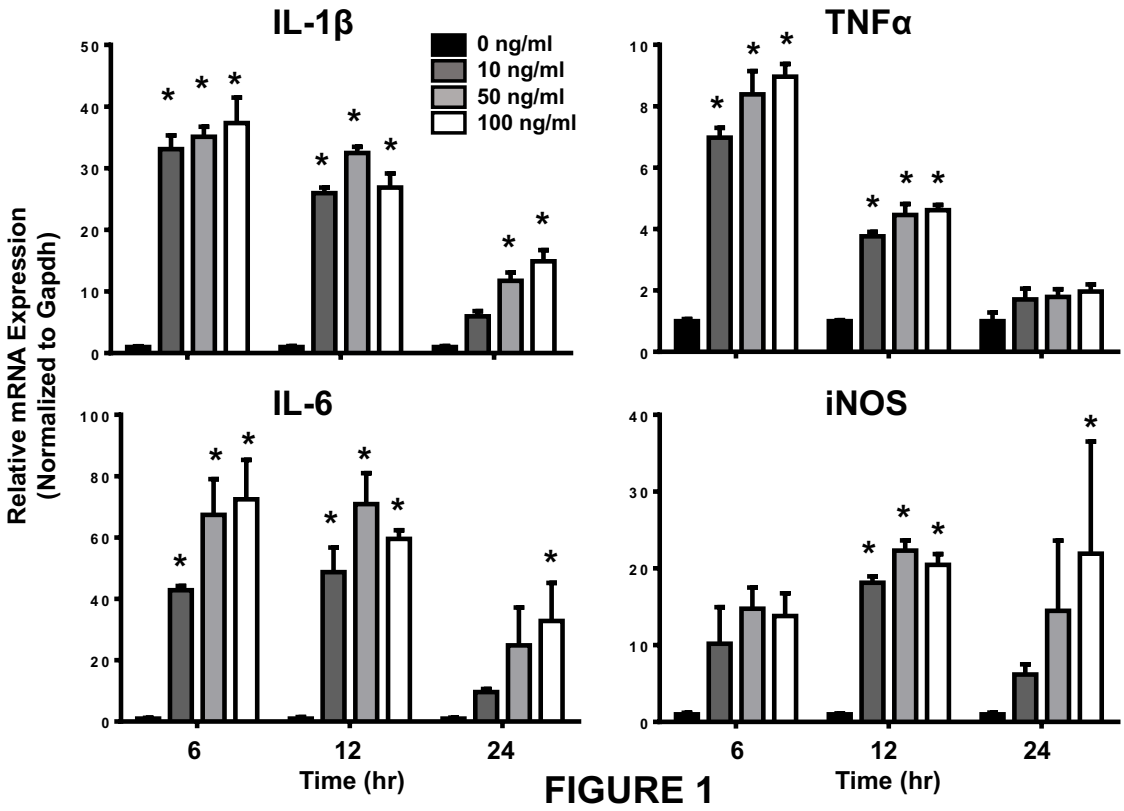


Figure 1. Quantification of Microglial Activation Model. BV-2 cell activation was analyzed through the relative mRNA expression of pro-inflammatory cytokines and one cytokine-stimulated enzyme. Microglia were exposed to 6, 12 and 24 hr periods of LPS treatment. Data were normalized to Gapdh mRNA and presented as mean relative mRNA expression ± SEM (n=3) compared to 0 ng/ml LPS. Asterisks (∗) represent statistically significant differences ($p < 0.05$) compared to 0 ng/ml LPS.

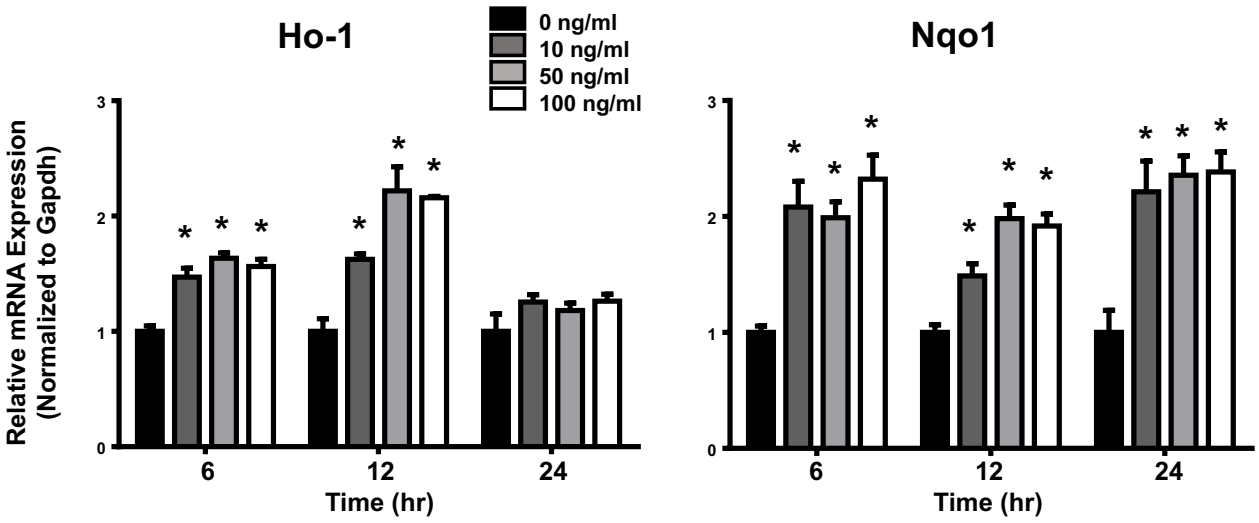


FIGURE 2

Figure 2. mRNA Expression of Nrf2 Target Genes in Activated Microglia. Relative mRNA expression of Nrf2 target genes, Ho-1 and Nqo1 were determined after 6, 12 and 24 hr incubations with LPS. Data were normalized to Gapdh mRNA and presented as mean relative mRNA expression ± SEM (n=3) compared to 0 ng/ml LPS. Asterisks (∗) represent statistically significant differences ($p < 0.05$) compared to 0 ng/ml LPS.

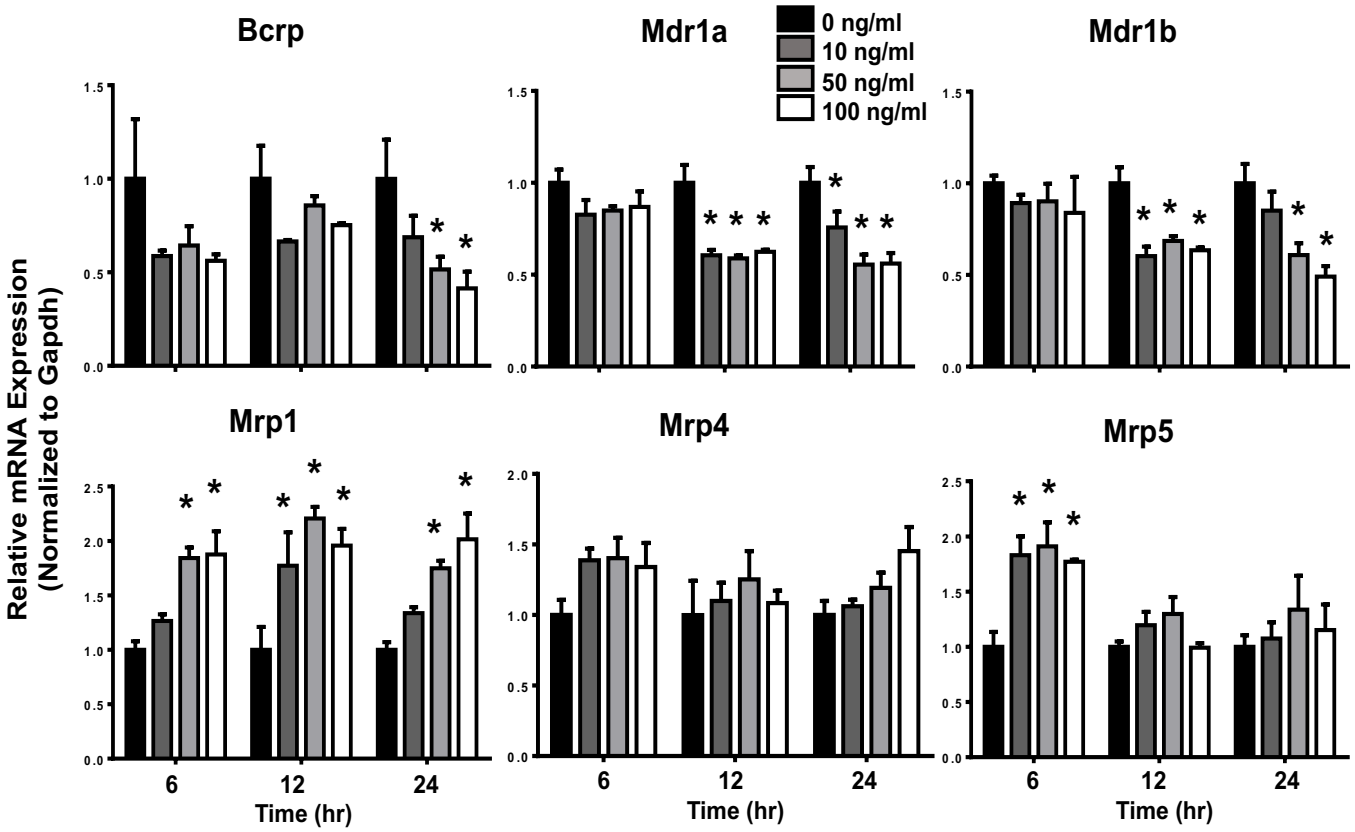


FIGURE 3

Figure 3. mRNA Expression of Efflux Transporters in Activated Microglia. Relative efflux transporter mRNA expression was quantified after 6, 12 and 24 hr incubations with LPS. Data were normalized to Gapdh mRNA and presented as mean relative mRNA expression ± SEM (n=3) compared to 0 ng/ml LPS. Asterisks (∗) represent statistically significant differences ($p < 0.05$) compared to 0 ng/ml LPS.

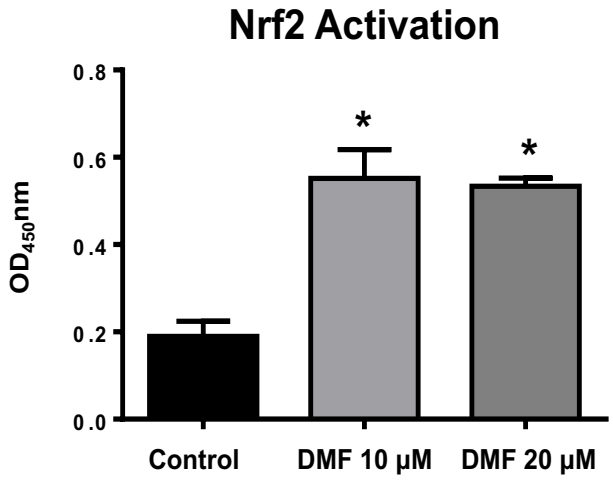


FIGURE 4

Figure 4. Quantification of the Nuclear Translocation of Nrf2. BV-2 cells were incubated with 10 and 20 μM DMF for 4 hr. The nuclear extracts were analyzed through a Nrf2 ELISA, and measured the enrichment and binding of Nrf2 in BV-2 cells. Data were subtracted from negative control and presented as mean absorbance value at 450 nm ± SEM (n=4) compared to 0 μM DMF. Asterisks (∗) represent statistically significant differences ($p < 0.05$) compared to 0 μM DMF.

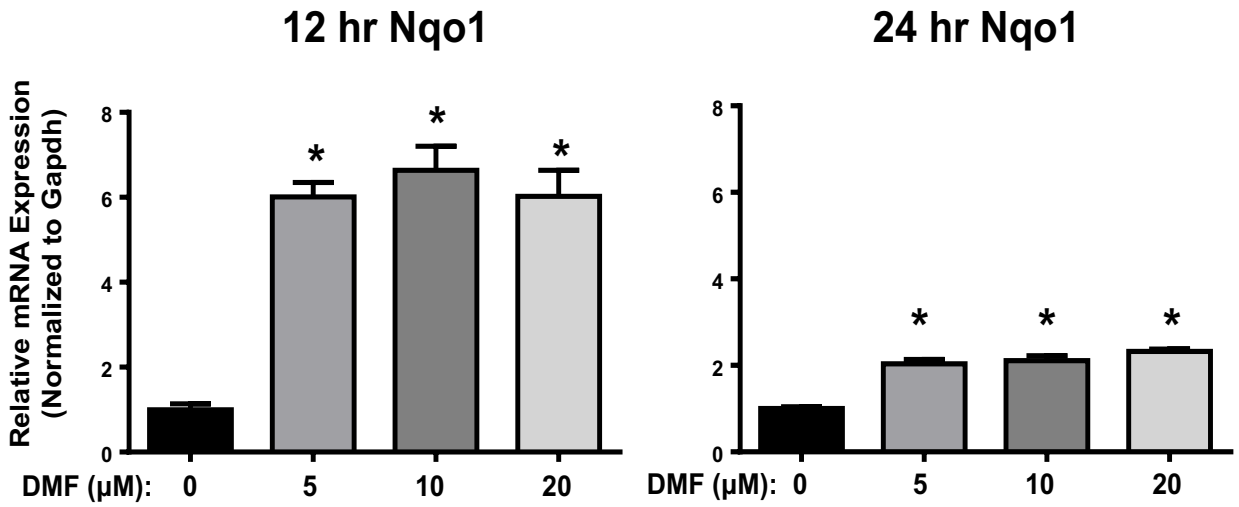


FIGURE 5

Figure 5. Assessment of Nqo1 mRNA Up-Regulation with DMF. Relative mRNA expression of Nrf2 target gene, Nqo1 was determined after 12 and 24 hr incubations with a range (0 - 20 μM) of DMF concentrations. Data were normalized to Gapdh mRNA and presented as mean relative mRNA expression ± SEM (n=3) compared to 0 μM DMF. Asterisks (∗) represent statistically significant differences ($p < 0.05$) compared to 0 μM DMF.

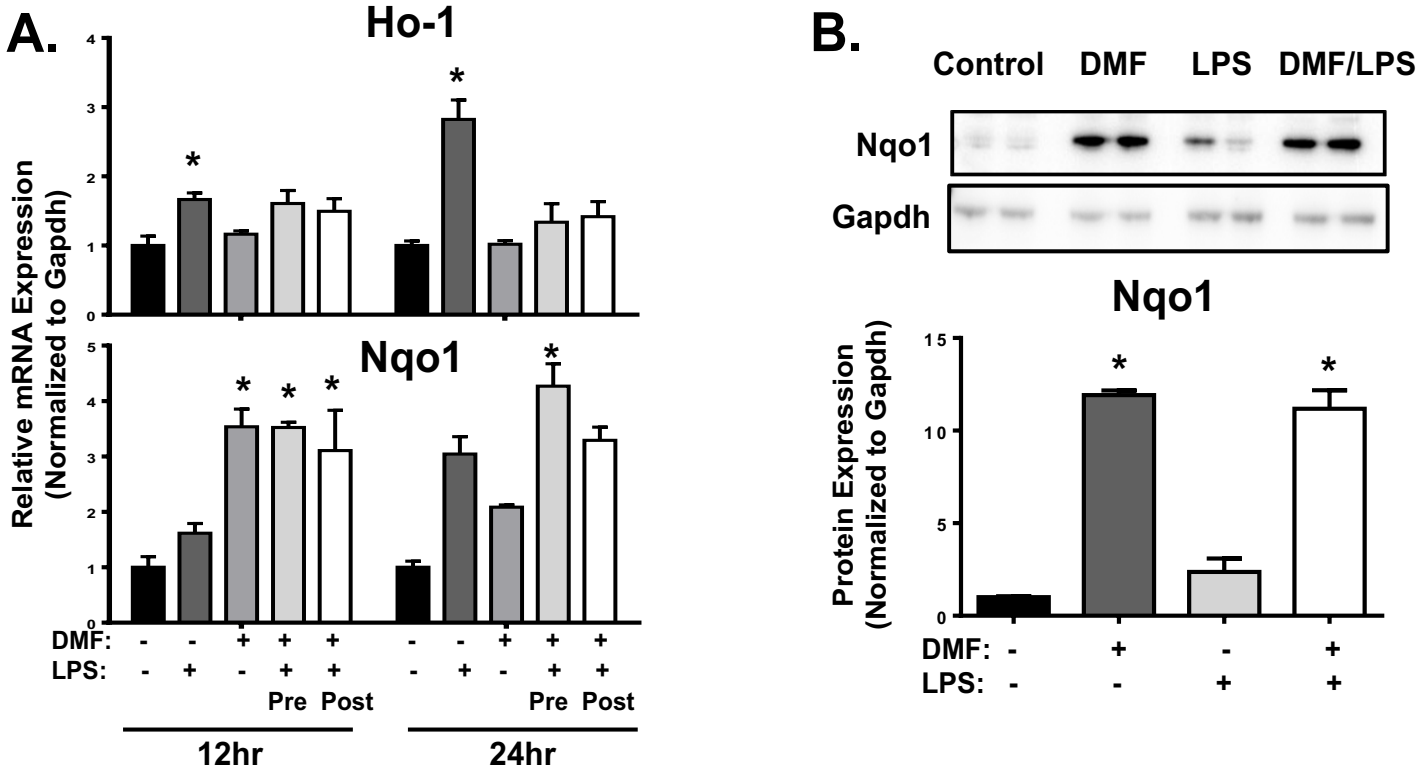


FIGURE 6

Figure 6. Nrf2 Pathway Activation with DMF Treatment. BV-2 cells were treated with DMF (20 μM) and LPS (100 ng/ml) alone as well as co-treated with LPS and DMF (30 mins pre- and post-DMF exposure). A) Relative mRNA expression of Ho-1 and Nqo1 were determined after 12 and 24 hr incubations with the appropriate treatment. B) Relative expression of Nqo1 protein was determined after 30 hr treatments with the appropriate chemicals. Data were normalized to Gapdh mRNA and presented as mean relative mRNA expression ± SEM (n=3) compared to control (no DMF or LPS). Semi-quantitative expression of protein was measured by densitometry from western blots. Representative western blots display changes in protein expression. Gapdh was used as the loading control. Data are presented as mean relative protein expression ± SEM (n=5). Asterisks (∗) represent statistically significant differences ($p < 0.05$) compared to control.

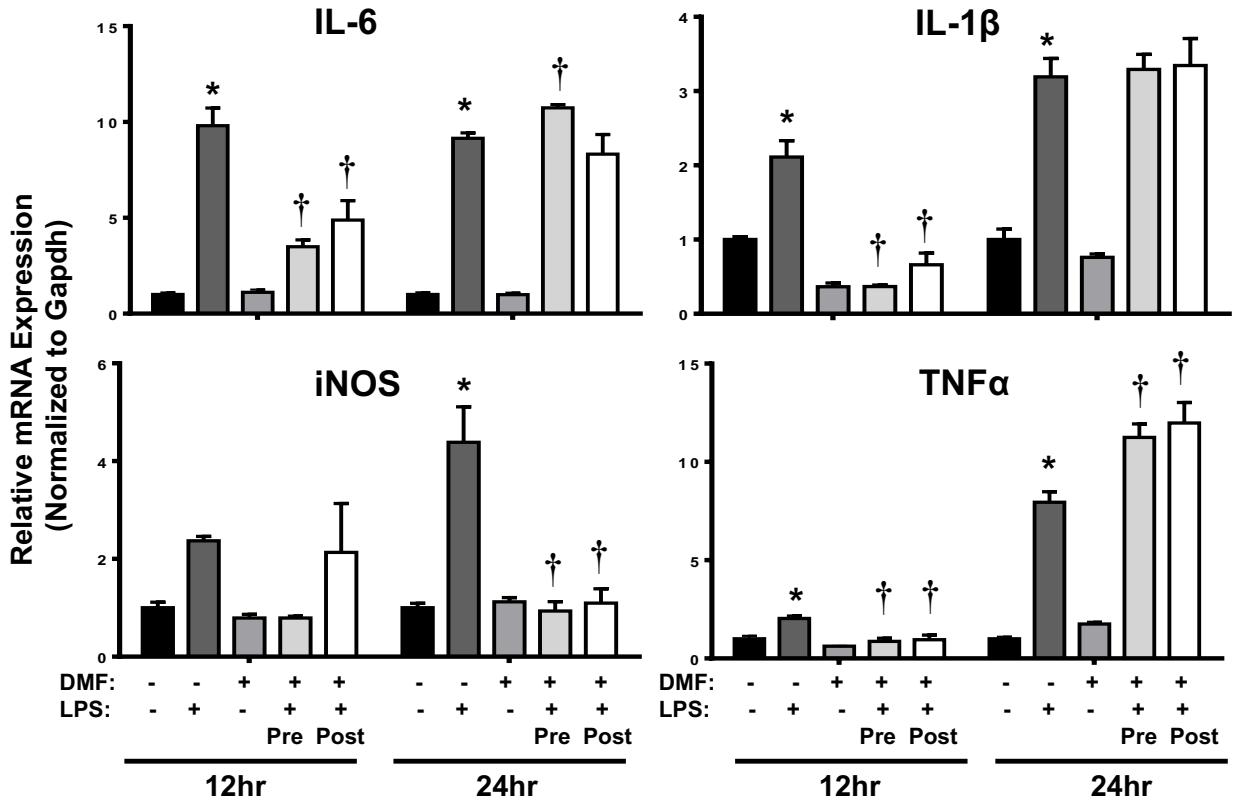


FIGURE 7

Figure 7. mRNA Expression of Pro-inflammatory Target Genes Following Nrf2 Activation. BV-2 cells were treated with DMF (20 μM) and LPS (100 ng/ml) alone as well as co-treated with LPS and DMF (30 mins pre- and post-DMF exposure). Relative mRNA expression of inflammatory cytokines and nitrative enzyme were determined after 12 and 24 hr incubations with the appropriate treatment. Data were normalized to Gapdh mRNA and presented as mean relative mRNA expression ± SEM (n=3) compared to control (no LPS or DMF). Asterisks (∗) represent statistically significant differences ($p < 0.05$) compared to control (no LPS or DMF). Single dagger (†) indicates statistically significant differences ($p < 0.05$) compared to LPS-treated cells (no DMF).

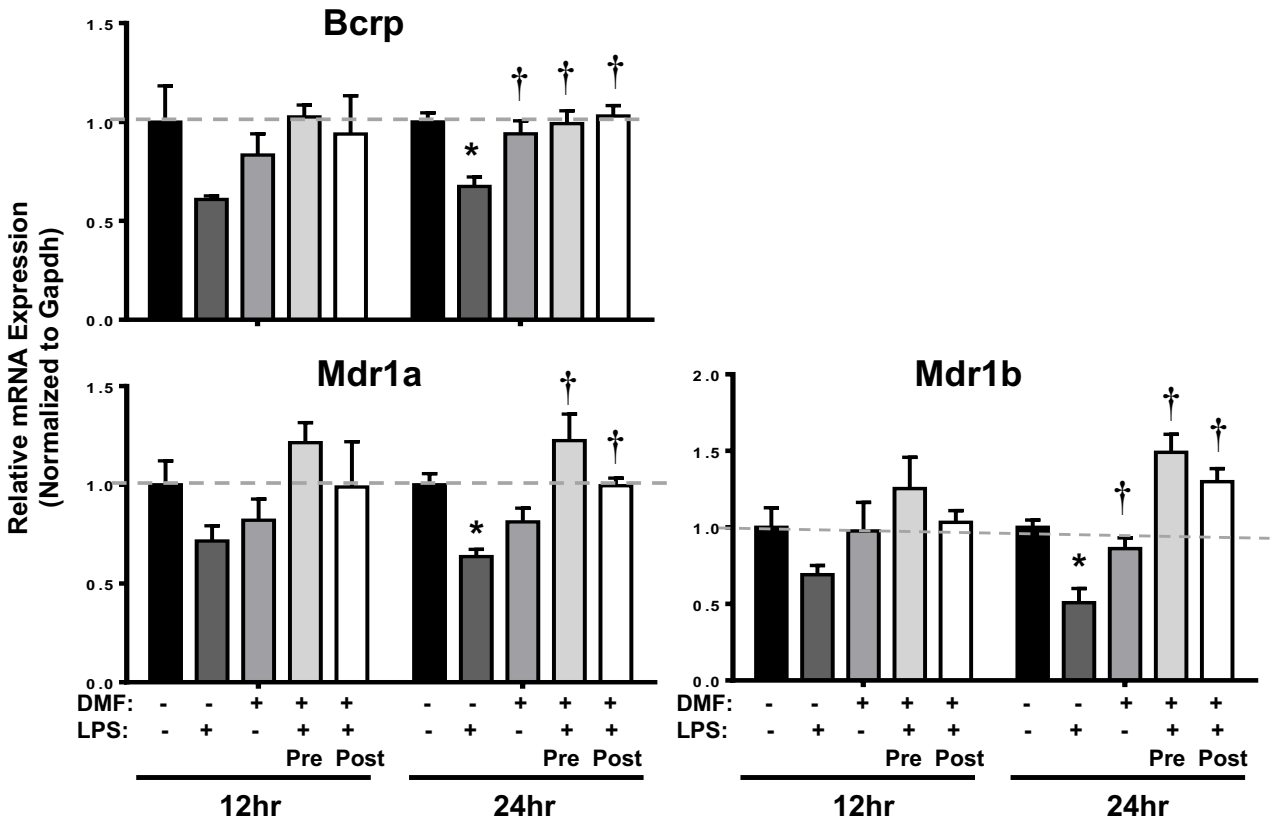


FIGURE 8

Figure 8. mRNA Expression of Efflux Transporters Following Nrf2 Activation. BV-2 cells were treated with DMF (20 μM) and LPS (100 ng/ml) alone as well as co-treated with LPS and DMF (30 mins pre- and post-DMF exposure). Relative mRNA expression of efflux transporters was determined after 12 and 24 hr incubations with the appropriate treatment. Data were normalized to Gapdh mRNA and presented as mean relative mRNA expression ± SEM (n=3) compared to control (no LPS or DMF). Dotted line at Y=1 represents control expression. Asterisks (∗) represent statistically significant differences ($p < 0.05$) compared to control (no LPS or DMF). Single dagger (†) indicates statistically significant differences ($p < 0.05$) compared to LPS-treated cells (no DMF).

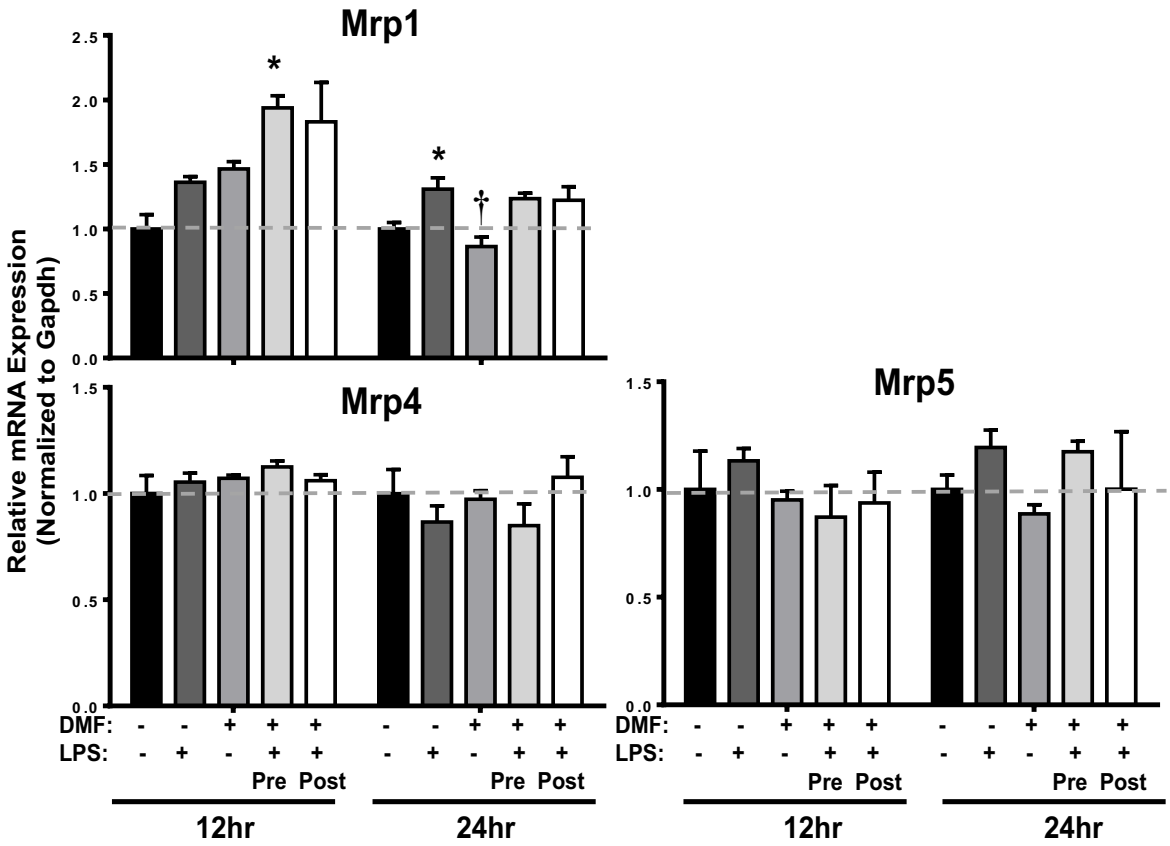


FIGURE 9

Figure 9. mRNA Expression of Efflux Transporters Following Nrf2 Activation. BV-2 cells were treated with DMF (20 μM) and LPS (100 ng/ml) alone as well as co-treated with LPS and DMF (30 mins pre- and post-DMF exposure). Relative mRNA expression of efflux transporters was determined after 12 and 24 hr incubations with the appropriate treatment. Data were normalized to Gapdh mRNA and presented as mean relative mRNA expression ± SEM (n=3) compared to control (no LPS or DMF). Dotted line at Y=1 represents control expression. Asterisks (∗) represent statistically significant differences ($p < 0.05$) compared to control (no LPS or DMF). Single dagger (†) indicates statistically significant differences ($p < 0.05$) compared to LPS-treated cells (no DMF).

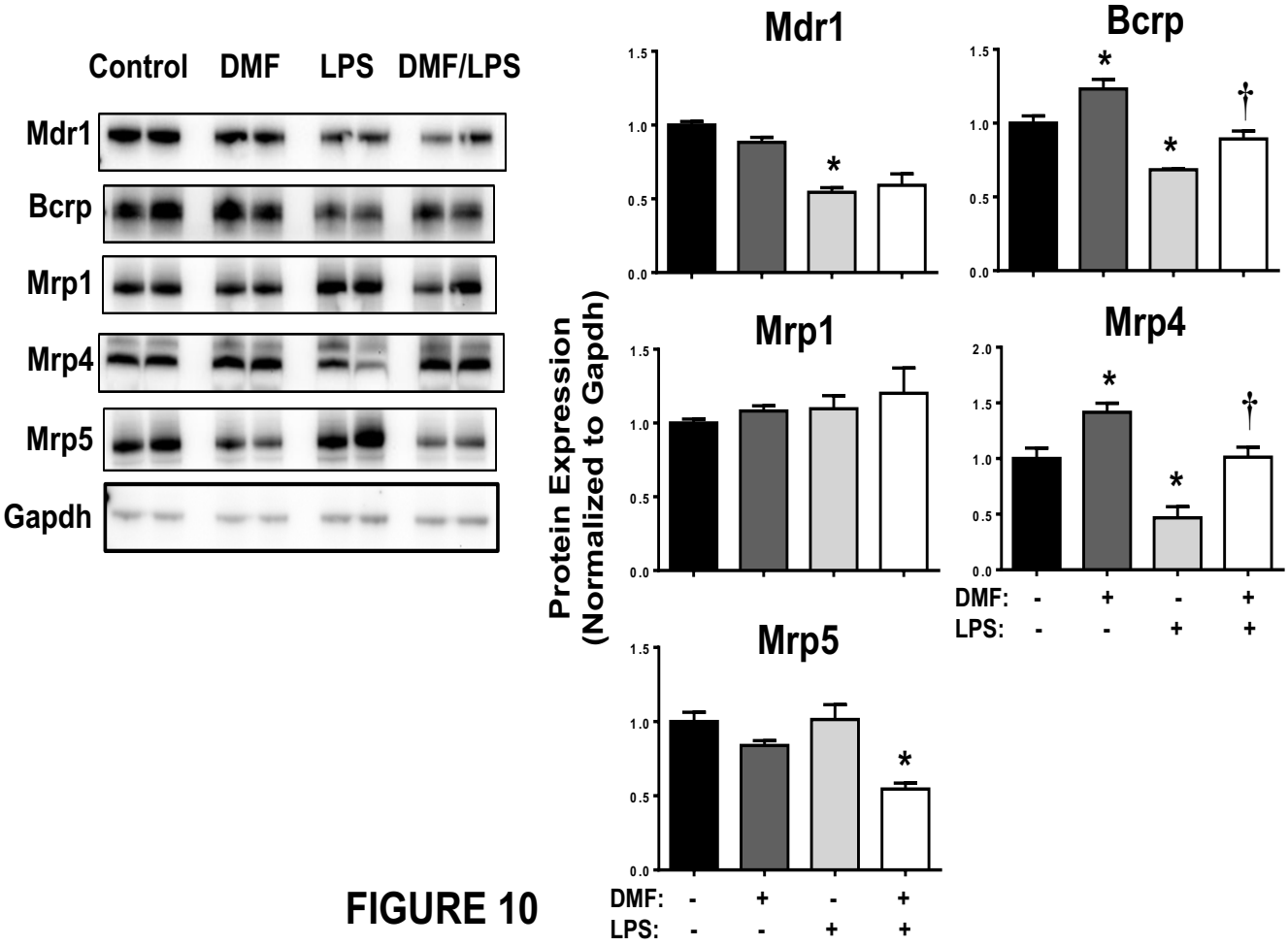
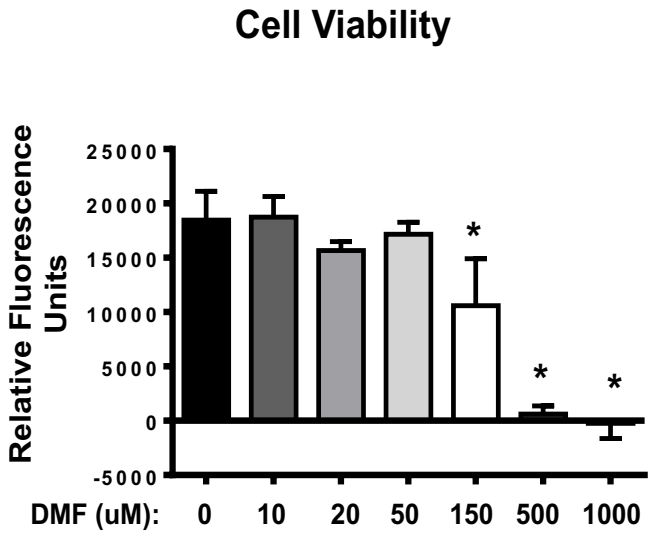


FIGURE 10

Figure 10. Protein Expression of Efflux Transporters Following Nrf2 Activation. BV-2 cells were treated with DMF (20 μM) and LPS (100 ng/ml) alone as well as co-treated with LPS and DMF (30 mins pre-DMF exposure). Relative expression of efflux transporter protein was determined after 30 hr treatments with the appropriate chemicals. Semi-quantitative expression of protein was measured by densitometry from western blots. Representative western blots display changes in protein expression. Gapdh was used as the loading control. Data are presented as mean relative protein expression ± SEM (n=5). Asterisks (∗) represent statistically significant differences ($p < 0.05$) compared to control (no LPS or DMF). Single dagger (†) indicates statistically significant differences ($p < 0.05$) compared to LPS-treated cells (no DMF).



SUPPLEMENTAL FIGURE 1

Supplemental Figure 1. Quantification of BV-2 Cell Viability. BV-2 cell viability was assessed through the Alamar Blue assay. Cells were treated with a range (0 - 1000 μM) of DMF concentrations for 24 hr. Cell viability was measured by relative fluorescent values. Increased fluorescence indicates the presence of viable cells. Data were subtracted from negative control and presented as mean fluorescent units ± SEM (n=8). Asterisks (∗) represent statistically significant differences ($p < 0.05$) compared to control (no DMF).

References

Abuznait, A. H., Cain, C., Ingram, D., Burk, D., and Kaddoumi, A. (2011). Up-regulation of P-glycoprotein reduces intracellular accumulation of beta amyloid: investigation of P-glycoprotein as a novel therapeutic target for Alzheimer's disease. *The Journal of pharmacy and pharmacology* **63**(8), 1111-8.

Ahuja, M., Ammal Kaidery, N., Yang, L., Calingasan, N., Smirnova, N., Gaisin, A., Gaisina, I. N., Gazaryan, I., Hushpulian, D. M., Kaddour-Djebbar, I.*, et al.* (2016). Distinct Nrf2 Signaling Mechanisms of Fumaric Acid Esters and Their Role in Neuroprotection against 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine-Induced Experimental Parkinson's-Like Disease. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **36**(23), 6332-51.

Alam, J., Stewart, D., Touchard, C., Boinapally, S., Choi, A. M., and Cook, J. L. (1999). Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. *The Journal of biological chemistry* **274**(37), 26071-8.

Aleksunes, L. M., Slitt, A. L., Maher, J. M., Augustine, L. M., Goedken, M. J., Chan, J. Y., Cherrington, N. J., Klaassen, C. D., and Manautou, J. E. (2008). Induction of Mrp3 and Mrp4 transporters during acetaminophen hepatotoxicity is dependent on Nrf2. *Toxicology and applied pharmacology* **226**(1), 74-83.

Alvarez-Erviti, L., Couch, Y., Richardson, J., Cooper, J. M., and Wood, M. J. (2011). Alpha-synuclein release by neurons activates the inflammatory response in a microglial cell line. *Neuroscience research* **69**(4), 337-42.

Bauer, B., Hartz, A. M., and Miller, D. S. (2007). Tumor necrosis factor alpha and endothelin-1 increase P-glycoprotein expression and transport activity at the blood-brain barrier. *Molecular pharmacology* **71**(3), 667-75.

Belinsky, M. G., Guo, P., Lee, K., Zhou, F., Kotova, E., Grinberg, A., Westphal, H., Shchaveleva, I., Klein-Szanto, A., Gallo, J. M.*, et al.* (2007). Multidrug resistance protein 4 protects bone marrow, thymus, spleen, and intestine from nucleotide analogue-induced damage. *Cancer research* **67**(1), 262-8.

Biber, K., Owens, T., and Boddeke, E. (2014). What is microglia neurotoxicity (Not)? *Glia* **62**(6), 841-54.

Blazquez, A. G., Briz, O., Romero, M. R., Rosales, R., Monte, M. J., Vaquero, J., Macias, R. I., Cassio, D., and Marin, J. J. (2012). Characterization of the role of ABCG2 as a bile acid transporter in liver and placenta. *Molecular pharmacology* **81**(2), 273-83.

Block, M. L., Zecca, L., and Hong, J. S. (2007). Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. *Nature reviews. Neuroscience* **8**(1), 57-69.

Bloom, F. E. (2013). Fundamentals of Neuroscience. In Fundamental Neuroscience (L. R. Squire, D. Berg, F. E. Bloom, S. d. Lac, A. Ghosh, and N. C. Spitzer, Eds.) 4 ed., pp. 3-13. Elsevier

Bomprezzi, R. (2015). Dimethyl fumarate in the treatment of relapsing-remitting multiple sclerosis: an overview. *Therapeutic advances in neurological disorders* **8**(1), 20-30.

Borst, P., and Schinkel, A. H. (1997). Genetic dissection of the function of mammalian P-glycoproteins. *Trends in genetics : TIG* **13**(6), 217-22.

Brandenburg, L. O., Kipp, M., Lucius, R., Pufe, T., and Wruck, C. J. (2010). Sulforaphane suppresses LPS-induced inflammation in primary rat microglia.

Inflammation research : official journal of the European Histamine Research Society ... [et al.] **59**(6), 443-50.

Brenn, A., Grube, M., Jedlitschky, G., Fischer, A., Strohmeier, B., Eiden, M., Keller, M., Groschup, M. H., and Vogelgesang, S. (2014). St. John's Wort reduces beta-amyloid accumulation in a double transgenic Alzheimer's disease mouse model-role of P-glycoprotein. *Brain pathology (Zurich, Switzerland)* **24**(1), 18-24.

Brennan, M. S., Matos, M. F., Richter, K. E., Li, B., and Scannevin, R. H. (2017). The NRF2 transcriptional target, OSGIN1, contributes to monomethyl fumarate-mediated cytoprotection in human astrocytes. *Scientific reports* **7**, 42054.

Brennan, M. S., Patel, H., Allaire, N., Thai, A., Cullen, P., Ryan, S., Lukashev, M., Bista, P., Huang, R., Rhodes, K. J.*, et al.* (2016). Pharmacodynamics of Dimethyl Fumarate Are Tissue Specific and Involve NRF2-Dependent and -Independent Mechanisms. *Antioxidants & redox signaling* **24**(18), 1058-71.

Chan, K., Han, X. D., and Kan, Y. W. (2001). An important function of Nrf2 in combating oxidative stress: detoxification of acetaminophen. *Proceedings of the National Academy of Sciences of the United States of America* **98**(8), 4611-6.

Chan, K., Lu, R., Chang, J. C., and Kan, Y. W. (1996). NRF2, a member of the NFE2 family of transcription factors, is not essential for murine erythropoiesis, growth, and development. *Proceedings of the National Academy of Sciences of the United States of America* **93**(24), 13943-8.

Chen, C. J., Clark, D., Ueda, K., Pastan, I., Gottesman, M. M., and Roninson, I. B. (1990). Genomic organization of the human multidrug resistance (MDR1) gene and origin of P-glycoproteins. *The Journal of biological chemistry* **265**(1), 506-14.

Chen, X. Q., Wu, S. H., Zhou, Y., and Tang, Y. R. (2013). Lipoxin A4-induced heme oxygenase-1 protects cardiomyocytes against hypoxia/reoxygenation injury via p38 MAPK activation and Nrf2/ARE complex. *PloS one* **8**(6), e67120.

Chin, J. E., Soffir, R., Noonan, K. E., Choi, K., and Roninson, I. B. (1989). Structure and expression of the human MDR (P-glycoprotein) gene family. *Molecular and cellular biology* **9**(9), 3808-20.

Choudhuri, S., and Klaassen, C. D. (2006). Structure, function, expression, genomic organization, and single nucleotide polymorphisms of human ABCB1 (MDR1), ABCC (MRP), and ABCG2 (BCRP) efflux transporters. *Int J Toxicol* **25**(4), 231-59.

Chufan, E. E., Kapoor, K., Sim, H. M., Singh, S., Talele, T. T., Durell, S. R., and Ambudkar, S. V. (2013). Multiple transport-active binding sites are available for a single substrate on human P-glycoprotein (ABCB1). *PloS one* **8**(12), e82463.

Cirrito, J. R., Deane, R., Fagan, A. M., Spinner, M. L., Parsadanian, M., Finn, M. B., Jiang, H., Prior, J. L., Sagare, A., Bales, K. R.*, et al.* (2005). P-glycoprotein deficiency at the blood-brain barrier increases amyloid-beta deposition in an Alzheimer disease mouse model. *The Journal of clinical investigation* **115**(11), 3285-90.

Cole, S. P., and Deeley, R. G. (2006). Transport of glutathione and glutathione conjugates by MRP1. *Trends in pharmacological sciences* **27**(8), 438-46.

Cordon-Cardo, C., O'Brien, J. P., Casals, D., Rittman-Grauer, L., Biedler, J. L., Melamed, M. R., and Bertino, J. R. (1989). Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. *Proceedings of the National Academy of Sciences of the United States of America* **86**(2), 695-8.

Dallas, S., Miller, D. S., and Bendayan, R. (2006). Multidrug resistance-associated proteins: expression and function in the central nervous system. *Pharmacological reviews* **58**(2), 140-61.

Dallas, S., Zhu, X., Baruchel, S., Schlichter, L., and Bendayan, R. (2003). Functional expression of the multidrug resistance protein 1 in microglia. *The Journal of pharmacology and experimental therapeutics* **307**(1), 282-90.

Dauchy, S., Dutheil, F., Weaver, R. J., Chassoux, F., Daumas-Duport, C., Couraud, P. O., Scherrmann, J. M., De Waziers, I., and Decleves, X. (2008). ABC transporters, cytochromes P450 and their main transcription factors: expression at the human blood-brain barrier. *Journal of neurochemistry* **107**(6), 1518-28.

Deo, A. K., Borson, S., Link, J. M., Domino, K., Eary, J. F., Ke, B., Richards, T. L., Mankoff, D. A., Minoshima, S., O'Sullivan, F.*, et al.* (2014). Activity of P-Glycoprotein, a beta-Amyloid Transporter at the Blood-Brain Barrier, Is Compromised in Patients with Mild Alzheimer Disease. *Journal of nuclear medicine : official publication, Society of Nuclear Medicine* **55**(7), 1106-11.

Dibbert, S., Clement, B., Skak-Nielsen, T., Mrowietz, U., and Rostami-Yazdi, M. (2013). Detection of fumarate-glutathione adducts in the portal vein blood of rats: evidence for rapid dimethylfumarate metabolism. *Archives of dermatological research* **305**(5), 447-51.

Dinkova-Kostova, A. T., and Talalay, P. (2010). NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1), a multifunctional antioxidant enzyme and exceptionally versatile cytoprotector. *Archives of biochemistry and biophysics* **501**(1), 116-23.

Doyle, L., and Ross, D. D. (2003). Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2). *Oncogene* **22**(47), 7340-58.

Doyle, L. A., Yang, W., Abruzzo, L. V., Krogmann, T., Gao, Y., Rishi, A. K., and Ross, D. D. (1998). A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proceedings of the National Academy of Sciences of the United States of America* **95**(26), 15665-70.

Elmshauser, S., Straehle, L. C., Kranz, J., Krebber, R., and Geyer, J. (2015). Brain penetration of emodepside is increased in P-glycoprotein-deficient mice and leads to neurotoxicosis. *Journal of veterinary pharmacology and therapeutics* **38**(1), 74-9.

Enokizono, J., Kusuhara, H., Ose, A., Schinkel, A. H., and Sugiyama, Y. (2008). Quantitative investigation of the role of breast cancer resistance protein (Bcrp/Abcg2) in limiting brain and testis penetration of xenobiotic compounds. *Drug metabolism and disposition: the biological fate of chemicals* **36**(6), 995-1002.

Enokizono, J., Kusuhara, H., and Sugiyama, Y. (2007). Effect of breast cancer resistance protein (Bcrp/Abcg2) on the disposition of phytoestrogens. *Molecular pharmacology* **72**(4), 967-75.

Enomoto, A., Itoh, K., Nagayoshi, E., Haruta, J., Kimura, T., O'Connor, T., Harada, T., and Yamamoto, M. (2001). High sensitivity of Nrf2 knockout mice to acetaminophen hepatotoxicity associated with decreased expression of ARE-regulated drug metabolizing enzymes and antioxidant genes. *Toxicological sciences : an official journal of the Society of Toxicology* **59**(1), 169-77.

Esser, L., Zhou, F., Pluchino, K. M., Shiloach, J., Ma, J., Tang, W. K., Gutierrez, C., Zhang, A., Shukla, S., Madigan, J. P.*, et al.* (2017). Structures of the Multidrug Transporter P-glycoprotein Reveal Asymmetric ATP Binding and the Mechanism of Polyspecificity. *The Journal of biological chemistry* **292**(2), 446-461.

Fan, Z., Okello, A. A., Brooks, D. J., and Edison, P. (2015). Longitudinal influence of microglial activation and amyloid on neuronal function in Alzheimer's disease. *Brain : a journal of neurology* **138**(Pt 12), 3685-98.

Fellner, L., Irschick, R., Schanda, K., Reindl, M., Klimaschewski, L., Poewe, W., Wenning, G. K., and Stefanova, N. (2013). Toll-like receptor 4 is required for alpha-synuclein dependent activation of microglia and astroglia. *Glia* **61**(3), 349-60.

Fojo, A. T., Ueda, K., Slamon, D. J., Poplack, D. G., Gottesman, M. M., and Pastan, I. (1987). Expression of a multidrug-resistance gene in human tumors and tissues. *Proceedings of the National Academy of Sciences of the United States of America* **84**(1), 265-9.

Foresti, R., Bains, S. K., Pitchumony, T. S., de Castro Bras, L. E., Drago, F., Dubois-Rande, J. L., Bucolo, C., and Motterlini, R. (2013). Small molecule activators of the Nrf2-HO-1 antioxidant axis modulate heme metabolism and inflammation in BV2 microglia cells. *Pharmacological research* **76**, 132-48.

Fromm, M. F., Kim, R. B., Stein, C. M., Wilkinson, G. R., and Roden, D. M. (1999). Inhibition of P-glycoprotein-mediated drug transport: A unifying mechanism to explain the interaction between digoxin and quinidine [seecomments]. *Circulation* **99**(4), 552-7.

Fuse, Y., and Kobayashi, M. (2017). Conservation of the Keap1-Nrf2 System: An Evolutionary Journey through Stressful Space and Time. *Molecules (Basel, Switzerland)* **22**(3).

Ghanem, C. I., Rudraiah, S., Bataille, A. M., Vigo, M. B., Goedken, M. J., and Manautou, J. E. (2015). Role of nuclear factor-erythroid 2-related factor 2 (Nrf2) in the transcriptional regulation of brain ABC transporters during acute acetaminophen (APAP) intoxication in mice. *Biochemical pharmacology* **94**(3), 203-11.

Gibson, C. J., Hossain, M. M., Richardson, J. R., and Aleksunes, L. M. (2012). Inflammatory regulation of ATP binding cassette efflux transporter expression and function in microglia. *The Journal of pharmacology and experimental therapeutics* **343**(3), 650-60.

Gillard, G. O., Collette, B., Anderson, J., Chao, J., Scannevin, R. H., Huss, D. J., and Fontenot, J. D. (2015). DMF, but not other fumarates, inhibits NF-kappaB activity in vitro in an Nrf2-independent manner. *Journal of neuroimmunology* **283**, 74-85.

Goralski, K. B., Hartmann, G., Piquette-Miller, M., and Renton, K. W. (2003). Downregulation of mdr1a expression in the brain and liver during CNS inflammation alters the in vivo disposition of digoxin. *British journal of pharmacology* **139**(1), 35-48.

Gum, S. I., and Cho, M. K. (2013). The amelioration of N-acetyl-p-benzoquinone imine toxicity by ginsenoside Rg3: the role of Nrf2-mediated detoxification and Mrp1/Mrp3 transports. *Oxidative medicine and cellular longevity* **2013**, 957947.

Haines, D. E., and Mihailoff, G. A. (2013). The Telencephalon. In Fundamental Neuroscience for Basic and Clinical Applications (4 ed., pp. 211-224. Saunders.

Haines, D. E., Raila, F. A., and Terrell, A. C. (2013). Orientation to the Structure and Imaging of the Central Nervous System. In Fundamental Neuroscience for Basic and Clinical Applications (4 ed., pp. 2-13. Saunders.

Hartmann, G., Vassileva, V., and Piquette-Miller, M. (2005). Impact of endotoxin-induced changes in P-glycoprotein expression on disposition of doxorubicin in mice. *Drug metabolism and disposition: the biological fate of chemicals* **33**(6), 820-8.

Hartz, A. M., Bauer, B., Fricker, G., and Miller, D. S. (2006). Rapid modulation of P-glycoprotein-mediated transport at the blood-brain barrier by tumor necrosis factor-alpha and lipopolysaccharide. *Molecular pharmacology* **69**(2), 462-70.

Hartz, A. M., Miller, D. S., and Bauer, B. (2010). Restoring blood-brain barrier P-glycoprotein reduces brain amyloid-beta in a mouse model of Alzheimer's disease. *Molecular pharmacology* **77**(5), 715-23.

Hayashi, A., Suzuki, H., Itoh, K., Yamamoto, M., and Sugiyama, Y. (2003). Transcription factor Nrf2 is required for the constitutive and inducible expression of multidrug resistance-associated protein 1 in mouse embryo fibroblasts. *Biochemical and biophysical research communications* **310**(3), 824-9.

Higgins, C. F., and Gottesman, M. M. (1992). Is the multidrug transporter a flippase? *Trends in biochemical sciences* **17**(1), 18-21.

Hirrlinger, J., Konig, J., and Dringen, R. (2002). Expression of mRNAs of multidrug resistance proteins (Mrps) in cultured rat astrocytes, oligodendrocytes, microglial cells and neurones. *Journal of neurochemistry* **82**(3), 716-9.

Hof, P. R., Kidd, G., DeFelipe, J., Vellis, J. d., Sosa, M. A. G., Elder, G. A., and Trapp, B. D. (2013). Cellular Components of Nervous Tissue. In Fundamental Neuroscience (L. R. Squire, D. Berg, F. E. Bloom, S. d. Lac, A. Ghosh, and N. C. Spitzer, Eds.) 4 ed., pp. 41-59. Elsevier

Huang, H. C., Nguyen, T., and Pickett, C. B. (2002). Phosphorylation of Nrf2 at Ser-40 by protein kinase C regulates antioxidant response element-mediated transcription. *The Journal of biological chemistry* **277**(45), 42769-74.

Ibbotson, K., Yell, J., and Ronaldson, P. T. (2017). Nrf2 signaling increases expression of ATP-binding cassette subfamily C mRNA transcripts at the blood-brain barrier following hypoxia-reoxygenation stress. *Fluids and barriers of the CNS* **14**(1), 6.

Ieiri, I., Takane, H., and Otsubo, K. (2004). The MDR1 (ABCB1) gene polymorphism and its clinical implications. *Clin Pharmacokinet* **43**(9), 553-76.

Iizuka, T., Ishii, Y., Itoh, K., Kiwamoto, T., Kimura, T., Matsuno, Y., Morishima, Y., Hegab, A. E., Homma, S., Nomura, A.*, et al.* (2005). Nrf2-deficient mice are highly susceptible to cigarette smoke-induced emphysema. *Genes to cells : devoted to molecular & cellular mechanisms* **10**(12), 1113-25.

Ishikawa, T., Hirano, H., Onishi, Y., Sakurai, A., and Tarui, S. (2004). Functional evaluation of ABCB1 (P-glycoprotein) polymorphisms: high-speed screening and structure-activity relationship analyses. *Drug metabolism and pharmacokinetics* **19**(1), 1-14.

Ito, T., Yano, I., Tanaka, K., and Inui, K. I. (1997). Transport of quinolone antibacterial drugs by human P-glycoprotein expressed in a kidney epithelial cell line, LLC-PK1. *J Pharmacol Exp Ther* **282**(2), 955-60.

Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., Oyake, T., Hayashi, N., Satoh, K., Hatayama, I.*, et al.* (1997). An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochemical and biophysical research communications* **236**(2), 313-22.

Itoh, K., Ishii, T., Wakabayashi, N., and Yamamoto, M. (1999). Regulatory mechanisms of cellular response to oxidative stress. *Free radical research* **31**(4), 319-24.

Jain, S., Rathod, V., Prajapati, R., Nandekar, P. P., and Sangamwar, A. T. (2014). Pregnane X Receptor and P-glycoprotein: a connexion for Alzheimer's disease management. *Molecular diversity* **18**(4), 895-909.

Jansen, R. S., Mahakena, S., de Haas, M., Borst, P., and van de Wetering, K. (2015). ATP-binding Cassette Subfamily C Member 5 (ABCC5) Functions as an Efflux Transporter of Glutamate Conjugates and Analogs. *The Journal of biological chemistry* **290**(51), 30429-40.

Ji, L., Li, H., Gao, P., Shang, G., Zhang, D. D., Zhang, N., and Jiang, T. (2013). Nrf2 pathway regulates multidrug-resistance-associated protein 1 in small cell lung cancer. *PloS one* **8**(5), e63404.

Johnson, R. A., Shepard, E. M., and Scotto, K. W. (2005). Differential regulation of MDR1 transcription by the p53 family members. Role of the DNA binding domain. *The Journal of biological chemistry* **280**(14), 13213-9.

Jonker, J. W., Buitelaar, M., Wagenaar, E., Van Der Valk, M. A., Scheffer, G. L., Scheper, R. J., Plosch, T., Kuipers, F., Elferink, R. P., Rosing, H.*, et al.* (2002). The breast cancer resistance protein protects against a major chlorophyll-derived dietary phototoxin and protoporphyria. *Proceedings of the National Academy of Sciences of the United States of America* **99**(24), 15649-54.

Kabba, J. A., Xu, Y., Christian, H., Ruan, W., Chenai, K., Xiang, Y., Zhang, L., Saavedra, J. M., and Pang, T. (2017). Microglia: Housekeeper of the Central Nervous System. *Cellular and molecular neurobiology* doi: 10.1007/s10571-017-0504-2.

Kang, M. I., Kobayashi, A., Wakabayashi, N., Kim, S. G., and Yamamoto, M. (2004). Scaffolding of Keap1 to the actin cytoskeleton controls the function of Nrf2 as key regulator of cytoprotective phase 2 genes. *Proceedings of the National Academy of Sciences of the United States of America* **101**(7), 2046-51.

Kastrati, I., Siklos, M. I., Calderon-Gierszal, E. L., El-Shennawy, L., Georgieva, G., Thayer, E. N., Thatcher, G. R., and Frasor, J. (2016). Dimethyl Fumarate Inhibits the Nuclear Factor kappaB Pathway in Breast Cancer Cells by Covalent Modification of p65 Protein. *The Journal of biological chemistry* **291**(7), 3639-47.

Keppler, D. (2011). Multidrug resistance proteins (MRPs, ABCCs): importance for pathophysiology and drug therapy. *Handbook of experimental pharmacology* doi: 10.1007/978-3-642-14541-4_8(201), 299-323.

Kim, S. S., Lim, J., Bang, Y., Gal, J., Lee, S. U., Cho, Y. C., Yoon, G., Kang, B. Y., Cheon, S. H., and Choi, H. J. (2012). Licochalcone E activates Nrf2/antioxidant response element signaling pathway in both neuronal and microglial cells: therapeutic relevance to neurodegenerative disease. *The Journal of nutritional biochemistry* **23**(10), 1314-23.

Kobayashi, A., Kang, M. I., Okawa, H., Ohtsuji, M., Zenke, Y., Chiba, T., Igarashi, K., and Yamamoto, M. (2004). Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. *Molecular and cellular biology* **24**(16), 7130-9.

Koh, K., Cha, Y., Kim, S., and Kim, J. (2009). tBHQ inhibits LPS-induced microglial activation via Nrf2-mediated suppression of p38 phosphorylation. *Biochemical and biophysical research communications* **380**(3), 449-53.

Kortekaas, R., Leenders, K. L., van Oostrom, J. C., Vaalburg, W., Bart, J., Willemsen, A. T., and Hendrikse, N. H. (2005). Blood-brain barrier dysfunction in parkinsonian midbrain in vivo. *Annals of neurology* **57**(2), 176-9.

Kraft, A. D., and Harry, G. J. (2011). Features of microglia and neuroinflammation relevant to environmental exposure and neurotoxicity. *International journal of environmental research and public health* **8**(7), 2980-3018.

Krishnamachary, N., and Center, M. S. (1993). The MRP gene associated with a non-P-glycoprotein multidrug resistance encodes a 190-kDa membrane bound glycoprotein. *Cancer research* **53**(16), 3658-61.

Lacher, S. E., Skagen, K., Veit, J., Dalton, R., and Woodahl, E. L. (2015). P-Glycoprotein Transport of Neurotoxic Pesticides. *The Journal of pharmacology and experimental therapeutics* **355**(1), 99-107.

Lankas, G. R., Cartwright, M. E., and Umbenhauer, D. (1997). P-glycoprotein deficiency in a subpopulation of CF-1 mice enhances avermectin-induced neurotoxicity. *Toxicology and applied pharmacology* **143**(2), 357-65.

Lee, G., Babakhanian, K., Ramaswamy, M., Prat, A., Wosik, K., and Bendayan, R. (2007). Expression of the ATP-binding cassette membrane transporter, ABCG2,

in human and rodent brain microvessel endothelial and glial cell culture systems. *Pharmaceutical research* **24**(7), 1262-74.

Leggas, M., Adachi, M., Scheffer, G. L., Sun, D., Wielinga, P., Du, G., Mercer, K. E., Zhuang, Y., Panetta, J. C., Johnston, B.*, et al.* (2004). Mrp4 confers resistance to topotecan and protects the brain from chemotherapy. *Molecular and cellular biology* **24**(17), 7612-21.

Li, W., Khor, T. O., Xu, C., Shen, G., Jeong, W. S., Yu, S., and Kong, A. N. (2008). Activation of Nrf2-antioxidant signaling attenuates NFkappaB-inflammatory response and elicits apoptosis. *Biochemical pharmacology* **76**(11), 1485-9.

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods (San Diego, Calif.)* **25**(4), 402-8.

Loboda, A., Damulewicz, M., Pyza, E., Jozkowicz, A., and Dulak, J. (2016). Role of Nrf2/HO-1 system in development, oxidative stress response and diseases: an evolutionarily conserved mechanism. *Cellular and molecular life sciences : CMLS* **73**(17), 3221-47.

Loe, D. W., Stewart, R. K., Massey, T. E., Deeley, R. G., and Cole, S. P. (1997). ATP-dependent transport of aflatoxin B1 and its glutathione conjugates by the product of the multidrug resistance protein (MRP) gene. *Molecular pharmacology* **51**(6), 1034-41.

Maher, J. M., Dieter, M. Z., Aleksunes, L. M., Slitt, A. L., Guo, G., Tanaka, Y., Scheffer, G. L., Chan, J. Y., Manautou, J. E., Chen, Y.*, et al.* (2007). Oxidative and electrophilic stress induces multidrug resistance-associated protein transporters via the nuclear factor-E2-related factor-2 transcriptional pathway. *Hepatology (Baltimore, Md.)* **46**(5), 1597-610.

Maliepaard, M., Scheffer, G. L., Faneyte, I. F., van Gastelen, M. A., Pijnenborg, A. C., Schinkel, A. H., van De Vijver, M. J., Scheper, R. J., and Schellens, J. H. (2001). Subcellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues. *Cancer research* **61**(8), 3458-64.

Martin, C., Berridge, G., Higgins, C. F., and Callaghan, R. (1997). The multi-drug resistance reversal agent SR33557 and modulation of vinca alkaloid binding to P-glycoprotein by an allosteric interaction. *British journal of pharmacology* **122**(4), 765-71.

Martin, C., Berridge, G., Higgins, C. F., Mistry, P., Charlton, P., and Callaghan, R. (2000a). Communication between multiple drug binding sites on P-glycoprotein. *Molecular pharmacology* **58**(3), 624-32.

Martin, C., Berridge, G., Mistry, P., Higgins, C., Charlton, P., and Callaghan, R. (2000b). Drug binding sites on P-glycoprotein are altered by ATP binding prior to nucleotide hydrolysis. *Biochemistry* **39**(39), 11901-6.

Martin, D., Rojo, A. I., Salinas, M., Diaz, R., Gallardo, G., Alam, J., De Galarreta, C. M., and Cuadrado, A. (2004). Regulation of heme oxygenase-1 expression through the phosphatidylinositol 3-kinase/Akt pathway and the Nrf2 transcription factor in response to the antioxidant phytochemical carnosol. *The Journal of biological chemistry* **279**(10), 8919-29.

McGuire, V. A., Ruiz-Zorrilla Diez, T., Emmerich, C. H., Strickson, S., Ritorto, M. S., Sutavani, R. V., Weibeta, A., Houslay, K. F., Knebel, A., Meakin, P. J.*, et al.* (2016). Dimethyl fumarate blocks pro-inflammatory cytokine production via inhibition of TLR induced M1 and K63 ubiquitin chain formation. *Scientific reports* **6**, 31159.

McMahon, M., Itoh, K., Yamamoto, M., and Hayes, J. D. (2003). Keap1-dependent proteasomal degradation of transcription factor Nrf2 contributes to the negative

regulation of antioxidant response element-driven gene expression. *The Journal of biological chemistry* **278**(24), 21592-600.

Mesev, E. V., Miller, D. S., and Cannon, R. E. (2017). Ceramide 1-Phosphate Increases P-Glycoprotein Transport Activity at the Blood-Brain Barrier via Prostaglandin E2 Signaling. *Molecular pharmacology* **91**(4), 373-382.

Meyer zu Schwabedissen, H. E., and Kroemer, H. K. (2011). In vitro and in vivo evidence for the importance of breast cancer resistance protein transporters (BCRP/MXR/ABCP/ABCG2). *Handbook of experimental pharmacology* doi: 10.1007/978-3-642-14541-4_9(201), 325-71.

Naftel, J. P., Ard, M. D., and Fratkin, J. D. (2013). The Cell Biology of Neurons and Glia In Fundamental Neuroscience for Basic and Clinical Applications (4 ed., pp. 14-31. Saunders.

Ni, Z., Bikadi, Z., Rosenberg, M. F., and Mao, Q. (2010). Structure and function of the human breast cancer resistance protein (BCRP/ABCG2). *Current drug metabolism* **11**(7), 603-17.

Nies, A. T., Jedlitschky, G., Konig, J., Herold-Mende, C., Steiner, H. H., Schmitt, H. P., and Keppler, D. (2004). Expression and immunolocalization of the multidrug resistance proteins, MRP1-MRP6 (ABCC1-ABCC6), in human brain. *Neuroscience* **129**(2), 349-60.

Okada, K., Shoda, J., Taguchi, K., Maher, J. M., Ishizaki, K., Inoue, Y., Ohtsuki, M., Goto, N., Takeda, K., Utsunomiya, H.*, et al.* (2008). Ursodeoxycholic acid stimulates Nrf2-mediated hepatocellular transport, detoxification, and antioxidative stress systems in mice. *American journal of physiology. Gastrointestinal and liver physiology* **295**(4), G735-47.

Okawa, H., Motohashi, H., Kobayashi, A., Aburatani, H., Kensler, T. W., and Yamamoto, M. (2006). Hepatocyte-specific deletion of the keap1 gene activates Nrf2 and confers potent resistance against acute drug toxicity. *Biochemical and biophysical research communications* **339**(1), 79-88.

Parodi, B., Rossi, S., Morando, S., Cordano, C., Bragoni, A., Motta, C., Usai, C., Wipke, B. T., Scannevin, R. H., Mancardi, G. L.*, et al.* (2015). Fumarates modulate microglia activation through a novel HCAR2 signaling pathway and rescue synaptic dysregulation in inflamed CNS. *Acta neuropathologica* **130**(2), 279-95.

Pauli-Magnus, C., von Richter, O., Burk, O., Ziegler, A., Mettang, T., Eichelbaum, M., and Fromm, M. F. (2000). Characterization of the major metabolites of verapamil as substrates and inhibitors of P-glycoprotein. *J Pharmacol Exp Ther* **293**(2), 376-82.

Peng, H., Li, H., Sheehy, A., Cullen, P., Allaire, N., and Scannevin, R. H. (2016). Dimethyl fumarate alters microglia phenotype and protects neurons against proinflammatory toxic microenvironments. *Journal of neuroimmunology* **299**, 35-44.

Poller, B., Drewe, J., Krahenbuhl, S., Huwyler, J., and Gutmann, H. (2010). Regulation of BCRP (ABCG2) and P-glycoprotein (ABCB1) by cytokines in a model of the human blood-brain barrier. *Cellular and molecular neurobiology* **30**(1), 63-70.

Powley, T. L. (2013). Central Control of Autonomic Functions: Organization of the Autonomic Nervous System. In Fundamental Neuroscience (L. R. Squire, D. Berg, F. E. Bloom, S. d. Lac, A. Ghosh, and N. C. Spitzer, Eds.) 4 ed., pp. 729-747. Elsevier

Purisai, M. G., McCormack, A. L., Cumine, S., Li, J., Isla, M. Z., and Di Monte, D. A. (2007). Microglial activation as a priming event leading to paraquat-induced dopaminergic cell degeneration. *Neurobiology of disease* **25**(2), 392-400.

Qin, H., Buckley, J. A., Li, X., Liu, Y., Fox, T. H., 3rd, Meares, G. P., Yu, H., Yan, Z., Harms, A. S., Li, Y*., et al.* (2016). Inhibition of the JAK/STAT Pathway Protects Against alpha-Synuclein-Induced Neuroinflammation and Dopaminergic Neurodegeneration. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **36**(18), 5144-59.

Qosa, H., Abuznait, A. H., Hill, R. A., and Kaddoumi, A. (2012). Enhanced brain amyloid-beta clearance by rifampicin and caffeine as a possible protective mechanism against Alzheimer's disease. *Journal of Alzheimer's disease : JAD* **31**(1), 151-65.

Rangasamy, T., Guo, J., Mitzner, W. A., Roman, J., Singh, A., Fryer, A. D., Yamamoto, M., Kensler, T. W., Tuder, R. M., Georas, S. N*., et al.* (2005). Disruption of Nrf2 enhances susceptibility to severe airway inflammation and asthma in mice. *J Exp Med* **202**(1), 47-59.

Rao, V. V., Dahlheimer, J. L., Bardgett, M. E., Snyder, A. Z., Finch, R. A., Sartorelli, A. C., and Piwnica-Worms, D. (1999). Choroid plexus epithelial expression of MDR1 P glycoprotein and multidrug resistance-associated protein contribute to the blood-cerebrospinal-fluid drug-permeability barrier. *Proceedings of the National Academy of Sciences of the United States of America* **96**(7), 3900-5.

Rappa, G., Lorico, A., Flavell, R. A., and Sartorelli, A. C. (1997). Evidence that the multidrug resistance protein (MRP) functions as a co-transporter of glutathione and natural product toxins. *Cancer research* **57**(23), 5232-7.

Reichard, J. F., Motz, G. T., and Puga, A. (2007). Heme oxygenase-1 induction by NRF2 requires inactivation of the transcriptional repressor BACH1. *Nucleic acids research* **35**(21), 7074-86.

Ritter, C. A., Jedlitschky, G., Meyer zu Schwabedissen, H., Grube, M., Kock, K., and Kroemer, H. K. (2005). Cellular export of drugs and signaling molecules by the ATP-binding cassette transporters MRP4 (ABCC4) and MRP5 (ABCC5). *Drug metabolism reviews* **37**(1), 253-78.

Roberts, D. J., and Goralski, K. B. (2008). A critical overview of the influence of inflammation and infection on P-glycoprotein expression and activity in the brain. *Expert opinion on drug metabolism & toxicology* **4**(10), 1245-64.

Rocchi, E., Khodjakov, A., Volk, E. L., Yang, C. H., Litman, T., Bates, S. E., and Schneider, E. (2000). The product of the ABC half-transporter gene ABCG2 (BCRP/MXR/ABCP) is expressed in the plasma membrane. *Biochemical and biophysical research communications* **271**(1), 42-6.

Rosenberg, M. F., Callaghan, R., Ford, R. C., and Higgins, C. F. (1997). Structure of the multidrug resistance P-glycoprotein to 2.5 nm resolution determined by electron microscopy and image analysis. *The Journal of biological chemistry* **272**(16), 10685-94.

Rosenberg, M. F., Callaghan, R., Modok, S., Higgins, C. F., and Ford, R. C. (2005). Three-dimensional structure of P-glycoprotein: the transmembrane regions adopt an asymmetric configuration in the nucleotide-bound state. *The Journal of biological chemistry* **280**(4), 2857-62.

Rudd, L. P., Kabler, S. L., Morrow, C. S., and Townsend, A. J. (2011). Enhanced glutathione depletion, protein adduct formation, and cytotoxicity following exposure to 4-hydroxy-2-nonenal (HNE) in cells expressing human multidrug resistance protein-1 (MRP1) together with human glutathione S-transferase-M1 (GSTM1). *Chemico-biological interactions* **194**(2-3), 113-9.

Rushworth, S. A., MacEwan, D. J., and O'Connell, M. A. (2008). Lipopolysaccharide-induced expression of NAD(P)H:quinone oxidoreductase 1 and heme oxygenase-1 protects against excessive inflammatory responses in human monocytes. *Journal of immunology (Baltimore, Md. : 1950)* **181**(10), 6730-7.

Saeki, T., Ueda, K., Tanigawara, Y., Hori, R., and Komano, T. (1993). Human P-glycoprotein transports cyclosporin A and FK506. *The Journal of biological chemistry* **268**(9), 6077-80.

Sauna, Z. E., and Ambudkar, S. V. (2001). Characterization of the catalytic cycle of ATP hydrolysis by human P-glycoprotein. The two ATP hydrolysis events in a single catalytic cycle are kinetically similar but affect different functional outcomes. *The Journal of biological chemistry* **276**(15), 11653-61.

Sauna, Z. E., and Ambudkar, S. V. (2000). Evidence for a requirement for ATP hydrolysis at two distinct steps during a single turnover of the catalytic cycle of human P-glycoprotein. *Proceedings of the National Academy of Sciences of the United States of America* **97**(6), 2515-20.

Scannevin, R. H., Chollate, S., Jung, M. Y., Shackett, M., Patel, H., Bista, P., Zeng, W., Ryan, S., Yamamoto, M., Lukashev, M.*, et al.* (2012). Fumarates promote cytoprotection of central nervous system cells against oxidative stress via the nuclear factor (erythroid-derived 2)-like 2 pathway. *The Journal of pharmacology and experimental therapeutics* **341**(1), 274-84.

Schinkel, A. H., Mayer, U., Wagenaar, E., Mol, C. A., van Deemter, L., Smit, J. J., van der Valk, M. A., Voordouw, A. C., Spits, H., van Tellingen, O.*, et al.* (1997). Normal viability and altered pharmacokinetics in mice lacking mdr1-type (drug-transporting) P-glycoproteins. *Proceedings of the National Academy of Sciences of the United States of America* **94**(8), 4028-33.

Schinkel, A. H., Wagenaar, E., van Deemter, L., Mol, C. A., and Borst, P. (1995). Absence of the mdr1a P-Glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin, and cyclosporin A. *The Journal of clinical investigation* **96**(4), 1698-705.

Schuetz, E. G., Yasuda, K., Arimori, K., and Schuetz, J. D. (1998). Human MDR1 and mouse mdr1a P-glycoprotein alter the cellular retention and disposition of erythromycin, but not of retinoic acid or benzo(a)pyrene. *Archives of biochemistry and biophysics* **350**(2), 340-7.

Shah, S. A., Khan, M., Jo, M. H., Jo, M. G., Amin, F. U., and Kim, M. O. (2017). Melatonin Stimulates the SIRT1/Nrf2 Signaling Pathway Counteracting Lipopolysaccharide (LPS)-Induced Oxidative Stress to Rescue Postnatal Rat Brain. *CNS neuroscience & therapeutics* **23**(1), 33-44.

Shan, Y., Lambrecht, R. W., Donohue, S. E., and Bonkovsky, H. L. (2006). Role of Bach1 and Nrf2 in up-regulation of the heme oxygenase-1 gene by cobalt protoporphyrin. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **20**(14), 2651-3.

Sharom, F. J. (2014). Complex Interplay between the P-Glycoprotein Multidrug Efflux Pump and the Membrane: Its Role in Modulating Protein Function. *Frontiers in oncology* **4**, 41.

Shilling, R. A., Venter, H., Velamakanni, S., Bapna, A., Woebking, B., Shahi, S., and van Veen, H. W. (2006). New light on multidrug binding by an ATP-binding-cassette transporter. *Trends in pharmacological sciences* **27**(4), 195-203.

Silverstein, P. S., Audus, K. L., Qureshi, N., and Kumar, A. (2010). Lipopolysaccharide increases the expression of multidrug resistance-associated protein 1 (MRP1) in RAW 264.7 macrophages. *Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology* **5**(4), 516-20.

Sparreboom, A., van Asperen, J., Mayer, U., Schinkel, A. H., Smit, J. W., Meijer, D. K., Borst, P., Nooijen, W. J., Beijnen, J. H., and van Tellingen, O. (1997). Limited oral bioavailability and active epithelial excretion of paclitaxel (Taxol) caused by

P-glycoprotein in the intestine. *Proceedings of the National Academy of Sciences of the United States of America* **94**(5), 2031-5.

Svirnovski, A. I., Shman, T. V., Serhiyenka, T. F., Savitski, V. P., Smolnikova, V. V., and Fedasenka, U. U. (2009). ABCB1 and ABCG2 proteins, their functional activity and gene expression in concert with drug sensitivity of leukemia cells. *Hematology (Amsterdam, Netherlands)* **14**(4), 204-12.

Swanson, L. W. (2013). Basic Plan of the Nervous System. In Fundamentals of Neuroscience (L. R. Squire, D. Berg, F. E. Bloom, S. d. Lac, A. Ghosh, and N. C. Spitzer, Eds.) 4 ed., pp. 15-38. Elsevier

Taguchi, K., Takaku, M., Egner, P. A., Morita, M., Kaneko, T., Mashimo, T., Kensler, T. W., and Yamamoto, M. (2016). Generation of a New Model Rat: Nrf2 Knockout Rats Are Sensitive to Aflatoxin B1 Toxicity. *Toxicological sciences : an official journal of the Society of Toxicology* **152**(1), 40-52.

Thevenod, F., Friedmann, J. M., Katsen, A. D., and Hauser, I. A. (2000). Up-regulation of multidrug resistance P-glycoprotein via nuclear factor-kappaB activation protects kidney proximal tubule cells from cadmium- and reactive oxygen species-induced apoptosis. *The Journal of biological chemistry* **275**(3), 1887-96.

Thiebaut, F., Tsuruo, T., Hamada, H., Gottesman, M. M., Pastan, I., and Willingham, M. C. (1987). Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proceedings of the National Academy of Sciences of the United States of America* **84**(21), 7735-8.

Townsend, B. E., and Johnson, R. W. (2016). Sulforaphane induces Nrf2 target genes and attenuates inflammatory gene expression in microglia from brain of young adult and aged mice. *Experimental gerontology* **73**, 42-8.

Trapp, B. D., and Herrup, K. (2011). Neurons and Neuroglia. In Youmans Neurological Surgery (H. R. Winn, Ed.) 6 ed., pp. 326-346. Saunders.

Udasin, R. G., Wen, X., Bircsak, K. M., Aleksunes, L. M., Shakarjian, M. P., Kong, A. N., Heck, D. E., Laskin, D. L., and Laskin, J. D. (2016). Nrf2 Regulates the Sensitivity of Mouse Keratinocytes to Nitrogen Mustard via Multidrug Resistance-Associated Protein 1 (Mrp1). *Toxicological sciences : an official journal of the Society of Toxicology* **149**(1), 202-12.

Ueda, K., Okamura, N., Hirai, M., Tanigawara, Y., Saeki, T., Kioka, N., Komano, T., and Hori, R. (1992). Human P-glycoprotein transports cortisol, aldosterone, and dexamethasone, but not progesterone. *The Journal of biological chemistry* **267**(34), 24248-52.

Umemura, T., Kuroiwa, Y., Kitamura, Y., Ishii, Y., Kanki, K., Kodama, Y., Itoh, K., Yamamoto, M., Nishikawa, A., and Hirose, M. (2006). A crucial role of Nrf2 in in vivo defense against oxidative damage by an environmental pollutant, pentachlorophenol. *Toxicological sciences : an official journal of the Society of Toxicology* **90**(1), 111-9.

Varadi, A., Szakacs, G., Bakos, E., and Sarkadi, B. (2002). P glycoprotein and the mechanism of multidrug resistance. *Novartis Foundation symposium* **243**, 54-65; discussion 65-8, 180-5.

Vilaboa, N. E., Galan, A., Troyano, A., de Blas, E., and Aller, P. (2000). Regulation of multidrug resistance 1 (MDR1)/P-glycoprotein gene expression and activity by heat-shock transcription factor 1 (HSF1). *The Journal of biological chemistry* **275**(32), 24970-6.

Villeneuve, N. F., Lau, A., and Zhang, D. D. (2010). Regulation of the Nrf2-Keap1 antioxidant response by the ubiquitin proteasome system: an insight into cullin-ring ubiquitin ligases. *Antioxidants & redox signaling* **13**(11), 1699-712.

von Wedel-Parlow, M., Wolte, P., and Galla, H. J. (2009). Regulation of major efflux transporters under inflammatory conditions at the blood-brain barrier in vitro. *Journal of neurochemistry* **111**(1), 111-8.

Wakabayashi, N., Itoh, K., Wakabayashi, J., Motohashi, H., Noda, S., Takahashi, S., Imakado, S., Kotsuji, T., Otsuka, F., Roop, D. R.*, et al.* (2003). Keap1-null mutation leads to postnatal lethality due to constitutive Nrf2 activation. *Nature genetics* **35**(3), 238-45.

Walker, J. E., Saraste, M., Runswick, M. J., and Gay, N. J. (1982). Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *The EMBO journal* **1**(8), 945-51.

Wang, R. B., Kuo, C. L., Lien, L. L., and Lien, E. J. (2003). Structure-activity relationship: analyses of p-glycoprotein substrates and inhibitors. *Journal of clinical pharmacy and therapeutics* **28**(3), 203-28.

Wang, X., Campos, C. R., Peart, J. C., Smith, L. K., Boni, J. L., Cannon, R. E., and Miller, D. S. (2014). Nrf2 upregulates ATP binding cassette transporter expression and activity at the blood-brain and blood-spinal cord barriers. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **34**(25), 8585-93.

Wang, Y. L., Chen, M., Huo, T. G., Zhang, Y. H., Fang, Y., Feng, C., Wang, S. Y., and Jiang, H. (2017). Effects of Glycyrrhetinic Acid on GSH Synthesis Induced by Realgar in the Mouse Hippocampus: Involvement of System [Formula: see text], System [Formula: see text], MRP-1, and Nrf2. *Molecular neurobiology* **54**(4), 3102-3116.

Wegiel, B., Nemeth, Z., Correa-Costa, M., Bulmer, A. C., and Otterbein, L. E. (2014). Heme oxygenase-1: a metabolic nike. *Antioxidants & redox signaling* **20**(11), 1709-22.

Wilms, H., Sievers, J., Rickert, U., Rostami-Yazdi, M., Mrowietz, U., and Lucius, R. (2010). Dimethylfumarate inhibits microglial and astrocytic inflammation by suppressing the synthesis of nitric oxide, IL-1beta, TNF-alpha and IL-6 in an in-vitro model of brain inflammation. *Journal of neuroinflammation* **7**, 30.

Wu, W., Li, Y., Wu, Y., Zhang, Y., Wang, Z., and Liu, X. (2015). Lutein suppresses inflammatory responses through Nrf2 activation and NF-kappaB inactivation in lipopolysaccharide-stimulated BV-2 microglia. *Molecular nutrition & food research* **59**(9), 1663-73.

Xiao, J., Wang, Q., Bircsak, K. M., Wen, X., and Aleksunes, L. M. (2015). In Vitro Screening of Environmental Chemicals Identifies Zearalenone as a Novel Substrate of the Placental BCRP/ABCG2 Transporter. *Toxicology research* **4**(3), 695-706.

Yates, M. S., Kwak, M. K., Egner, P. A., Groopman, J. D., Bodreddigari, S., Sutter, T. R., Baumgartner, K. J., Roebuck, B. D., Liby, K. T., Yore, M. M.*, et al.* (2006). Potent protection against aflatoxin-induced tumorigenesis through induction of Nrf2-regulated pathways by the triterpenoid 1-[2-cyano-3-,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole. *Cancer research* **66**(4), 2488-94.

Yu, C., Argyropoulos, G., Zhang, Y., Kastin, A. J., Hsuchou, H., and Pan, W. (2008). Neuroinflammation activates Mdr1b efflux transport through NFkappaB: promoter analysis in BBB endothelia. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* **22**(5-6), 745-56.

Zhang, D. D., and Hannink, M. (2003). Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by

chemopreventive agents and oxidative stress. *Molecular and cellular biology* **23**(22), 8137-51.

Zhang, D. D., Lo, S. C., Cross, J. V., Templeton, D. J., and Hannink, M. (2004). Keap1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex. *Molecular and cellular biology* **24**(24), 10941-53.

Zhang, Y., Wang, H., Unadkat, J. D., and Mao, Q. (2007). Breast cancer resistance protein 1 limits fetal distribution of nitrofurantoin in the pregnant mouse. *Drug metabolism and disposition: the biological fate of chemicals* **35**(12), 2154-8.

Zhou, L., Naraharisetti, S. B., Wang, H., Unadkat, J. D., Hebert, M. F., and Mao, Q. (2008). The breast cancer resistance protein (Bcrp1/Abcg2) limits fetal distribution of glyburide in the pregnant mouse: an Obstetric-Fetal Pharmacology Research Unit Network and University of Washington Specialized Center of Research Study. *Molecular pharmacology* **73**(3), 949-59.

Zhou, S. F. (2008). Structure, function and regulation of P-glycoprotein and its clinical relevance in drug disposition. *Xenobiotica; the fate of foreign compounds in biological systems* **38**(7-8), 802-32.

Zipper, L. M., and Mulcahy, R. T. (2002). The Keap1 BTB/POZ dimerization function is required to sequester Nrf2 in cytoplasm. *The Journal of biological chemistry* **277**(39), 36544-52.