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REGULATION OF EFFLUX TRANSPORTER EXPRESSION AND FUNCTION IN
NEURAL INJURY AND INFLAMMATION

By

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A dissertation submitted to

The Graduate School of New Brunswick

And

The Graduate School of Biomedical Sciences

Rutgers, The State University of New Jersey

In partial fulfillment of requirements

For the degree of

Doctor of Philosophy

Joint Graduate Program in Toxicology

Written under the direction of

Lauren M. Aleksunes and Jason R. Richardson

And approved by

New Brunswick, New Jersey

January, 2015

ABSTRACT OF THE DISSERTATION
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Parkinson disease (PD) is the second most common neurodegenerative disorder of aging worldwide. There is mounting evidence that both genetic and environmental factors contribute to the development of PD. The main hallmarks of PD pathology include loss of dopamine neurons in the substantia nigra pars compacta (SNc), activation of microglia, production of pro-inflammatory cytokines, and accumulation of oxidized lipids and proteins. Epidemiologic studies suggest that exposure to pesticides may contribute to the development of PD, particularly in individuals with a genetic polymorphism in the Multidrug Resistance Transporter 1 (MDR1) which removes chemicals from the brain. The herbicide paraquat is used to study features of PD in rodents, namely dopamine neuron loss and microglial activation. The work presented in this dissertation examines the role that the MDR1 efflux transporter plays in preventing neuronal death from paraquat-induced toxicity, as well as immunologic factors that modulate this protective function. Following exposure to a single dose of paraquat, mice lacking the *Mdr1a/1b* genes have extensive dopamine neuron loss and microglial activation in the SNc, whereas wild-type mice require at least two doses. It is hypothesized that MDR1 is an efflux transporter for

paraquat thereby preventing paraquat accumulation and toxicity in the brain. Experiments using an MDR1-transfected cell line or human cerebral endothelial cells naturally expressing MDR1 demonstrate that accumulation of paraquat is reduced in cells with functional MDR1. Work in this dissertation also investigates the regulation of Mdr1 and other transporters in microglia. Activation of microglia with lipopolysaccharide (LPS) leads to the down-regulation of Mdr1 and Bcrp and up-regulation of Mrp1 and Mrp5 transporters. Using fluorescent substrates, it is observed that LPS activation of microglia reduces the function of Mdr1 and Bcrp. Lastly, the mechanisms that regulate Mdr1 in activated microglia are explored. Using inhibitors and activators of several cellular pathways, including NF- κ B, Nrf2, and cytokines, it is found that Mdr1 is strongly influenced by TNF and NF- κ B pathways. This thesis demonstrates the ability of the Mdr1 transporter to protect against pesticide-induced neural injury and characterizes the role of inflammatory factors in regulating Mdr1 expression and function in microglia and the blood-brain barrier.

ACKNOWLEDGEMENTS

There are many people that have provided guidance and inspiration along the way. Firstly, I would like to thank my graduate advisors Dr. Lauren Aleksunes and Dr. Jason Richardson for their guidance and encouragement. In addition to providing me with the skills and opportunities that come with being in their labs, they have given me broader knowledge of science and the critical thinking skills needed to excel. I would also like to thank Dr. Kenneth Reuhl for encouraging me to pursue toxicology, introducing me to Drs. Richardson and Aleksunes, and for providing the ideal environment for studying for the pathology certifying exam early on. For this, I can't thank him enough. I also thank Dr. Keith Cooper and Dr. Long-Jun Wu for serving as my graduate thesis advisors and providing insight and suggestions for the dissertation. I am also grateful to Dr. James Rottman for encouraging me to pursue a Ph.D following my pathology residency training.

I have been very fortunate to be surrounded by a wonderful group of people at Rutgers including my lab partners, Lindsay Yacovino, Kristin Bircsak, and Jamie Moscovitz who have all provided me with constant entertainment. Xia Wen and Myrna Trumbauer for their patience and always taking the time to teach me what I need to know as well as our conversations. I am lucky to have gotten to know you all. I would like to thank everyone else at Rutgers who have helped me along the way including the crew at Gordon Rd., administrators, faculty, RATS, and the staff that make our lives easier.

I am mostly thankful to my loving, understanding wife, Erin, who never questioned the decision to continue my education and happily followed me around the country while putting up with the long hours, early mornings, and frequent absent weekends.

And to my boys Thomas and Andrew, I tried my best to not let the long, hard work these past several years interfere with my time with you. I thank you mostly for providing a constant, daily perspective of what is really important.

Everything I have I owe to my parents and brother for making me who I am. You are my inspiration.

Most of the work presented in Chapter 3 has been published previously:

Gibson CJ, Hossain MM, Richardson JR, Aleksunes LM. (2012). Inflammatory regulation of ATP binding cassette efflux transporter expression and function in microglia. *J Pharmacol Exp Ther*, 343(3): 650-660.

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CHAPTER 1.

INTRODUCTION.

Brain Organization and Composition.

Evolutionary Development of the CNS. Nerve cells (called neurons) are the basic functional units of all nervous systems. Primitive neural systems first appeared in aquatic coelenterates, which include corals, jellyfish, and sea anemones. In these animals, nerve cells transmit signals, as a simple reflex, from sensory cells to contractile epithelial cells. The basic transmission of an impulse across synapses to an effector cell does not require a control center. Because of the radial symmetry of these lower animals, the nervous system is organized into many local circuits that form nerve nets. As animals became more complex and developed bilateral symmetry, as in the flat worms, the nervous system also became more intricate. Further development of the nervous system was needed in these early 'predators' to coordinate the integration of muscles and organ systems. The coordination of multiple functions occurs in higher neural organs, such as the brain or head ganglion, which act predominantly as relay centers. Over time, arthropods with their segmented bodies and specialized features developed centralized nervous systems that allowed for greater control of coordinated movements and increased sensory processing. In these animals, the brain was no longer just a relay center but rather a critical regulator of adaptive and social behavior as well as specialized functions. Chordates, including amphioxus, developed an even more centralized nervous system analogous to the spinal cord observed in higher vertebrates. Advanced cephalization in mammals, birds, and fishes occurred out of necessity to coordinate the activity of spinal

neurons with their respective with muscle groups and to allow modulation by diverse sensory (sight, hearing, taste, smell, touch) inputs (Squire 2013).

Structural Organization of the CNS. Development of a centralized nervous system allows for greater control and a more efficient ability to modulate complex functions. It also enables separation of processes by grouping together nerves that perform similar functions. Broadly divided, the central nervous system (CNS) is composed of the spinal cord, brainstem, cerebellum and cerebrum. The brain stem in mammals is further subdivided into four parts: the myelencephalon, metencephalon, mesencephalon, and diencephalon. The myelencephalon, also known as the medulla oblongata, is continuous with the spinal cord. It is responsible for essential visceral reflexes that control respiration and cardiovascular function. This region also controls vestibular and postural reflexes. The metencephalon is composed of the pons and cerebellum. It receives nerve impulses from all levels of the CNS and is responsible for arousal, balance, cardiac reflexes, fine muscle movement, muscle tone, and sleep. The metencephalon can be thought of as an integration center that helps regulate motor coordination. The mesencephalon, or midbrain, contains visual-auditory relay nuclei and controls responses to sight, eye movements (tectum), and body movements including righting and postural reflexes (tegmentum). The diencephalon marks the division of the forebrain and the brain stem. The diencephalon contains major sensory relay nuclei to the cortex and subcortical nuclei. This region is involved in the autonomic regulation of visceral motor activity that occurs via the peripheral nervous system. It also integrates the endocrine system and the nervous system, and assists the limbic system with modulating emotions and memory.

The telencephalon is the anterior portion of the brain and covers most of the brainstem structures. It is composed of the cerebral hemispheres and the basal ganglia. Basal ganglia regulate voluntary motor control, stereotyped automatic muscle movements and muscle tonus (Squire 2013).

Cellular Components of the CNS. The brain is responsible for coordinating complex functions including movement, homeostasis, thermoregulation, emotion, learning memory, and behavior to name but a few. This functional diversity is achieved through comparatively few cell types. There are only four main cell groups that, together, serve the myriad functions of the brain. These cells are neurons, astrocytes, oligodendrocytes, and microglia. The majority of brain functions are accomplished by the neurons. There are many different forms of neurons including motor neurons, sensory neurons, and interneurons to name a few, and they are either inhibitory, excitatory, or modulatory in nature. Diversity of neural function is also accomplished through the production and release of multiple neurotransmitters and the establishment of interneuronal connections and synapses. Neurons are terminally differentiated and, for the most part, cannot be regenerated once they are lost. This means that proper nervous system function relies on protecting and maintaining neuronal populations. Consequently, the other cell types within the CNS function coordinately to support neuronal development, protect neurons from damage, and ensure proper neuronal function. Neurons can only function within a narrow set of local environmental parameters. They require a microenvironment that is tightly regulated with proper electrolyte and nutrient balance, and the efficient removal of waste products and toxicants. A microenvironment that is not ideal can lead to neuronal

damage and death. Neuroglia, which include astrocytes and oligodendrocytes, are essential for maintaining homeostasis and rapidly responding to fluctuations in the microenvironment. Microglia are the only resident immune cell in the central nervous system. These cells are constantly monitoring the microenvironment and respond rapidly to various stimuli (infection, injury, etc) within the CNS. Other cell types in the CNS contribute to the ideal microenvironment for neuronal function by regulating nutrients, wastes, and metabolites. These include choroid plexus epithelial cells, ependymal lining cells, capillary endothelial cells, and pericytes. The common and collective function of all cells within the CNS is to support neuronal health; the dysfunction of any one of them can contribute to neurologic disease.

Protecting the CNS

The best means of protecting neurons from damage by external insults is to completely isolate them with an impermeable barrier. However, complete isolation would also lead to the buildup of toxic metabolic byproducts and prevent the passage of required nutrients to neurons. Therefore, protecting neurons while allowing access to nutrients requires a specialized, tightly controlled, semi-permeable barrier. The CNS has several specialized barriers that offer protection and maintain homeostasis.

Barriers of the Brain. As mentioned above, neurons have limited regenerative capacity. One of the primary ways to ensure neuronal health is to prevent damage by external factors. To accomplish this, a system of barriers strictly control the neuronal extracellular environment. Neuron function is highly influenced by and responsive to the local

environmental composition of the surrounding extracellular fluid (Lajtha and Reith 2007). Perturbations in the composition of the microenvironment can result in excitability, inhibition, or damage. Maintaining adequate oxygen and ionic composition within the narrow limits of neuron survival also requires an intricate and rapidly adjustable system. The main structures in place to allow for such tight regulation of the extracellular compartment are the blood-brain barrier (BBB), the blood-cerebrospinal fluid (CSF) barrier and the brain-CSF barrier. The BBB allows for both the protection of neurons and provision of systemic nutrients to neurons. It acts as a physical and biochemical barrier that separates neurons from the external environment. The blood-CSF barrier is mainly composed of the choroid plexus epithelial cells, while the ependymal lining cells and arachnoid villi make up the brain-CSF barrier. These barriers also work together to ensure a suitable microenvironment for neurons. They prevent neuronal damage from chemicals that might gain entry through the CSF. These barriers are established early in development when blood vessels invade the brain.

The Blood-Brain Barrier. Arteries of the brain and spinal cord are surrounded by connective tissue of the pia and arachnoid mater. Smaller branches of the arteries penetrating the nervous tissue only have a thin neuroglial membrane that continues along capillaries. Brain capillaries are the site of nutrient and oxygen exchange as well as removal of waste products from the brain. The capillary endothelium, basement membrane and astrocytic processes are the only structures that separate neurons from plasma. Astrocytic feet cover approximately 85% of the area of the total basal capillary surface. Brain microvasculature is unique from all other capillary networks of the body.

The brain microvasculature needs to tightly control permeability from the blood to the extracellular space within the brain. It is able to accomplish this through the interaction of specialized endothelial cells (capillary endothelium) along with surrounding pericytes, astrocytes and neurons that form the functional unit of the BBB, known as the neurovascular unit (Willis 2011).

Capillary Endothelium. Physiologic properties of the brain capillary endothelium are unique from that of other organs and include the ability to form a completely continuous layer without fenestrations, low pinocytotic activity, and the presence of very high-resistance tight junctions between cells. Formation of the BBB is under strict control and is highly influenced by products released from astrocytes, including glial derived neurotrophic factor (GDNF) and src-suppressed C-kinase substrate (SSeCKS) (Lee, Kim et al. 2003, Lajtha and Reith 2007). Formation of the basal lamina is under the control of pericytes and is influenced by the interaction of platelet derived growth factor – B (PDGF-B) released by endothelial cells and PDGF-R β receptor on pericytes. The BBB phenotype is in fact not determined by the endothelial cells themselves, but rather by the neuronal environment in which they reside (Lajtha and Reith 2007). In addition to forming a continuous, non-fenestrated layer, brain capillary endothelium is able to maintain a selective barrier quality through both transporters and tight junctions.

Transporters. Brain endothelial cells, responsible for regulating the passage of essential nutrients to neurons, contain plasma membrane transport proteins and receptors that provide routes of entry for polar solutes. These include the glucose transporter type 1

(GLUT-1) for glucose transport, the Na⁺/K⁺ ATPase and Na⁺ and K⁺ channels for cation exchange, as well as receptors for insulin, transferrin and other macromolecules that trigger endocytosis of these larger molecules. Endothelial cells also contain a complement of efflux transport proteins, most commonly ATP binding cassette (ABC) transporters that enable the removal of metabolic waste products and toxicants, discussed in detail below (Fig. 1). Briefly, ABC transporters line the basolateral and luminal plasma membrane, protecting the brain from neurotoxicants and limiting the access of therapeutics. The ABC transporters present on brain capillary endothelial cells of greatest significance for the efflux of drugs are ABCB1 (Pgp/MDR1), ABCC1, 4, and 5 (MRP1, 4, 5), and ABCG2 (BCRP) (Dallas 2006). In fact, MDR1 is one of the first proteins expressed in human endothelial cells undergoing differentiation to the BBB phenotype and is required for its differentiation (Lajtha 2007, Lippmann, Azarin et al. 2012). MDR1 and BCRP are located on the luminal surface while the various MRP isoforms are present on both the luminal and/or basal surface. Brain endothelium also contains transport proteins of the solute carrier (SLC) class to actively transport solutes (including glucose and amino acids) into the endothelial cells from the plasma or from the brain parenchyma interstitium. Members of the organic anion (OAT) and organic cation (OCT) transporters are largely responsible for the influx of solutes (Dauchy, Dutheil et al. 2008).

Regulation of BBB Permeability

The Wnt Pathway. The molecular mechanisms that coordinate the formation of the BBB are only partially understood. It is clear that pericytes are critical for normal formation

and function of tight junctions as well as the recruitment of astrocytic foot processes. The Wnt signaling pathway has recently been shown to be integral to the maintenance and function of the BBB (L'Episcopo, Tirolo et al. 2011). As reviewed by L'Episcopo and coworkers, the Wnt canonical pathway is responsible for cell fate, proliferation, survival and organogenesis. Wnts are a family of 19 extracellular matrix (ECM) glycoproteins that modulate intracellular signaling through either the frizzled receptor or low-density lipoprotein related protein (LRP). Ligation of these receptors leads to the inactivation of a destruction complex that includes glycogen synthase kinase-3 β (GSK-3 β), axin and adenomatous polyposis coli (APC). The destruction complex ordinarily phosphorylates β -catenin marking it for polyubiquitination and proteasomal degradation. However, when the Wnt pathway is activated, β -catenin is free to translocate to the nucleus, bind to transcription factors, and alter cell transcription. Wnt/ β -catenin signaling is essential for proper vascular formation by influencing angiogenesis and modulating endothelial cell morphology and function. Embryos lacking β -catenin show alteration in vascular lumens, defects in endothelial remodeling, and hemorrhages. β -catenin deficiency also results in defective endocardial cushion formation and abnormal cardiac valve development due to altered endothelial-mesenchymal transformation (Paolinelli, Corada et al. 2011). Wnt activity is particularly prominent in vessels penetrating the brain in embryonic and postnatal mice (Paolinelli, Corada et al. 2011). Equally important to CNS angiogenesis, Wnts are also involved in the formation of the BBB phenotype in brain endothelial cells by organizing tight junctions and regulating glucose transport (L'Episcopo, Tirolo et al. 2011).

Tight Junctions. Brain capillary endothelial cells have two specialized molecular structures that limit paracellular permeability: adherens junctions and tight junctions. Adherens junctions initiate cell-to-cell contact and promote maturation and the subsequent formation of tight junctions. Adherens junctions are composed of molecules called cadherins which form intercellular bridges essential for the proper formation of tight junctions. Tight junctions act between cells to limit the paracellular passage of ions and solutes. Tight junctions are particularly abundant in brain endothelial cells and form extremely tight intercellular junctions that limit the permeability of polar solutes into the brain (Lajtha 1957, Lajtha and Reith 2007).

Tight junction adhesion is mediated by specialized transmembrane proteins that include occludin, nectin, claudins (Cldn) and junctional adhesion molecules. Junctional adhesion molecules, such as zonula occludens, are linked to the cytoskeleton and can act as signaling proteins (see below). There are 20 known Cldn but only a few are expressed in the brain (Goncalves, Ambrosio et al. 2013). Deficiency of Cldn 3 and 5 loosens the tight junctions between endothelial cells and cause increased BBB permeability in a model of the human BBB (Winger, Koblinski et al. 2014). In addition to providing normal vascular development, Wnt activation also increases Cldn 3 expression, reinforcing the BBB by providing more impermeable tight junctions (Abbott, Revest et al. 1992, L'Episcopo, Tirolo et al. 2011).

Selective BBB permeability is essential to the preservation of neuronal microenvironment homeostasis. Tight junctions and membrane transporters maintain constant, selective

permeability during steady state. However, rapid fluctuations in neuronal activity demand that the selective permeability of the BBB also be able to adapt rapidly. The BBB is capable of rapid modulation of tight junctions through signaling molecules, including Ca^{2+} , cAMP, protein kinase C, G-proteins, calmodulin, phospholipase C, and tyrosine kinases. Signaling pathways transduced by the Ras-related small GTPase Rho family such as Rho and Rac-1 also regulate tight junctions through actions on actin polymerization. Pathologic signals, including many inflammatory cytokines, can also alter expression and function of tight junction proteins and overall BBB permeability (Abbott 2002).

Drug Transporters. Drug transporters are abundantly expressed in tissues with barrier functions including the intestines, kidneys, liver, placenta, and brain. In these areas, drug transporters are arranged along both the apical and basolateral surfaces of cells to facilitate the unidirectional and bidirectional transport of chemicals (Klaassen and Aleksunes 2010). Transcellular transport is carried out by both uptake and efflux transporters. Movement of substrates can occur through passive facilitated diffusion, or by active transport against substrate concentration gradients. Most drug transporters are classified as members of the ABC (ATP-binding cassette) or SLC (solute carrier) families of transporters. ABC transporters support the efflux of substrates and require ATP hydrolysis to provide the energy required to move substrates across the membrane. These transporters have a nucleotide binding domain that catalyzes the hydrolysis of ATP. The most common ABC transporters are the multidrug resistance protein 1 (MDR1/*ABCB1*/P-glycoprotein), multidrug resistance-associated proteins (MRP/*ABCC*),

and the breast cancer resistance protein (BCRP/*ABCG2*). SLC transporters do not have ATP-binding sites and therefore either facilitate the movement of substrates down their electrochemical gradient or utilize ion gradients and act as secondary active transporters to couple the movement of substrates against the electrochemical gradient. The SLC transporters are a large family of proteins that include the organic anion transporters (OATs/*SLC22A6-11*), organic anion transporting polypeptides (OATPs/*SLCO1-6*), organic cation transporters (OCTs/*SLC22A1-3*), organic cation/carnitine transporters (OCTNs/*SLC22A4, 5, and 21*), peptide transporters, monocarboxylate transporters, nucleoside transporters, and some bile acid transporters (reviewed in (Klaassen and Aleksunes 2010)).

ABC Efflux Transporter Structure and Function. ABC efflux transporters are membrane localized proteins that are responsible for the efflux of many endogenous substrates. As a result of their broad substrate specificity, ABC efflux transporters play a significant role in the absorption, distribution, and excretion of xenobiotics and can regulate target organ toxicity.

ABC efflux transporters share many similarities in their structure with most containing α -helical transmembrane spanning domains. Some of these transmembrane domains contain kinks and bends which largely determine substrate specificity (Lajtha and Reith 2007). Some ABC transporters, including MDR1, have multiple binding regions within their transmembrane domains enabling wide substrate specificity.

Transport of substrates generally begins with substrate entering and binding to the transporter. The act of substrate binding leads to a conformational change that results in the ability of ATP to bind to the nucleotide binding domains. Bound ATP produces the most profound conformational change, resulting in transfer of the substrate to the extracellular face of the plasma membrane concurrent with reduced affinity and release of the substrate. Subsequent hydrolysis of ATP returns the protein to its original conformation ready to begin the process again. Individual transporter isoforms are discussed in more detail below.

ABCB1/MDR1/Pgp. MDR1 was one of the first members of the ABC transporter family to be discovered and remains the best studied. Over-expression of MDR1 has been linked to a multidrug resistance phenotype observed in mammalian cells and human cancers. Genes encoding MDRs are comprise two isoforms in humans and three isoforms in rodents (Juliano and Ling 1976). Human MDR1/*ABCB1* and mouse *Mdr1a/Abcb1a* and *Mdr1b/Abcb1b* are drug efflux transporters. Human MDR3/*ABCB4* and mouse *Mdr2/Abcb4* are responsible for exporting phosphatidylcholine into the bile and are exclusively found on bile canaliculi (Cui, Cheng et al. 2009). MDR1 is expressed at low levels in most tissues in humans and rodents but is highly expressed on the apical surface of epithelial cells lining the intestine, pancreatic ductules, bile ductules, kidney proximal tubules and in the adrenal gland (Jette, Beaulieu et al. 1996, Ernest, Rajaraman et al. 1997, Rost, Mahner et al. 2002, Scheffer, Kool et al. 2002). MDR1 was the first ABC transporter to be discovered in the BBB and was subsequently found to be located on the luminal surface of endothelial cells of the blood-brain barrier, blood-testes barrier, blood-

mammary tissue barrier, and blood-inner ear barrier (Schinkel, Smit et al. 1994, Rao, Dahlheimer et al. 1999, Zhang, Saito et al. 2000, Bart, Hollema et al. 2004, Roberts, Black et al. 2008). MDR1 is highly expressed on the luminal surface of the pregnant endometrium and the placenta (Lankas, Wise et al. 1998, St-Pierre, Serrano et al. 2000). MDR1 is also present on the surface of hematopoietic cells. In this setting, its role is not entirely known, however, several cytokines including IL-2, IL-4, IFN γ and TNF are substrates for MDR1 (Albermann, Schmitz-Winnenthal et al. 2005, Pawlik, Baskiewicz-Masiuk et al. 2005).

The tissue and cellular localization of MDR1 suggests that its main role is the protection of susceptible tissues from toxicants, secretion of toxic metabolites from tissues and release of hormones from the adrenal gland, uterus and possibly other endocrine organs. Additional evidence of its protective role has been characterized in multiple species and transgenic models. Mice lacking functional *Mdr1a* are phenotypically indistinguishable from their wild-type counterparts under normal circumstances (Schinkel, Mayer et al. 1997). However, knockout mice are extremely sensitive to the neurotoxic effects of ivermectin and other xenobiotics (Goralski, Hartmann et al. 2003, Geyer, Gavrilova et al. 2009, Elmshausen, Straehle et al. 2014). Inhibition of *Mdr1* in the BBB of rainbow trout alters ivermectin toxicity and swimming behavior (Kennedy, Tierney et al. 2014). Additionally, *Mdr1* has long been known to be involved in the toxicity of avermectins (lethargy, ataxia, coma, tremors, seizures, blindness) in dogs and cats (Merola and Eubig 2012). It was discovered that a 4-bp deletion results in a frame shift that creates stop codons that prematurely terminate *Mdr1* synthesis. Collie dogs that are homozygous for

the deletion display the ivermectin-sensitive phenotype, while homozygous-normal or heterozygous do not display increased sensitivity to ivermectin (Mealey, Bentjen et al. 2001).

The *MDR1* gene and subsequent MDR1 protein (P-glycoprotein, Pgp) expression are up-regulated in response to various environmental insults (Dauchy, Dutheil et al. 2008, Rigalli, Ruiz et al. 2011, Ménez, Mselli-Lakhal et al. 2012). Enhanced expression of MDR1 could potentially be mediated either through stabilization of mRNA or through increased MDR1 gene transcription. Transcription is determined by response elements in the gene promoter region and the accessibility and activity of corresponding transcription factors. Regulation is complex, with multiple pathways and response elements involved in activation of MDR1 transcription. The most common pathway for activation involves the promoter region in the middle of exon 1. Here, an inverted CCAAT box interacts with the transcription factors NF-Y, Sp1, and Sp3 to initiate transcription (Choudhuri and Klaassen 2006). MDR1 expression can also be increased as part of general cellular stress response. Signals such hypoxia, inflammation, and ionizing radiation can lead to downstream MDR1 transcription. Studying MDR1 regulation is difficult as it acts as the downstream effector of many pathways including protein kinase C, MAP kinase, and NF- κ B (Choudhuri and Klaassen 2006, Dauchy, Dutheil et al. 2008). A recent crystal structure of mouse Mdr1 revealed a large internal cavity open to the cytosol and internal leaflet of the plasma membrane (Aller, Yu et al. 2009). The large cavity has multiple hydrophobic and polar residues that account for its wide specificity for hydrophobic and amphipathic substrates. The location of the binding pocket supports the hypothesis that

Mdr1 functions mainly as a flippase, exporting substrates that have partitioned into the plasma membrane (Sharom 2008).

Multidrug Resistance-Associated Proteins (MRPs). The multidrug resistance-associated proteins (MRPs) belong to the *ABCC* subfamily of ABC transporters, and are one of the subfamilies, along with MDR1 and BCRP, that is responsible for the efflux of chemicals from cells (reviewed in (Klaassen and Aleksunes 2010)). The human *ABCC* subfamily consists of 12 members, 9 of which are MRPs. These transporters contain two cytoplasmic nucleotide binding domains and either two or three transmembrane spanning domains. Like MDR1, the MRPs facilitate efflux of many endogenous and xenobiotic lipophilic substrates (Dallas 2006). Most substrates for MRPs are compounds conjugated with glutathione, glucuronate, or sulfate. MRPs are often located on intestinal and renal epithelium, hepatocytes, and the brain where they function as unidirectional efflux pumps for conjugated and unconjugated organic anions (Scheffer, Kool et al. 2002, Nies, Jedlitschky et al. 2004, Potschka 2012).

Breast Cancer Resistance Protein (BCRP). The BCRP transporter belongs to the *ABCG* subfamily of the ABC transporters. It was initially discovered by several groups independently while studying drug resistance in cancer cell lines and drug transport in the placenta (Allikmets, Schriml et al. 1998, Rabindran, He et al. 1998, Hazlehurst, Foley et al. 1999). BCRP is a half-transporter, with a single nucleotide binding domain and a single membrane spanning domain, and requires homodimerization in order to function (reviewed in (Klaassen and Aleksunes 2010)). A high level of resistance to the anti-

cancer drug mitoxantrone is a hallmark of cells overexpressing BCRP (Doyle, Yang et al. 1998, Allen, Brinkhuis et al. 1999). Additionally, many chemotherapeutic agents are known substrates for BCRP (Takenaka, Morgan et al. 2007, Potschka 2012). BCRP mRNA is highly expressed in human placenta with lower levels in the brain, prostate, small intestine, testes, ovary, colon, and liver (Gutmann, Hruz et al. 2005, Fetsch, Abati et al. 2006). Little is known about the mechanisms controlling BCRP expression, however, an estrogen response element has been identified in the upstream region of the *ABCG2* gene (Hartz, Madole et al. 2010). Controversy exists over the exact role of steroids in BCRP expression, but there is some evidence that sex hormones contribute to its regulation, particularly in reproductive tissues (Zhang, Zhou et al. 2006, Zhang, Wang et al. 2010). The BCRP gene also contains a hypoxia response element in the promoter region; in turn, periods of low oxygen concentration typically induce the transcription of BCRP by the hypoxia-inducible-factor-1 α (Krishnamurthy and Schuetz 2005).

ABC Transporters in the Brain. Introduced above, ABC transporters are an essential component of the BBB and function to regulate solute entry into the brain. Many ABC transporters are expressed on both the endothelial cells of the BBB and the epithelial cells of the choroid plexus comprising the blood-CSF barrier. There are no comprehensive studies of the expression or function of ABC transporters in ependymal cells of the brain-CSF barrier. The main ABC transporters present in the human brain are MDR1, MRPs 1, 4, and 5, and BCRP (reviewed by (Dallas 2006). The anatomic and cellular localization of the individual transporters determine their major function in the brain (Dauchy, Dutheil et al. 2008, Shawahna, Uchida et al. 2011).

MDR1 is the prototypical drug transporter and as discussed above is a major determinant of barrier phenotype of capillary endothelial cells in the brain. Of the two Mdr1 isoforms expressed in rodents, Mdr1a is the predominant form found in brain capillary endothelial cells while the Mdr1b isoform is predominantly localized to brain parenchymal cells such as astrocytes and neurons (Lajtha and Reith 2007). Although most groups agree that Mdr1 expression is confined to the apical/luminal surface, Mdr1 may also be expressed in intracellular compartments including cytoplasmic vesicles (Meschini, Calcabrini et al. 2000, Arancia, Molinari et al. 2001). It is believed that intracellular localization of Mdr1 may serve as an alternate mechanism of protection by sequestering toxicants away from subcellular targets. On the plasma membrane, a large proportion of Mdr1 proteins are concentrated in specialized regions called caveolae (Lajtha 1993). Within the brain, Mdr1 is also located in the mouse choroid plexus (Fig. 2A). There is very low expression of Mdr1 within astrocytes but its expression is enhanced in astrocytes and neurons in patients with epilepsy and gliomas (Takamiya, Abe et al. 1997, Dauchy, Dutheil et al. 2008, Potschka 2012). Several groups have shown functional Mdr1 in microglia in several species (Ballerini, Di Iorio et al. 2002, Hirrlinger, Konig et al. 2002, Dallas 2003, Lee, Babakhanian et al. 2007). As discussed previously, mice lacking Mdr1 have a disrupted BBB and are much more susceptible to the pesticide ivermectin (Geyer, Gavrilova et al. 2009). MDR1 substrates include drugs that are used for treating cancer, as well as HIV protease inhibitors, analgesics, immunosuppressive agents, etc. limiting the effectiveness of these drugs in treating central nervous system disorders.

MRP1 is present in the brain but its location and function are somewhat controversial. MRP1 is found on the basolateral surface of choroid plexus epithelial cells (Fig. 2B), thereby limiting entry of substances into the CSF (Roberts, Black et al. 2008). MRP1 protein localization is controversial and has been shown to be present on the luminal surface of human brain capillary endothelial cells (Nies, Jedlitschky et al. 2004, Dauchy, Dutheil et al. 2008). However, others have reported that it is not present at all in human microvascular endothelium (Daood, Tsai et al. 2009). Additionally, Mrp1 has been localized basolaterally in rat brain endothelium (Roberts, Black et al. 2008). *In vivo* and *in vitro* functional studies largely support the presence of high mRNA in microvessels but agree that MRP1 function in the BBB is limited (Cisternino, Rousselle et al. 2003, Shawahna, Uchida et al. 2011). Specifically, MRP1 knockout mice do not have increased brain accumulation of known MRP1 substrates including the cancer drug etoposide (Cisternino, Rousselle et al. 2003) or the dye fluorescein (Sun, Johnson et al. 2001). MRP1 mRNA has also been described in rat neurons, oligodendrocytes, and microglia and both mRNA and protein expression have been observed in rat and mouse astrocytes (Hirrlinger, Moeller et al. 2005, Dallas 2006). Additionally, MRP1 protein has been demonstrated in both neonatal and adult choroid plexus, ventricular ependyma, and large pyramidal neurons by immunostaining (Daood, Tsai et al. 2009).

MRP2 mRNA has only limited expression in the brain of some species under basal conditions. Brain expression of Mrp2 has been investigated in few species for particular diseases including capillaries in the hippocampus of humans with epilepsy (Potschka 2012) and in normal rats. MRP2 protein is not detected in the normal human brain (Nies,

Jedlitschky et al. 2004) but is present in the microvasculature and astrocytes of rats (Dallas 2006). Studies designed to study Mrp2 function in the brain are limited to knock out rats and rat strains that naturally lack Mrp2 protein, like the TR⁻, and Eisai hyperbilirubinemic rats, which are models for Dubin-Johnson syndrome (Potschka, Fedrowitz et al. 2003).

MRP4 protein has been identified, using immunohistochemistry, in the apical portions of human mouse and rat brain capillary endothelial cells (Fig. 2C), and basolateral aspect of choroid plexus epithelial cells (Fig. 2D) (Dallas 2006, Dauchy, Dutheil et al. 2008). MRP4 mRNA is also expressed in rat and mouse microglia astrocytes (Hirrlinger, König et al. 2002).

MRP5/Mrp5 protein has been detected in human and rodent astrocytes and pyramidal neurons in addition to capillary endothelial cells where it is present on the luminal surface (Dauchy, Dutheil et al. 2008). Mrp5 protein is highly expressed in rodent choroid plexus epithelium. Mrp5 mRNA has also been observed in rat neurons, astrocytes, oligodendrocytes, and microglia (Dallas 2006).

BCRP expression in the brain has significant overlap with MDR1 expression. Using RT-PCR, western blot, and immunofluorescence, it has been demonstrated that BCRP is expressed in the brain capillary endothelium where it localizes to the luminal surface (Aronica, Gorter et al. 2005). Aronica and coworkers demonstrated higher expression of BCRP in the microvasculature within human epileptic brains compared to healthy

controls (Aronica, Gorter et al. 2005). Using transcriptional analysis, it has been shown that there is 20 times more BCRP mRNA in human microvessels than in the cortex (Dauchy, Dutheil et al. 2008, Shawahna, Uchida et al. 2011). Others have shown an even greater difference in BCRP expression between microvessels and cortex in *Mdr1* knockout mice (Cisternino, Rousselle et al. 2001).

Regulation of MDR1 at the BBB. Introduced above was the concept that ABC transporter expression, including that of MDR1, is altered during disease states. Many transporters are increased up to 1000% in the microvessels of patients with epilepsy (Aronica, Gorter et al. 2005, Potschka 2012). Using positron emission tomography, it has also been established that MDR1 expression and function is reduced in the elderly by demonstrating a 30% increase in [¹¹C]-verapamil. This decreased function may contribute to the development or progression of neurodegenerative diseases (Bartels, Kortekaas et al. 2009, ElAli and Hermann 2011). In general, regulation of MDR1 mRNA stability or transcription influences its expression and can be observed in many disease states (Albermann, Schmitz-Winnenthal et al. 2005, Choudhuri and Klaassen 2006, Dauchy, Dutheil et al. 2008, Ronaldson, Ashraf et al. 2010, Dönmez and Gündüz 2011, Rigalli, Ruiz et al. 2011, Yu, Di et al. 2011, Ménez, Mselli-Lakhal et al. 2012). The following examples illustrate the numerous signaling pathways implicated in regulating ABC transporters, in particular MDR1. Each of these signaling mechanisms provides possible targets for therapeutic intervention to maintain proper transporter function. It must be kept in mind that each of these pathways is a small piece of the puzzle and not all mechanisms may be present in each tissue. In fact, it is more likely that regulation of

MDR1 is cell-specific. For instance, transcription factors that regulate MDR1 in the liver include PXR, CAR, and AhR, whereas only AhR has been shown to be present in isolated human brain capillaries (Dauchy, Dutheil et al. 2008).

Cellular oxidative stress mechanisms may contribute to MDR1 regulation in some tissues. Signaling through phospholipase C, protein kinase C, MAP kinase, intracellular Ca^{2+} , cytokines, NF- κ B, and Hsp-1 have been shown to regulate Mdr1 mRNA or function (Hartz 2005, Wang 2006, Dauchy, Dutheil et al. 2008, Morgan, Goralski et al. 2008, Yu, Di et al. 2011). Using isolated rat brain capillaries, Hartz and coworkers demonstrated rapid, dose-dependent reduction of Mdr1 efflux of fluorescent substrates by TNF via activation of the TNF-R1 receptor (Hartz 2005). Endothelin-1 release and endothelin-B receptor signaling influence short-term Mdr1 mRNA expression in porcine brain endothelial cells (von Wedel-Parlow, Wolte et al. 2009). Elevated glutamate increases Mdr1 protein expression through an NMDA receptor mechanism in a rodent seizure model (Yu, Di et al. 2011). Inflammation and intraventricular injection of rats with LPS reduced Mdr1 protein expression and function (Goralski, Hartmann et al. 2003).

Inflammatory Regulation of ABC Transporters. Many experimental tools, such as LPS, turpentine and exogenous cytokines, have been used *in vitro* and *in vivo* to study the regulation of drug transporters in response to inflammation (Ando, Nishio et al. 2001, Hartmann, Vassileva et al. 2005, Hartz 2005). Although there are some discrepancies, a majority of the studies demonstrate that drug transporters, including MDR1, are differentially expressed during inflammation. Depending upon the experimental model,

altered regulation during inflammation can occur at the mRNA, protein, and/or functional level. Inflammation stimulated by LPS, inflammatory cytokines, or turpentine reduces Mdr1a mRNA and protein expression in rodent liver (Hartmann, Vassileva et al. 2005). Functional studies have demonstrated reduced biliary elimination of the Mdr substrate, rhodamine 123, after the systemic administration of LPS to rats (Ando, Nishio et al. 2001). Interestingly, some groups have demonstrated reduced Mdr1b protein expression during systemic inflammation (Piquette-Miller, Pak et al. 1998), where others have demonstrated an increase in levels (Vos, Hooiveld et al. 1998, Ando, Nishio et al. 2001).

The effects of systemic inflammation on regulating brain Mdr1 are less clear. Hartmann and coworkers showed that systemic inflammation causes a minor reduction in Mdr1 in mouse brain and increased brain:plasma ratios of doxorubicin (Hartmann, Vassileva et al. 2005). The effect of neuroinflammation on BBB Mdr1 and other drug transporters is not as clear. Gorsalski and coworkers demonstrated that localized neuroinflammation induced by intraventricular injection of LPS not only causes rapid downregulation of Mdr1a mRNA in the brain but also in the liver (Goralski, Hartmann et al. 2003). Transport of the Mdr1 substrate digoxin was also reduced *in vivo* in both the brain and liver. The pathways involved in Mdr1 regulation in the brain during neuroinflammation are poorly understood.

NF- κ B Pathway

Diversity of Signaling Through NF- κ B. NF- κ B transcriptional signaling is the primary pathway involved in the coordination of inflammatory responses (reviewed in (Hayden

and Ghosh 2012)). The components responsible for this complex signaling pathway are only partially known. Adding to the complexity, multiple upstream stimuli can have various and often opposite downstream results. An overview of the pathway will be described with emphasis placed on how NF- κ B can regulate ABC transporters. As discussed above, ABC transporter expression, and in particular MDR1, can be reduced by pro-inflammatory cytokines; however, the causal relationship underlying this phenomena is not simple. The complex functions of NF- κ B are not only involved in inflammation but also influence cell survival, proliferation and differentiation. The diversity of NF- κ B functions are in part the result of multiple subunits with different effects (Hayden and Ghosh 2012).

In the resting state, NF- κ B is inactive in the cytoplasm as either a homodimer or heterodimer and bound to the inhibitory molecule, I κ B (Fig. 3) (Tang, Inohara et al. 2003, Tergaonkar, Correa et al. 2005). Stimulation of NF- κ B through various pathways activates an I κ B kinase complex, resulting in phosphorylation, ubiquitination, and proteasomal degradation of I κ B (Alkalay, Yaron et al. 1995, Chen, Hagler et al. 1995). NF- κ B dimers are subsequently released and translocate to the nucleus, where they bind to specific DNA sequences to begin transcription of target genes (Baeuerle and Henkel 1994). Termination of NF- κ B signaling is achieved through inactivation of the I κ B kinase complex, resynthesis of I κ B, and displacement of NF- κ B dimers from DNA. The types of genes transcribed, duration of response, and overall effect of NF- κ B pathway activation depends on which NF- κ B dimer combinations are activated (Bauer, Villunger et al. 2006, Siggers, Chang et al. 2012). The five families of NF- κ B proteins that make up the dimers

are RelA/p65, RelB, c-Rel, p105/p50(NF- κ B1), and p100/p52(NF- κ B2) (Sanjabi, Williams et al. 2005, Shih, Kearns et al. 2009). NF- κ B proteins bind to DNA sequences either as homodimers or heterodimers which can have positive or negative transcription effects, increasing the diversity of function of this family of proteins (Tang, Inohara et al. 2003). All members have a Rel homology domain (RHD) allowing DNA binding and dimerization. Only p65, c-Rel, and RelB contain transactivation domains (TAD) that support transcription (Sanjabi, Williams et al. 2005, Siggers, Chang et al. 2012). Therefore, p50 and p52 can either positively regulate transcription through heterodimerization with a TAD-containing subunit, or negatively regulate transcription through homodimerization (Saccani, Pantano et al. 2003, Tang, Inohara et al. 2003).

All NF- κ B subunits are held in check by I κ B proteins. There are several I κ B proteins, adding to the complexity of NF- κ B regulation (Basak, Kim et al. 2007). Some of the I κ B family members include, I κ B α , I κ B β , I κ B ϵ , I κ B γ , BCL-3, and I κ Bn (Beg and Baldwin 1993, DiDonato, Hayakawa et al. 1997, McKenzie, Connelly et al. 2000, Doerre, Mesires et al. 2005). Also included in this family are the precursor proteins p105(NF- κ B1) and p100(NF- κ B2). The best understood member of the family is I κ B α which is rapidly degraded during the activation of the canonical NF- κ B pathway (Pahl 1999). I κ B α degradation typically results in the release and translocation of p65:p50 heterodimers (Beg and Baldwin 1993). The nuclear localization signal on p65 is masked by I κ B α which also contains a nuclear export signal resulting in the cytoplasmic localization of the I κ B α :p65:p50 complex (Chen, Mu et al. 2002). When I κ B α is degraded by the

proteasome, the p65:p50 dimer is free to translocate to the nucleus and accumulate, resulting in DNA binding and transcription (Hoffmann, Natoli et al. 2006).

Upstream NF- κ B signaling, leading to I κ B phosphorylation, is broadly divided into canonical and non-canonical pathways. The canonical, or classical, pathway utilizes ligand-receptor initiation as is the case for the TNF, IL-1 β , and TLR4 receptors (Wang, Westerheide et al. 2000, Chen, Chio et al. 2008, Yamauchi, Ito et al. 2010). Ligand binding to these membrane-bound receptors triggers an intracellular signaling cascade that activates IKK β . IKK β exists in a complex with IKK α and a regulatory protein, NEMO (Agou, Ye et al. 2002). Activated IKK β phosphorylates I κ B α leading to its degradation (Delhase, Hayakawa et al. 1999). The non-canonical, or alternative, pathway relies on direct activation of IKK α and is not dependent on NEMO or IKK β (Poyet, Srinivasula et al. 2000). Cytokines using the non-canonical pathway include some members of the TNF cytokine family such as CD40 ligand, BAFF, and lymphotoxin- β (Yamauchi, Ito et al. 2010, Sun 2011). Activation of IKK α results in phosphorylation of p100 and formation of p52:RelB complexes (Senftleben, Cao et al. 2001, Courtois and Israel 2011). IKK α and IKK β are key enzymatic components of NF- κ B signaling but are also involved in the p53, MAP kinase, and IRF signaling pathways, facilitating cross-talk between these pathways (Oeckinghaus, Hayden et al. 2011). In addition to the large number of receptor ligands that activate the NF- κ B pathway, there are a growing number of intracellular signals that also stimulate NF- κ B including signals through nucleotide oligomerizing domains (NODs), DNA damage signals, and reactive oxygen species (Pahl

1999). No matter the upstream signal, the downstream process of IKK complex activation is fairly conserved (Torgerson, Colosia et al. 1998, Hayden and Ghosh 2012).

IKK Complex Activation. Following receptor activation, signaling proceeds through the binding of a series of adapter proteins through protein:protein interactions. These adapter proteins also contain death domains (DDs), caspase activation and recruitment domains (CARDs), RIP homotypic interaction domains (RHIMs) and Toll IL-1R (TIR) domains (Hur, Lewis et al. 2003). These large protein complexes recruit and activate kinases that are responsible for the phosphorylation of I κ B. I κ B is replenished via a negative feedback loop where NF- κ B transcribes additional I κ B (Brown, Park et al. 1993). The duration of the NF- κ B response is heavily dependent on the kinetics of the feedback loop. I κ B α , I κ B β , and I κ B ϵ are each different in their speed of degradation and resynthesis. I κ B ϵ degradation and resynthesis occurs with delayed kinetics compared to I κ B α (Torgerson, Colosia et al. 1998, Hayden and Ghosh 2012).

The Nrf2 Pathway

Antioxidant Response. The transcription factor Nrf2 is a key regulator of antioxidant response element (ARE)-dependent gene expression in response to oxidative stimuli (Higgins and Hayes 2011). Nrf2 is ubiquitinated by ketch-like ECH-associated protein 1 (Keap1) and degraded through the proteasome (Cullinan, Gordan et al. 2004). Nrf2 is also present in the nucleus under basal conditions for constitutive expression of NAD(P)H:quinone oxidoreductase 1 (Nqo1) and heme oxygenase-1 (Ho-1) (He, Chen et al. 2008). Nrf2 is activated by electrophiles and pro-oxidants as well as antioxidants.

Following activation, Nrf2 is recruited to the nucleus where it dimerizes with small musculoaponeurotic fibrosarcoma (MAF) proteins on AREs on promoter regions of target genes where it regulates transcription of over 200 genes (Ma, Kinnear et al. 2004). Nrf2 is primarily responsible for regulating genes that encode antioxidant proteins including glutathione peroxidase, thioredoxin, thioredoxin reductase 1, peroxyredoxins and Ho-1 in addition to Nqo1 (Ishii, Itoh et al. 2000). It also regulates transcription of some ABC transporters including Mrp1, 2, 3, and 4 and controls the ability to generate NADPH in mice (Klaassen and Slitt 2005, Maher, Dieter et al. 2007, Higgins and Hayes 2011). The Nrf2 signaling pathway is intimately connected to many other signaling pathways including NF- κ B.

Using mouse embryo fibroblasts from wild type and Nrf2-null mice, Hayashi and coworkers demonstrated that both the constitutive and inducible expression of Mrp1 mRNA and protein are governed by Nrf2. Knock-out of Nrf2 reduced the uptake of the Mrp1 substrate [3 H]2,4-dinitrophenyl-S-glutathione in isolated membrane vesicles (Hayashi, Suzuki et al. 2003). Additionally, Mrp2 and Mrp3 mRNAs are significantly lower in livers of Nrf2-null mice (Anwar-Mohamed, Degenhardt et al. 2011, Aleksunes and Klaassen 2012). Treatment with the Nrf2 activator oltipraz increases Mrp2, 3, and 4 mRNA expression in livers of wild-type mice, but not Nrf2-null mice (Maher, Dieter et al. 2007).

Expression and activation of Nrf2 is neuroprotective in animal models of cerebral ischemia, traumatic brain injury, hemorrhage, and spinal cord injury (Yan, Wang et al.

2008, Alfieri, Srivastava et al. 2011, Chen, Fang et al. 2011, Mao, Wang et al. 2012). Activation of Nrf2 at the BBB and blood-CSF barrier using sulforaphane increases protein expression and luminal accumulation of a fluorescent substrate of Mdr1 in isolated rat brain and spinal cord capillaries (Wang, Campos et al. 2014). Similar elevation in protein expression was observed in wild-type but not Nrf2-null mice. Further investigation revealed that Mdr1 protein expression and function are altered indirectly through Nrf2. Sulforaphane is only able to up-regulate Mdr1 expression and function when p53 and NF- κ B pathways are intact (Wang, Campos et al. 2014). Additional evidence for cross-talk between Nrf2 and NF- κ B is highlighted by reduced proinflammatory and profibrogenic factors in RAW264.7 macrophages by the Nrf2 activator, resveratrol (He, Wang et al. 2012).

Parkinson Disease.

Overview. Parkinson disease (PD) is a common neurodegenerative disease of aging. Of all neurodegenerative diseases worldwide, the incidence of PD is second only to Alzheimer disease. PD presents clinically as bradykinesia, muscular rigidity, resting tremors, and postural instability, all of which are the direct result of degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNc). With the number of Americans over 65 rapidly increasing, it is inevitable that there will be a significant increase in PD cases over the next 20 years (Dorsey, Constantinescu et al. 2007). Treatment for PD is currently limited to symptomatic treatment of the movement disorder since, at the time of diagnosis, no more than approximately 25% of dopamine neurons of the SNc remain. Little progress has been made in the early diagnosis and/or treatment of

PD because no single cause has been identified. PD most likely results from the interaction of multiple genetic and environmental factors.

Parkinson Disease and Pesticides. Epidemiological studies have reported that individuals exposed to pesticides have an increased risk for PD (Table 1) (Ho, Woo et al. 1989, Semchuk, Love et al. 1991, Hubble, Cao et al. 1993, Le Couteur, McLean et al. 1999, Priyadarshi, Khuder et al. 2000, Ascherio, Chen et al. 2006, Kamel, Tanner et al. 2007, Hancock, Martin et al. 2008, Richardson, Shalat et al. 2009, Richardson, Roy et al. 2011, Tanner, Kamel et al. 2011). However, the identity of specific pesticides is often not mentioned in these studies. Nonetheless, one pesticide that has received significant attention as a potential contributor to PD is the herbicide paraquat. Several epidemiological studies have linked paraquat exposure to an increased risk of PD (Liou, Tsai et al. 1997, Firestone, Smith-Weller et al. 2005, Li, Mink et al. 2005, Tanner, Kamel et al. 2011). However, the odds ratios for these associations are small (1.67 – 3.22), suggesting that paraquat exposure, alone, is not a singular contributor to the development of PD.

Paraquat Neurodegeneration. Although the epidemiological associations between paraquat exposure and PD continue to be investigated, paraquat is routinely used in animal models to study mechanisms of dopaminergic neurodegeneration (Brooks, Chadwick et al. 1999, Richardson, Quan et al. 2005, Peng, Stevenson et al. 2009, Roede, Hansen et al. 2011, Jiao, Lu et al. 2012). In rodents, paraquat exposure leads to loss of a subset of dopamine neurons, increased α -synuclein levels in the brain, oxidative damage,

and neuroinflammation in the form of microglia activation and up-regulation of NADPH oxidase, all of which are hallmarks of PD (Berry, La Vecchia et al. 2010). It is important to note that more than one dose of paraquat is required to induce neuronal loss. Alternatively, treatment with LPS prior to paraquat administration can similarly sensitize mice to paraquat-induced neurodegeneration. Furthermore, mice lacking gp91-Phox, the catalytic subunit of microglial NADPH oxidase, are resistant to paraquat-induced neuronal cell loss (Purisai, McCormack et al. 2007). Together, these and other data suggest neuroinflammation is a central 'priming event' for subsequent paraquat neurotoxicity, but the mechanism underlying this sensitization is unknown.

Astrocytes activated during inflammation can augment pro-inflammatory signals leading to further damage. Astrocytes have high expression of the uptake transporter organic cation transporter (OCT) 3/*SLC22A3*. OCT3, in conjunction with the dopamine transporter (DAT/*SLC6A3*), was shown to play a role in paraquat trafficking between microglia, astrocytes and dopaminergic neurons (Rappold, Cui et al. 2011). Paraquat was shown to be transported by both DAT and OCT3 after a one-electron reduction by microglial NADPH oxidase (Rappold, Cui et al. 2011).

NADPH Oxidase and Inflammation. NADPH oxidase is composed of multiple subunits that are sequestered into different subcellular compartments when the enzyme is inactive. When microglia are exposed to a variety of stimuli, these subunits are phosphorylated and assemble into active NADPH oxidase which then catalyzes the production of superoxide by a one-electron reduction of oxygen (Babior 2004). Superoxide reacts with

cellular components forming reactive oxygen species and lipid radicals. Cellular defense against oxidative damage is through the transcription of antioxidant mechanisms via the Nrf2 signaling pathway (Hayashi, Suzuki et al. 2003, Brandenburg, Kipp et al. 2009). The extent to which NADPH oxidase, reactive oxygen species, lipid radicals, and Nrf2 are involved in the pathogenesis or adaptation to neurodegeneration is not clear.

Summary.

ABC transporters are present in the BBB where they limit chemical accumulation in the brain. Despite recent advances in our understanding of drug transport at the BBB, little is known about the ability of ABC proteins to efflux environmental chemicals from the brain, their expression in cell populations other than endothelial cells, or their contribution to the pathogenesis of neurodegenerative diseases.

In humans, loss of dopaminergic cells in the SNc is one of the key features of PD. Possible exposure to pesticides, including paraquat, may contribute to PD. Hypofunctional polymorphisms in the MDR1/*ABCB1* gene have been linked to PD in individuals exposed to pesticides, although the contribution of paraquat has not been specifically examined (Drozdik, Bialecka et al. 2003). Together, these findings suggest that MDR1 may contribute to the protection of the brain from paraquat-induced neurotoxicity.

Inflammation is a central component of neurodegeneration. Neuronal degeneration is accompanied by microglial activation in animal models and patients with PD. The goals

of this thesis are to determine whether paraquat is a substrate of MDR1 and whether neuroinflammation can alter the expression and function of transporters in the brain. The overall hypothesis of this dissertation is that *efflux transporters expressed in the brain limit the accumulation and toxicity of paraquat and are differentially regulated in response to neuroinflammation.*

Studies described will address the specific aims:

Specific Aim 1 – To determine the role of MDR1/Mdr1 in the transport and neurotoxicity of paraquat.

Specific Aim 2 – To examine how neuroinflammation alters the expression and function of ABC transporters.

Specific Aim 3 – To identify signaling pathways that contribute to the down-regulation of MDR1/Mdr1 during neuroinflammation.

This research will address whether neuroinflammation is a mechanism by which ABC transporter function is altered at the BBB and in other cells types leading to subsequent xenobiotic-induced neural injury. Reduced transporter function in these models may provide a mechanistic link between neuroinflammation, exposure to environmental chemicals, and neuronal degeneration. This knowledge may provide useful information for possible therapeutic interventions to enhance transporter function in the brain and limit further neural damage.

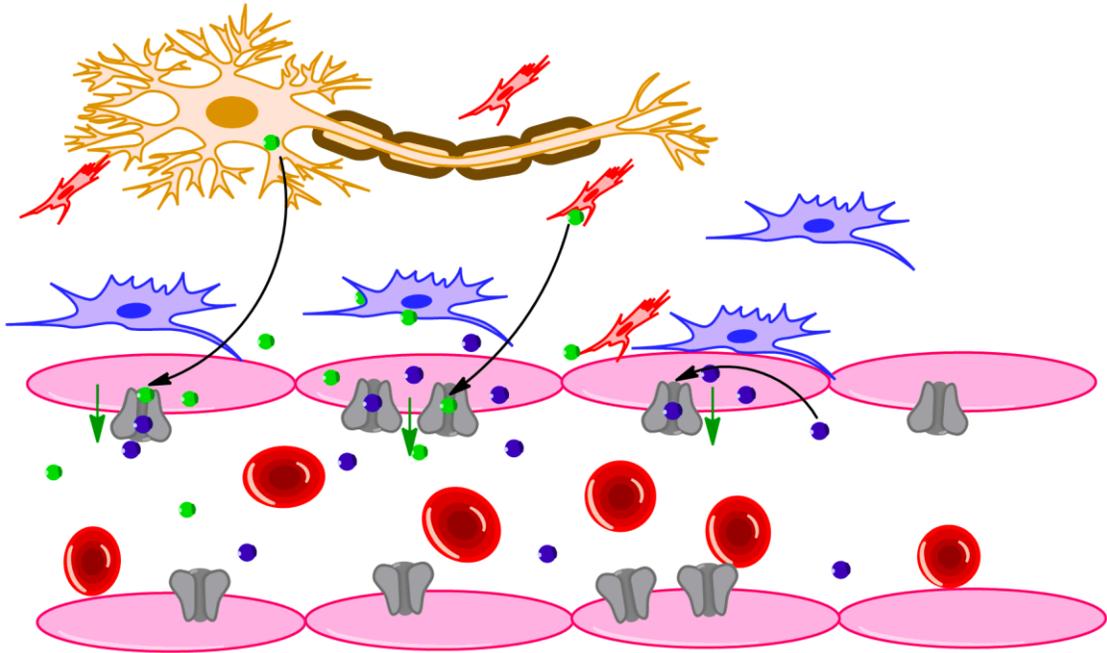


Figure 1-1

Figure 1-1. Transporters in the blood-brain barrier. The blood-brain barrier is comprised of specialized capillary endothelial cells with tight junctions and efflux transporters along with astrocytes. Efflux transporters on endothelial cells prevent exogenous compounds (*blue*) from entering the brain, and help prevent the buildup of endogenous compounds (*green*).

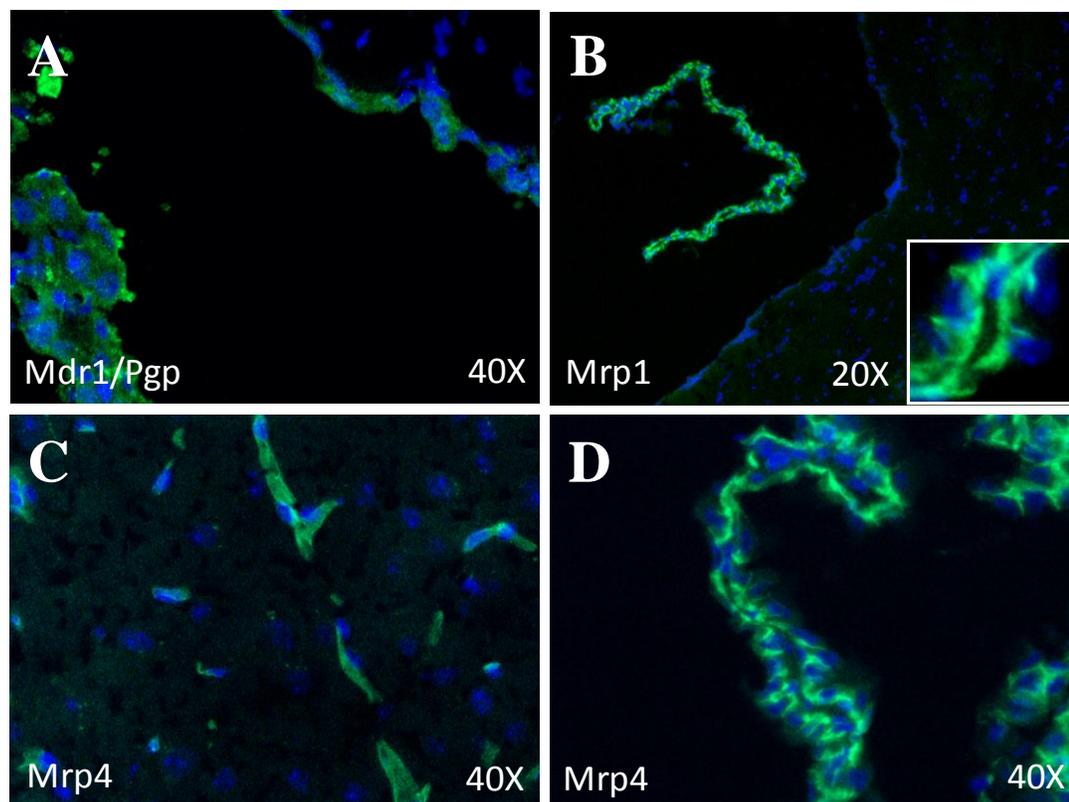


Figure 1-2

Figure 1-2. Localization of efflux transporters in the mouse brain. Mdr1 is highly expressed in choroid plexus epithelium predominantly along the apical border, and within ependymal lining cells. Mrp1 is highly expressed along the basolateral portion (see inset) of the choroid plexus epithelium. Mrp4 is also present along the basolateral surface of choroid plexus epithelium and highly expressed in brain capillaries.

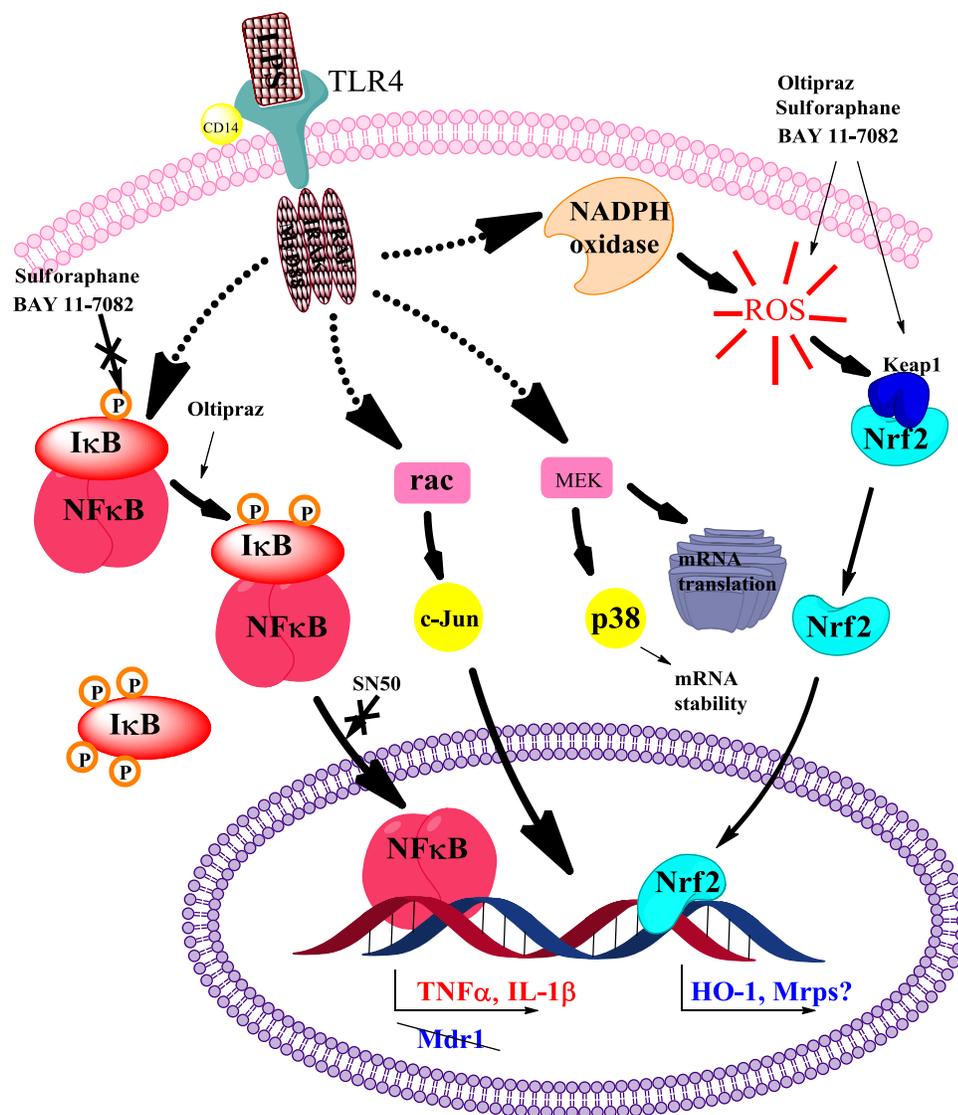


Figure 1-3

Figure 1-3. Interaction between the NF-κB and Nrf2 pathways. Inhibitors and activators of each pathway are shown.

Table 1-1. Odds ratios for studies examining associations between pesticide exposure and Parkinson Disease.

Authors (year)	Study Details	Odds Ratio (95% CI)
Ho, Woo et al. 1989	Hong Kong - Association with rural areas, farming, herbicides , pesticides, consumption of raw vegetables	3.6 (1.0 - 12.9)
Semchuk, Love et al. 1991	Calgary - Association with rural living, farming, well water, pesticides	3.06
Hubble, Cao et al. 1993	USA - Association with rural populations, male behaviors, pesticides	3.15 (1.30 - 6.63)
Le Couteur, McLean et al. 1999	Review of pesticides and PD	1 - 7.0
Priyadarshi, Khuder et al. 2000	Meta-analysis of pesticides and PD	1.94 (1.49 - 2.53)
Ascherio, Chen et al. 2006	USA - Cancer Prevention Study II: Nutritional Cohort – pesticides	1.7 (1.2 - 2.3)
Kamel, Tanner et al. 2007	Agricultural Health Study - Association with lifetime pesticide use	2.3 (1.2 - 4.5)
Hancock, Martin et al. 2008	Family-based pesticide exposure	1.61 (1.13 - 2.29)
Richardson, Shalat et al. 2009	Organochlorine pesticides in serum	4.39 (1.67 - 11.6)
Richardson, Roy et al. 2011	Beta-hexachlorocyclohexane	2.85 (1.8 - 4.48)
Tanner, Kamel et al. 2011	Agriculture Health Study - Lifetime pesticide use – Paraquat	2.5 (1.4 - 4.7)
Liou, Tsai et al. 1997	Taiwan - Association with pesticide use – Paraquat	3.22 (2.41 - 4.31)
Firestone, Smith-Weller et. al. 2005	Group Health Cooperative - Western Washington State – Paraquat	1.67 (0.22 - 12.76)

CHAPTER 2

**Multidrug Resistance Transporter 1 (MDR1/P-glycoprotein) Protects
Against Paraquat Neurotoxicity****Abstract**

Repeat administration of the herbicide paraquat causes dopaminergic neuron degeneration in the brains of rodents. Access of paraquat to the brain is largely restricted by the blood-brain barrier and only a small fraction of paraquat enters the brain and initiates redox cycling and oxidative stress leading to neuron loss. The purpose of this study was to determine whether the multidrug resistance transporter 1 (MDR1/Mdr1, *ABCB1* or P-glycoprotein) in capillary endothelial cells participates in the removal of paraquat at the blood-brain barrier and protects against neurodegeneration. Paraquat transport was quantified *in vitro* using an immortalized human brain capillary endothelial cell line (hCMEC/D3) which endogenously expresses efflux transporters. In hCMEC/D3 cells, reduction of MDR1 transport using the inhibitor PSC833 or siRNA knock-down increased cellular accumulation of paraquat by up to 200%. Mdr1a/1b-null mice were used to determine whether the absence of Mdr1 expression alters susceptibility to paraquat-induced neurotoxicity. Mdr1a/1b-null mice had slightly higher brain paraquat levels than WT mice following a single dose. Mdr1a/1b-null and wild-type mice were given one or two doses of paraquat (10mg/kg, i.p.). Dopamine neurons stained for tyrosine hydroxylase (TH) in the substantia nigra pars compacta were counted using unbiased stereology. Mdr1a/1b-null mice treated with a single dose of paraquat exhibited a 38% loss of TH positive neurons and enhanced microglia activation that was not

observed in wild-type mice. These studies demonstrate that MDR1 transports paraquat *in vitro* and protects against paraquat-induced neurotoxicity *in vivo*.

Abbreviations

ABC, ATP binding cassette; BCRP, breast cancer resistance protein; DAT, Dopamine transporter; hCMEC/D3, human cerebral microvascular endothelial cells; HEK293 cells, human embryonic kidney 293 cells; Ho-1, Heme oxygenase-1; LDH, lactate dehydrogenase; MATE1/Mate1, multidrug and toxin extrusion 1 protein; MDR1/Mdr1, multidrug resistance protein 1; MRP, multidrug resistance-associated protein; Nqo1, NAD(P)H quinone oxidoreductase 1; OCT, organic cation transporter; PBS, phosphate-buffered saline; PBS/T, phosphate buffered saline with Tween 20; PD, Parkinson Disease; ROS, reactive oxygen species; TH, Tyrosine hydroxylase; TNF, tumor necrosis factor; WT, wild-type

Introduction

Parkinson disease (PD) is a common neurodegenerative disease of aging worldwide, second only to Alzheimer disease. PD presents clinically as bradykinesia, muscular rigidity, resting tremor, and postural instability, all of which are the direct result of degeneration and loss of dopaminergic neurons in the substantia nigra pars compacta (SNc). With the number of Americans over 65 rapidly increasing, it is inevitable that there will be a significant increase in PD cases over the next 20 years (Dorsey, Constantinescu et al. 2007). Little progress has been made in the early diagnosis or treatment of PD largely because no single cause has been identified. The main difficulty in finding a treatment lies in the fact that 1) PD likely results from the interaction of genetic and environmental factors (Schapira and Jenner 2011), 2) the disease does not become apparent clinically until more than 60% of dopaminergic neurons of the SNc have been lost.

Epidemiological studies have reported that individuals exposed to pesticides have an increased risk for PD (Ho, Woo et al. 1989, Semchuk, Love et al. 1991, Hubble, Cao et al. 1993, Le Couteur, McLean et al. 1999, Priyadarshi, Khuder et al. 2000, Ascherio, Chen et al. 2006, Kamel, Tanner et al. 2007, Hancock, Martin et al. 2008, Richardson, Shalat et al. 2009, Richardson, Roy et al. 2011, Tanner, Kamel et al. 2011). However, the identity of specific pesticides is often not mentioned in these studies. Nonetheless, one particular pesticide that has received significant attention as a potential contributor to PD is the herbicide paraquat. Several epidemiological studies have linked paraquat exposure to an increased risk of PD (Liou, Tsai et al. 1997, Firestone, Smith-Weller et al. 2005, Li,

Mink et al. 2005, Tanner, Kamel et al. 2011). However, the odds ratios for these associations are generally small and other studies have not confirmed the association between paraquat and PD.

Although the epidemiological associations between paraquat exposure and PD continue to be investigated, paraquat has been used in animal models to study mechanisms of dopaminergic neurodegeneration (Brooks, Chadwick et al. 1999, Richardson, Quan et al. 2005, Peng, Stevenson et al. 2009, Roede, Hansen et al. 2011, Jiao, Lu et al. 2012). Paraquat exposure in rodents leads to loss of a subset of dopamine neurons, increased α -synuclein levels, oxidative damage, and neuroinflammation in the form of microglia activation and up-regulation of NADPH oxidase, all hallmarks of PD (Berry, La Vecchia et al. 2010). Paraquat, a soluble cation, is thought to be transported across the BBB by a neutral amino acid transporter or possibly by an organic cation transporter (OCT). Once in the brain, paraquat remains detectable for weeks in mice (Prasad, Tarasewicz et al. 2009). OCT3 on astrocytes in conjunction with the dopamine transporter (DAT) were shown to play a role in paraquat trafficking between microglia, astrocytes and dopaminergic neurons (Rappold, Cui et al. 2011). Paraquat is transported by both DAT and OCT3 only after being reduced by microglial NADPH oxidase.

A number of ABC efflux drug transporters are expressed by the BBB to prevent the passage and accumulation of toxic chemicals in the brain (Nies, Jedlitschky et al. 2004, Dallas 2006). One of the more abundant transporters responsible for the integrity and function of the BBB is multidrug resistance protein 1 (MDR1)/P-glycoprotein. Inhibition

or reduced function of MDR1 can lead to excessive uptake of toxicants, such as ivermectin/avermectin, resulting in neurotoxicity (Merola and Eubig 2012). MDR1 in humans and Mdr1a and 1b in rodents are responsible for the efflux of many chemicals, including some pesticides (Bircsak, Richardson et al. 2013), and loss of MDR1 function by pesticides results in the accumulation of potentially toxic substrates (Sreeramulu, Liu et al. 2007). Moreover, transporter expression and function can be influenced by inflammatory cytokines, of which TNF, IL-1 β , and IL-6 have been shown to modify MDR1 expression and function in the brain (Morgan, Goralski et al. 2008). It is unclear whether impairment of MDR1 by inflammation could contribute to the priming events that sensitize mice to paraquat neurotoxicity upon repeated administration. Additionally, polymorphisms in MDR1 were present in some PD patients exposed to pesticides (Zschiedrich, König et al. 2009).

The purpose of this study was to assess the ability of the MDR1/Mdr1 transporter to protect against paraquat-induced neurodegeneration. It was hypothesized that MDR1 transports paraquat from brain capillary endothelial cells and that mice lacking Mdr1 would have greater brain paraquat concentrations and more extensive paraquat-induced neurodegeneration and neuroinflammation than wild-type mice.

Materials and Methods

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

Cell Culture. Immortalized human cerebral microvascular endothelial cells (hCMEC/D3) were obtained from Dr. Babette Weksler (Weill Cornell Medical College, New York, NY) (Weksler, Subileau et al. 2005). All cell culture flasks and plates were coated with rat collagen I (R&D systems, Minneapolis, MN) and incubated for 1 hour at 37°C prior to addition of medium and cells. hCMEC/D3 cells were grown in EBM-2 basal medium (Lonza, Allendale, NJ), 5% fetal bovine serum “Gold” (PAA, GE healthcare Bio-Sciences, Pittsburgh, PA), 1% penicillin-streptomycin (Life Technologies), 1.4µM hydrocortisone, 5µg/mL ascorbic acid, 1% chemically defined lipid concentrate (Life Technologies), 10mM HEPES, and 1ng/mL basic fibroblast growth factor.

Knock-down of MDR1 in hCMEC/D3 cells was achieved using the human MDR1 siRNA transfection kit (Santa Cruz Biotechnology, Santa Cruz, CA) according to the manufacturer’s protocol. Transfection efficiency and negative control siRNA transfections were determined using FITC-labeled scrambled sequence siRNA (Santa Cruz Biotechnology). Transfection efficiency in hCMEC/D3 cells was 60% as determined by intracellular fluorescence of the FITC-labeled sequence using the Cellometer Vision fluorescent cell counter, using filter cube VB-535-402 (FITC; Excitation / Emission: 475nm/535nm) (Nexcelom Bioscience LLC., Lawrence, MA).

Knockdown of MDR1 protein (-40%) expression and function were confirmed by western blot and transport of rhodamine 123, respectively.

Western Blot Analysis. Cultured cells were collected and transferred to microcentrifuge tubes and lysed in cell lysis buffer containing 20 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton 100 and 1% protease inhibitor cocktail. Protein concentration was determined using the bovine serum albumin protein quantification kit (Thermo Fisher Scientific). Cell lysates (30 μ g protein/well) were separated by SDS-PAGE electrophoresis on NuPage Tris-Acetate 4% gels (Invitrogen). Proteins were transferred overnight to polyvinylidene fluoride membranes. Membranes were blocked in 5% nonfat dry milk in phosphate-buffered saline (PBS) with 0.5% Tween-20. Primary antibodies were diluted in 2% nonfat dry milk and incubated with membranes for 3 hours at the following concentrations: 1:1000 for Mdr1a/b (C219, Novus Biologicals, Littleton, CO), Multidrug and toxin extrusion 1 (Mate1) (sc-138983), 1:1000 for Oct2 (sc-292622) (Santa Cruz Biotechnology, Santa Cruz, CA), 1:2000 for β -actin (Abcam) and 1:5000 for Gapdh (Millipore, Temecula, CA). Primary antibodies were probed using species-appropriate secondary antibodies (Sigma) and detected using SuperSignal West Dura blotting reagents (Thermo-Scientific). Detection and semi-quantification of protein bands were performed using a FluorChem E imager (Protein Simple, Santa Clara, CA) and normalized to β -actin (for *in vitro* studies) or GAPDH (for *in vivo* studies).

MDR1 Transporter Efflux Assay. MDR1 protein function was determined in cells by measuring the retention of the fluorescent substrate Rhodamine123 as described in a

previous protocol (Gibson, Hossain et al. 2012, Bircsak, Richardson et al. 2013). Briefly, transporter function was quantitated on a Cellometer Vision cell counter using fluorescent rhodamine 123 MDR1 substrate (2 μ g/ml) with or without the MDR1 inhibitor PSC833 (2 μ M) for 30 minutes at 37°C, 5% CO₂ (*uptake period*). Cells were then washed, centrifuged, resuspended, and incubated in substrate-free medium in the presence and absence of PSC833 (2 μ M) for 1 hour (*efflux period*). Cells were then washed, resuspended in cold phosphate-buffered saline, and fluorescence was quantified using the Cellometer Vision using filter cube VB-595-502 (Rhodamine 123; Excitation / Emission: 525nm/595nm). The total number of cells analyzed for each sample ranged from 500 to 2000. Raw fluorescence intensity for each cell was normalized to cell size. The average fluorescence for each sample was determined and the treatment average was based on four independent samples for each treatment.

Paraquat Efflux Assay. Preliminary experiments demonstrated that the MDR1 specific inhibitor, PSC833, did not significantly alter the uptake of paraquat in hCMEC/D3 cells (data not shown). To assess the ability of MDR1 to efflux paraquat from cells, hCMEC/D3 cells were seeded onto 6-well tissue culture plates with growth medium until they became 80-90% confluent. Growth medium was changed every 3 days until cells were ready to use. Cells were preloaded with paraquat by incubating them in 100 μ M paraquat in HBSS in the presence and absence of PSC833 (2 μ M) for 60 minutes. Following the incubation period, cells were washed 3 times with ice cold HBSS. One mL of fresh, warm HBSS +/- PSC833 was then added to each well. 200 μ L of supernatant was removed from each well after 0, 1, 5, 15, 30 and 60 minutes and stored at

-20°C. Cells were washed 3 times with ice cold HBSS. After removal of HBSS, 200µL of cell lysis buffer was added. Cells were scraped and cell lysates were collected and stored at -20°C until analysis. All cell culture experiments were conducted in triplicate and each experiment was performed at least three times on different days.

Paraquat Quantification Using Enzyme-Linked Immunosorbent Assay (ELISA).

Frozen samples from cell efflux experiments or serum samples were thawed. Twenty-five microliters of sample or standard was added to each well of a 96-well ELISA plate coated with anti-paraquat antibody. Samples were diluted in HBSS as needed to fall within the linear range of the standard curve. Subsequent steps were performed according to the manufacturer's instructions (Abnova, Taiwan).

Cytotoxicity Assay. Cytotoxicity was determined in hCMEC/D3 cells by measuring lactate dehydrogenase release (LDH) 72 hours following incubation with paraquat or paraquat plus PSC833 (2µM) using the LDH release assay (Sigma) according to the manufacturer's protocol.

Animal Care and Treatment. C57BL/6 WT mice were purchased from Charles River (Wilmington, MA). Mdr1a/1b-null mice were obtained from Taconic Laboratories (Hudson, NY) and backcrossed to the C57Bl/6 background until 99% congenic. Congenic analysis was performed by the Bionomics Research and Technology Core at Rutgers University (New Brunswick, NJ). Mice were acclimated for one week prior to the start of experiments and maintained in a 12 hour light/dark cycle, temperature- and

humidity-controlled environment. Paraquat or 0.9% sterile saline vehicle was administered (10 mg/kg; 5 ml/kg, i.p.) to adult male wild-type and Mdr1a/1b-null mice as a single dose or two doses 1 week apart. All animal studies were conducted in accordance with the Rutgers University Institutional Animal Care and Use Committee.

Mice were assigned into one of six groups to assess the neurotoxicity of paraquat. Each group contained between 5 and 8 mice. Three groups of Mdr1a/1b-null mice and WT mice included: saline control (2 doses, 1 week apart, i.p.), paraquat single dose (saline, 10mg/kg paraquat, 1 week apart, i.p.), two doses of paraquat (10 mg/kg, 1 week apart, i.p.). Blood samples were collected by tail vein 24 hours following each dose of paraquat. Mice were monitored daily for signs of toxicity. One week following the last dose, mice were anesthetized using sodium pentobarbital (0.03ml/mouse, i.p. Fort Dodge Animal Health, Fort Dodge, IA). When mice reached a surgical plane of anesthesia, a transverse ventral incision was made in the cranial abdomen just caudal to the diaphragm. The diaphragm was incised at its attachment to the ventral body wall and incisions were continued cranially through the thorax to expose the heart. Blood collected from the right ventricle was used for serum paraquat quantification. A 25-gauge needle connected to a syringe containing phosphate-buffered saline was inserted into the left ventricle and the right atrium was incised. The syringe was placed in a perfusion pump and the mice were perfused at a rate of 3ml per minute for a total of 15 mls. Adequate perfusion was assessed by blanching of the liver and extremities. Mice were then perfused with 15 ml of freshly prepared 4% paraformaldehyde over 5 minutes. Adequate perfusion was assessed by stiffening of the tail and extremities. Brains were removed whole and placed in 4%

paraformaldehyde for 24 hours. Brains were then immersed in 30% sucrose in PBS for 48 hours (changing sucrose solution every 24 hours) until they sank. Samples were then cryopreserved until further use in 30% sucrose/30% ethylene glycol and stored at -20°C.

For quantification of brain paraquat concentrations, Mdr1a/1b-null or WT mice received a single dose of paraquat (10mg/kg, i.p.) and serum and brain were collected after 2, 6, 12, 24, 48, 72 and 168 hours. Mice were deeply anesthetized using pentobarbital and perfused with PBS as above. Adequate perfusion of the brain was confirmed by lack of visible meningeal blood vessels. The brain was removed and sections of cortex, striatum, midbrain, hippocampus, and remaining brain were collected and snap frozen in liquid nitrogen. Frozen midbrain sections were weighed and placed in tubes on dry ice until needed for paraquat quantification by LC/MS.

Liquid Chromatography-Coupled Mass Spectrometry.

Paraquat concentrations in the midbrains and serum of WT and Mdr1a/1b-null mice were quantified by LC/MS as described previously (Wen, Gibson et al. 2014). Briefly, samples (20 mg of midbrain) or standards, 120 µl of extraction buffer (13.3% acetic acid) and 10 µl of internal standard (10 ppb of 1-Methyl-4-phenylpyridinium iodide, MPP⁺ iodide, from Sigma) were sonicated for 30 min, then microwaved in vessels (CEM, Matthews, NC) for 30 min at 300 W (52% power) twice. The liquid phase solutions were transferred to Nanosep[®] centrifugal devices with a membrane cutoff of 10 kDa (VWR, Bridgeport, NJ) and centrifuged at 12,000 g at 4°C for 60 min. The filtrates (100 µl) were used for paraquat LC/MS analysis.

HPLC/MS was performed using a Thermo Finnigan LTQ Mass Spectrometer system equipped with a Thermo Finnigan Surveyor MS pump Plus and a Surveyor Autosampler Plus system (Thermo Fisher Scientific, San Jose, CA). A gradient program with 0.1% formic acid water (mobile phase A) and 0.1% formic acid methanol (mobile phase B) was applied into a reversed phase Onyx Monolithic C18 column (50mmX 2.1mm ID, Phenomenex). An electrospray interface operated in positive mode. Parent ions of m/z 186 for paraquat and m/z 170 for MPP⁺ were selected with an isolation width of 1 m/z . Selected reaction monitoring (SRM) mode with window 1 m/z used for monitoring fragment ions of m/z 171 for paraquat and m/z 128 and m/z 154 for MPP⁺. Quantitation was achieved by comparing the detected peak areas with the standard.

Dopamine Neuron Quantification.

Dopaminergic neurons of the SNc were quantified by indirect immunofluorescent labeling of free floating, 40 μ m thick, cryotome cut sections of midbrain using rabbit anti-mouse tyrosine hydroxylase (1:1000, Abcam, Cambridge, MA) followed by Alexafluor 488 anti-rabbit IgG secondary (Life Technologies). Sections were mounted using Prolong Gold with DAPI (Life Technologies). Stereologic analysis was performed using a Leica DM2500 (Leica Microsystems, Chicago, IL), and Stereologer computer assisted stereology system (Stereology Resource Center, St. Petersburg, FL). Every sixth section was used for subsequent stereologic analysis.

Reference spaces (SNc) were outlined under low magnification (5X) and TH⁺ neurons were counted at high magnification (63X oil immersion) with a guard distance of 2 μ m to avoid surface artifacts. The probes parameters were 10 μ m³ and spaced 150 μ m apart.

The counting criteria were TH immunoreactivity in the cytoplasm of cells with neuronal morphology, including a clear nuclear membrane and nucleolus. The optical fractionators was used to determine total TH⁺ neuron number with the following sampling fractions: section sampling fraction, area sampling fraction, and total sampling fraction.

Immunofluorescent Staining for Activated Astrocytes and Microglia. Free-floating serial midbrain sections were washed in PBS, then in PBS with 0.1% triton X-100 and blocked with 5% goat serum in PBS with 0.1% triton X-100. Slides were then incubated in primary antibody (GFAP 1:500, Abcam; Mac-1 1:400, Abcam), washed, incubated with the secondary antibody-conjugated to AlexaFluor 568 or AlexaFluor 488 (Life Technologies), washed, mounted on charged glass slides and dried. Coverslips were mounted with Prolong Gold containing 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies).

Statistical Analysis. GraphPad Prism© version 5 software (GraphPad Software, La Jolla, CA) was used for statistical analysis. Differences among groups were determined by either a two-tailed t test when only two groups were compared, or a one-way analysis of variance with a Newman-Keul's posthoc test. Differences were considered statistically significant at $p < 0.05$.

Results

Accumulation and efflux of paraquat in a human cerebral microvascular endothelial cell line (hCMEC/D3). To determine whether paraquat is transported by MDR1 in a cell line naturally expressing MDR1, a cell line modeling the human blood brain barrier was used. The hCMEC/D3 cell line was previously characterized with regards to presence of efflux transporters (Poller, Gutmann et al. 2008). We confirmed the presence of MDR1 protein in these cells by western blot (Fig. 2-1A) and assessed function using the MDR1 substrate, rhodamine 123, and pharmacological and genetic knockdown of transport activity (Fig. 2-1B). Minimal intracellular rhodamine fluorescence was detected in hCMEC/D3 cells (-PSC833), but was increased 900% in the presence of the MDR1 specific inhibitor PSC833, indicating these cells exhibited functional MDR1. To further confirm that MDR1 was responsible for the efflux of rhodamine 123, MDR1/P-glycoprotein expression was knocked down using siRNA. MDR1/P-glycoprotein knockdown in hCMEC/D3 cells was confirmed by western blot (55% decrease in protein levels) (Fig. 2-1C), and reduced function was confirmed by enhanced retention of Rhodamine 123 (52% increase in fluorescence) compared to the scrambled sequence siRNA (Fig. 2-1D).

Transport of paraquat was assessed in the hCMEC/D3 cells by pre-loading cells with paraquat and measuring residual intracellular paraquat and effluxed paraquat following a 30 minute efflux period (Fig. 2-2). hCMEC/D3 cells preloaded with 100 μ M paraquat were able to efficiently efflux paraquat (Control) and had little residual intracellular paraquat (Fig. 2-2A). In contrast, hCMEC/D3 cells preloaded with paraquat in the

presence of PSC833 were not able to efflux paraquat as efficiently and had 100% more intracellular paraquat. hCMEC/D3 cells with reduced MDR1 levels following siRNA knock-down had 300% more intracellular retention of paraquat than control. For efflux into the media, hCMEC/D3 with functional MDR1 were able to transport paraquat into the medium (Fig 2-2B). hCMEC/D3 cells in the presence of the inhibitor, PSC833, or with MDR1 knock-down had less than 50% of paraquat present in the medium compared to control.

Reduced cytotoxicity in hCMEC/D3 endothelial cells. Although the BBB is not considered a primary pathologic target for paraquat toxicity, the lactate dehydrogenase (LDH) release assay was used to determine if MDR1 generally protects cells from paraquat-induced injury. Following incubation of hCMEC/D3 cells with paraquat (up to 100 mM) for 4 hours, cells co-treated with the MDR1 inhibitor PSC833 exhibited greater LDH release than those without PSC33 at 48hrs (Fig. 2-3).

Paraquat concentrations in the midbrains of Mdr1a/1b-null and wild-type mice. Lack of Mdr1 protein in Mdr1a/1b-null mice was confirmed by western blot (Fig. 2-4A). Paraquat was quantified using LC/MS in the midbrains of mice to determine if Mdr1 prevents paraquat from crossing the BBB (Fig. 2-4B). Paraquat levels in the midbrains of Mdr1a/1b null mice were not significantly different at 2, 4, 12, 24, or 48 hours, than those of wild-type mice with functional Mdr1a/1b, however slightly higher paraquat levels (+40%) were observed in Mdr1a/1b-null mice at 2 hours. Mate1 and Oct2 proteins, which are known renal paraquat transporters, were not detected by western blot in midbrain

samples of wild-type (WT) or Mdr1a/1b-null mice but were present in kidneys (positive control, data not shown).

Paraquat-induced neurodegeneration in mice. Using unbiased stereology, total dopamine neurons stained by tyrosine hydroxylase (TH⁺) neurons were quantified in the SNc (Figure 2-5A) and shown as representative images (Figure 2-5B). Saline-treated Mdr1a/1b-null mice and WT mice had comparable numbers of TH⁺ neurons in the substantia nigra. Wild-type mice treated with a single dose of paraquat (10mg/kg, i.p.) had no change in the number of TH⁺ neurons (*green*) in the substantia nigra compared to saline-treated WT mice. Following a second dose of paraquat, WT mice exhibited a 30% loss of TH⁺ neuron staining compared to saline-treated WT mice, which is similar to prior reports (McCormack, Thiruchelvam et al. 2002). In contrast, Mdr1a/1b-null mice receiving a single dose of paraquat exhibited a 38% loss of TH⁺ neurons that was unchanged by a second injection of paraquat.

Neuroinflammation is enhanced in PQ-treated Mdr1 knockout mice

Neuroinflammation was assessed by GFAP staining of astrocytes and Mac1 staining of microglia (Fig.2-6, 2-7). GFAP staining (*red*) within the SNc was increased in all groups receiving paraquat, but was generally more prominent in Mdr1a/1b-null mice receiving either one or two doses of paraquat. Microglial activation, assessed by short, thickened processes, was observed in all mice receiving paraquat with particular prominence in WT and Mdr1a/1b-null mice receiving two doses of paraquat.

Discussion

In the present study, the capability of the MDR1/Mdr1 transporter to protect against paraquat-induced toxicity and neurodegeneration was assessed in human BBB cells and in Mdr1a/1b-null mice. The data demonstrate that MDR1 prevents the accumulation of paraquat in hCMEC/D3 endothelial cells by enhancing efflux. Functional MDR1 inhibited or reduced by the specific inhibitor, PSC833 or siRNA, respectively, resulted in elevated intracellular paraquat levels and increased cytotoxicity. As previously reported, wild-type mice require two doses of paraquat, typically 7 days apart, in order to achieve loss of approximately one-third of dopamine neurons in the SN (Purisai, McCormack et al. 2007). Here, only a single dose of paraquat was needed to cause toxicity to dopaminergic neurons in mice lacking Mdr1a and Mdr1b. Moreover, Mdr1a/1b-null mice exhibited greater staining of activated microglia and astrocytes following a single dose of paraquat than wild-type counterparts. Collectively, these data demonstrate the importance of MDR1/Mdr1 in the efflux of paraquat at the BBB and protection of dopamine neurons from degeneration.

The root cause of dopamine neuron loss in Parkinson disease is unknown but is likely the result of both genetic and environmental factors leading to persistent damage and loss of neurons. Genetic and environmental interactions are known for pesticide toxicity in other species (Merola and Eubig 2012, Kennedy, Tierney et al. 2014). For example, the ABCB1-1 Δ mutation in the Mdr1 gene predisposes dogs to neurotoxicity associated with ivermectin and other macrocyclic lactones (Merola and Eubig 2012). Additionally, in a study of 599 European PD patients, the 3435C/T single nucleotide polymorphism in MDR1 differed in patients exposed to pesticides (odds ratio = 4.74) (Zschiedrich, König

et al. 2009). Other studies also showed that the 3435C/T MDR1 polymorphism was associated with pesticide exposure in PD patients. A meta-analysis of pesticide-induced gene mutations, found more MDR1 mutations in PD patients compared to healthy controls (Liu, Ma et al. 2013). Although paraquat was not specifically examined in these studies, these findings suggest that non-functional Mdr1/MDR1 may play an important role in neurodegeneration.

Because MDR1 has such broad substrate specificity, there has been ongoing research aimed at determining whether paraquat is a substrate for MDR1. Several previous studies have suggested a protective role of MDR1 against paraquat-induced toxicities in various cell lines and *in vivo* animal models (Dinis-Oliveira, Duarte et al. 2006, Dinis-Oliveira, Remiao et al. 2006, Silva, Carmo et al. 2010, Zerín, Kim et al. 2012), including work by our laboratories demonstrating MDR1-mediated protection of the kidney from paraquat toxicity (Wen et al 2014). However, there is a paucity of data regarding the role of Mdr1 and protection against the neurotoxicity of pesticides. In the current study, we demonstrated paraquat efflux in hCMEC/D3 endothelial cells and HEK293-MDR1 overexpressing cells (data not shown) by determining the intracellular vs. extracellular concentration of paraquat following MDR1 inhibition (Fig. 2-2). We conclude that the differences in paraquat concentration in hCMEC/D3 cells were directly related to efflux by MDR1. In contrast, a recent study by Lacher and co-workers concluded that paraquat was not a substrate for MDR1 (Lacher, Gremaud et al. 2014). In their study, paraquat failed to stimulate ATP hydrolysis in plasma membranes expressing MDR1 and did not inhibit ATP hydrolysis in the presence of a known MDR1 substrate. However, our laboratory has observed false negatives using the ATPase assay including for the known

MDR1 substrate, paliperidone (Bircsak, Richardson et al. 2013). In contrast to the lack of differences in cytotoxicity in LLC and LLC-MDR1 expressing cells in the study by Lacher *et. al.*, we observed significant cytotoxicity (as assessed by LDH release) in hCMEC/D3 cells exposed to paraquat in the presence of the MDR1 specific inhibitor, PSC833 (Fig. 2-3). The differences observed between the two groups may be due to differences in the cell lines used, transfection efficiencies with resulting differences in MDR1 expression between the cell lines, paraquat concentrations and incubation times used. It should be noted that similar to their study, we did not observe significant differences in brain concentrations of paraquat between wild-type and *Mdr1a/1b*-null mice. This was unanticipated given the other findings demonstrating MDR1-mediated efflux of paraquat and protection from toxicity but may be due to the high amount of variance, the time points assessed, the need for more extensive studies investigating free versus bound paraquat, or using brain microdialysis.

Because paraquat in serum is a divalent cation (PQ^{2+}), it is largely prevented from crossing the BBB. However, sufficient amounts enter the brain and are able to activate microglia (Purisai, McCormack et al. 2007). Microglial NADPH oxidase reduces PQ^{2+} to the monovalent cation, PQ^+ . The monovalent PQ^+ is a substrate for the dopamine uptake transporter on neurons where it accumulates and causes cytotoxicity. It is also a substrate for the uptake transporter, organic cation transporter-3 present on astrocytes where it is hypothesized to act as a sink, preventing uptake by neurons (Rappold, Cui et al. 2011). Our results suggest that *Mdr1* is involved in preventing the initial uptake of paraquat into the brain by effluxing paraquat at the BBB. These data add to the knowledge regarding the overall disposition of paraquat in the brain.

Inflammation alters the expression of Mdr1 in isolated brain capillaries (Hartz 2005) and function of Mdr1 in mouse microglial BV-2 cells (Gibson, Hossain et al. 2012) and in vivo (Goralski, Hartmann et al. 2003). Taken together, the down-regulation of Mdr1 may be the priming event that allows for neurodegeneration by paraquat.

Additionally, some substances released or produced during neuronal death following paraquat toxicity may themselves be substrates for Mdr1/MDR1. The accumulation of these byproducts resulting from Mdr1/MDR1 dysfunction could propagate further neurodegeneration. Ceramide is a bioactive lipid derived from cell membrane sphingolipids that is involved in apoptosis (Hannun and Obeid 2008). It is a substrate for Mdr1 and accumulation of ceramide occurs with Mdr1 inhibition (Lee, Torchalski et al. 2011). Beta amyloid is another substrate of Mdr1 that is implicated in neurodegeneration (Yang, Shiao et al. 2011, Deo, Borson et al. 2014). Whether other mediators including, neuroprostanes, neurofurans, and protein carbonyls are substrates for Mdr1 is not known. Further studies are needed to determine the multiple protective roles of MDR1 in persistent neurodegeneration. Reducing neuroinflammation and restoring a functional BBB following neurotoxicity may represent a therapeutic approach to reduce ongoing disease.

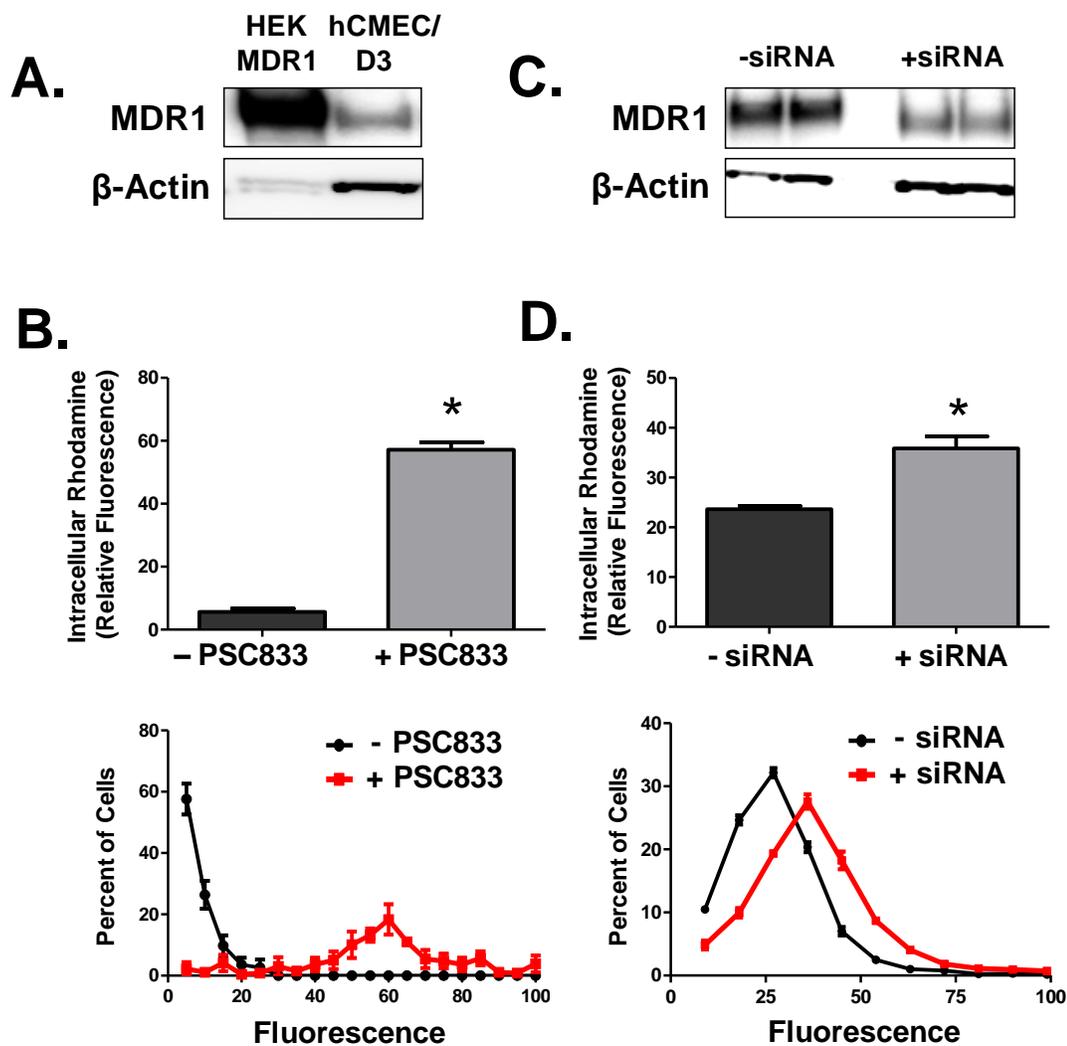


Figure 2-1

Figure 2-1. MDR1 expression and function in hCMEC/D3 cells. A) Western blot of MDR1 protein in hCMEC/D3 cells and HEK293 cells overexpressing MDR1 (positive control). β -actin was used as a loading control. B) Accumulation of the fluorescent MDR1 substrate, rhodamine 123, was quantified using the Nexcelom Cellometer Vision in the presence and absence of the specific inhibitor, PSC833 (2 μ M) C) Western blot of MDR1 protein in hCMEC/D3 cells transfected with MDR1 siRNA (+ siRNA) to knock

down MDR1 expression or FITC-tagged random sequence siRNA (-siRNA). D) Accumulation of the fluorescent MDR1 substrate, rhodamine 123, was quantified in the hCMEC/D3 cells with MDR1 knockdown (+ siRNA) or control sequence siRNA (-siRNA). Data (bar graphs) are presented as mean relative fluorescence \pm SE (n=3-4) normalized to cell size. Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control. Line graphs represent the distribution of individual cell fluorescence. Each point represents the mean percent of cells \pm SE (n=3 samples) exhibiting a quantity of fluorescence. The number of cells counted for each sample ranged from 200 to 600 cells.

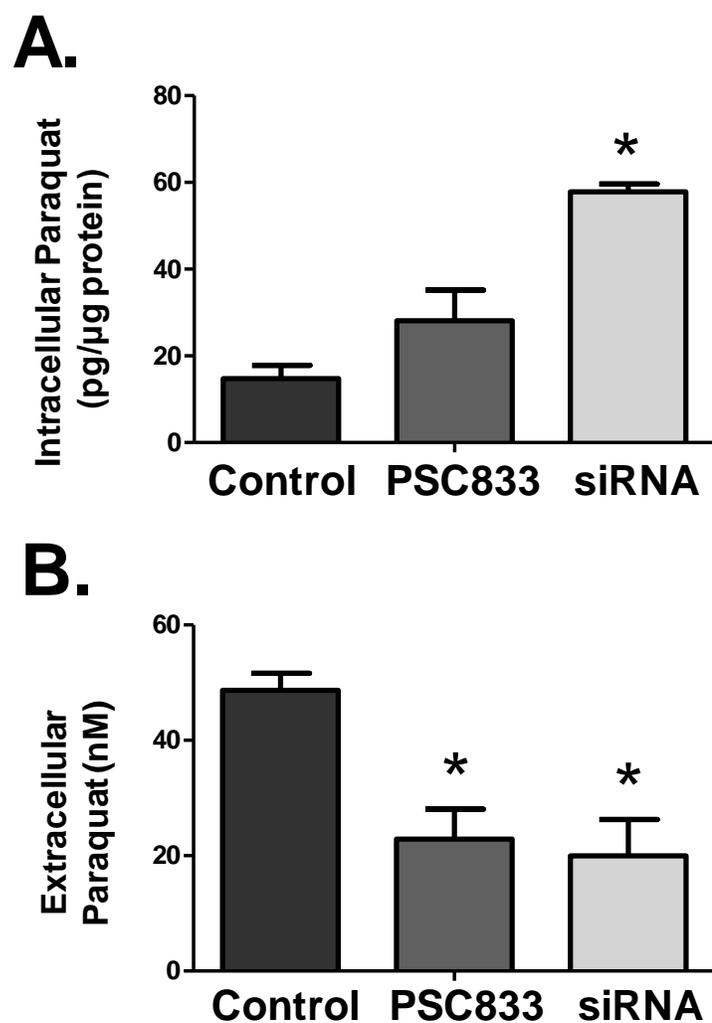


Figure 2-2

Figure 2-2. Paraquat transport in hCMEC/D3 cells. A) Intracellular paraquat was quantified following a 60 minute efflux period in control hCMEC/D3 cells, cells treated with the MDR1 specific inhibitor PSC833 (2μM), and in cells with knock down of MDR1 (siRNA). B) Extracellular paraquat was quantified in the culture media. Data are presented as mean paraquat levels normalized to protein (intracellular) or paraquat

concentration (extracellular) \pm SE (n=3). Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control.

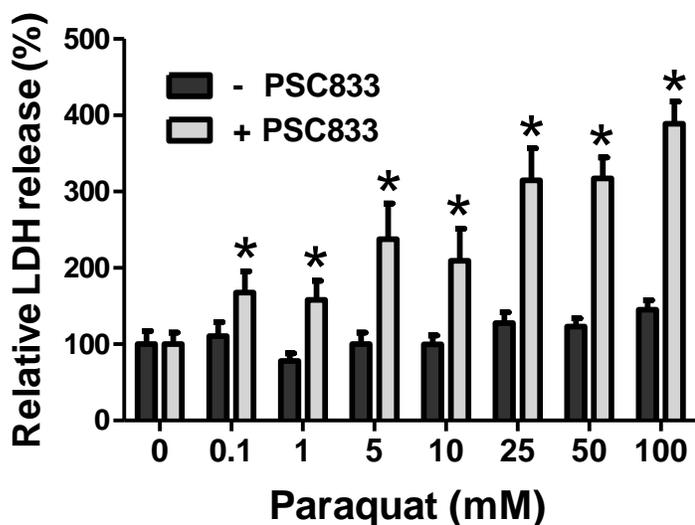


Figure 2-3

Figure 2-3. Paraquat cytotoxicity in hCMEC/D3 endothelial cells. Paraquat cytotoxicity in the presence and absence of the specific inhibitor, PSC833 (2 μ M) was assessed in hCMEC/D3 cells by measuring lactate dehydrogenase (LDH) release. Cells were incubated with paraquat for 4 hours and then placed with fresh medium. Lactate dehydrogenase release was measured 48 hours later. Data are presented as mean percent lactate dehydrogenase release relative to control (media only) \pm SE (n=5). Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to no PSC833.

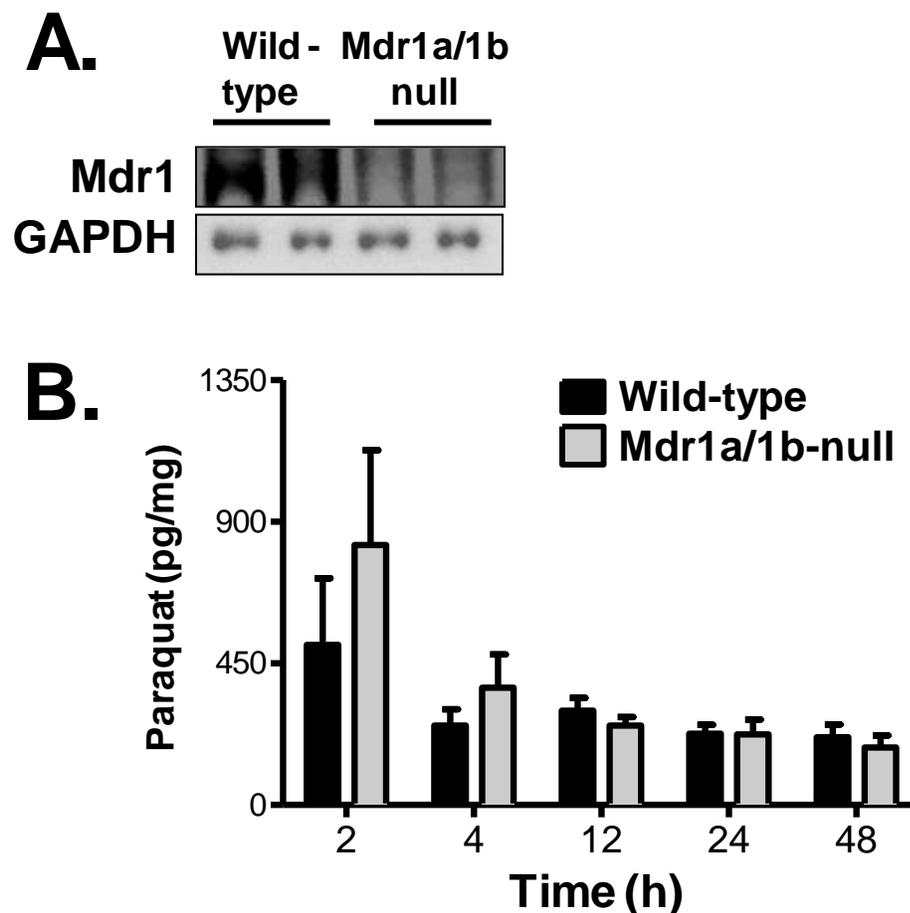


Figure 2-4

Figure 2-4. Paraquat quantification by LC/MS in the midbrains of wild-type and Mdr1a/1b-null mice. A) Western blot analysis confirms the absence of Mdr1 protein in Mdr1a/1b-null mice. B) Paraquat concentration was quantified 2, 4, 12, 24, and 48 hours in the midbrains of wild-type and Mdr1a/1b-null mice following a single intraperitoneal dose (10 mg/kg). Data are presented as mean paraquat concentration \pm SE (n=5-8).

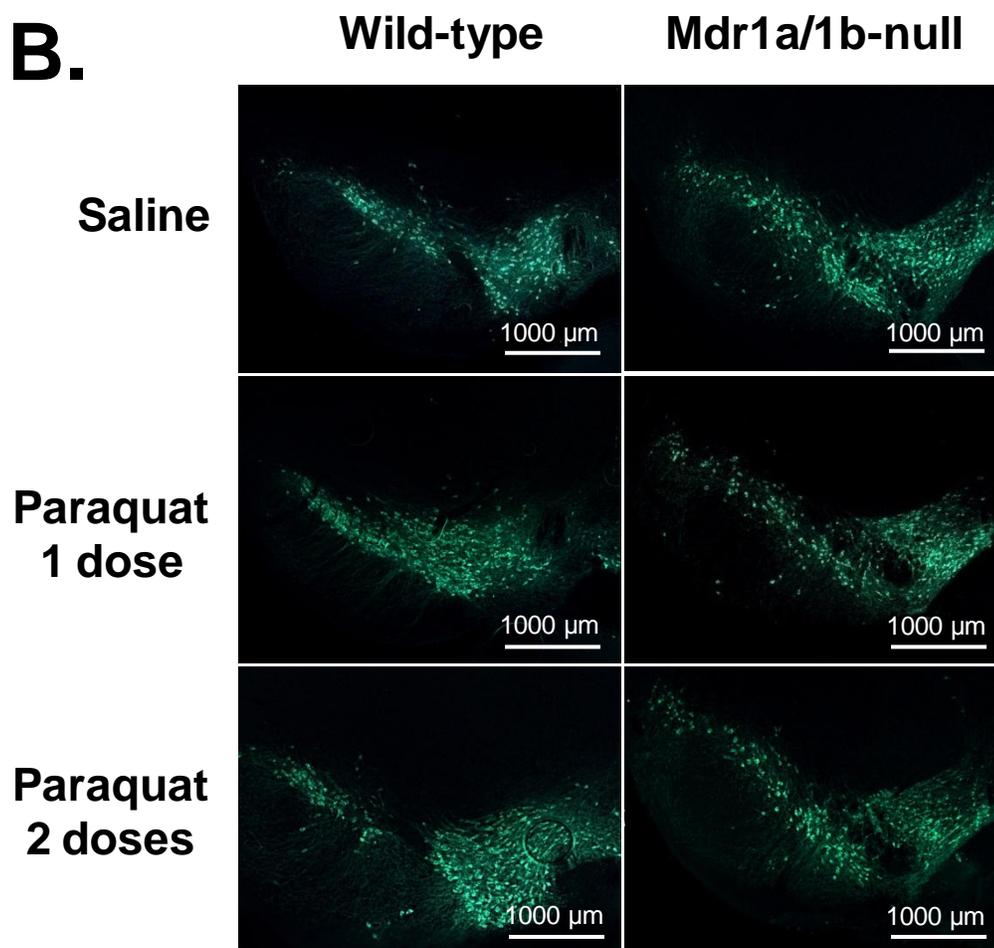
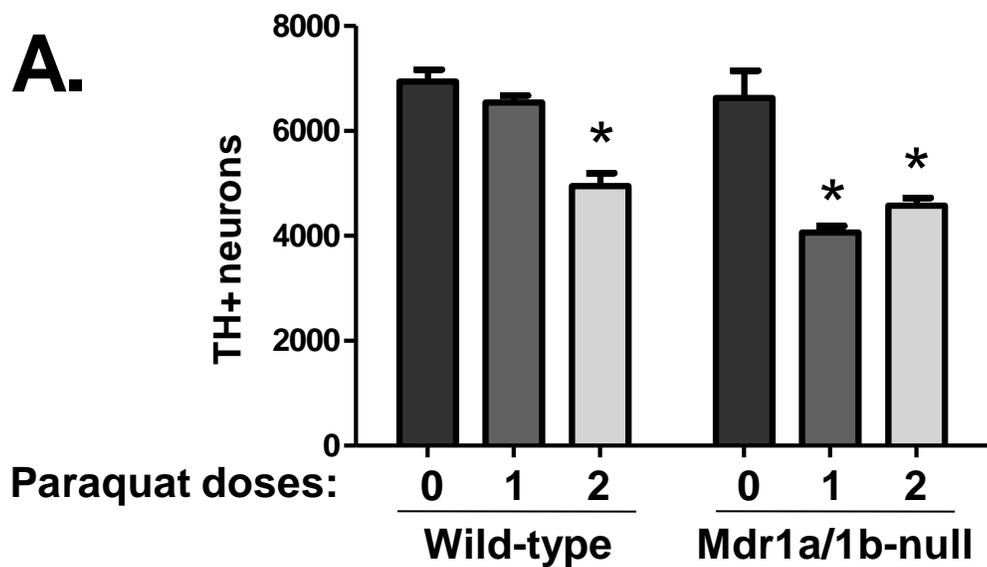


Figure 2-5

Figure 2-5. Paraquat dopamine neuron toxicity in the midbrains of wild-type and Mdr1a/1b-null mice after paraquat treatment. A) Cryosections (40 μ m) of midbrains of wild-type and Mdr1a/1b-null mice were stained for tyrosine hydroxylase (TH) 7 days after saline vehicle (5 ml/kg, i.p.) one or two doses of paraquat (10 mg/kg, i.p.). Stereology was used to quantify TH⁺ neurons in every 6th section B) Representative images of TH⁺ staining (*green*) in midbrains of wild-type and Mdr1a/1b-null mice. Data are presented as mean total numbers of TH⁺ neurons \pm SE (n=5).

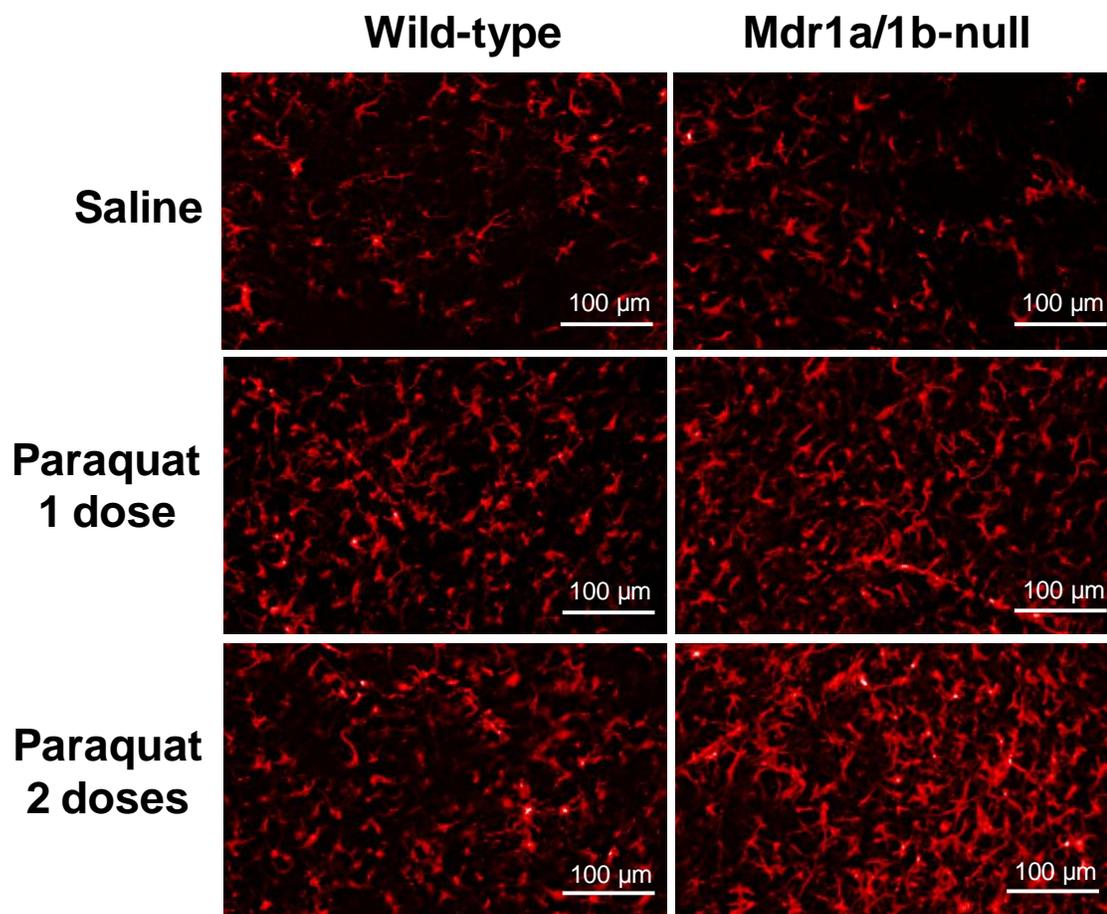


Figure 2-6

Figure 2-6. Astrocyte activation in the midbrains of wild-type and Mdr1a/1b-null mice after paraquat treatment. Cryosections (40 μm) of midbrains of wild-type and Mdr1a/1b-null mice were stained for glial fibrillary acidic protein (GFAP) protein (*red*) 7 days after saline vehicle (5 ml/kg, i.p.) one or two doses of paraquat (10 mg/kg, i.p.). Astrocyte activation was determined by increased numbers and intensity of astrocytic processes in GFAP⁺ cells.

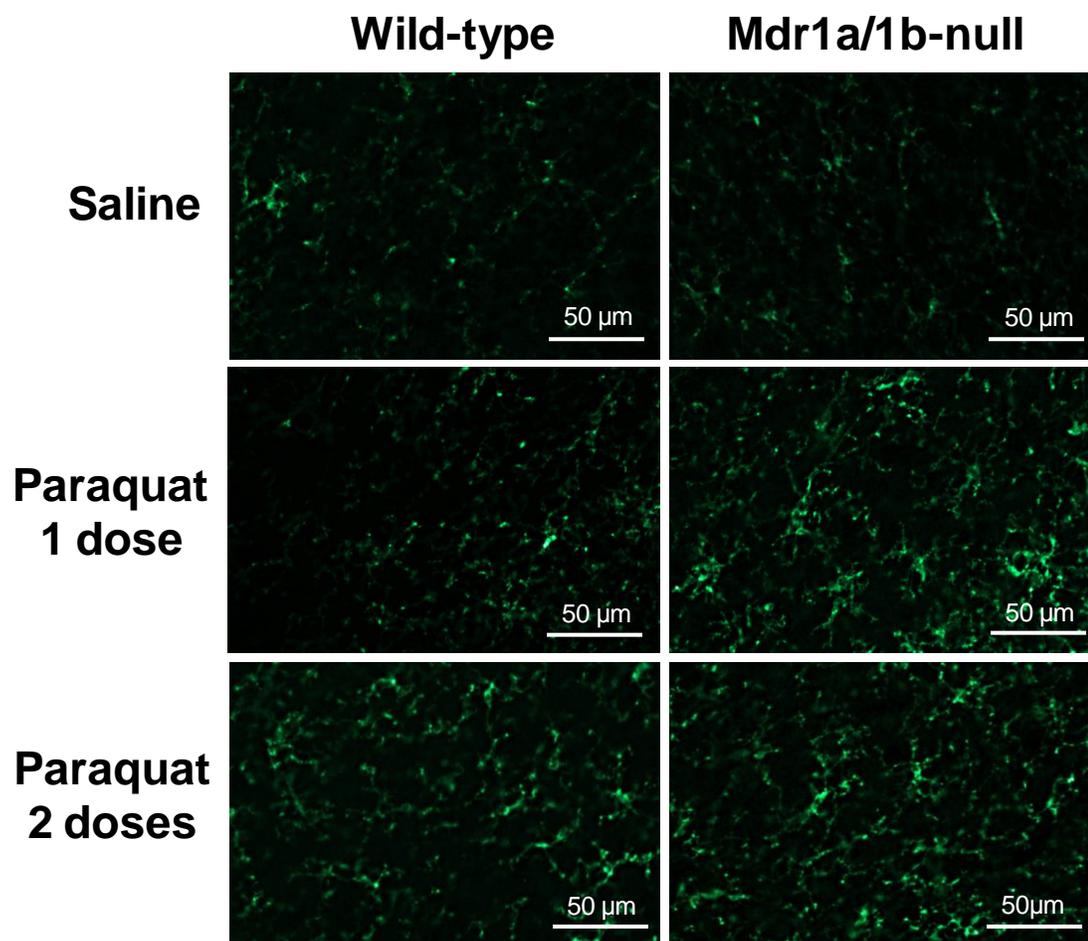
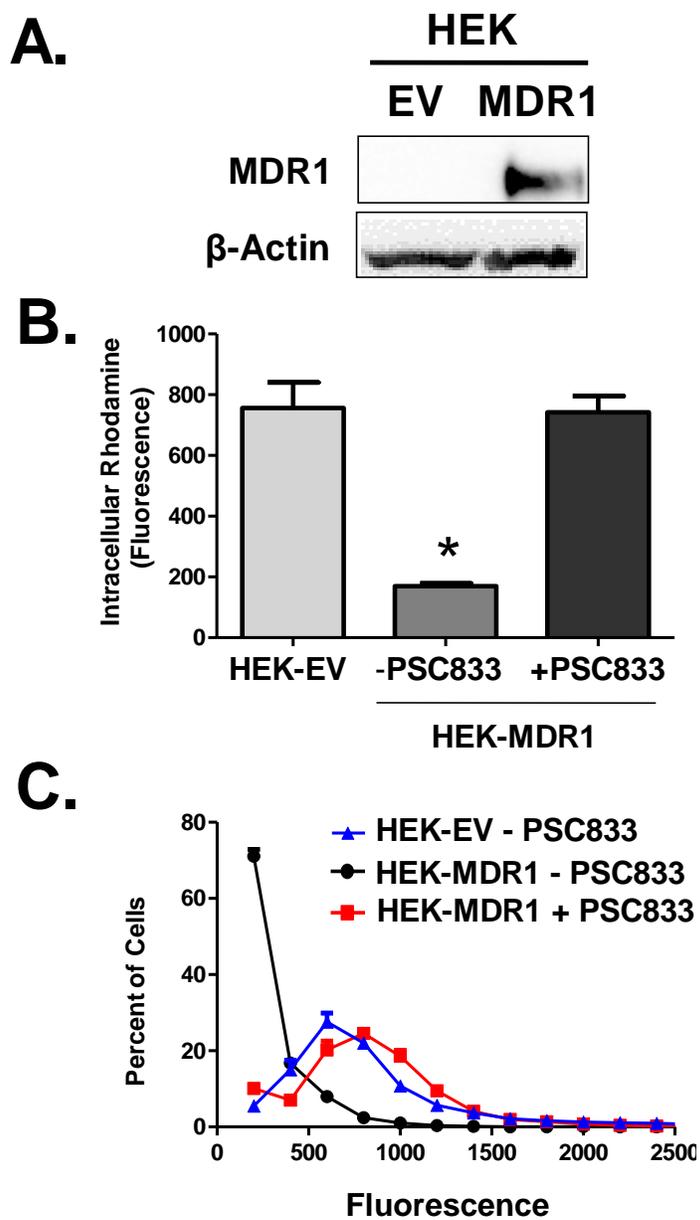


Figure 2-7

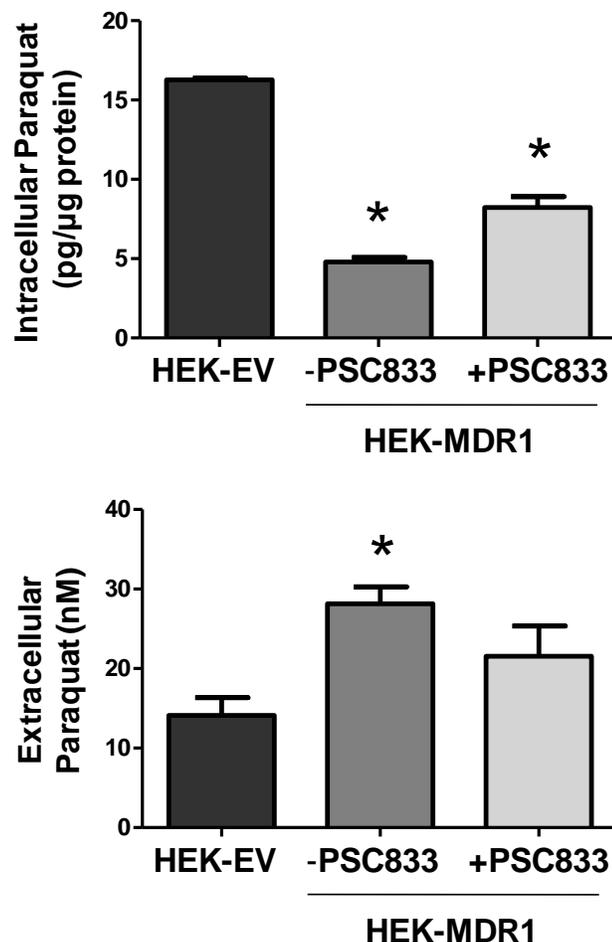
Figure 2-7. Microglial activation in the midbrains of wild-type and Mdr1a/1b-null mice after paraquat treatment. Cryosections (40 μm) of midbrains of wild-type and Mdr1a/1b-null mice were stained for Mac-1 protein (*green*) 7 days after saline vehicle (5 ml/kg, i.p.) one or two doses of paraquat (10 mg/kg, i.p.). Microglial activation was determined by larger cell bodies and thick, stubby cytoplasmic projections in Mac-1+ cells.



Supplemental Figure 2-1

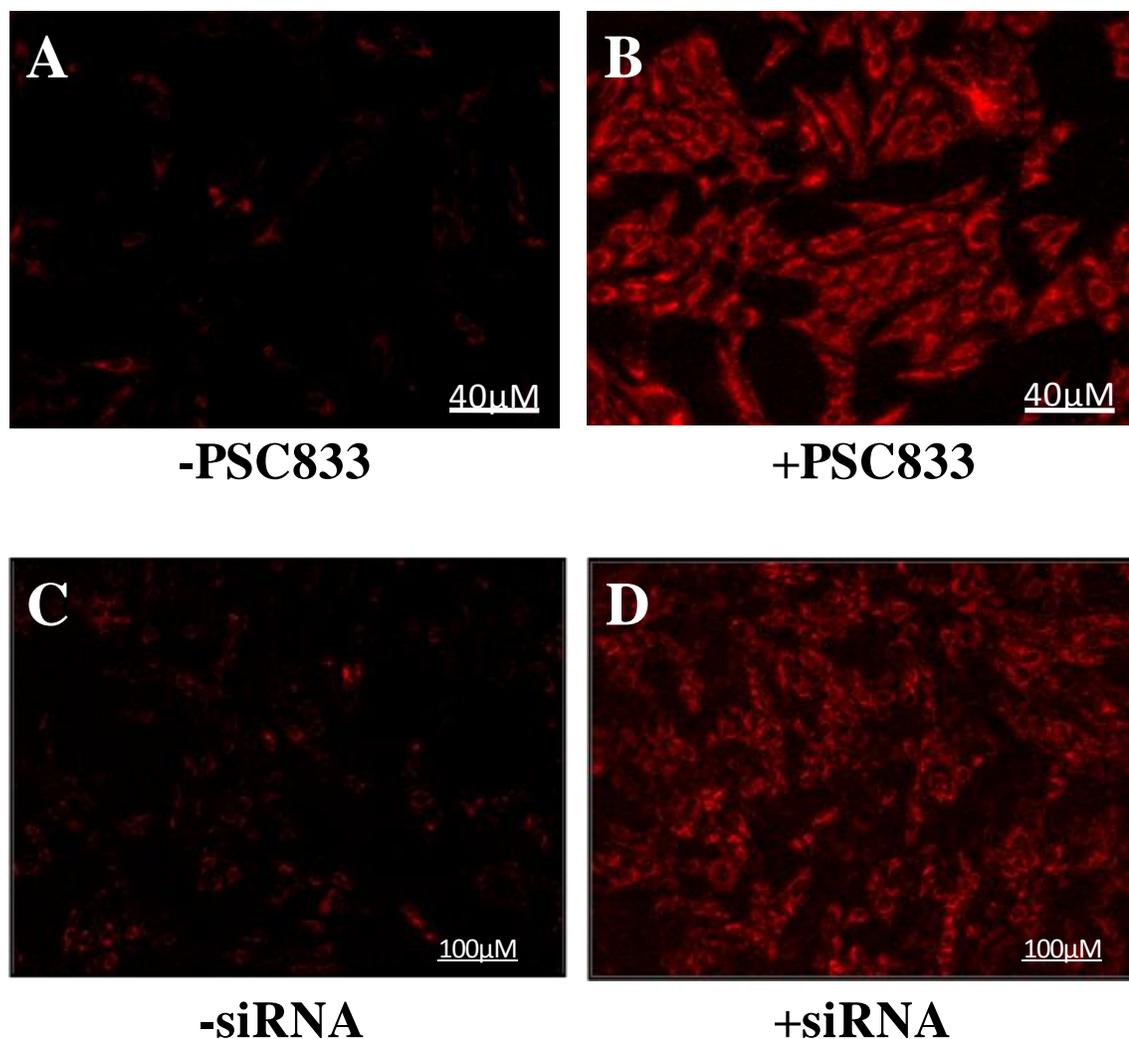
Supplemental Figure 2-1. MDR1 expression and function in HEK293 transfected cells. Initial characterization of MDR1 expression and function in HEK-EV and HEK-MDR1 transfected cells was performed. A) Western blots of cell culture lysates

demonstrate the presence of MDR1 protein in HEK-MDR1 transfected cells. B) and C) Function of MDR1 in HEK293 cells was evaluated using the Nexcelom Cellometer Vision. Fluorescent substrate, rhodamine 123, was used to characterize function. The specific inhibitor, PSC833 (1 μ M) was used as a positive control for functional inhibition. B) Data (bar graphs) are presented as mean relative fluorescence \pm SE (n=3-4). Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control. C) Line graphs represent the distribution of individual cell fluorescence. Each point represents the mean percent of cells \pm SE (n=3 samples) exhibiting a quantity of fluorescence. The number of cells counted for each sample ranged from 200 to 600 cells.



Supplemental Figure 2-2

Supplemental Figure 2-2. Paraquat transport in HEK cells. A) Intracellular paraquat was quantified following a 60 minute efflux period in control HEK cells, HEK-MDR1 cells, and HEK-MDR1 cells treated with the MDR1 specific inhibitor PSC833 (2 μ M). B) Extracellular paraquat was quantified in the culture media. Data are presented as mean paraquat levels normalized to protein (intracellular) or paraquat concentration (extracellular) \pm SE (n=3). Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control.



Supplemental Figure 2-3

Supplemental Figure 2-3. Direct fluorescent imaging of rhodamine 123 in cultured hCMEC/D3 cells. A) Following a 30 minute efflux period, little rhodamine 123 remains within hCMEC/D3 cells. B) Addition of the specific MDR1 inhibitor, PSC833, allows rhodamine 123 accumulation. C) Following a 30 minute efflux period, little rhodamine 123 is present in hCMEC/D3 cells transfected with scrambled siRNA. D) hCMEC/D3 cells with siRNA knock-down of MDR1 have retention of rhodamine 123.

CHAPTER 3

Inflammatory Regulation of ABC Efflux Transporter Expression and Function in Microglia**Abstract**

ATP-binding cassette (ABC) efflux transporters, including the multidrug resistance protein 1 (Mdr1), breast cancer resistance protein (Bcrp), and multidrug resistance-associated proteins (Mrps) extrude chemicals from the brain. While ABC transporters are critical for blood-brain barrier integrity, less attention has been placed on the regulation of these proteins in brain parenchymal cells such as microglia. Prior studies demonstrate that inflammation following lipopolysaccharide (LPS) treatment alters transporter expression in the livers of mice. Here, we sought to determine the effects of inflammation on the expression and function of transporters in microglia. To test this, the expression and function of ABC efflux transport proteins were quantified in mouse BV-2 microglial cells in response to activation with LPS. Intracellular retention of fluorescent rhodamine 123, Hoechst 33342, and calcein AM was increased in LPS-treated microglia suggesting that the function of Mdr1, Bcrp, and Mrps were decreased, respectively. LPS reduced Mdr1, Bcrp, and Mrp4 mRNA and protein expression between 40 and 70%. Conversely, LPS increased expression of Mrp1 and Mrp5 mRNA and protein. Immunofluorescent staining confirmed reduced Bcrp and Mrp4 and elevated Mrp1 and Mrp5 protein in activated microglia. Pharmacological inhibition of NF- κ B transcriptional signaling attenuated down-regulation of Mdr1a mRNA and potentiated up-regulation of Mrp5 mRNA in LPS-treated cells. Together, these data suggest that LPS stimulates

microglia and impairs efflux of prototypical ABC transporter substrates by altering mRNA and protein expression, in part through NF- κ B signaling. Decreased transporter efflux function in microglia may lead to retention of toxic chemicals and aberrant cell-cell communication during neuroinflammation.

Abbreviations

ABC, ATP binding cassette; Mdr, multidrug resistance protein; Mrp, multidrug resistance associated protein; Bcrp, breast cancer resistance protein; LPS, lipopolysaccharide; NF- κ B, nuclear factor kappa B; I κ B, inhibitor of kappa B; Nrf2, nuclear factor-e2 related factor 2; TNF, tumor necrosis factor- α ; IL, interleukin; BODIPY, boron-dipyrromethene; NBD, nitrobenzoxadiazole; DAPI, 4',6-Diamidino-2-phenylindole; Nqo1, NAD(P)H quinone oxidoreductase 1; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

Introduction

ATP-binding cassette (ABC) efflux transporters are a large group of transmembrane proteins responsible for the efflux of endogenous substances and xenobiotics. Although a significant amount of research has focused on the expression and regulation of these transporters in the liver and kidneys (Klaassen and Aleksunes 2010), research has recently focused on the localization and function of these transporters in the brain (Dallas 2006). In the brain, efflux proteins are expressed on brain capillary endothelial cells and are critical in the blood-brain-barrier extrusion of toxic chemicals and drugs from the brain parenchyma (Warren, Zerangue et al. 2009, ElAli and Hermann 2011). Members from major classes of transporters [multidrug resistance protein 1 (MDR1/ABCB1), multidrug resistance-associated protein 1-5 (MRP1-5/ABCC1-5), and the breast cancer resistance protein (BCRP/ABCG2)] have been found in one or more cell types in the brain, including neurons, astrocytes, and microglia (Klaassen and Aleksunes 2010).

Microglia are the resident immune cells of the central nervous system and have important responsibilities including neuron support and nutrition as well as immune surveillance (Streit 2002). In adult animals, microglia are generally present in a stationary, ramified state and continuously monitor the neural parenchyma through finger-like cytoplasmic projections (Czeh, Gressens et al. 2011). Microglia can be stimulated by infections, cerebral ischemia, traumatic brain injury, or neuronal/parenchymal damage to remove necrotic and apoptotic debris and coordinate immune responses (Rogers, Mastroeni et al. 2007, Henn, Lund et al. 2009, Badoer 2010, Glass, Saijo et al. 2010, Wee Yong 2010, Cao, Li et al. 2011, Czeh, Gressens et al. 2011). Activated microglia progressively

increase in number during the normal aging process and are also commonly present in neurodegenerative diseases. Activation of microglia is characterized by the production of proinflammatory cytokines as well as morphological changes, including retraction of cell processes, increased size, and an amoeboid shape (Kim and Joh 2006). The degree of activation, localization, and types of cytokines produced varies by the type of insult (Czeh, Gressens et al. 2011). After resolution of an acute response, some microglia undergo apoptosis while others return to the ramified state (Cao, Li et al. 2011). However, under certain circumstances, including neurodegenerative diseases, microglia can persist in an activated state (Kim and Joh 2006, Yasuda, Shinagawa et al. 2007, Long-Smith, Sullivan et al. 2009, Vazquez-Claverie, Garrido-Gil et al. 2009). Recently, a role for these transporters in neurodegenerative diseases was postulated since some ABC isoforms can efflux mediators of neuronal injury such as β -amyloid (Hartz 2005). Likewise, the expression of ABC transporters can be modulated by inflammation. For example, the hepatic expression and function of Mdr1a/1b are reduced in rodent models of inflammation using exogenous administration of LPS or pro-inflammatory cytokines (Ando, Nishio et al. 2001, Hartmann, Kim et al. 2001).

With recent evidence suggesting that microglia play a prominent role in brain inflammation and neurodegenerative diseases, we hypothesized that ABC transporter function is disrupted in activated microglia, which could alter cell-cell communication and chemical sequestration in the brain. *In vitro* exposure of mouse BV-2 microglia cells to lipopolysaccharide (LPS) increases expression of cytokines and alters cell morphology, which are hallmark features of microglial activation (Henn, Lund et al.

2009, Cao, Li et al. 2011). One signaling pathway implicated in microglial activation is the nuclear factor kappa B (NF- κ B) transcription factor (Cao, Kaur et al. 2011). Likewise, NF- κ B has been previously shown to regulate expression of efflux transporters (Ronaldson, Ashraf et al. 2010, Yu, Di et al. 2011). Therefore, the purpose of the present study was to 1) quantify ABC transporter function and expression in BV-2 microglia cells, 2) investigate whether transporter function and expression are altered in response to activation with LPS, and 3) determine whether inhibition of NF- κ B signaling alters transporter mRNA regulation in activated microglia.

Materials and Methods

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

Cell Culture. The immortalized mouse (C57Bl/6) microglia cell line, BV-2, was used for all experiments (Henn, Lund et al. 2009). Cells were grown in modified Eagle's medium (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 5% sodium pyruvate (Mediatech), 5% non-essential amino acids (Mediatech), and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA). Cells were incubated at 37°C with 5% CO₂. Cells were used in experiments when they reached 80 to 90% confluence. BV-2 cells were activated with LPS from *E. coli* 026:B6 (Sigma-Aldrich, St. Louis, MO) for up to 24 hours. LPS was dissolved in sterile water at a concentration of 1 mg/ml (stock solution). An equal volume of sterile water was added to all control treatments.

Cell Morphology. Cell morphology was evaluated visually with phase contrast imaging using an inverted microscope (Zeiss Axio Observer, Thornwood, NY). Cell morphology was also characterized in real-time using an xCELLigence RTCA DP analyzer (Roche, Pleasanton, CA). Briefly, BV-2 cells were trypsinized, counted, and seeded evenly (5 x 10³ cells) into each well of an E-Plate 16 (Roche). Impedance measurements were recorded every minute during the first two hours of the attachment phase and then every 15 minutes overnight. The next morning, medium was removed and cells were treated with either fresh medium containing vehicle (sterile water) or LPS (100 ng/ml) (0 h time

point). Impedance was measured every minute for 100 minutes and then every 15 minutes for 24 hours. Impedance measurements were recorded as a combined cellular index of proliferation, viability, and morphology changes.

Quantitative Transporter Function. Transporter function was quantitated on a Cellometer Vision (Nexcelom Bioscience LLC., Lawrence, MA) cell counter using fluorescent substrates. The technique was adapted from Robey and coworkers (Robey, Lin et al. 2011). Briefly, cells were grown in T75 flasks to 80% confluence, trypsinized, and seeded into round bottom wells of a 96-well plate. Cells were then centrifuged at 500 x g for 5 minutes at 5°C, resuspended, and loaded with fluorescent substrate with or without inhibitor for 30 minutes at 37°C, 5% CO₂ (*uptake period*). Fluorescent substrate and inhibitor concentrations are provided in Table 1. All transporter substrates and inhibitors were initially dissolved in dimethyl sulfoxide (DMSO). Equal volumes of DMSO were added to all controls when appropriate (final concentration of DMSO was less than 0.5%). Cells were then washed, centrifuged, resuspended, and incubated in substrate-free medium in the presence and absence of inhibitor for 1 hour (*efflux period*). Cells were then washed, resuspended in cold phosphate-buffered saline, and fluorescence quantified using the Nexcelom Vision cellometer. Twenty microliters of cell suspension were applied to the cell counting chamber and each sample was analyzed using the bright-field images for cell size and cell number. The intensity of fluorescence for each cell was subsequently analyzed using filter cubes VB-450-302 (Hoechst 33342; Excitation / Emission: 375nm/450nm), VB-535-402 (Calcein, BODIPY, NBD; Excitation / Emission: 475nm/535nm), or VB-595-502 (Rhodamine 123; Excitation / Emission:

525nm/595nm). The total number of cells analyzed for each sample ranged from 500 to 2000. Raw fluorescence intensity for each cell was normalized to cell size to account for any changes in cell size during microglial activation. The average fluorescence for each sample was determined and the treatment average was based on four independent samples for each treatment.

RNA Isolation and Real-Time Quantitative PCR (qPCR). Cultured BV-2 cells (5×10^5 cells/well) were plated onto 6-well tissue culture plates and incubated overnight until attachment. Quadruplicates were treated with vehicle, 1, 10, 100, or 500 ng/ml LPS in fresh medium. After 12 hours of incubation, cell cultures were washed twice with PBS and lysed with Buffer RLT (Qiagen, Valencia, CA) containing 1% β -mercaptoethanol. Total RNA was isolated using the RNeasy mini kit (Qiagen). Complimentary DNA (cDNA) was generated with the First Strand SuperScript cDNA synthesis kit (Invitrogen). cDNA purity and concentration were assessed using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Rockford, IL). For qPCR, specific forward and reverse primers (Integrated DNA Technologies, Coralville, IA) for each gene were added to one microgram of cDNA from each sample. Sybr Green (Applied Biosystems, Carlesbad, CA) was used for detection of amplified products. qPCR was performed in a 384-well plate format using the ABI 7900HT PCR system (Applied Biosystems). Ct values were converted to delta delta Ct values by comparing to a reference gene, ribosomal protein 13a (Rpl13a). Primer sequences for all target mRNAs are provided in Supplemental Table 1.

Western Blot Analysis. BV-2 cells (1×10^6 cells) were plated onto 60mm cell culture dishes and allowed to attach overnight. Triplicates were treated with fresh media containing vehicle, 1, 10, 100, or 500 ng/ml LPS and incubated at 37°C for 24 hours. Cells were then washed twice in PBS. Cells were collected, transferred to a microcentrifuge tube, and centrifuged at 1000 x g for 10 minutes. The supernatant was removed and the pellet was resuspended in cell lysis buffer with protease inhibitors. Protein concentration was determined using the bovine serum albumin protein kit (Thermo-Scientific). Western blot analysis of ABC transporters was performed using 50 µg protein homogenate per well by SDS-PAGE on NuPage 8% gels (Invitrogen). Proteins were transferred overnight to a polyvinylidene fluoride membrane. Membranes were blocked in 5% nonfat dry milk in phosphate-buffered saline (PBS) with 0.5% Tween-20. Primary antibodies were diluted in 2% nonfat dry milk and incubated with membranes for 3 hours at the following concentrations: 1:1000 for Mdr1a/b (C219, Novus Biologicals, Littleton, CO), 1:2000 for Mrp1 (MRPr1, Alexis/Enzo Life Sciences, Farmingdale, NY), 1:2000 for Mrp4 (M₄I-80, Alexis/Enzo), 1:500 for Mrp5 (M₅I-10, G. Scheffer, VU Medical Center, Amsterdam, The Netherlands), 1:5000 for Bcrp (BXP-53, Alexis/Enzo), and 1:5000 for Gapdh (Millipore, Temecula, CA). Primary antibodies were probed using species-appropriate secondary antibodies (Sigma) and detected using SuperSignal West Dura blotting reagents (Thermo-Scientific). Detection and semi-quantification of protein bands was performed using a FluorChem imager (Alpha Innotech, San Leandro, CA).

Indirect Immunofluorescent Staining. BV-2 cells (5×10^5 cells) were seeded into chambers of a chamber slide (Fisher Scientific) and allowed to attach overnight. The following day, cells were washed twice in PBS. Chambers were either filled with medium alone or medium plus 100 ng/ml LPS and incubated for 24 hours. Slides were fixed with 4% paraformaldehyde, washed twice in PBS, once in PBS with 0.1% triton X-100 and then blocked with 5% goat serum in PBS with 0.1% triton X-100. Slides were then incubated in primary antibody or 5% goat serum, washed, incubated again with the secondary antibody-conjugated to AlexaFluor 488 (Molecular Probes, Eugene, OR), washed, and dried. Slides were coverslipped with Prolong Gold containing 4',6-Diamidino-2-phenylindole (DAPI) (Molecular Probes). Images were acquired on a Zeiss Observer D1 microscope (Zeiss Inc., Thornwood, NY) with an X-Cite series 120Q fluorescent illuminator and a Jenoptik camera with ProgRes CapturePro 2.8 software (Jenoptik, Easthampton, MA). Images were cropped and brightness and contrast were adjusted equally in Adobe Photoshop CS2 (San Jose, CA). All sections were both stained and imaged under uniform conditions for each antibody. Negative controls without primary antibody were included to ensure minimal non-specific staining (data not shown).

NF- κ B inhibition. Cultured BV-2 cells (5×10^5 cells/well) were pre-incubated with vehicle (DMSO) or the NF- κ B inhibitor (5 μ M) InSolution™ BAY 11-7082 (EMD Biosciences, San Diego, CA) for 30 minutes in triplicate. After pre-incubation, cells were treated with vehicle or 100 ng/ml LPS. After 12 hours of incubation, cell culture medium was removed and frozen for quantification of tumor necrosis factor (TNF) protein. Cell

cultures were washed and lysed. RNA isolation, cDNA synthesis, and qPCR were performed as described above.

TNF protein quantification. TNF levels were quantified in cell culture medium using a mouse ELISA kit (Invitrogen) according to the manufacturer's instructions.

Statistical Analysis. GraphPad Prism© version 5 software (GraphPad Software, La Jolla, CA) was used for statistical analysis. Differences among groups were determined by either a two-tailed t test when only two groups were compared, or a one-way analysis of variance with a Newman-Keul's posthoc test. Differences were considered statistically significant at $p < 0.05$.

Results

Basal expression and function of ABC efflux transporters in BV-2 cells. Resting BV-2 microglia express ABC transporter mRNA including Mrp1-5, Mdr1a, Mdr1b, and Bcrp. Messenger RNA expression in BV-2 cells was compared to mouse liver after normalizing to the reference gene Rpl13a (Table 3-2). The expression of transporter mRNA differed between BV-2 microglia and mouse liver. After normalizing to Rpl13a, BV-2 microglia had negligible amounts of Mrp2 mRNA compared to mouse liver. BV-2 cells also expressed lower levels of Mrp3, and Bcrp mRNA. In contrast, microglia expressed high levels of Mrp1, Mrp4, Mrp5, Mdr1a, and Mdr1b mRNA compared to mouse liver. Basal transporter function was quantified in control BV-2 cells using a fluorescent cell counter (Fig. 3-1) as described by Robey and coworkers. A panel of fluorescent chemicals (rhodamine, calcein AM, and Hoechst 33342) and inhibitors against various ABC transporters were tested (Table 3-1). Inhibition of MDR1 with PSC833 significantly increased accumulation of rhodamine 123 in BV-2 cells by 84%. Compared to control cells, MK-571, a general inhibitor of MRPs, significantly enhanced calcein AM fluorescence by 108%. The BCRP inhibitor KO143 significantly increased Hoechst 33342 levels by 51% (Fig 3-1A). Images demonstrating fluorescent dye retention in BV-2 cells in the presence of prototypical inhibitors are shown in Fig 3-1B.

Lipopolysaccharide activation of BV-2 microglia. Altered BV-2 cell morphology in response to LPS was assessed quantitatively by changes in impedance using real-time measurements (Fig 3-2A) and qualitatively by light microscopy (Supplemental Fig. 3-1). Greater electrical impedances across interdigitating microelectrodes on the bottom of the

culture plate reflect collective changes in cell morphology, viability, and proliferation. As cells occupy a larger surface area on the electrodes, the path that a small electrical current must travel through the cell membrane is longer which results in increased electrical impedance. In the present study, the early attachment and longer growth phases of plated BV-2 cells were clearly observed with slow, gradual increases in impedance over 24 hours (Time = -24 to 0 hours). Upon addition of 100 ng/ml LPS (Time = 0 hours), an immediate and dramatic change in the impedance was detected in LPS-treated cells (*blue*) as compared to control cells (*red*). The peak of the impedance difference between control and LPS-treated cells occurred 4 hours after addition of LPS. Morphological changes were confirmed at 6 hours with phase contrast microscopy (Supplemental Figs. 3-1A and B). BV-2 cells without exposure to LPS were 10 to 15 μm in diameter, uniformly round, with occasional thin cytoplasmic projections. After LPS activation, BV-2 cells were much larger in diameter (25 to 30 μm), polymorphic, with many exhibiting thick cytoplasmic projections. Compared to vehicle-treated cells, incubation with 100 ng/ml LPS for 24 hours did not alter cell number or cell viability as determined by the alamar Blue assay (data not shown). These data suggest that the quantitative differences in cell impedance were largely due to the observed morphologic changes. By 20 hours, cell impedance values were similar between control- and LPS-treated cells. In addition to morphologic changes, activated microglia produced pro-inflammatory cytokines including TNF, interleukin-1 β (IL-1 β), and interleukin-6 (IL-6). Messenger RNA levels of TNF, IL-1 β , and IL-6 were dose-dependently increased in BV-2 cells in response to LPS at 12 hours (Fig. 3-2B). Stimulation of TNF cytokine mRNA expression was achieved even at the lowest concentration of LPS (1 ng/ml).

Activation of microglia decreases efflux transporter function and increases intracellular retention of substrates. Activation of BV-2 microglia with 100 ng/ml LPS for 24 hours enhanced intracellular accumulation of all fluorescent substrates tested (Figs. 3-3A and 3-3B). Retention of rhodamine 123, an Mdr1 substrate, was increased approximately 25% in LPS-activated microglia (Fig. 3-3A). The distribution curve shows a larger percentage of cells had more fluorescence indicated by a right shift in the curve. Retention of calcein AM, an Mrp substrate, increased 110% in LPS-treated microglia. Most control BV-2 cells had minimal calcein AM fluorescence suggesting efficient efflux during the 1 hour incubation period. In contrast, the majority of activated microglia exhibited strong retention of calcein AM, indicated by a right shift in the distribution of cellular fluorescence. Hoechst 33342, a Bcrp substrate, also shows greater average fluorescence and retention in activated microglia, although at a much lower degree than the other efflux transporters. Two additional chemicals including a pharmaceutical and an endogenous signaling molecule were included as examples of relevant ABC efflux substrates. Efflux of the α -adrenergic antagonist, prazosin, and the pro-apoptotic molecule, ceramide, was also inhibited by LPS treatment (Fig. 3-3B). Mean fluorescence for prazosin and ceramide increased 39% and 90%, respectively, after microglial activation with LPS. Images demonstrating fluorescent dye retention in control- and LPS-treated BV-2 cells are shown in Supplemental Fig. 3-2. Uptake of all substrates tested was not altered by LPS treatment (data not shown).

Decreased efflux transporter function is paralleled by down-regulation of transporter mRNA. Messenger RNA expression of ABC transporters was quantified after microglial activation with LPS treatment (Fig. 3-4). Transporter mRNA expression of most efflux transporters decreased by 12 hours after initiation of LPS treatment. Mrp2 and Mrp3 mRNA decreased 60% at the lowest concentration (1 ng/ml LPS) with no further declines observed at higher LPS concentrations. All other efflux transporters exhibited concentration-dependent changes with increasing LPS concentrations. In BV-2 cells treated with 500 ng/ml LPS, levels of Mrp1 and Mrp5 mRNA rose by 150% and 300%, respectively, whereas Mdr1a, Mdr1b, and Bcrp mRNAs were reduced by 70%, 75%, and 55%, respectively.

Transporter protein expression is consistent with altered function and mRNA levels.

Western blot analysis of transporter proteins showed high basal protein expression of Mdr1, Bcrp, Mrp1, and Mrp4 in cultured BV-2 microglia (Fig. 3-5). Mouse liver, kidney, and placenta homogenates were used as positive controls for each transporter (data not shown). Mrp5 basal protein expression was relatively low. Upon LPS stimulation of BV-2 microglia for 24 hours, Mrp4, Mdr1, and Bcrp proteins were significantly decreased by up to 55%, 42%, and 42%, respectively. Mrp1 and Mrp5 proteins were elevated up to 140% and 210%, respectively, after LPS treatment. Protein expression for all transporters followed the same pattern as their respective mRNAs. Notably, Mrp2 and Mrp3 proteins were neither present in control cells nor induced by LPS (data not shown). Qualitative transporter expression was assessed in paraformaldehyde-fixed BV-2 cells using specific transporter antibodies (Fig. 3-6). Treatment of BV-2 cells with 100 ng/ml

LPS for 24 hours intensified the diffuse and plasma membrane staining of Mrp1 and Mrp5 proteins and resulted in multiple intensely fluorescent foci. Decreased staining of Mrp4 and Bcrp proteins was observed in LPS-treated cells. There are no commercial antibodies capable of selectively staining Mdr1 protein in cells of mouse origin.

Inhibition of NF- κ B attenuates LPS-induced Mdr1a down-regulation and potentiates Mrp5 up-regulation. LPS activation of the toll-like receptor-4 results in downstream NF- κ B activation through phosphorylation of I κ B, the inhibitory protein of NF- κ B. NF- κ B is then free to enter the nucleus where it increases the mRNA transcription of several genes including TNF, IL-1 β , and IL-6. BAY 11-7082 inhibits the phosphorylation of I κ B, resulting in the stabilization of the I κ B/NF- κ B complex and inhibition of NF- κ B-mediated transcription (Min et al., 2011). Compared to LPS alone, co-treatment of BV-2 cells with LPS and BAY 11-7082 attenuated IL-1 β and IL-6 cytokine mRNA induction by approximately 75% (Fig. 3-7A and B). Surprisingly, TNF α mRNA was increased further by co-treatment of cells with LPS and BAY 11-7082 (Fig 3-7C). However, secreted TNF protein in the cell culture media of BAY 11-7082 treated cells was decreased by approximately 75%, confirming inactivation of NF- κ B signaling (Fig. 3-7D).

Inhibition of NF- κ B with BAY 11-7082 attenuated the decrease in Mdr1a mRNA that resulted from LPS activation of microglia (Fig 3-8). Treatment with BAY 11-7082 did not affect the LPS-induced down-regulation of Bcrp mRNA. BAY 11-7082 also caused an increase in Mrp5 mRNA in control cells and potentiated the increase of Mrp5 in LPS-

treated cells (Fig. 3-9). Although not statistically significant, Mrp1 mRNA showed a similar trend to Mrp5 using BAY 11-7082. In order to further investigate the enhancement of Mrp5 mRNA by BAY 11-7082, the mRNA expression of the oxidative stress transcription factor nuclear factor e2-related factor 2 (Nrf2) and its target genes NAD(P)H quinone oxidoreductase 1 (Nqo1) and heme oxygenase-1 were quantified. This signaling pathway has been previously shown to regulate expression of some Mrps (Maher, Dieter et al. 2007). Interestingly, BAY 11-7082 increased Nrf2 mRNA in LPS-treated cells and elevated mRNA levels of Nqo1 (Fig 3-9) and heme oxygenase-1 (data not shown) in control- and LPS-treated cells.

Discussion

The present study investigated the regulation of ABC efflux transporter function in activated microglia. Significant reductions in the cellular accumulation of fluorescent substrates after a 60-minute efflux period demonstrated basal activity of Mrps, Mdr1, and Bcrp transporters in BV-2 cells (Fig. 3-1). On the other hand, LPS-treated microglia had greater intracellular fluorescence after the same time interval, which suggested that microglial activation decreased the function of efflux transporters (Fig. 3-3 and Supplemental Fig. 3-2, LPS). The retention of fluorescent substrates was similar to, and in some cases greater than, the retention observed in BV-2 cells following incubation with known transporter inhibitors. These data indicate that efficient efflux of chemicals in resting microglia is impaired during microglial activation and neuroinflammation.

BV-2 microglia as a model for microglial activation. BV-2 cells are immortalized mouse microglia that produce a wide variety of cytokines and other substances, similar to *in vivo* inflammatory responses, making them an ideal tool for mechanistic studies (Henn, Lund et al. 2009). In this study, we confirmed the presence of ABC efflux transporters in BV-2 cells and showed that they are similar to primary microglia isolated from other species as well as results from *in vivo* rodent studies. Several groups have shown that cultured rat primary microglia and the rat MLS-9 microglia cell line express functional Mrp1, Mrp4, Mrp5, and Mdr1 proteins but are deficient in Mrp2 protein (Lee, Schlichter et al. 2001, Ballerini, Di Iorio et al. 2002, Hirrlinger, König et al. 2002, Dallas 2003, Dallas 2004). Likewise, the MLS-9 cell line has been shown to lack Bcrp-mediated efflux of mitoxantrone. This is similar to our data demonstrating modest differences in Bcrp-

mediated Hoechst 33342 retention in control, LPS-treated, and KO143-treated BV-2 cells (Lee, Babakhanian et al. 2007). Additionally, Dauchy and coworkers determined that MRP2 and MRP3 mRNA are not present in human cortex or isolated microvessels and concluded that BCRP and MDR1 represent the primary ABC transporters in human microvessels with relatively low amounts of MRP1, MRP4, and MRP5 (Dauchy, Dutheil et al. 2008). Human whole cerebral cortex samples, on the other hand, had greater expression of MRP5, BCRP, and MRP1 mRNA, suggesting that other cell types including microglia express a unique set of efflux transporters (Dauchy, Dutheil et al. 2008, Warren, Zerangue et al. 2009).

ABC transporter protein response to inflammation. Although ABC transporters function similarly to export substrates from cells, there are likely different regulatory mechanisms for each transporter. This is supported by the fact that Mrp1 and Mrp5 mRNA and protein were up-regulated by LPS whereas all other transporters were decreased. The decreased function of Mrp1 (calcein AM retention, Fig. 3-3), despite the up-regulation of mRNA and protein, may be due to altered membrane trafficking, loss of a transport co-substrate, or a compensatory response. The multiple fluorescent foci of Mrp1 staining in LPS-treated cells (Fig. 3-6) suggest that trafficking to the plasma membrane may be altered despite an increase in total protein levels. This pattern of staining was not seen with any other treatment. Alternatively, functional inhibition of Mrp1 may result from the depletion of glutathione (GSH), which is a transport co-substrate. Resting microglia have high amounts of GSH which is rapidly oxidized in activated microglia that are producing reactive oxygen species (Hirrlinger, Gutterer et al. 2000, Rudd, Kabler et al. 2011). GSH

depletion could lead to decreased function of Mrp1. It is also conceivable that up-regulation of Mrp1 mRNA and protein may be a compensatory response to impaired function. Further investigation into these mechanisms is needed to elucidate the discrepancy observed between increased Mrp1 transporter expression and reduced function after LPS treatment.

Inflammation causes similar changes in transporter expression in other tissues such as the liver. Interestingly, Mrp1 and Mrp5 mRNA in livers of mice exposed to LPS are up-regulated, similar to activated BV-2 microglia (Lickteig, Slitt et al. 2006). LPS also decreases the function, mRNA, and protein expression of Mdr1 in rodent livers (Piquette-Miller, Pak et al. 1998). Additional studies point to IL-6 and/or TNF as key cytokines responsible for hepatic Mdr1 down-regulation in rodents during inflammation (Ando, Nishio et al. 2001, Hartmann, Kim et al. 2001). Regulation of Mdr1 expression in the brain by LPS is less consistent; however, there appears to be a uniform decline in function in human cell culture models and rodent studies regardless of expression levels (Goralski, Hartmann et al. 2003, Hartz 2005).

Further investigation into the mechanism underlying diminished function in LPS-treated microglia revealed that, with the exception of Mrp1, transporter function paralleled time-dependent decreases in mRNA and protein expression. Cytokine mRNA (TNF, IL-1 β , and IL-6) were up-regulated 6 hours after LPS activation (data not shown) which coincided with morphologic changes in BV-2 microglia. It was not until 12 hours that mRNA changes in transporter genes began to be observed. The earliest alteration of

transporter protein expression occurred with 24 hour incubation with LPS. These temporal changes suggest that impaired transporter function results from altered transcriptional regulation possibly in response to pro-inflammatory cytokines.

Role of transcription factors in ABC transporter regulation during inflammation.

Although several studies have explored mechanism(s) of inflammation-mediated alteration in P450 enzymes, few studies have investigated the signaling mechanisms underlying regulation of Mdr1 and other transporters in response to LPS treatment (Morgan, Goralski et al. 2008, Teng and Piquette-Miller 2008). From these limited studies, however, it is likely that LPS alters transcriptional regulation by modulating transcription factors and nuclear receptors. Here, inhibition of the NF- κ B transcription factor pathway, which regulates cytokine production, attenuated Mdr1a mRNA down-regulation (Fig. 3-7 and 3-8). Evidence of impaired NF- κ B signaling by BAY 11-7082 was observed in TNF protein and IL-6 and IL-1 β mRNA. Somewhat paradoxically, TNF mRNA was induced in co-treated cells at 24 h, possibly through alternate pathways to compensate for lower protein levels.

In contrast to Mdr1, Mrp1 was not affected by inhibition of the NF- κ B. In primary rat astrocytes, Ronaldson and coworkers found that expression of Mrp1 protein increased 2.7-fold after treatment with TNF and was accompanied by increased Mrp-mediated transport of the substrate 2,7-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) (Ronaldson, Ashraf et al. 2010). This finding is in contrast to the functional decrease of Mrp1 in microglial cells, as determined by increased retention of calcein AM

(Fig. 3-3). This discrepancy may be related to the inherently different functional responses of astrocytes and microglia to TNF and LPS, respectively. From a mechanistic standpoint, inhibition of NF- κ B did not prevent Mrp1 expression changes in astrocytes treated with TNF (Ronaldson, Ashraf et al. 2010). Similarly, we found that inhibiting NF- κ B in LPS-treated microglia did not interfere with Mrp1 induction, indicating that Mrp1 mRNA is not directly regulated by NF- κ B.

Nrf2 has been shown to be required for the constitutive and inducible expression of Mrp1 in cultured mouse fibroblasts (Hayashi, Suzuki et al. 2003). Likewise, pharmacological activation of Nrf2 increases Mrp5 mRNA levels in mouse liver (Maher 2005). In the present study, mRNA expression of the Nrf2 target, Nqo1, was up-regulated by LPS supporting Nrf2 activation in the current model and providing a candidate transcription factor for Mrp1 and Mrp5 up-regulation. Nrf2 activation in the LPS model is likely a result of NF- κ B, cytokine production, and oxidative stress associated with microglial activation. Others have shown Nrf2 to be important in dampening LPS-induced microglial activation and lowering the production of pro-inflammatory cytokines in BV-2 cells (Koh, Cha et al. 2009, Koh, Kim et al. 2011, Lee, Ryu et al. 2012). Interestingly, inhibition of NF- κ B in microglia using BAY 11-7082 resulted in the up-regulation of Nqo1 rather than down-regulation as would be expected if there was direct cross-talk between NF- κ B and Nrf2. One possible explanation is that BAY 11-7082 also acts independent of NF- κ B to activate Nrf2. In fact, BAY 11-7082 has been shown to increase mRNA and protein of Nrf2 responsive genes in a Nrf2-dependent manner in the

HT29 human colorectal cell line likely through production of reactive oxygen species (Min, Lee et al. 2011).

Implications of altered ABC transport function in neuroinflammation. Our study provides novel information regarding efflux processes in activated microglia that may alter signaling with other neuronal cells. Amyloid beta, lipid peroxides, prostaglandins, pesticides, and other chemicals have been found to either influence the expression of, or are themselves substrates for, ABC efflux transporters (Sreeramulu, Liu et al. 2007, Oosterhuis, Vukman et al. 2008, Kania, Wijesuriya et al. 2011, Rudd, Kabler et al. 2011, Yang, Shiao et al. 2011). Interestingly, altered cyclic AMP and GMP levels, substrates of Mrp4 and Mrp5, have been observed in the striatum of experimental models of Parkinson disease (Giorgi, Melchiorri et al. 2011). The pro-apoptotic protein ceramide was also markedly retained in LPS-activated microglia in the present study. Collectively, these findings suggest the potential ability of ABC transporters to regulate the accumulation of potentially detrimental endogenous compounds in various cells within the brain.

Activation of microglia was initially believed to be a secondary response to neurodegeneration. However, it is now widely accepted that microglia play a more active or even primary role in the initiation of neuronal injury (Kim and Joh 2006, Rogers, Mastroeni et al. 2007, Long-Smith, Sullivan et al. 2009, Wee Yong 2010, Yokoyama, Uchida et al. 2010, Cao, Li et al. 2011, Czeh, Gressens et al. 2011, Ros-Bernal, Hunot et al. 2011). Activated microglia have been detected in living Parkinson disease patients (Gerhard, Pavese et al. 2006) and observed in postmortem brain tissue from Parkinson

Disease patients (McGeer, Itagaki et al. 1988, Mirza, Hadberg et al. 2000). This phenomenon has also been observed in samples from humans with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinson Disease years after the initial insult (Langston, Forno et al. 1999, McGeer, Schwab et al. 2003) and in monkeys treated with MPTP. Activated microglia are typically implicated in direct neuronal degeneration through production and release of cytokines, reactive oxygen species, and lipid peroxidation products (Block, Zecca et al. 2007). Understanding how microglia communicate, react, and interact with the neuronal environment, particularly through the expression of transporters, may help in determining mechanisms of neurodegeneration and development of possible interventions.

Conclusions

We have demonstrated that activation of microglia with LPS alters chemical transport, which could influence direct cell-cell signaling during neurodegenerative diseases. Future studies should aim to verify altered ABC transporter expression and function in activated primary microglia and in animal models of neurodegenerative diseases.

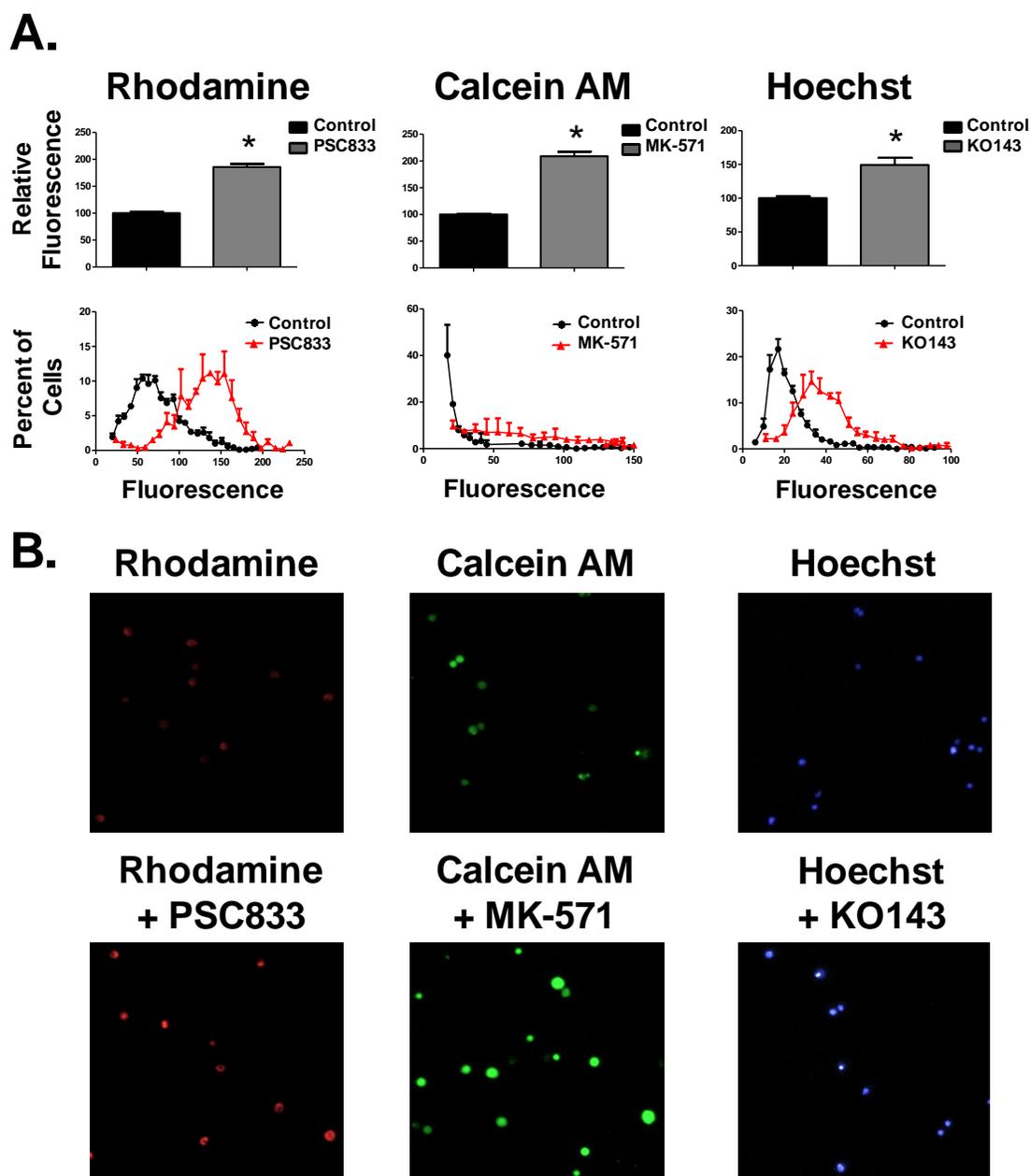


Figure 3-1

Figure 3-1. Functional characterization of efflux transporters in resting microglia.

The ability of microglia to efflux fluorescent substrates for specific transporters was evaluated using the Nexcelom Cellometer Vision. A) Fluorescent dyes known as specific

ABC transporter substrates were used to characterize function. Specific inhibitors (PSC833, MK571, KO143) were used as a positive control for functional inhibition. B) Representative fluorescent images of resting microglia incubated with ABC efflux transporter substrates or substrate plus inhibitor. Data (bar graphs) are presented as mean relative fluorescence \pm SE (n=3) normalized to cell size. Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control. Line graphs represent the distribution of individual cell fluorescence. Each point represents the mean percent of cells \pm SE (n=3 samples) exhibiting a quantity of fluorescence. The number of cells counted for each sample ranged from 200 to 600 cells.

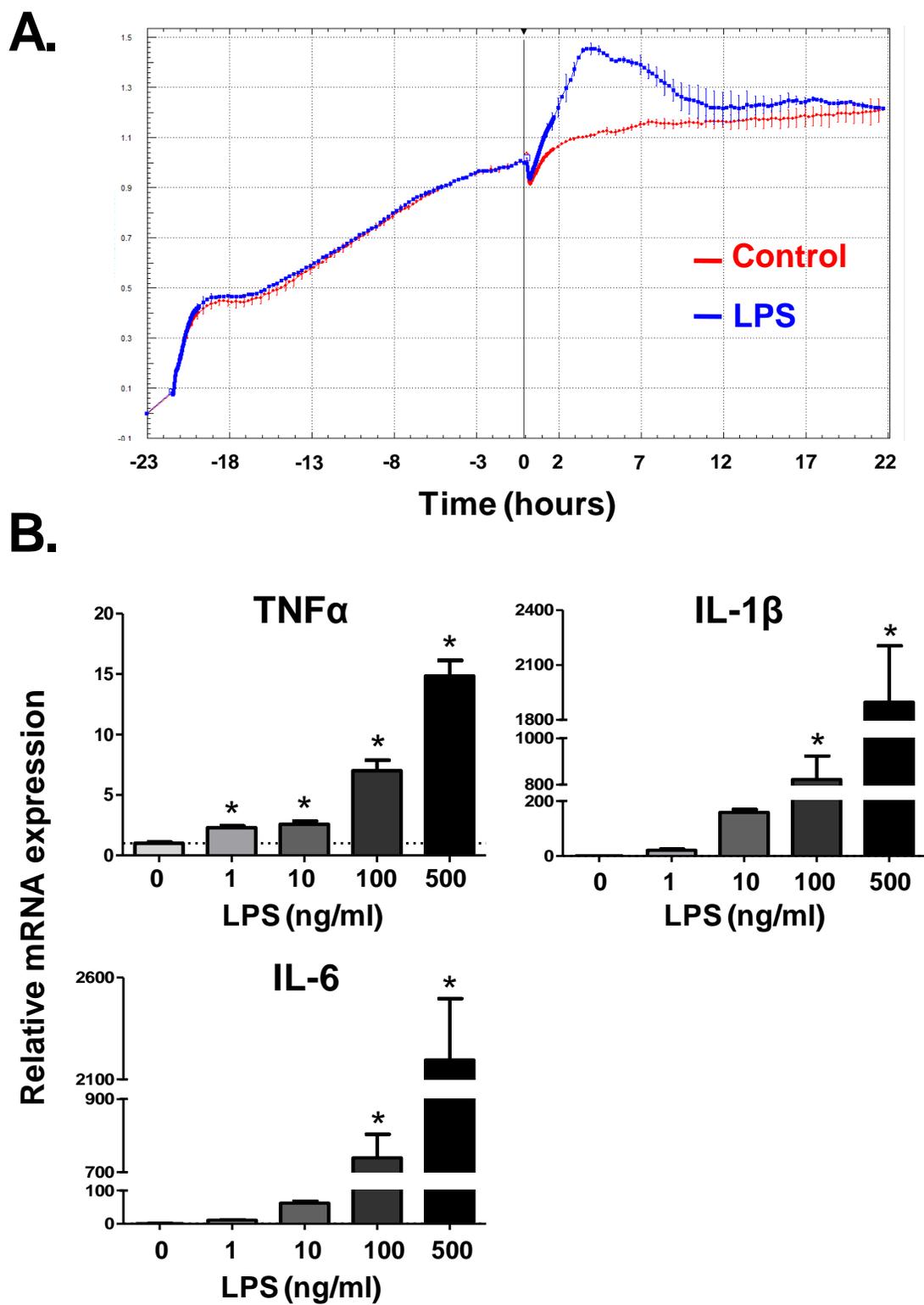


Figure 3-2

Figure 3-2. Quantitative microglial activation. BV-2 cell activation was examined using A) the xCELLigence RTCA DP analyzer before and after LPS activation. Real time impedance measurements demonstrate the attachment (T=-21 to -20), and growth (T=-16 to T=0) of BV-2 microglia as gradual increases in cell impedance. Addition of LPS to microglia occurred at T=0. LPS-treated microglia are in blue and vehicle-treated control cells are in red. Data are presented as mean \pm SE (n=4). B) Microglial activation after 12 hour incubation with LPS was confirmed by quantifying pro-inflammatory cytokine mRNA expression. Data were normalized to Rpl13A mRNA and presented as mean relative mRNA expression \pm SE (n=4) compared to 0 ng/ml LPS. Dotted line at Y=1 represents control expression. Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to 0 ng/ml LPS.

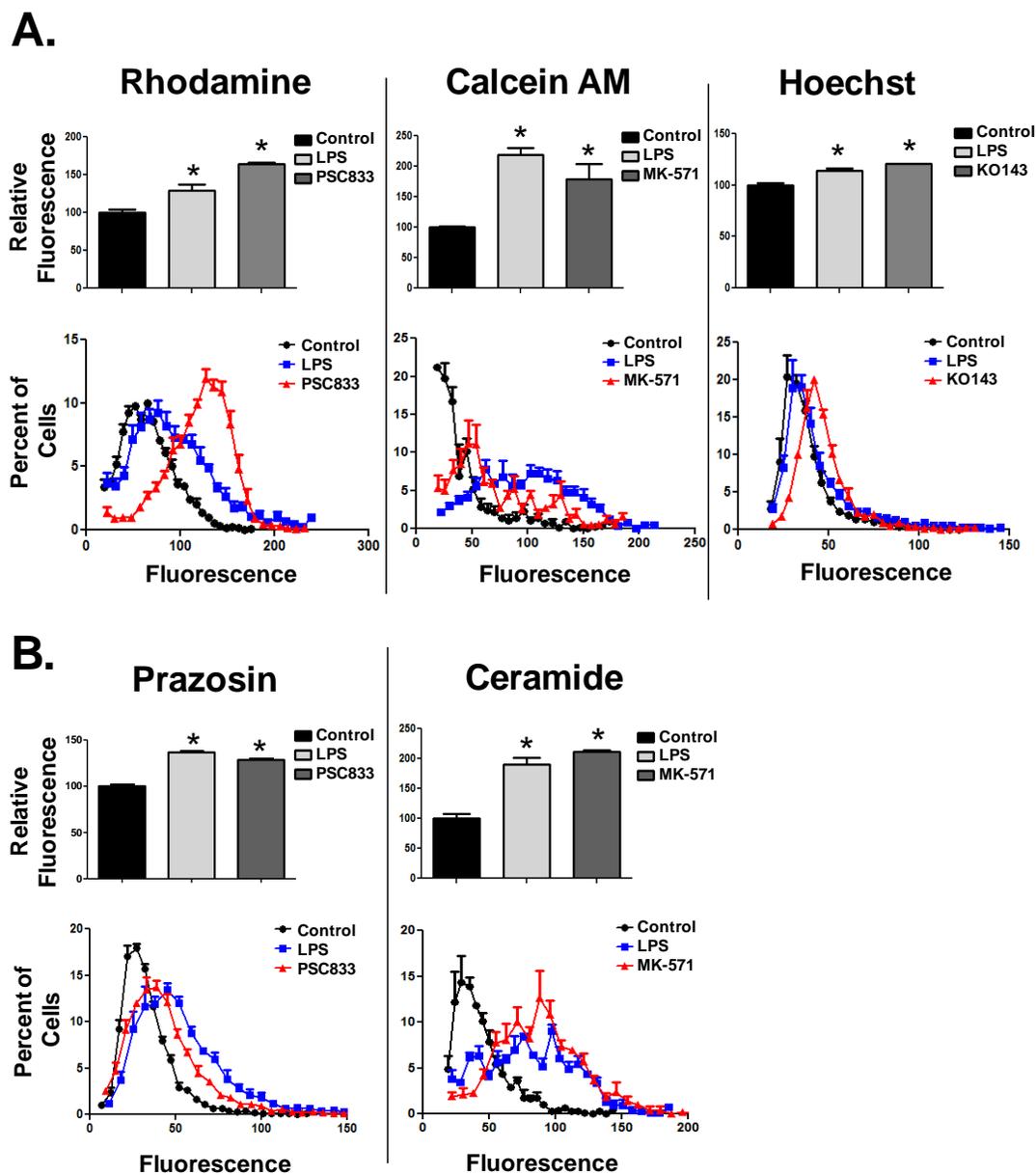


Figure 3-3

Figure 3-3. Functional characterization of efflux transporters in activated microglia.

The ability of specific transporters to efflux substrates from activated microglia (100 ng/ml LPS for 24 hours) was characterized using fluorescent substrates and the Nexcelom Cellometer Vision. A) Fluorescent dyes known as specific ABC transporter substrates

were used to characterize function. Specific inhibitors (PSC833, MK571, KO143) were used as a positive control for functional inhibition. B) Exogenous and endogenous substrates were also examined for confirmation of transporter function. Data (bar graphs) are presented as mean relative fluorescence \pm SE (n=3) normalized to cell size. Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control. Line graphs represent the distribution of individual cell fluorescence. Each point represents the mean percent of cells \pm SE (n=3 samples) exhibiting a quantity of fluorescence. The number of cells counted for each sample ranged from 500 to 2000 cells.

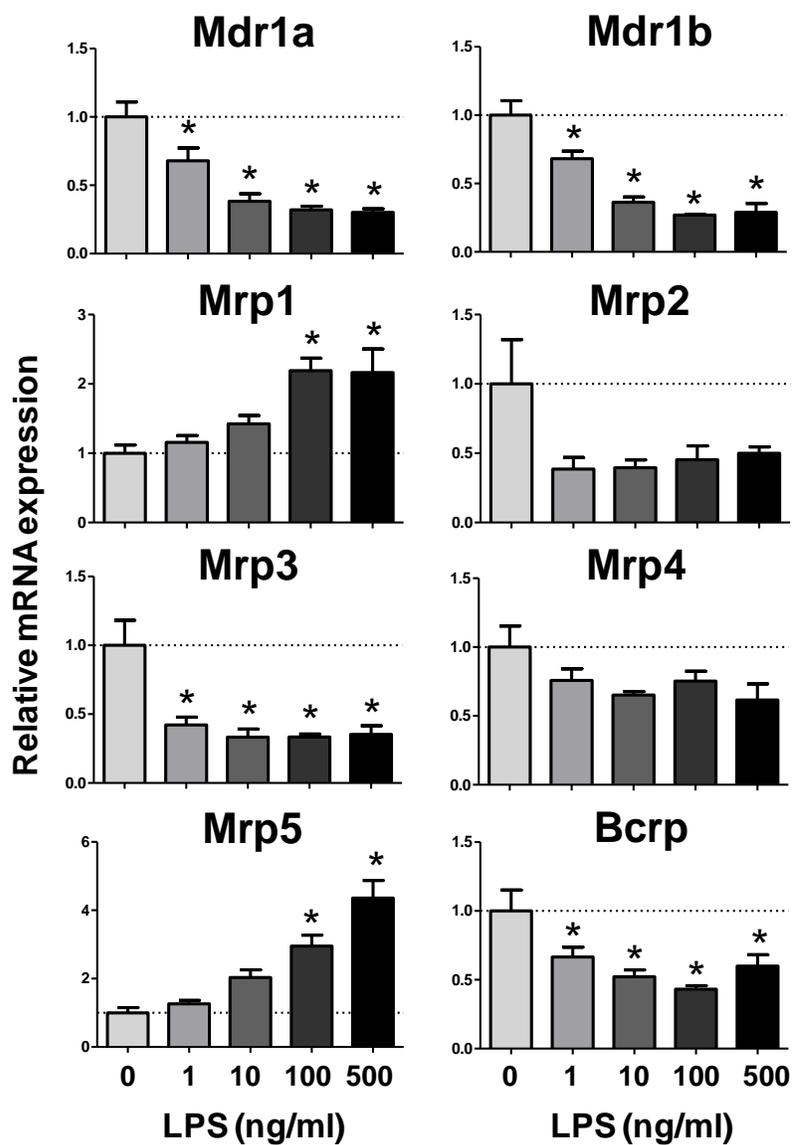


Figure 3-4

Figure 3-4. mRNA expression of efflux transporters. Relative efflux transporter mRNA was determined after 12 hour incubation of BV-2 microglia with LPS. Data were normalized to Rpl13A and presented as mean relative mRNA expression \pm SE (n=4). Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control. Dotted line at Y=1 represents control expression.

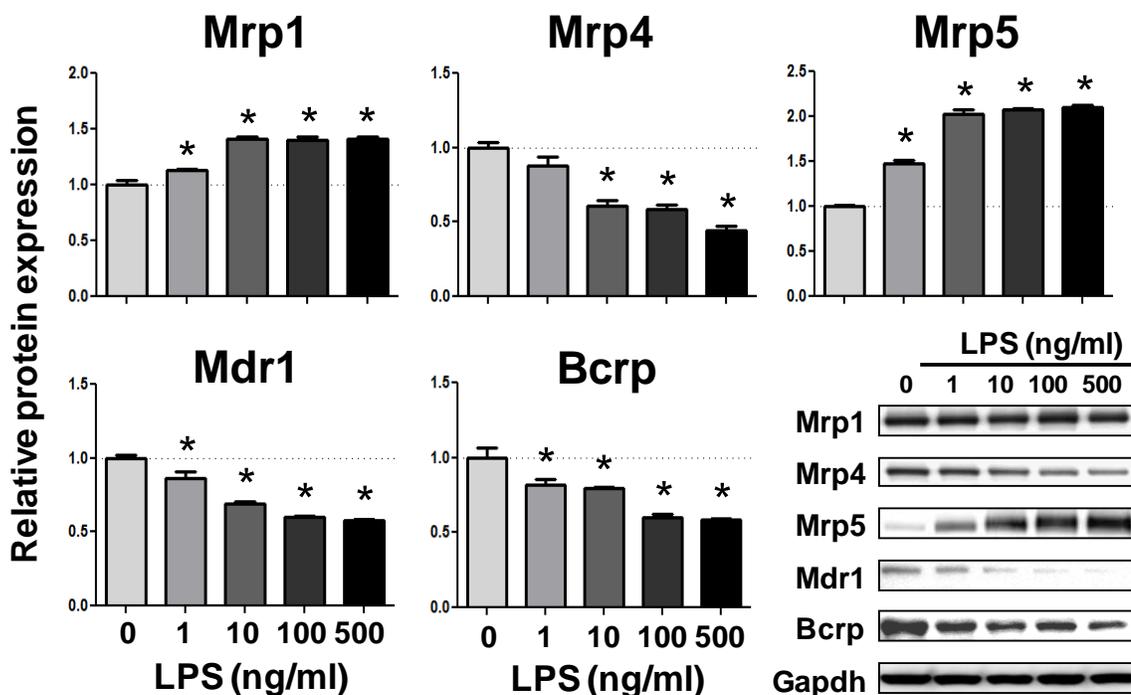


Figure 3-5

Figure 3-5. Protein expression of efflux transporters. Relative expression of efflux transporter protein was determined after 24 hours of microglial activation with LPS. Semi-quantitative expression of protein was determined by densitometry from western blots. Representative western blots exhibit changes in protein expression. Gapdh was used as loading control. Data are presented as mean relative protein expression \pm SE (n=3). Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control.

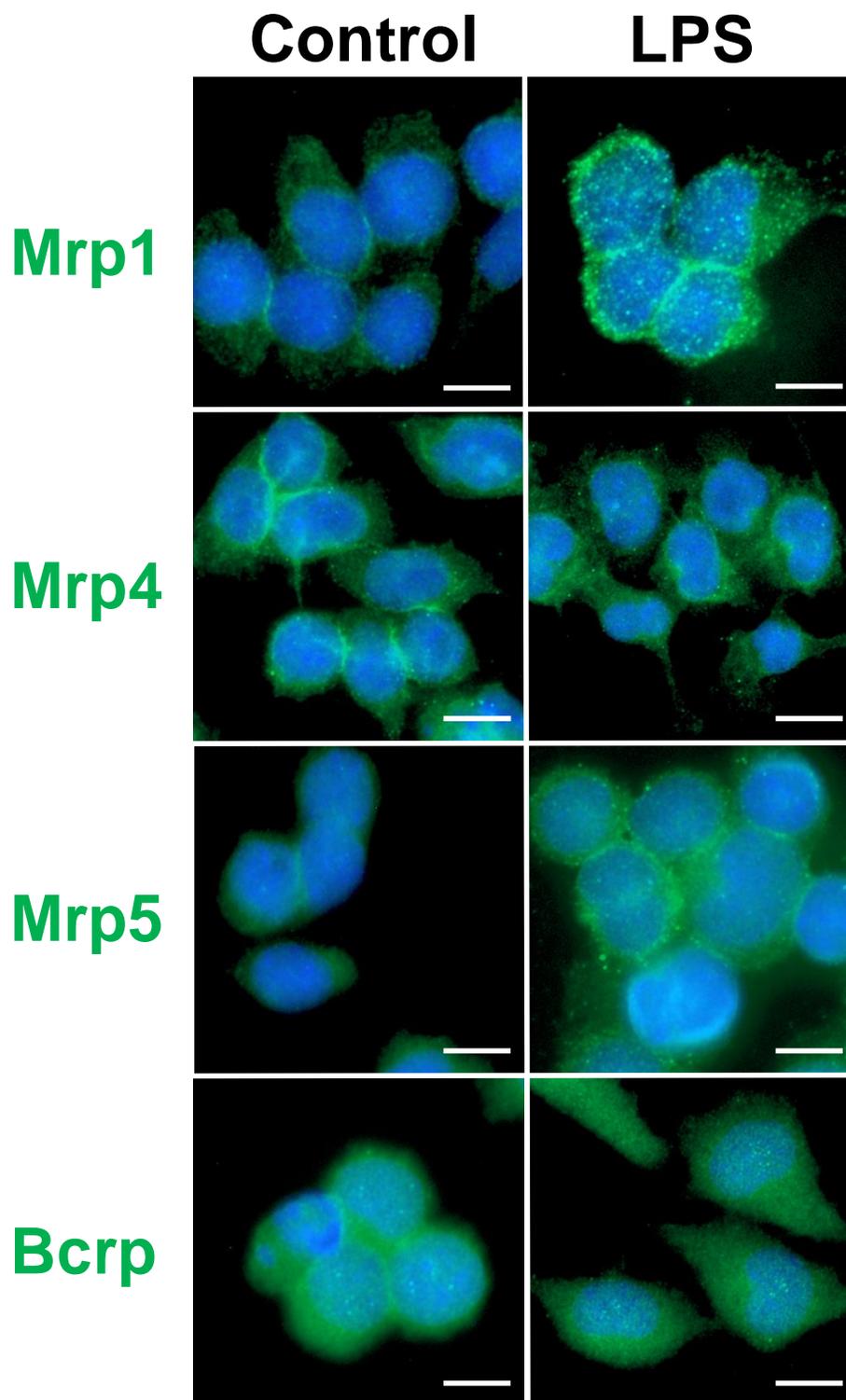


Figure 3-6

Figure 3-6. Transporter protein immunofluorescence in activated BV-2 microglia.

BV-2 microglia were grown on chamber slides and incubated with vehicle or 100ng/ml LPS or fresh medium for 24 hours. Slides were fixed in 4% paraformaldehyde and incubated with primary antibodies specific for each efflux transporter and appropriate fluorescent AlexaFluor488-labeled secondary antibodies (*green*). Nuclei (*blue*) were stained with DAPI. Bar = 10 μ m.

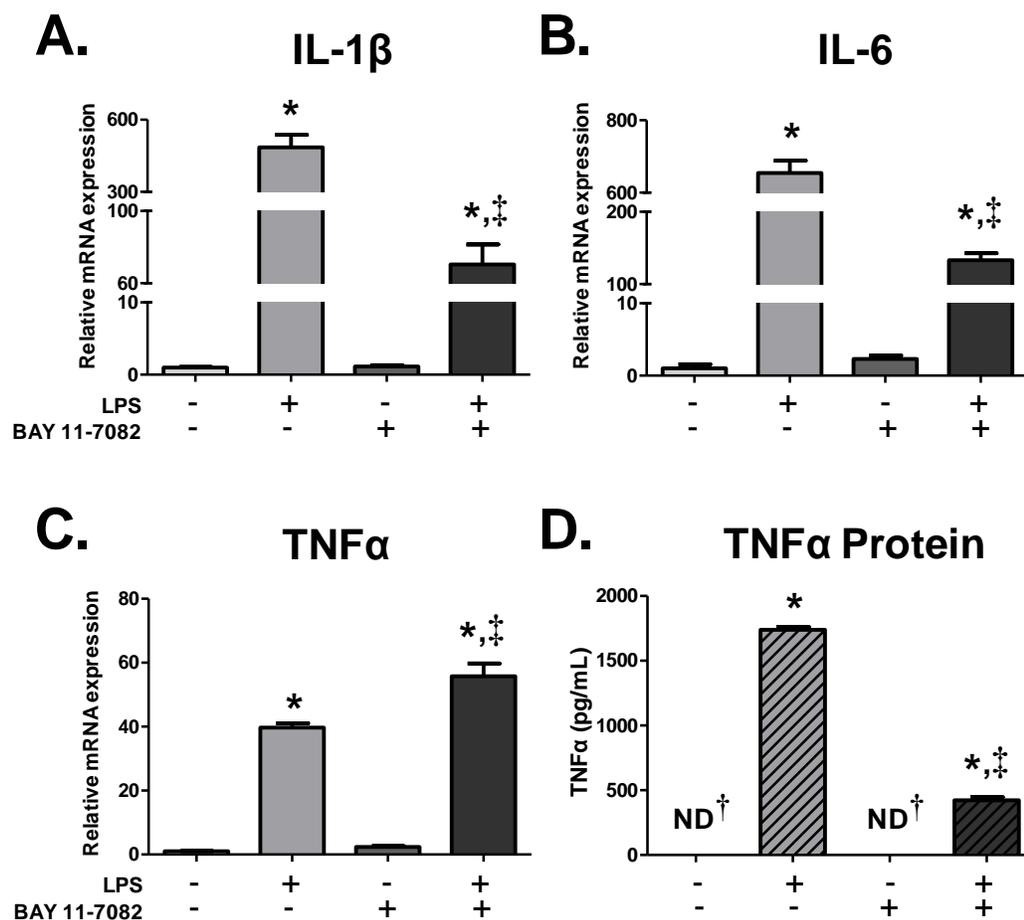


Figure 3-7

Figure 3-7. Expression of inflammatory cytokines following NF- κ B inhibition. BV-2 microglia were incubated with vehicle or 5 μ M BAY 11-7082 30 minutes prior to the addition of vehicle or 100ng/ml LPS. Cells were collected after 12 hours and cell lysates were analyzed for mRNA expression of A) IL-1 β , B) IL-6, and C) TNF. D) Cell culture medium was analyzed for TNF protein by ELISA. mRNA data were normalized to Rpl13A mRNA and presented as mean relative mRNA expression \pm SE (n=3). Protein data are presented as mean protein levels \pm SE (n=3). Single dagger (†) indicates that

protein concentration was below the detection level of 3 pg/mL (ND). Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control cells (no LPS, no BAY 11-7082). Double dagger (‡) represents statistically significant differences ($p < 0.05$) compared to LPS-treated cells (no BAY 11-7082).

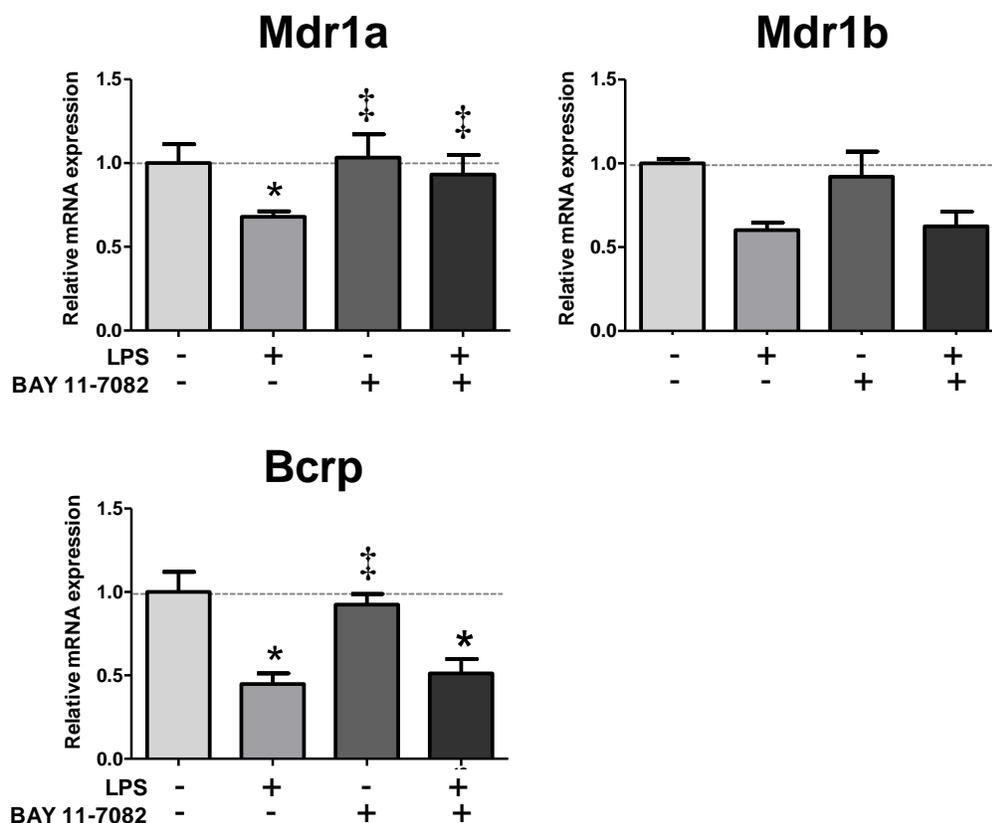


Figure 3-8

Figure 3-8. mRNA expression of efflux transporters following NF- κ B inhibition.

BV-2 microglia were incubated with 5 μ M BAY 11-7082 or DMSO 30 minutes prior to the addition of vehicle or 100ng/ml LPS. Cells were collected after 12 hours and cell lysates were analyzed for mRNA expression of Mdr1a, Mdr1b, and Bcrp. mRNA data were normalized to Rpl13A mRNA and presented as mean relative mRNA expression \pm SE (n=3). Dotted line at Y=1 represents control expression. Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control cells (no LPS, no BAY

11-7082). Double dagger (‡) represents statistically significant differences ($p < 0.05$) compared to LPS-treated cells (no BAY 11-7082).

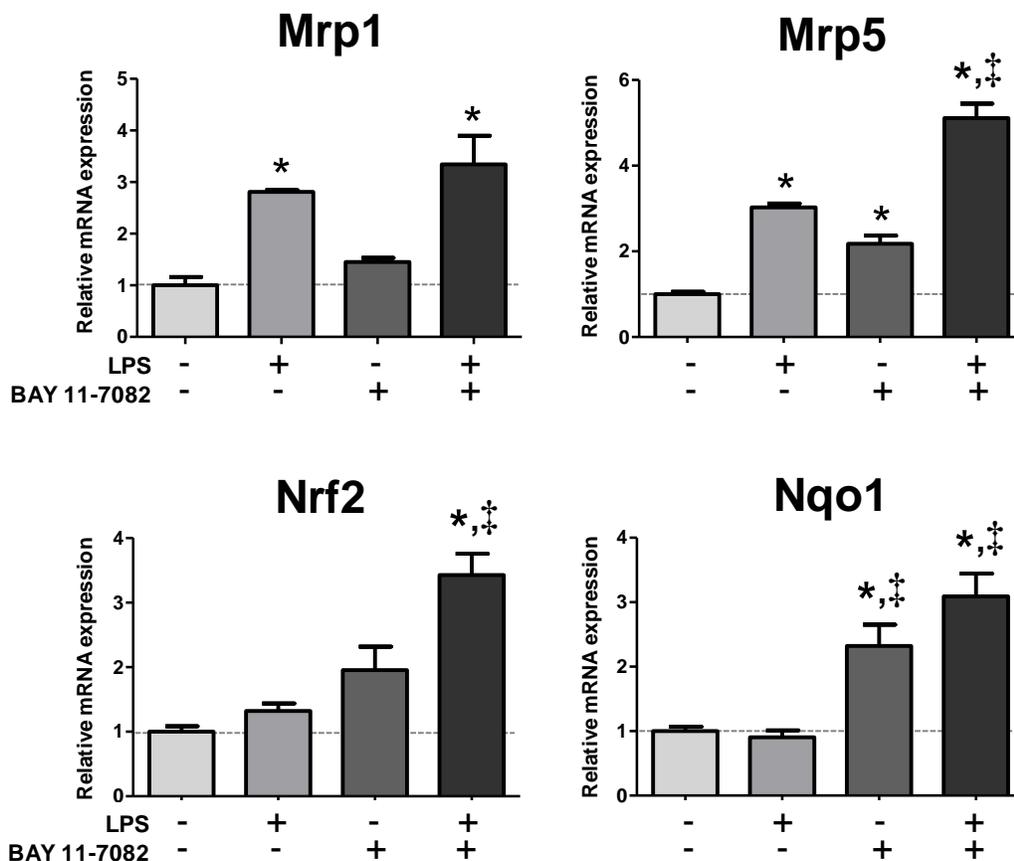
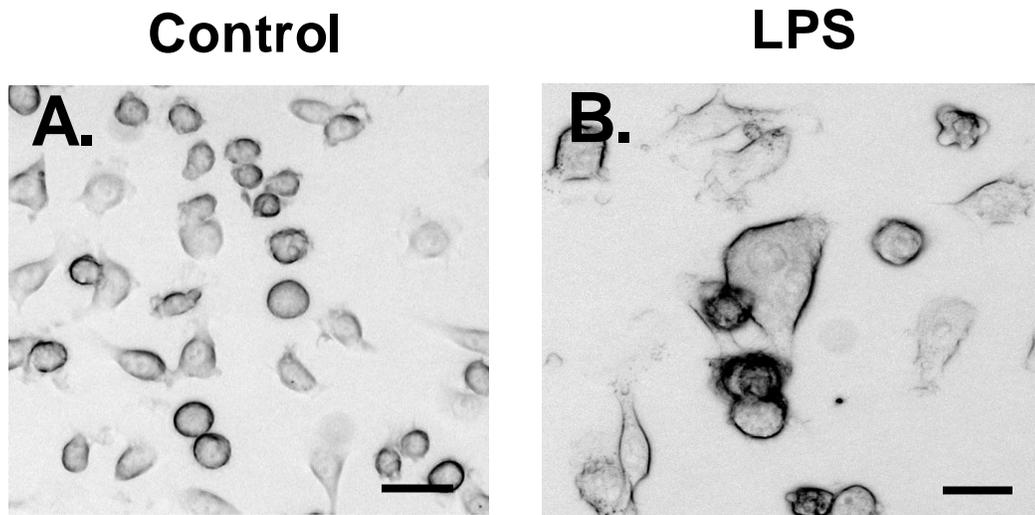


Figure 3-9

Figure 3-9. Mrp and Nrf2 activation following NF- κ B inhibition with BAY 11-7082.

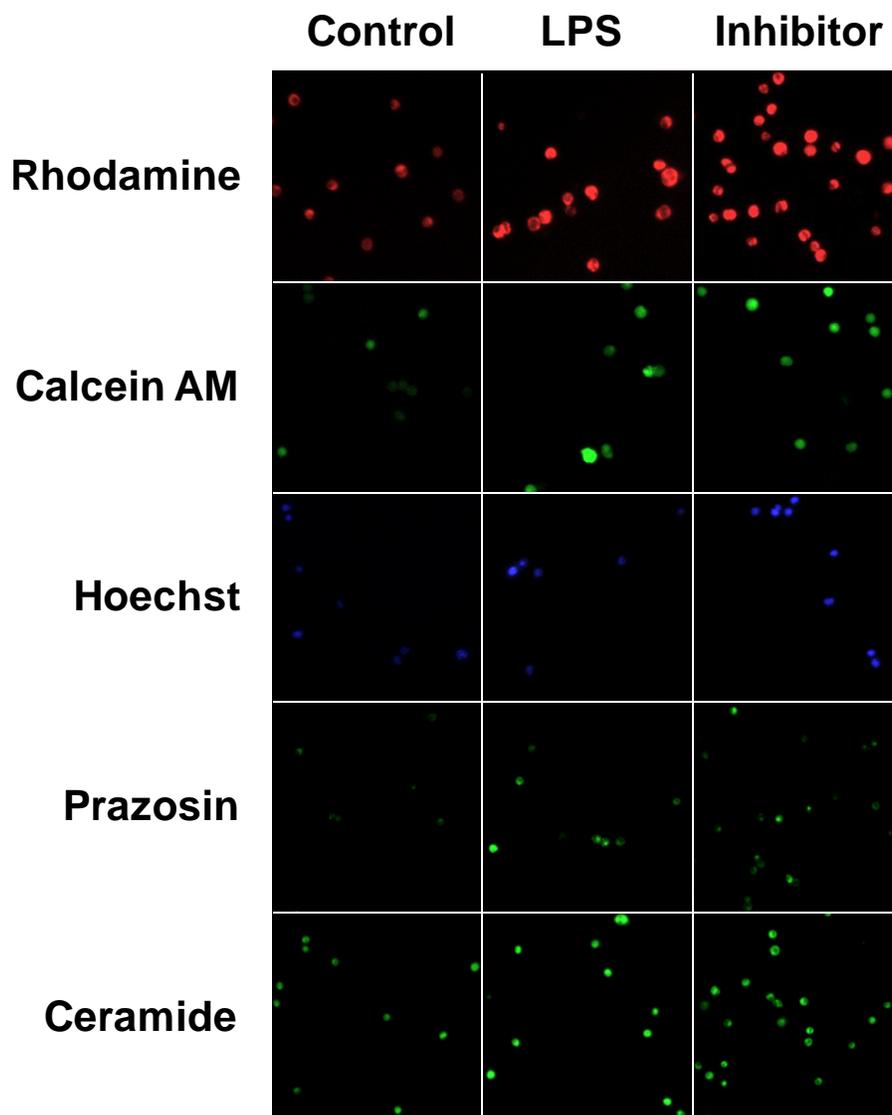
BV-2 microglia were incubated with 5 μ M BAY 11-7082 or DMSO 30 minutes prior to the addition of vehicle or 100ng/ml LPS. Cells were collected after 12 hours and cell lysates were analyzed for mRNA expression of Nrf2, the Nrf2 responsive gene, Nqo1, and the efflux transporters Mrp1, and Mrp5. mRNA data were normalized to Rpl13A mRNA and presented as mean relative mRNA expression \pm SE (n=3). Dotted line at Y=1 represents control expression. Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control cells (no LPS, no BAY 11-7082). Double dagger (‡)

represents statistically significant differences ($p < 0.05$) compared to LPS-treated cells (no BAY 11-7082).



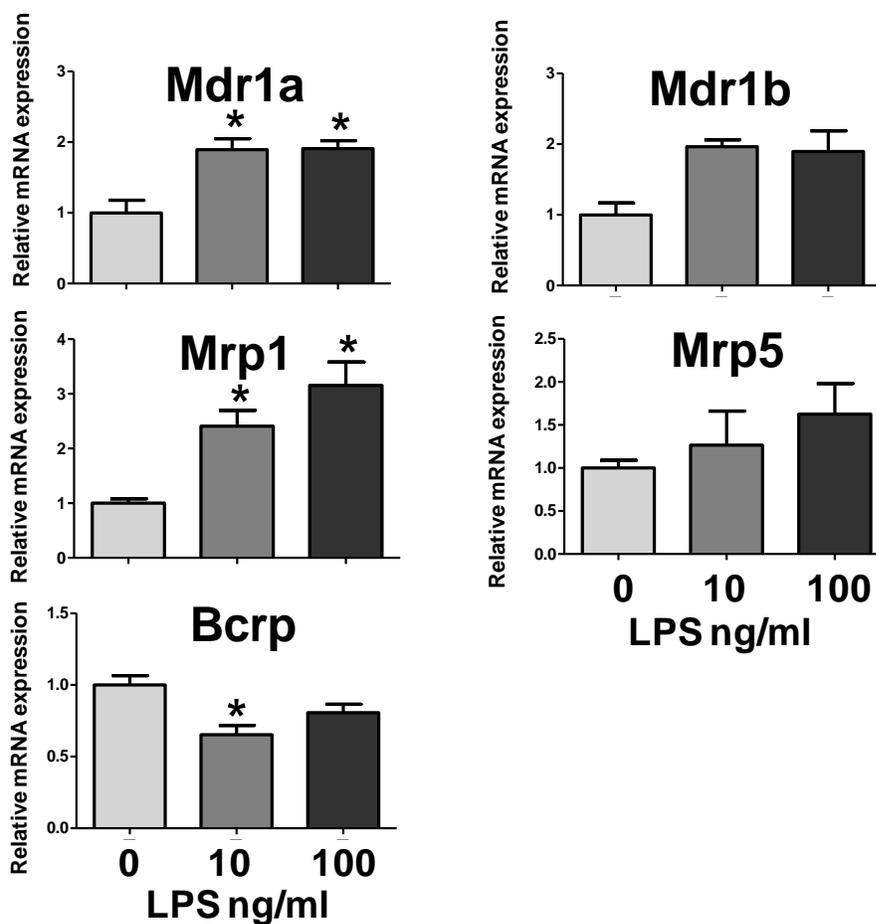
Supplemental Figure 3-1

Supplemental Figure 3-1. BV-2 microglia morphology. Confirmation of altered morphology was determined visually (T = 6 hours) by examining live cell cultures grown in B) fresh medium or C) 100 ng/ml LPS. Bar = 20 μ m.



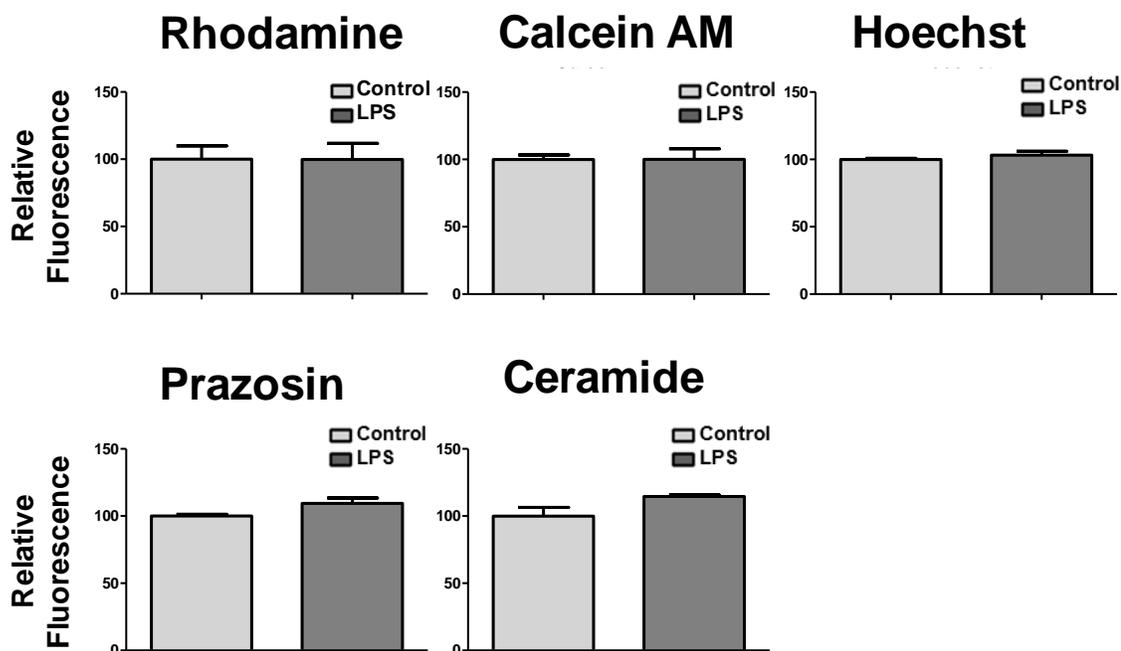
Supplemental Figure 3-2

Supplemental Figure 3-2. Intracellular retention of fluorescent substrates in activated microglia. Representative fluorescent images of activated BV-2 microglia incubated with ABC efflux transporter substrates (*LPS*) or control BV-2 microglia incubated with substrate (*Control*) or substrate plus inhibitor (*Inhibitor*).



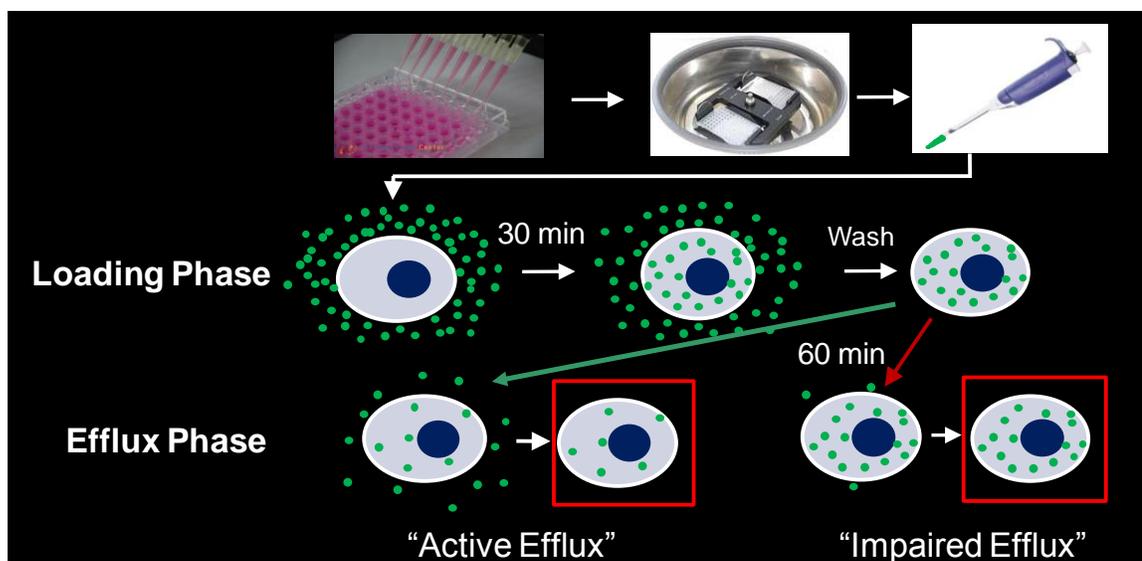
Supplemental Figure 3-3

Supplemental Figure 3-3. mRNA expression of efflux transporters in activated primary microglia. Relative efflux transporter mRNA was determined after 12 hour incubation of neonatal mouse (C57Bl/6) primary microglia with LPS. Data were normalized to Rpl13a and presented as mean relative mRNA expression \pm SE (n=3). Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control.



Supplemental Figure 3-4

Supplemental Figure 3-4. Cellular uptake of fluorescent substrates. The relative amount of fluorescent substrate uptake in control and activated microglia (100 ng/ml LPS for 24 hours) was characterized using the Nexcelom Cellometer Vision. Cellular fluorescence was quantified immediately following a 30 minute incubation with each substrate. Data are presented as mean relative fluorescence \pm SE (n=3) normalized to cell size. There was no statistical difference ($p < 0.05$) in the amount of fluorescent substrate uptake between control and LPS-activated microglia.



Supplemental Figure 3-5

Supplemental Figure 3-5. Method of intracellular fluorescence quantification.

Representation of the method used to quantify intracellular fluorescence.

Table 3-1. Fluorescent transporter substrates and inhibitors

Substrate	Concentration	Source
Rhodamine 123	5 μ M	Sigma-Aldrich (St. Louis, MO)
Calcein AM	2 μ M	Sigma-Aldrich
bisBenzimide H 33342 trihydrochloride (Hoechst)	10 μ M	Sigma-Aldrich
BODIPY Prazosin	2 μ M	Invitrogen/Molecular Probes (Eugene, OR)
NBD-Ceramide	5 μ M	Avanti Polar Lipids (Alabaster, AL)
Inhibitor		
PSC833	1 μ M	XenoTech LLC. (Lenexa, KS)
MK-571	10 μ M	Sigma-Aldrich
KO143	5 μ M	Sigma-Aldrich

Table 3-2. Normalized mRNA expression in BV-2 microglia

Gene	Ct value microglia	Percent difference from liver ¹
<i>Mrp1/Abcc1</i>	21.81	8029.2%
<i>Mrp2/Abcc2</i>	26.44	0.4%
<i>Mrp3/Abcc3</i>	23.69	6.3%
<i>Mrp4/Abcc4</i>	24.52	5509.9%
<i>Mrp5/Abcc5</i>	22.84	304.6%
<i>Mdr1a/Abcb1a</i>	22.55	776.5%
<i>Mdr1b/Abcb1b</i>	22.59	1279%
<i>Bcrp/Abcg2</i>	24.05	9.6%

¹Basal expression of microglial mRNA was compared to C57BL/6 mouse liver and normalized to the reference gene, Rpl13a.

Supplemental Table 3-1. qPCR primer sequences

Target	Forward Primer (5' → 3')	Reverse Primer (5' → 3')
<i>TNF</i> ^a	AGCCACGTCGTAGCAAACCAC	AGGAGCACGTAGTCGGGGCA
<i>IL-1β</i> ^b	GACCTTCCAGGATGAGGACA	AGCTCATATGGGTCCGACAG
<i>IL-6</i> ^b	AGTTGCCTTCTTGGGACTGA	CAGAATTGCCATTGCACAAC
<i>Rpl13a</i> ^a	CTGTGAAGGCATCAACATTTCTG	GACCACCATCCGCTTTTTCTT
<i>Mdr1a/Abcb1a</i> ^a	TGCCCCACCAATTTGACACCCT	ATCCAGTGCGGCCTGAACCA
<i>Mdr1b/Abcb1b</i> ^a	GTGTTAAAGGGGCGATGGGCG	AGGCTTGGCCAGACAACAGCTT
<i>Mrp1/Abcc1</i> ^a	GCTGTGGTGGGCGCTGTCTA	CCCAGGCTCAGCCACAGGAA
<i>Mrp2/Abcc2</i> ^b	AGCAGGTGTTTCGTTGTGTGT	AGCCAAGTGCATAGGTAGAGAAT
<i>Mrp3/Abcc3</i> ^b	CTGGGTCCCCTGCATCTAC	GCCGTCTTGAGCCTGGATAAC
<i>Mrp4/Abcc4</i> ^b	CCAGACCCTCGTTGAAAGAC	TGAAGCCGATTCTCCCTTC
<i>Mrp5/Abcc5</i> ^a	AGGGCAGCTTGTGCAGGTGG	TGCTGTTCCCGCTTCCTTGCT
<i>Bcrp/Abcg2</i> ^a	GCGGAGGCAAGTCTTCGTTGC	TCTTCACTGTCAGGGTGCCCA
<i>Nrf2</i>	TCTTGGAGTAAGTCGAGAAGTGT	GTTGAAACTGAGCGAAAAAGGC
<i>Nqo1</i>	AGGATGGGAGGTACTCGAATC	AGGCGTCCTTCCTTATATGCTA

CHAPTER 4

Microglial-Derived Factors Down-Regulate MDR1/ABCB1**Transporter Expression During Neuroinflammation****Abstract**

Mdr1/Abcb1 is an efflux pump expressed on microglia and endothelial cells of the brain that is responsible for the extrusion of chemicals. As resident immune cells of the brain, microglia coordinate inflammatory events by secreting cytokines and neurotoxic factors and communicating with other cell types. Prior studies demonstrate that neuroinflammation reduces the expression and function of Mdr1 in microglial and endothelial cells, although the paracrine signaling and intracellular molecular mechanisms are not clear. Here, we sought to determine whether microglia-derived secreted factors, including the cytokine TNF, could regulate Mdr1 expression in naive microglia, endothelial cells, and whole brain and to identify the contribution of NF- κ B and Nrf2 transcription factor signaling to altered Mdr1 levels. To test this, Mdr1 expression was quantified in 1) mouse BV-2 microglia treated with lipopolysaccharide (LPS, 500 ng/ml), conditioned media (media containing factors secreted from activated microglia), and recombinant TNF 2) human brain hCMEC/D3 endothelial cells exposed to conditioned media and 3) brains from LPS-treated (5 mg/kg, i.p.) mice. Treatment of microglia with LPS, conditioned media, and recombinant TNF reduced Mdr1 mRNA and protein expression by 40-50% at 24 h. Further, down-regulation of Mdr1 mRNA in LPS-treated microglia was attenuated by inhibition of NF- κ B and activation of Nrf2 signaling using SN50 and sulforaphane, respectively. MDR1/Mdr1 expression was also reduced in human hCMEC/D3 endothelial cells treated with conditioned media and in the brains of

mice treated with LPS. These results demonstrate that factors released from activated microglia can affect the regulation of ABC transporters in neighboring microglia and the capillary endothelium.

Non-Standard Abbreviations

ABC, ATP binding cassette; Mdr, multidrug resistance protein; Mrp, multidrug resistance-associated protein; Bcrp, breast cancer resistance protein; LPS, lipopolysaccharide; NF- κ B, nuclear factor kappa B; I κ B, inhibitor of kappa B; Nrf2, nuclear factor-e2 related factor 2; TNF, tumor necrosis factor; IL, interleukin; BODIPY, boron-dipyrromethene; NBD, nitrobenzoxadiazole; DAPI, 4',6-Diamidino-2-phenylindole; Nqo1, NAD(P)H quinone oxidoreductase 1; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

Introduction

Neuroinflammation is a common feature of many neurodegenerative diseases including Alzheimer disease, Parkinson disease (PD), multiple sclerosis, and amyotrophic lateral sclerosis (Glass, Saijo et al. 2010). Chronic inflammation contributes to neurodegeneration by several mechanisms including the production of pro-inflammatory cytokines and reactive oxygen species (ROS), formation of free radicals, and lipid peroxidation (Hopkins and Rothwell 1995, Mosley, Benner et al. 2006, Farooqui and Farooqui 2011). These inflammatory pathways are important for maintaining normal homeostasis but can be detrimental when uncontrolled and can lead to neuronal damage and death. Microglia are the resident macrophage cells present in the brain and are a major contributor to neuroinflammation. PD is the second most common neurodegenerative disease and uncontrolled chronic inflammation along with activated microglia, as evidenced by NF- κ B activation and NADPH oxidase expression, are major post mortem findings (Glass, Saijo et al. 2010).

Dopamine neurons of the substantia nigra pars compacta (SNc) are particularly vulnerable to damage in PD because they undergo oxidative processes related to the synthesis of dopamine making them susceptible to oxidative stress (Mosley, Benner et al. 2006, Glass, Saijo et al. 2010). Microglia are the principal source of pro-inflammatory cytokines, interleukin (IL)-1 β and tumor necrosis factor (TNF), in the brain and these cytokines are elevated in the brains of PD patients, particularly in the SNc and striatum, as well as serum and cerebral spinal fluid (Mogi, Harada et al. 1994, Kim and Joh 2006, Mosley, Benner et al. 2006). These cytokines coordinate immune responses through cell-

cell communication and are produced and released by activated microglia responding to various stimuli.

Microglia contain mechanisms to counteract the potential damage from producing free radicals and oxidants. They contain high levels of the antioxidant glutathione (GSH) and express the efflux transporter multidrug resistance protein 1 (MDR1/Mdr1). Mdr1/MDR1 is an ATP-binding cassette (ABC) transporter responsible for the efflux of a large number of exogenous and endogenous substrates. This transporter is expressed in the plasma membrane of microglia and brain capillary endothelial cells, where it is an essential component of the blood-brain barrier (BBB). Mdr1/MDR1 regulates solute accumulation in the brain. During inflammation, however, Mdr1 expression and function are impaired (Goralski, Hartmann et al. 2003, Morgan, Goralski et al. 2008, Gibson, Hossain et al. 2012). Systemic inflammation reduces Mdr1 expression in the mouse BBB resulting in increased brain-to-plasma ratios for known substrates, such as digoxin (Goralski, Hartmann et al. 2003). Signaling by the cytokine TNF has been proposed as one mechanism that contributes to the down-regulation of Mdr1. For example, Mdr1 function in isolated mouse brain capillaries is rapidly reduced by TNF through interaction with TNFR1 (Hartz 2005).

Regulation of TNF and other cytokines is tightly controlled in the CNS. In the brain, cytokine production mainly occurs in microglia. When microglia are activated by a stimulus, pro-inflammatory cytokines are up-regulated through activation of the transcription factor, nuclear factor- κ B (NF- κ B) pathway and subsequent gene transcription (Hayden and Ghosh 2012). NF- κ B also regulates both apoptotic and anti-apoptotic genes and acts as a sensor of oxidative stress. The transcription factor Nrf2 is a

key transcriptional regulator of antioxidant response element-dependent gene expression. Nrf2 is present in the nucleus under basal conditions and regulates the constitutive expression of NAD(P)H:quinone oxidoreductase 1 (Nqo1) and heme oxygenase 1 (Ho-1) (He, Chen et al. 2008). Nrf2 is activated by electrophiles and pro-oxidants and recruited to antioxidant response elements on DNA, and plays a role in the regulation of over 200 genes. Nrf2 is primarily responsible for the transcription of genes for antioxidant proteins, but also regulates the transcription of some ABC transporters (Higgins and Hayes 2011).

Because of its neuroprotective role as an efflux pump, identification of the cell-cell and intracellular signaling pathways involved in Mdr1/MDR1 down-regulation during neuroinflammation may allow for manipulation of this pathway to maintain homeostasis. Our laboratory has previously demonstrated that activation of mouse microglial BV-2 cells with lipopolysaccharide (LPS) reduces Mdr1 transporter expression and function (Gibson, Hossain et al. 2012). Therefore, the purpose of this study was to 1) determine whether microglia-secreted factors can regulate Mdr1 in naïve microglia and endothelial cells, 2) characterize the contribution of TNF, NF- κ B and Nrf2 signaling pathways to the regulation of Mdr1 in microglia, and 3) quantify whole brain Mdr1 expression in mice during neuroinflammation.

Materials and Methods

Chemicals and Reagents. All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Cell Culture. The immortalized mouse (C57BL/6) microglia cell line, BV-2, was used for all microglia experiments (Gibson, Hossain et al. 2012). Cells were grown in modified Eagle's medium (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 5% sodium pyruvate (Mediatech), 5% non-essential amino acids (Mediatech), and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA). Cells were incubated at 37°C with 5% CO₂. BV-2 cells were activated with LPS (500ng/ml) from *E. coli* 026:B6 (Sigma-Aldrich, St. Louis, MO) for up to 24 hours. LPS was dissolved in sterile water at a concentration of 1 mg/ml (stock solution). An equal volume of sterile water was added to all control treatments.

The human cerebral microvascular endothelial cell line, hCMEC/D3, was used as an *in vitro* model of the BBB. These cells have been well-characterized with regard to their BBB properties and response to cytokine stimulation (Weksler, Subileau et al. 2005, Poller, Drewe et al. 2009, Tai, Reddy et al. 2009, Warren, Zerangue et al. 2009, Fasler-Kan, Suenderhauf et al. 2010). Growth medium for hCMEC/D3 cells consists of EBM-2 basal medium (Lonza, Allendale, NJ), 5% fetal bovine serum "Gold" (PAA, GE healthcare Bio-Sciences, Pittsburgh, PA), 1% penicillin-streptomycin (Life Technologies), 1.4µM hydrocortisone, 5µg/mL ascorbic acid, 1% chemically defined

lipid concentrate (Life Technologies), 10mM HEPES, and 1ng/mL basic fibroblast growth factor.

Cell Treatments. BV-2 microglia used for mRNA analysis were grown in 6-well culture plates (4-6 wells/group, 1×10^5 cells/well). When 80% confluent, cells were treated with fresh medium, LPS, test compound, or test compound and LPS. Test compounds were chosen to block or activate specific signaling pathways as described in Table 1. The Nrf2 activator, sulforaphane, required extended pre-incubation (4 hours) prior to incubation with LPS. After 12 hours, supernatant was collected and cells were lysed in RLT buffer (QIAGEN) for RNA isolation and quantification. For protein analysis, cells were grown in 60-mm cell culture dishes (4 dishes/ group, 5×10^5 cells/dish) and treated for 24 hours. Cells were homogenized in lysis buffer according to the protocol for western blot quantification.

Conditioned medium for treatment of hCMEC/D3 cells was prepared activating BV-2 cells with LPS in hCMEC/D3 complete medium. Preliminary experiments were performed to confirm appropriate BV-2 microglial survival, activation, and cytokine production in hCMEC/D3 medium (data not shown). BV-2 microglia were activated by treating with LPS for 4 hours. Cells were washed 3 times with PBS to remove any residual LPS. They were then incubated in fresh hCMEC/D3 medium. Pooled medium was collected 24 hours later through a $0.2\mu\text{m}$ filter and used as the conditioned medium (CM).

Animals and Dosing. Wild type C57Bl/6 mice were purchased from Charles River (Wilmington, MA) between the ages of 8 and 12 weeks of age. Mice received a single i.p. injection of vehicle (0.9% saline, 5ml/kg) or lipopolysaccharide (LPS, E. coli, 5mg/kg, 5ml/kg). Brains were collected 24 hours later. Brains were dissected to collect specific brain regions (cortex, midbrain) and flash frozen in liquid nitrogen. All animal studies were performed with the approval of the Rutgers University Institutional Animal Care and Use Committee.

RNA Isolation and Real-Time Quantitative Polymerase Chain Reaction (PCR). For animal studies, C57Bl/6 mice were treated with LPS (5mg/kg, i.p.) dissolved in 0.9% saline (5ml/kg) and tissues collected at 24 hours. Sections of cortex (20mg) were homogenized using a TissueLyser LT (QIAGEN, Valencia, CA) with stainless steel beads in 0.5 ml RNABee (Thermo Fisher Scientific, Waltham, MA). 100 μ l of 100% chloroform (Sigma-Aldrich) was added to each tube and vortexed. Samples were placed on ice for 5 minutes and then centrifuged at 13,000 rpm for 15 minutes at 4°C. The upper aqueous phase was collected from each tube and mixed with 250 μ l of 70% ethanol at room temperature. Total RNA was isolated by using the RNeasy mini kit (QIAGEN). For cell culture experiments, cells were directly lysed on culture plates with Buffer RLT (QIAGEN) containing 1% β -mercaptoethanol. Lysates were collected and homogenized using QIAshredder (QIAGEN). Total RNA was isolated by using the RNeasy mini kit (QIAGEN).

Complimentary DNA (cDNA) was generated with the high capacity cDNA reverse transcription kit with RNase inhibitor (Life Technologies, Carlsbad, CA). cDNA purity and concentration were assessed using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Rockford, IL). For qPCR, specific forward and reverse primers (Integrated DNA Technologies, Coralville, IA) for each gene were added to one microgram of cDNA from each sample. Sybr Green (Applied Biosystems, Carlsbad, CA) was used for detection of amplified products. qPCR was performed in a 384-well plate format using the ViAA 7 PCR system (Applied Biosystems). Ct values were converted to delta delta Ct values by comparing to a reference gene, ribosomal protein 13a (Rpl13a). Primer sequences for all target mRNAs are provided in Supplemental Table 1.

Quantification by Western Blot. Cultured cells were collected in 1.5ml PBS, transferred to microcentrifuge tubes and centrifuged at 1000g for 10 minutes. The supernatant was removed and pellets were resuspended and homogenized in cell lysis buffer containing protease inhibitors. Protein concentration was determined using the bovine serum albumin protein quantification kit (Thermo Fisher Scientific). Western blot analysis of Mdr1/MDR1 was performed by using 30µg protein homogenate per well by SDS-PAGE on NuPage Tris-Acetate 4% gels (Invitrogen). Proteins were transferred overnight to a polyvinylidene fluoride membrane. Membranes were blocked in 5% nonfat dry milk in phosphate-buffered saline (PBS) with 0.5% Tween-20. Primary antibodies were diluted in 2% nonfat dry milk and incubated with membranes for 3 hours at the following concentrations: 1:1000 for Mdr1a/b (C219, Novus Biologicals, Littleton, CO), and

1:5000 for Gapdh (Millipore, Temecula, CA). Primary antibodies were probed using species-appropriate secondary antibodies (Sigma) and detected using SuperSignal West Dura blotting reagents (Thermo-Scientific). Detection and semi-quantification of protein bands was performed using a FluorChem E imager (Protein Simple, Santa Clara, CA).

TNF Protein Quantification. Supernatants from cultured cells used for the experiments above were collected to confirm the presence of cytokines in conditioned medium (CM). TNF concentration was quantified using an enzyme-linked immunosorbent assay (Life Technologies). Samples of media were added to 96-well ELISA plates and TNF was quantified using the manufacturer's protocol.

Statistical Analysis. GraphPad Prism© version 5 software (GraphPad Software, La Jolla, CA) was used for statistical analysis. Differences among groups were determined by either a two-tailed t test when only two groups were compared, or a one-way analysis of variance with a Newman-Keul's posthoc test. Differences were considered statistically significant at $p < 0.05$.

Results

Mdr1 Expression in Microglia Exposed to Conditioned Media. Our laboratory previously demonstrated that BV-2 microglia are activated in response to LPS stimulation as evidenced by morphological changes and the up-regulation of pro-inflammatory cytokine mRNA (TNF, IL-1 β , and IL-6), and that this activation was accompanied by down-regulation of Mdr1 transporter expression and function (Gibson, Hossain et al. 2012). In the present study, we hypothesized that secreted factors from activated microglia were involved in regulating Mdr1. Therefore, we determined whether conditioned medium from activated BV-2 microglia altered Mdr1 transporter expression in naïve resting cells. LPS treatment was used as a positive control. As previously observed, BV-2 microglia incubated with medium containing LPS (500ng/ml) had dramatic elevations in pro-inflammatory cytokine mRNA, whereas cells exposed to conditioned media for 12 hours had no change in expression (Figure 4-1A). Similar to cytokine expression, the mRNA level of the Nrf2 target gene, Ho-1, increased 600% in BV-2 microglia exposed to LPS, but was unchanged in BV-2 microglia exposed to conditioned medium. Down-regulation of Mdr1 in BV-2 microglia exposed to LPS was consistent with our previous report (Gibson, Hossain et al. 2012) demonstrating a 50% reduction in Mdr1a and Mdr1b mRNA. Interestingly, BV-2 microglia incubated in conditioned medium had a similar decrease, showing a 35-40% decrease in Mdr1a and 1b mRNA. Protein expression of Mdr1 exhibited a 40-50% loss when cultured in LPS or conditioned medium (Figure 4-1C).

Mdr1 Expression in hCMEC/D3 Cells Exposed to Conditioned Media. Cells derived from human cerebral microvascular endothelium were used to determine if soluble mediators present in conditioned medium from activated BV-2 microglia could also alter Mdr1 transporter expression in other cell types (Fig 4-2). Incubation with conditioned medium caused a modest 4- to 6-fold increase in cytokine IL-1B and IL-6 mRNA in hCMEC/D3 endothelial cells similar to LPS alone (Fig 4-2A). Both conditioned medium and LPS caused a decrease in HO-1 mRNA in hCMEC/D3 endothelial cells. Conditioned medium, but not LPS, reduced MDR1 mRNA expression by 30%. Protein expression of MDR1 by western blot supported the mRNA results (Fig 4-2B).

Mdr1 Expression in Microglia Stimulated with TNF. Analysis of the conditioned media revealed TNF concentrations of approximately 700 pg/ml (SE \pm 150 pg/ml) (data not shown). To determine the contribution of TNF secretion on Mdr1 transporter down-regulation, recombinant mouse TNF was used to directly stimulate BV-2 cells. Cells were treated with recombinant mouse TNF (0, 10, 150, and 500 pg/ml) for 12 h or 24 h (Fig. 4-3A). In response to TNF, pro-inflammatory cytokine mRNA levels (TNF, IL-1 β , and IL-6) were not significantly changed at 10 pg/ml but were increased significantly between 50 and 400% at 150 and 500 pg/ml. The maximum elevation in mRNA for pro-inflammatory cytokines was 200%. It should be noted that these increases were modest when compared to the cytokine up-regulation observed with LPS stimulation (Fig. 4-1A). Stimulation of BV-2 cells with TNF also caused a 60% rise in the Nrf2 target gene Ho-1. Both Mdr1a and 1b mRNAs exhibited dose-dependent decreases with a maximal loss of 55% at the highest dose of TNF (500pg/ml) (Fig. 4-3B). Similarly, TNF treatment caused

a dose-dependent decrease in Mdr1 protein with 40% lower mRNA levels at the highest dose (Fig. 4-3C).

Mdr1 Expression in Activated Microglia Following Inhibition of NF- κ B Signaling.

We have previously shown that inhibiting NF- κ B dissociation from I κ B and subsequent translocation into the nucleus using the chemical inhibitor BAY 11-7082 attenuates TNF protein release and prevents the down-regulation of Mdr1a mRNA (Gibson, Hossain et al. 2012). Because BAY 11-7082 can also activate Nrf2 signaling (Min, Lee et al. 2011), we used the cell permeable peptide, SN50, as an additional method of more selectively inhibiting NF- κ B by blocking nuclear translocation. The inactive peptide, SN50ip (IP) was used as a negative control. LPS dramatically increased pro-inflammatory cytokine mRNAs, increased Ho-1 mRNA (Fig. 4-4A), and reduced Mdr1a and 1b mRNAs (Fig. 4-4B), as previously observed. When SN50 was added to BV-2 microglia incubated with LPS, there was a slight attenuation of pro-inflammatory cytokine mRNA elevations (notably, TNF and IL-1 β), with no change in Ho-1. In spite of this, SN50 completely prevented the down-regulation of Mdr1a mRNA without altering Mdr1b levels. SN50 by itself had no effect on any of the genes quantified. The negative control inactive peptide did not change pro-inflammatory cytokine or Mdr1 transporter mRNA alone nor affected the BV-2 microglia response to LPS stimulation.

Mdr1 Expression in Activated Microglia Following Activation of Nrf2 Signaling.

BV-2 cells were exposed to sulforaphane for 4 h prior to LPS activation and collected for

quantification of cytokines and Mdr1 levels. Sulforaphane activates the Nrf2 pathway but can also inhibit the NF- κ B pathway (Guerrero-Beltrán, Mukhopadhyay et al. 2012). Sulforaphane dramatically reduced the induction of TNF, IL-1 β , and IL-6 mRNA in LPS-treated BV-2 cells (Fig 4-5A). TNF secretion into the medium was also reduced to negligible levels (Fig 4-5A). Sulforaphane alone and in combination with LPS increased the expression of Ho-1 by more than 700%. As expected, LPS caused a 40-50% reduction in Mdr1a and 1b mRNAs (Fig 4-5B). Pre-incubation with sulforaphane prevented the LPS-induced down-regulation of Mdr1a, but not Mdr1b. These mRNA results were confirmed at the protein level by western blot (Fig 4-5C).

Mdr1 Expression in the Brains of LPS-Treated Mice. Mice were treated with vehicle or a single dose of LPS (5mg/kg, i.p.). Expression of mRNA was quantified after 24 hours using qPCR (Fig. 4-6). TNF and IL-1 β mRNAs were up-regulated (1300% and 500%, respectively) in LPS-treated mice compared to saline controls. GFAP and Mac-1 mRNA expression were also elevated indicating both astrocyte and microglial activation, respectively (Fig.4-6 and data not shown). Ho-1 exhibited a slight but non-significant increase in LPS-treated mice ($p= 0.16$). Rather the Nrf2 target gene, Nqo1, was significantly up-regulated 50% in the brains of LPS-treated mice. The main ABC transporter expressed in the BBB, Mdr1a, demonstrated slightly reduced levels of mRNA expression in LPS-treated mice (Fig. 4-6A). In addition, Mdr1 protein was significantly reduced by 50% (Fig. 4-7B).

Discussion

The present study investigated the transcriptional and paracrine regulation of the Mdr1 transporter in microglia and endothelial cells in response to inflammatory stimuli. Specifically, we demonstrated that Mdr1/MDR1 was reduced in naïve microglia and brain capillary endothelial cells when cultured with LPS or microglia-secreted factors in conditioned medium. NF- κ B inhibition and Nrf2 activation abrogated LPS-mediated Mdr1a down-regulation in microglia. Furthermore, elevated pro-inflammatory cytokine and Nqo-1 mRNA in the brain accompany lowered Mdr1 protein in LPS-treated mice. Taken together, these results suggest that Mdr1/MDR1 is reduced in multiple brain cell types during inflammation and that the Nrf2 and NF- κ B pathways provide opposing mechanisms to regulate Mdr1/MDR1 levels.

Cell-cell signaling is an important mechanism for immune system regulation and function. It is also important in the brain for normal development, maintaining homeostasis, and mounting responses to injury (Abbott, Ronnback et al. 2006, Willis 2011). During development, formation of the BBB is under strict control and is highly influenced by products released from astrocytes, including glial derived neurotrophic factor (GDNF) and src-suppressed C-kinase substrate (SSeCKS) (Lajtha 1957, Abbott 2002). Homeostasis is maintained by constant cell-cell interactions and communication between astrocytes, microglia, neurons, oligodendrocytes, and endothelial cells. These cells dictate the permeability of the endothelium to limit access of many neurotoxic agents and, together, form the neurovascular unit (Willis 2011). When mounting a response to injury, cytokines TNF, IL-1 β , and IL-6 are important mediators of cellular responses (reviewed by (Hopkins and Rothwell 1995, Rothwell and Hopkins 1995).

Cytokines can protect and maintain neuronal health by triggering production of nerve growth factor and other neurotrophins, or can cause tissue damage directly through cytokine receptors. Ultimately, cytokines influence the actions of resident cells in an attempt to return to homeostasis.

The brain capillary endothelium is an important component of the BBB and maintains the ideal neuronal microenvironment. It achieves this by creating an impermeable barrier using the physical properties of the endothelial cells (tight junctions, low pinocytosis, lack of fenestrations), and active efflux from transport mechanisms such as MDR1. Communication between cells of the brain and the capillary endothelium can modulate the barrier capabilities by altering tight junctions as well as transporter expression and function (Dehouck, Meresse et al. 1990). Culturing endothelial cells in the presence of astrocytes allows endothelial cells to maintain tight junctions, high electrical resistance, inulin and sucrose impermeability, and active transport of leucine (Dehouck, Meresse et al. 1990). In fact, most *in vitro* models of the BBB require co-culture of endothelial cells with astrocytes in order to maintain barrier properties (Dehouck, Meresse et al. 1990, El Hafny, Chappey et al. 1997, Abbott 2002, Abbott, Ronnback et al. 2006). Studies using immortalized RBE4 rat brain endothelial cells suggest that factors produced by astrocytes modestly increase the basal expression and function of Mdr1 in these cells (El Hafny, Chappey et al. 1997). The authors speculated that these factors include basic fibroblast growth factor and transforming growth factor β . Wang and coworkers demonstrated, using mouse astrocyte and endothelial cell cultures, that IL-1 β produced by microglia increases BBB permeability by suppressing astrocytic sonic hedgehog production (Wang, Jin et al. 2014). Sonic hedgehog from astrocytes

promotes BBB integrity by up-regulating tight junctions in endothelial cells (Alvarez, Dodelet-Devillers et al. 2011). Together these studies demonstrate the importance of cell-cell communication in the maintenance of tight junctions and transporters for integrity of the BBB phenotype.

Microglia have the ability to rapidly produce and release a variety of soluble factors in response to activation (Badoer 2010). Our laboratory has previously demonstrated the reduced expression and function of Mdr1 in BV-2 microglia cultured with LPS (Gibson, Hossain et al. 2012). In the current study, we further show that soluble factors released by activated microglia are also capable of down-regulating Mdr1 in naïve BV-2 microglia and in capillary endothelial cells. Demonstrating that microglia-derived mediators are adept at regulating transporters in capillary endothelium, independent from astrocytes, is important in understanding BBB permeability during inflammation. Studies using porcine brain capillaries have similarly shown that exogenous administration of TNF and IL-1 β reduce Mdr1 protein expression after 24 to 48 hours (von Wedel-Parlow, Wolte et al. 2009). Others demonstrated using microglia/rat brain endothelial cell co-cultures that NADPH oxidase, produced by microglia, is involved in early Mdr1 dysfunction (Matsumoto, Dohgu et al. 2012). This study assessed Mdr1 function early (6 h) following microglial activation and prior to loss of Mdr1 protein. Together, these studies demonstrate that inflammation can affect Mdr1 function through multiple mechanisms and can have immediate inhibitory effects or delayed effects on function through lowered transcription.

Nrf2 initiates a redox-sensitive pathway that is intimately connected to cellular oxidative stress. Many antioxidant enzymes and drug efflux transporters are regulated in

tissues by the Nrf2 pathway (Aleksunes, Goedken et al. 2010, Klaassen and Aleksunes 2010). Recently, Wang and co-workers (2014) performed detailed experiments using isolated brain capillaries from wild-type, Nrf2-null, and p53-null mice to determine how these pathways participate in Mdr1 regulation. They demonstrated that the Nrf2 activator, sulforaphane, increased transport of verapamil, an Mdr1 substrate, in isolated capillaries of wild-type but not from those of Nrf2-null mice. However, increased Mdr1 function was dependent on p53 whereby inhibiting p53-dependent gene transcription, blocked the ability of sulforaphane to increase Mdr1 transport activity and protein expression. Additionally, inhibition of NF- κ B prevented sulforaphane-induced Mdr1 transport activity in isolated rat brain capillaries (Wang, Campos et al. 2014). In contrast, our current study demonstrates that inhibition of NF- κ B abolishes LPS-induced down-regulation of Mdr1 mRNA and protein in microglia. We also demonstrate that the Nrf2 activator, sulforaphane, does not induce Mdr1 in BV-2 microglia, but completely prevents TNF protein release from activated microglia and prevents LPS-induced up-regulation of TNF, IL-1 β , and IL-6 mRNA, suggesting that sulforaphane inhibits NF- κ B. The reasons for the contrasting effects of sulforaphane and NF- κ B in these studies may be due to several factors. Importantly, NF- κ B is composed of multiple interchangeable subunits and depending on which dimers are active can have profoundly different downstream effects (Siggers, Chang et al. 2012). Therefore, NF- κ B in microglia, which are innate immune cells, may have signaling differences than NF- κ B in capillary endothelial cells. Also, our current study evaluated Mdr1 regulation during inflammation as opposed to the basal state, as in the studies by Wang and co-workers. The fact that

basal regulation of Mdr1 may be different highlights the dramatic impact that soluble mediators produced by activated microglia can have on cell signaling.

The consequence of reduced MDR1 protein and transporter function at the BBB is highlighted by studies that demonstrate increased brain accumulation of potentially neurotoxic MDR1 substrates including digoxin, ivermectin, and doxorubicin (Goralski, Hartmann et al. 2003, Hartmann, Vassileva et al. 2005, Geyer, Gavrilova et al. 2009). MDR1 function is also important for limiting the brain accumulation of a wide variety of xenobiotics and therapeutic drugs (Schinkel, Smit et al. 1994, Miller, Nobmann et al. 2000, Loscher and Potschka 2005, Geyer, Gavrilova et al. 2009, Asakawa, Ogawa et al. 2011). MDR1 is increasingly being evaluated with regards to the transport and elimination of endogenous neurotoxic substances including β -amyloid (Deo, Borson et al. 2014). It is clear that MDR1 expression and function is not static. The regulation of MDR1 in the BBB is complex, and understanding the roles of various pathways that control transcriptional as well as paracrine signaling will help in fully understanding MDR1 modulation.

Conclusion

Regulation of Mdr1/MDR1 at the BBB is important in understanding how neuroinflammation can impact brain exposure to harmful chemicals. Some cell-cell interactions in the brain, such as astrocyte-endothelial communication, are important for BBB development and maintaining homeostasis. Others, such as microglial-endothelial interactions have the potential to be detrimental and increase BBB permeability, in part

by altering Mdr1 expression and function. Understanding these interactions and the mechanisms involved will allow for the development of therapeutics that can either enhance or modify the barrier properties of the brain capillary endothelium.

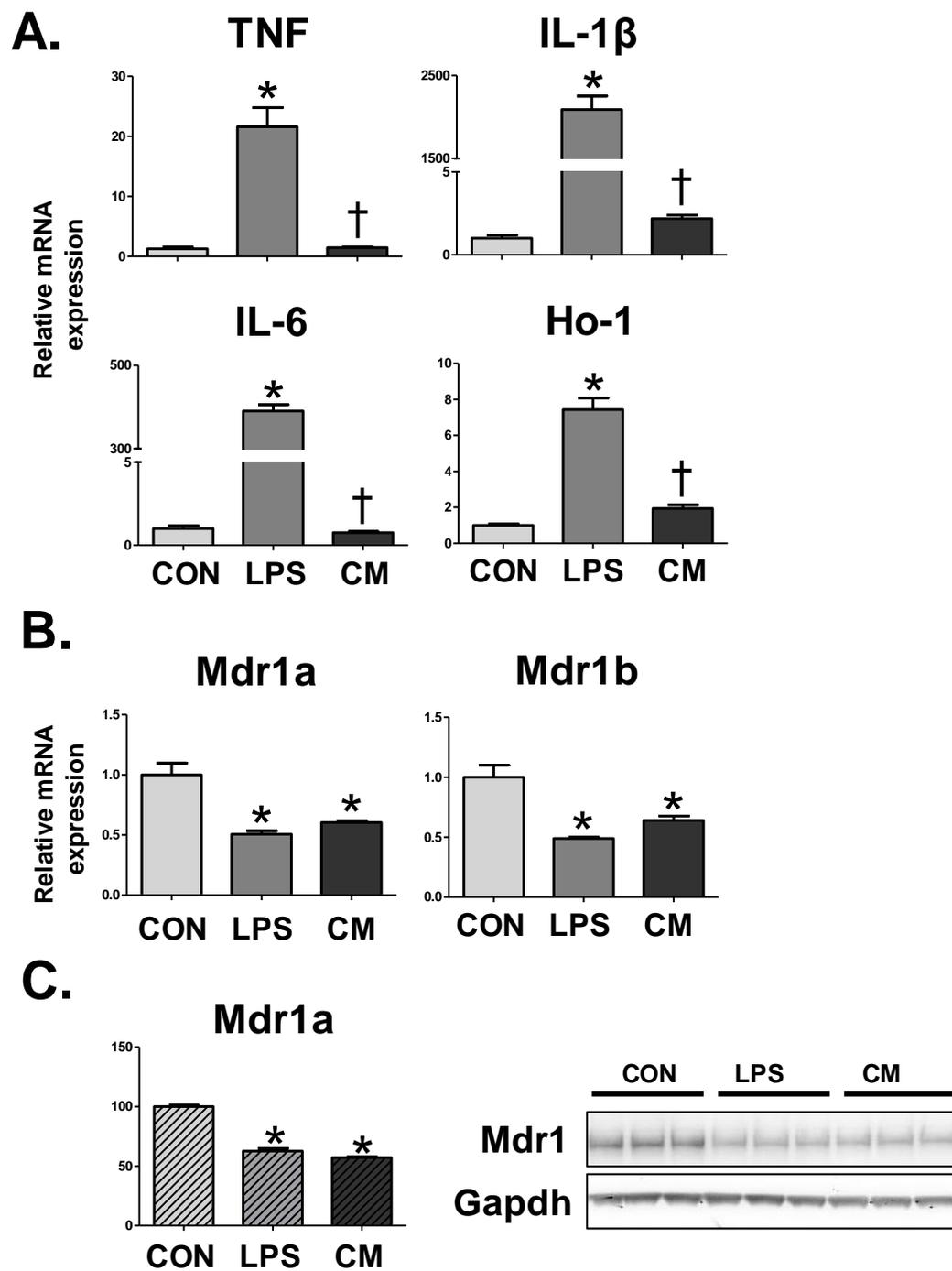


Figure 4-1

Figure 4-1. Regulation of Mdr1 in BV-2 microglia exposed to conditioned medium.

Resting microglia were treated for 12 h with either LPS (500 ng/ml) or conditioned medium (CM) from activated microglia. A) Pro-inflammatory cytokine and Ho-1 mRNAs were quantified by qPCR. B) Mdr1a and Mdr1b mRNA were quantified by qPCR, and C) Mdr1 protein expression was semi-quantified by western blot at 24 h. Data were normalized to Rpl13A and presented as mean expression \pm SE (n= 3-6). Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control.

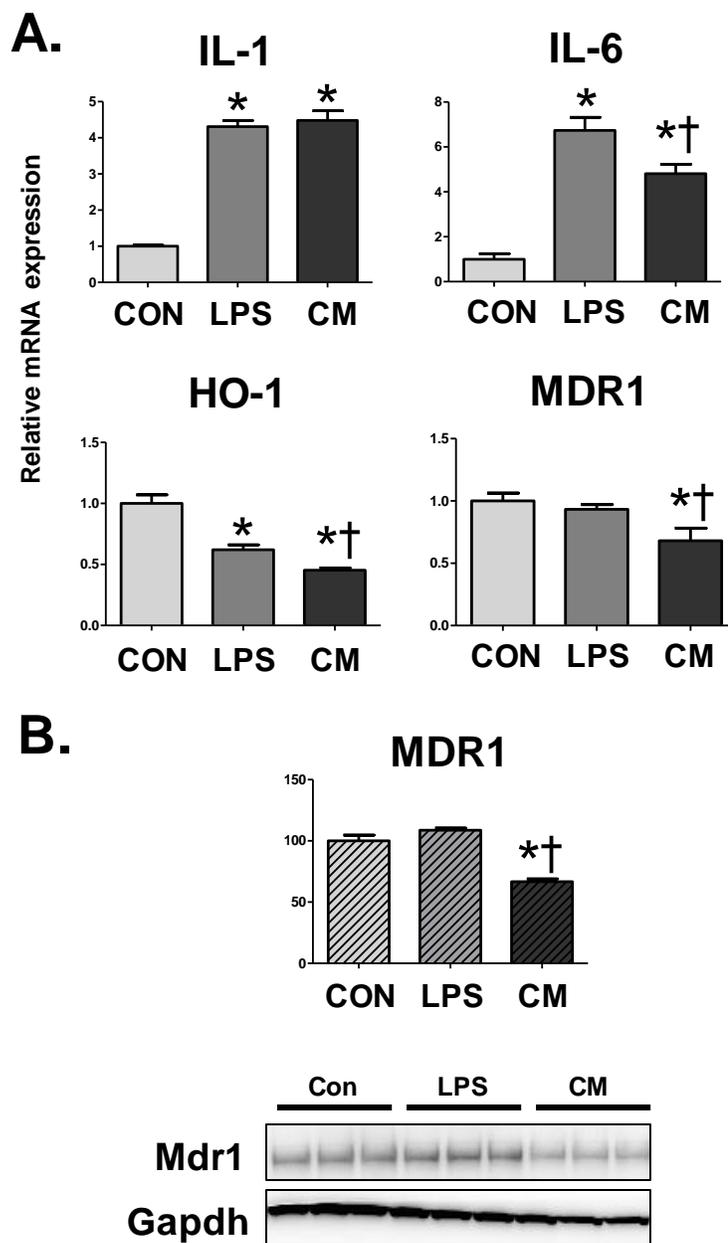


Figure 4-2

Figure 4-2. Regulation of MDR1 in hCMEC/D3 endothelial cells exposed to conditioned medium. Human microvascular endothelial cells (hCMEC/D3) were treated with conditioned medium (CM) from LPS-activated BV-2 microglial cells. Relative pro-inflammatory cytokine, MDR1, and Nrf2 responsive gene mRNA was determined after

12 hour incubation of hCMEC/D3 cells with CM or LPS. Data were normalized to Rpl13A and presented as mean relative mRNA expression \pm SE (n= 4-6). B) Mdr1 protein expression was semi-quantified by western blot at 24 h. Representative western blots exhibit changes in protein expression. Gapdh was used as loading control. Data are presented as mean relative protein expression \pm SE (n=3). Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control. Single dagger (†) represent statistically significant differences ($p < 0.05$) compared to LPS.

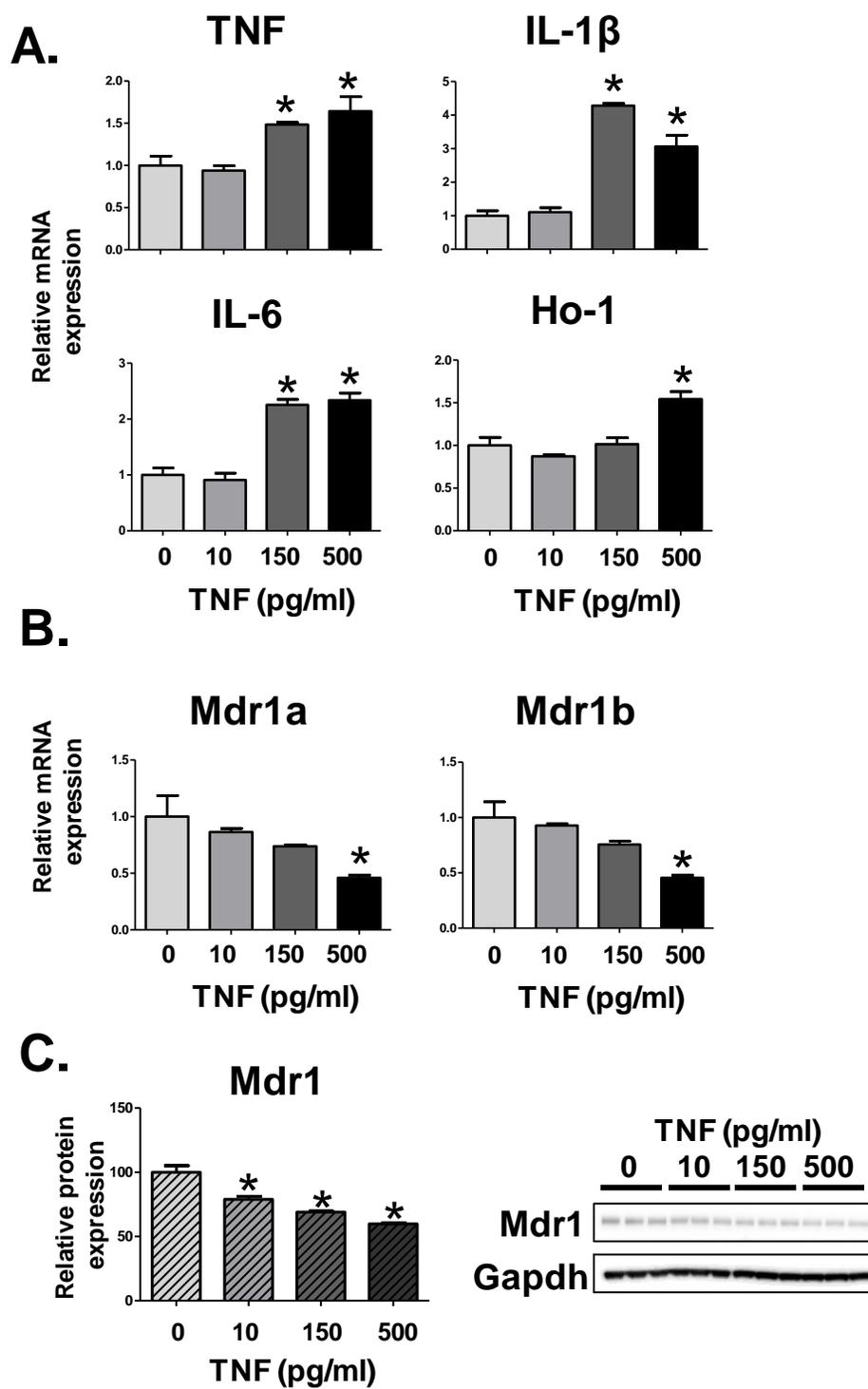


Figure 4-3

Figure 4-3. Regulation of Mdr1 in BV-2 microglia exposed to recombinant TNF.

Resting microglia were treated for 12 h with recombinant mouse TNF (0, 10, 150, 500 pg/ml). A) Relative pro-inflammatory cytokines and Ho-1 mRNAs were quantified by qPCR. B) Mdr1a and Mdr1b mRNA were quantified using qPCR, and C) Mdr1 protein expression was semi-quantified by western blot at 24 h. Data were normalized to Rpl13A and presented as mean \pm SE (n= 3-6). Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control.

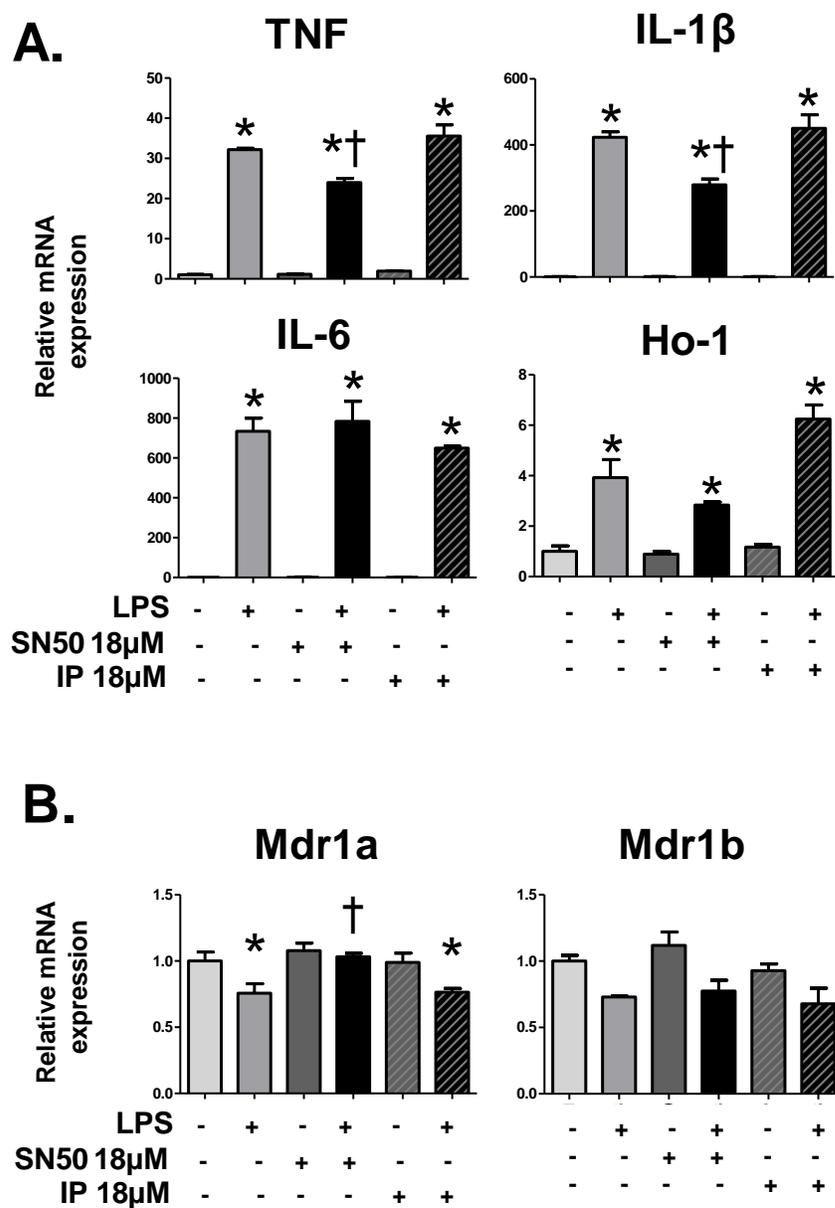


Figure 4-4

Figure 4-4. Regulation of Mdr1 in activated BV-2 microglia following NF- κ B inhibition. Microglia were treated with vehicle or the NF- κ B inhibitor, SN50 (18 μ M) or its inactive peptide (IP) in the presence and absence of LPS (500 ng/ml) for 12 h. A) Relative pro-inflammatory cytokine, and Nrf2 responsive gene mRNA were quantified

by qPCR. B) Mdr1a and Mdr1b mRNA were quantified using qPCR. Data were normalized to Rpl13A and presented as mean relative mRNA expression \pm SE (n= 4-6). Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control. Single dagger (†) represent statistically significant differences ($p < 0.05$) compared to LPS treatment alone.

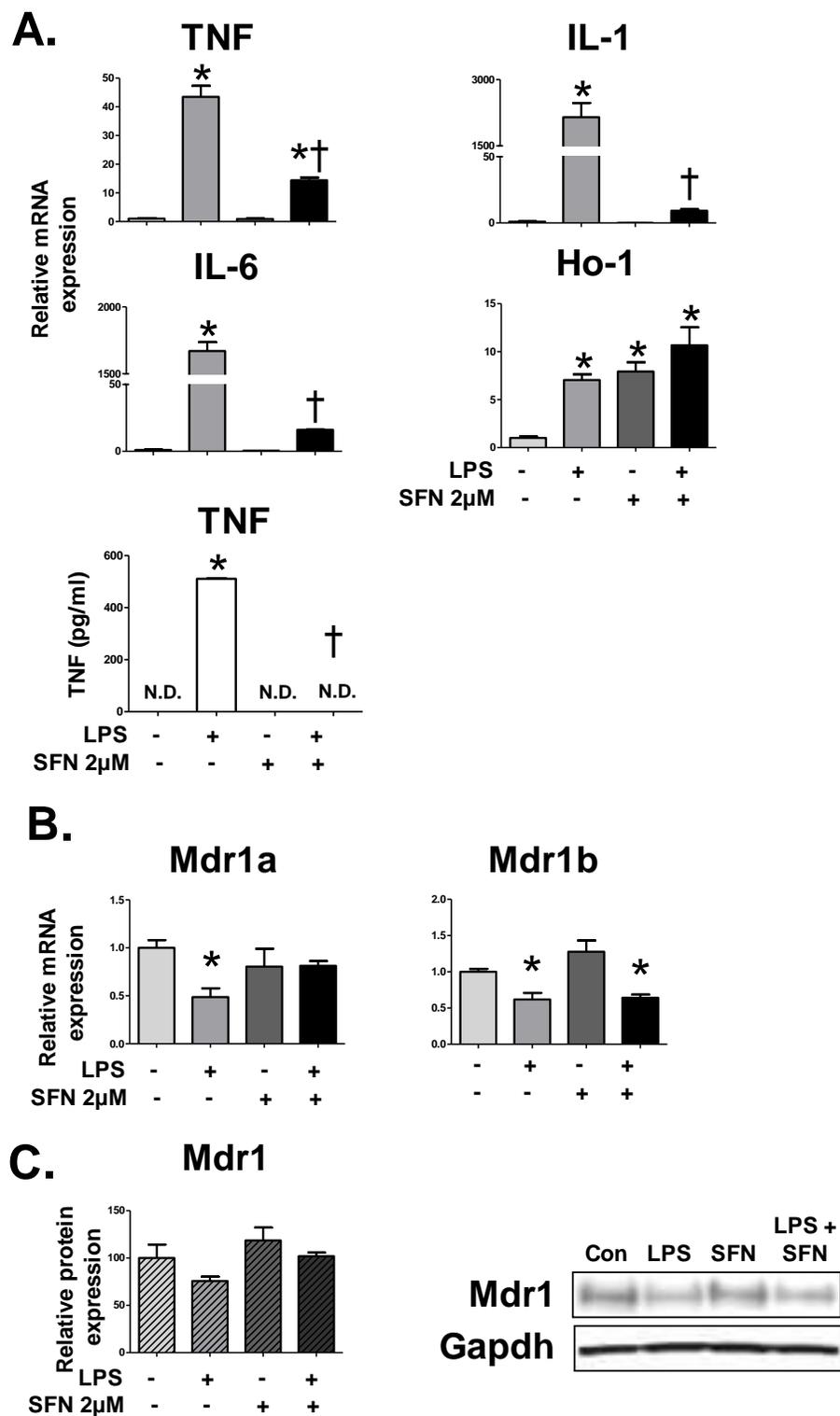


Figure 4-5

Figure 4-5. Regulation of Mdr1 in activated BV-2 microglia following Nrf2 activation. BV-2 microglia were treated with vehicle or sulforaphane (2 μ M) in the presence and absence of LPS (500 ng/ml) for 12 hours. A) Pro-inflammatory cytokine and Ho-1 mRNAs were quantified by qPCR and TNF release was quantified using ELISA. B) Mdr1a and Mdr1b mRNA were quantified using qPCR. Data were normalized to Rpl13A and presented as mean relative mRNA expression \pm SE (n= 3). C) Mdr1 protein expression was semi-quantified by western blot at 24 h. Representative western blots exhibit changes in protein expression. Gapdh was used as loading control. Data are presented as mean relative protein expression \pm SE (n=3). Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control. Single dagger (†) represent statistically significant differences ($p < 0.05$) compared to LPS.

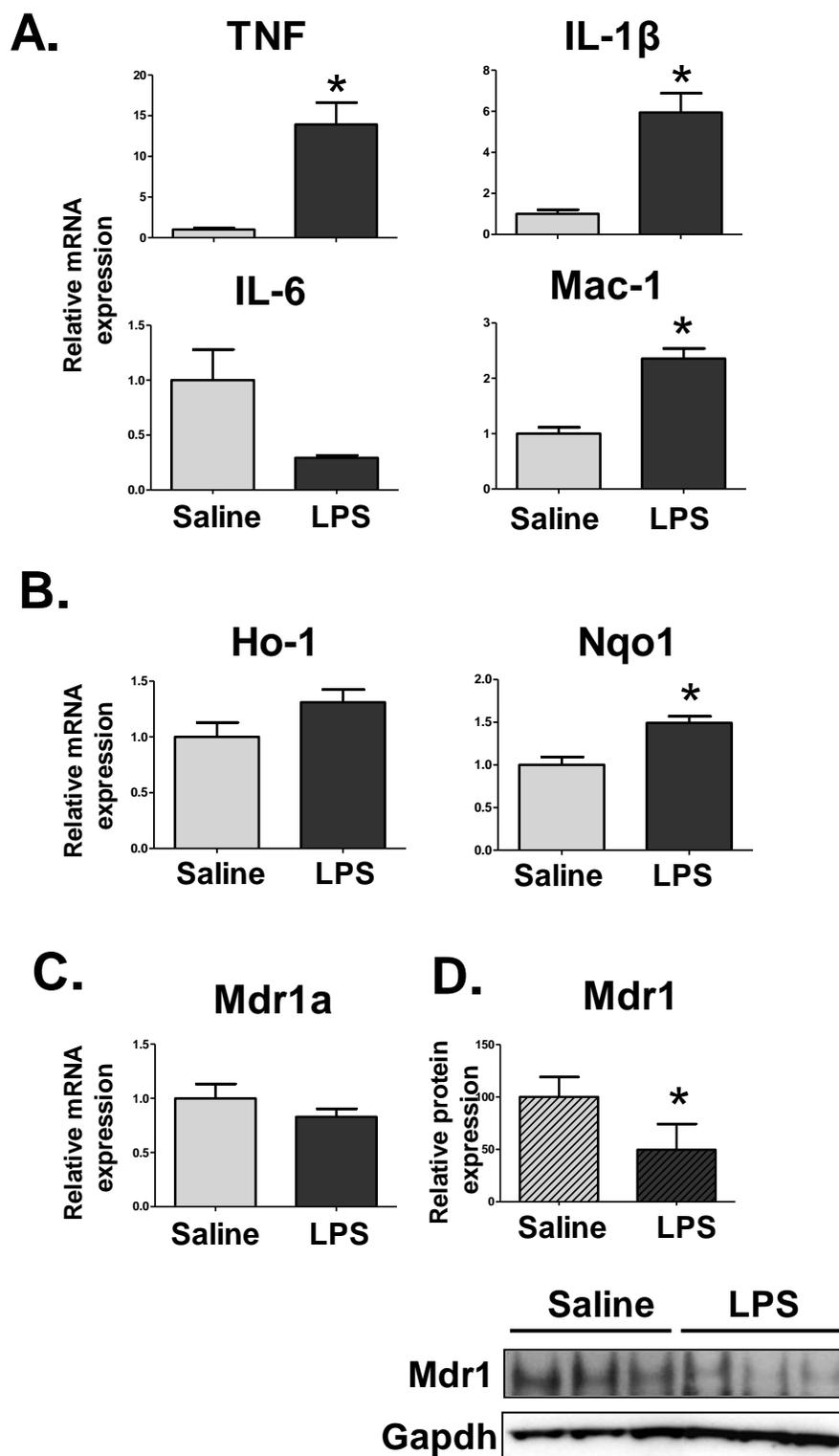


Figure 4-6

Figure 4-6. Regulation of Mdr1 in brains from mice treated with lipopolysaccharide.

The ability of systemic administration of LPS to mice to initiate inflammation in the brain was evaluated. Mice were treated with a single i.p. dose of saline vehicle (5ml/kg) or LPS (5 mg/kg) and tissues were collected after 24 hours. Quantitative PCR was used to measure (A) pro-inflammatory cytokine mRNA (TNF, IL-1 β , IL-6), microglial activation (Mac-1) and (B) activation of the antioxidant Nrf2 pathway (Ho-1, Nqo1, and Nrf2). Data were normalized to Rpl13A and presented as mean relative mRNA expression \pm SE, (n = 4/ group). (C) MDR1 mRNA and (D) protein expression were determined by qPCR and western blot, respectively. Gapdh was used as loading control. Data are presented as mean relative protein expression \pm SE (n=3-4). Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control.

Table 4-1. Inhibitors and activators of intracellular signaling pathways.

Pathway	Chemical name	Concentration	Preincubation	Mechanism	Alternate Mechanism
NF-κB	SN50	18 μ M	15 minutes	Inhibit NF- κ B translocation	
Nrf2	Sulforaphane	2 μ M	4 hours	Activate Nrf2	Inhibit NF- κ B

Supplemental Table 4-1. qPCR primer sequences

Target	Forward Primer (5' → 3')	Reverse Primer (5' → 3')
<i>mTNF</i> ^a	AGCCACGTCGTAGCAAACCAC	AGGAGCACGTAGTCGGGGCA
<i>mIL-1β</i> ^b	GACCTTCCAGGATGAGGACA	AGTCATATGGGTCCGACAG
<i>mIL-6</i> ^b	AGTTGCCTTCTTGGGACTGA	CAGAATTGCCATTGCACAAC
<i>mRpl13a</i> ^a	CTGTGAAGGCATCAACATTTCTG	GACCACCATCCGCTTTTTCTT
<i>mMdr1a/Abcb1a</i> ^a	TGCCCCACCAATTTGACACCCT	ATCCAGTGCGGCCTGAACCA
<i>mMdr1b/Abcb1b</i> ^a	GTGTTAAAGGGGCGATGGGCG	AGGCTTGGCCAGACAACAGCTT
<i>mHo-1</i> ^a	GGTGACAGAAGAGGCTAAGACCGC	GCAGTATCTTGCACCAGGCTAGCA
<i>mNqo1</i> ^a	AGGATGGGAGGTACTIONCGAATC	AGGCGTCCTTCCTTATATGCTA
<i>hIL-1β</i> ^a	CTCGCCAGTGAAATGATGGCT	GTCGGAGATTCGTAGCTGGAT
<i>hIL-6</i> ^a	AAATTCGGTACATCCTCGACGG	GGAAGGTTTCAGGTTGTTTTCTGC
<i>hMDR1/ABCB1</i> ^a	TTGAAATGAAAATGTTGTCTGG	CAAAGAAACAACGGTTCGG
<i>hNQO1</i> ^a	CAAAGGACCCTTCCGGAATAA	ACTTGGAAGCCACAGAAATGC
<i>hHO-1</i> ^a	CTGCTCAACATCCAGCTCTTTG	AGTGTAAGGACCCATCGGAGA

^a Integrated DNA Technologies (Coralville, IA)

^b University of Rhode Island, Kingston, RI, Dr. Angela Slitt

CHAPTER 5

Summary

There is mounting evidence that both genetic and environmental factors contribute to the development of PD. Dopamine neuron loss in the substantia nigra is one of the hallmarks of PD. More recent data suggest that neuroinflammation, through the production of pro-inflammatory mediators by microglia, actively contributes to the pathogenesis of PD. Epidemiologic studies examining the relationship between PD and pesticide exposure often have mixed results but generally conclude that PD is a multifactorial disorder with a potential contributory role for certain pesticides. Building upon these studies, researchers have suggested that there may be an interaction between pesticide exposure and genetic variants in proteins that regulate pesticide disposition, which sensitizes individuals to PD. Elucidation of these interactions may help to identify sensitive populations at risk of developing PD due to an impaired ability to restrict the brain accumulation of toxic chemicals. The work presented in this dissertation examined the role that the MDR1 efflux transporter plays in preventing dopaminergic neuron death from chemical-induced toxicity, as well as immunologic factors that modulate this protective function.

Pesticides are capable of producing neuronal degeneration and/or neuroinflammation in rodent and non-human primate models (Mulcahy, Walsh et al. 2011, Muroyama, Kobayashi et al. 2011, Swarnkar, Singh et al. 2011, Walsh, Finn et al. 2011, Blesa, Phani et al. 2012, Jackson-Lewis, Blesa et al. 2012). One pesticide often used to model specific components of PD in rodents is the herbicide paraquat. The

interest in paraquat began when people accidentally exposed to the compound MPTP developed parkinsonian-like symptoms (Langston and Ballard 1984, Ballard, Tetud et al. 1985). MPTP and paraquat have very similar chemical structures, therefore it was hypothesized that environmental exposure to paraquat may be responsible for sporadic cases of PD. While it has since been demonstrated that MPTP and paraquat have different mechanisms of toxicity, paraquat continues to be used as a tool to study neurodegeneration (Richardson, Quan et al. 2005). Since the initial interest in paraquat, there have been many published reports examining paraquat-induced neurodegeneration in rodents (McCormack, Thiruchelvam et al. 2002, Peng, Mao et al. 2004, Richardson, Quan et al. 2005, Purisai, McCormack et al. 2007, Mangano and Hayley 2009, Mangano, Litteljohn et al. 2012, Mosley, Jiao et al. 2012).

Preliminary studies in our laboratory suggested that paraquat was a substrate for the efflux transporter, MDR1. In those studies, HEK293 cells transfected with the human *MDR1* gene demonstrated lower retention of paraquat than parental HEK293 cells. Based on these results, experiments in **Chapter 2** further characterized paraquat transport *in vitro* and examined neuronal degeneration in *Mdr1a/1b*-null mice exposed to paraquat. In addition to the characterization of paraquat transport in MDR1 over-expressing HEK293 cells, *in vitro* experiments described in Chapter 2 used immortalized human microvascular endothelial cells (hCMEC/D3) that endogenously express functional MDR1. These cells were used because they basally express MDR1 and are a relevant model of the human BBB (Weksler, Subileau et al. 2005, Poller, Drewe et al. 2009, Tai, Reddy et al. 2009).

As demonstrated in **Chapter 2**, hCMEC/D3 endothelial cells with endogenous MDR1 expression had low intracellular accumulation of paraquat. Genetic knock-down of MDR1 by siRNA silencing and pharmacological inhibition of MDR1 using the specific inhibitor PSC833 increased intracellular accumulation of paraquat relative to control hCMEC/D3 cells. Additionally, hCMEC/D3 endothelial cells treated with PSC833 had increased cytotoxicity when exposed to paraquat. From these studies, we concluded that human MDR1 prevented paraquat accumulation in BBB cells. These data are supported by other studies that have also suggested paraquat is an MDR1 substrate (Dinis-Oliveira, Duarte et al. 2006, Zerlin, Kim et al. 2012). Since MDR1 is highly expressed at the BBB and is able to reduce the accumulation of many chemicals in the brain, we sought to determine whether Mdr1a/1b is responsible for preventing paraquat accumulation in the mouse brain. The majority of systemically-administered paraquat does not cross the BBB (Bartlett, Holden et al. 2009, Prasad, Tarasewicz et al. 2009). Despite restricted access to the brain parenchyma, paraquat is still able to cause neuroinflammation and neurodegeneration but only when multiple doses are administered (Mangano and Hayley 2009, Mangano, Litteljohn et al. 2012). Brain concentrations of paraquat were measured in a time-course (2, 4, 12, 24, and 48h) but only the 2 hour time point demonstrated slightly increased paraquat in the midbrains of Mdr1a/1b-null mice. The difference from wild-type mice was not significant but suggests that most paraquat enters the brain very early (<2hr).

The most impressive result from the *in vivo* paraquat study was demonstrated by stereologic counting of dopaminergic neurons in the midbrains of wild-type and Mdr1a/1b-null mice. In this experiment, Mdr1a/1b-null mice had dramatically reduced

numbers of dopamine neurons in the SNc 7 days following a single intraperitoneal injection of paraquat. In contrast, wild-type mice did not demonstrate any neuronal loss following a single dose. This response is consistent with other published results demonstrating that either a priming event or multiple paraquat doses are required for paraquat-induced neuronal loss in wild-type mice (Mangano and Hayley 2009). Two doses of paraquat did not further enhance neuronal loss in Mdr1a/1b-null mice, but did decrease the number of dopaminergic neurons in wild-type mice. Additionally, a single paraquat exposure in Mdr1a/1b-null mice caused microglial activation, as demonstrated by increased Mac-1 immunofluorescence and morphologic changes associated with an activated phenotype. Astrocyte activation was also apparent 7 days following paraquat exposure in both wild-type and Mdr1a/1b-null mice. These *in vivo* results clearly show that Mdr1/1b-null mice have significant neuronal loss following a single paraquat exposure. Exploration of the molecular events that occur during a single paraquat exposure in Mdr1a/1b-null mice are needed. Loss of Mdr1a/1b may be an important priming event for subsequent neuronal death following paraquat exposure. Characterization of Mdr1a/1b expression and function in wild-type mice following a single paraquat exposure could support this conclusion. Additionally, *in vivo* chemical inhibition of Mdr1a/1b, using PSC833, prior to paraquat would be useful in investigating functional Mdr1 loss as a possible priming event. Additional work evaluating downstream effects of dopamine neuron loss (i.e. striatal dopamine levels, behavioral assessments) would build on these initial studies.

The experiments in **Chapter 2** also tested whether MDR1 transports paraquat at the BBB and protects against its neurotoxic effects in mice. There was strong evidence to

show that paraquat is a substrate for human MDR1 *in vitro*, however, it could not be concluded that paraquat is a substrate for mouse Mdr1a/1b in the brain. Although there was a trend for increased paraquat brain accumulation in Mdr1a/1b-null mice, the difference was not statistically significant at the time points measured. In contrast, 4 h after paraquat treatment, renal concentrations of paraquat in the kidneys of Mdr1a/1b-null mice were 750% higher than wild-type mice (Wen, Gibson et al. 2014). The reason for significant accumulation of paraquat in the kidneys and not the brain at these time points may be partially explained by the difference in active uptake and the relative abundance of proposed paraquat uptake transporters. A more extensive examination of the active uptake transporters may also reveal important determinants of early paraquat pharmacokinetics. Subsequent studies are needed to better characterize paraquat accumulation in the brains of wild-type and Mdr1a/1b-null mice. Earlier time points (30, 60, 90 minutes) may help in better understanding how Mdr1a/1b is involved in preventing paraquat access to the brain. Future experiments examining paraquat transport using isolated mouse brain capillaries would help answer questions regarding specifics about paraquat movement across the mouse BBB. The *in vitro* studies in this dissertation characterized paraquat transport by human MDR1. Additional *in vitro* characterization of paraquat transport and affinity using mouse Mdr1a and Mdr1b may be helpful in determining the translatability of paraquat efflux between mouse Mdr1a/Mdr1b and human MDR1. Identifying differences in the kinetics of paraquat transport by Mdr1a versus Mdr1b are also necessary to better understand the distribution of paraquat within different cellular compartments in the mouse brain.

Neuronal loss observed in paraquat-treated Mdr1a/1b-null mice may also be partially explained by indirect effects of paraquat and subsequent byproducts of toxicity. Paraquat undergoes redox cycling, producing ROS, oxidized proteins, and oxidized lipids. Since MDR1 is responsible for the transport of a large variety of chemicals it is possible that endogenous, neurotoxic byproducts from redox cycling and inflammation are not adequately cleared from the brains of Mdr1a/1b-null mice. Additional studies are needed to determine if neuronal degeneration in Mdr1a/1b-null mice is directly related to paraquat or to potential neurotoxic Mdr1a/1b substrates that are secondarily produced during microglial activation and inflammation. Lipid peroxides are one potential toxic byproduct known to accumulate following paraquat exposure in mice (Prasad, Winnik et al. 2007, Navarro, González-Álvarez et al. 2011). It is possible that some oxidized lipids are either substrates for MDR1, inhibit MDR1 function, or both. Experiments investigating the impact of lipid composition on MDR1 function would be valuable in understanding indirect toxic effects of paraquat on MDR1. Performing transport studies in the presence of saturated fatty acids, cholesterol, oxidized lipids, or benzyl alcohol would help elucidate the role of membrane fluidity and oxidation in MDR1 transporter function. Evidence supporting this hypothesis has been provided by *in vitro* experiments demonstrating that MDR1 function is highly influenced by membrane lipid composition (Gelsomino, Corsetto et al. 2013, Sharom 2014). Additional experiments examining paraquat-induced production and accumulation of malondialdehyde, ceramide or β -amyloid *in vitro* and *in vivo* would add to our understanding of indirect toxicity of paraquat, as both of these are substrates of MDR1 (Lee, Torchalski et al. 2011, Gibson, Hossain et al. 2012, Deo, Borson et al. 2014). Additional studies using fluorescent-

labeled oxidized lipid substrates could also be useful for *in vitro* experiments.

Because inflammation is one of the hallmarks of PD and pro-inflammatory cytokines can cause neuronal degeneration, studies in **Chapter 3** were designed to interrogate the interplay between inflammation and ABC efflux transporter regulation. After observing robust microglial activation in mice treated with paraquat, we used the mouse microglial BV-2 cell line to investigate ABC transporter expression and function in an *in vitro* model of neuroinflammation. BV-2 cells were used because they are easy to culture and mount strong inflammatory responses in response to LPS treatment, including morphological changes and production of cytokines (Henn, Lund et al. 2009, Alvarez-Erviti, Couch et al. 2011). The experiments in this chapter investigated the regulation of Mdr1 and other ABC efflux transporters in microglia. Because little prior research on this topic had been performed in BV-2 cells, we characterized baseline transporter function as well as the mRNA, protein, and function of transporters in response to LPS stimulation.

To characterize ABC transporter function, fluorescent substrates specific to Mdr1, Mrps, or Bcrp were selected (Rhodamine 123, Calcein AM, and Hoechst, respectively). Additionally, specific inhibitors for each class of transporter were used to demonstrate transporter function in resting BV-2 microglia. Initial studies showed that resting BV-2 microglia had functional Mdr1, Mrps, and Bcrp activity as observed by the retention of their respective fluorescent substrates with the use of specific inhibitors. BV-2 microglia were exposed to LPS and pro-inflammatory cytokine expression was determined at 12 and 24 hours to confirm activation of BV-2 microglia. Fluorescent substrate accumulation was measured and activated BV-2 microglia exhibited reduced Mdr1, Mrp, and Bcrp function in LPS-treated cells. ABC transporter protein and mRNA expression

were quantified to determine if the loss of function was due to reduced expression. Interestingly, activated BV-2 microglia had lower Mdr1a, Mdr1b, Mrp4, and Bcrp protein and mRNA expression compared to resting cells. However, Mrp1 and Mrp5 mRNA and protein were significantly elevated in activated BV-2 microglia. These results are consistent with reports that endotoxin/LPS and pro-inflammatory cytokines cause decreased expression and function of Mdr1 in mouse liver (Ando, Nishio et al. 2001) and BBB using rat brain microvessels (Hartz 2005). To further determine if ABC transporter regulation was directly related to microglial activation and pro-inflammatory cytokine production, an NF- κ B inhibitor, Bay 11-7082, was used. In these studies, IL-1 β , IL-6, and TNF production in response to LPS were markedly attenuated with Bay11-7082. Resulting effects on transporter expression varied. NF- κ B inhibition prevented the LPS-induced down-regulation of Mdr1a only. Changes previously observed in Mdr1b, Bcrp, and Mrp1 mRNA levels in activated BV-2 microglia were not affected by NF- κ B inhibition. However, Mrp5 mRNA expression was elevated by Bay 11-7082 in resting microglia. Additionally, activated microglia treated with Bay 11-7082 had greater Mrp5 expression than LPS alone. Examination of the transcription factor Nrf2 and the Nrf2 responsive gene, Nqo1, demonstrated that Bay 11-7082 likely affected Mrp5 expression by activating the antioxidant response pathway. It is well-established that some members of the ABC transporter family are regulated by Nrf2 (Maher, Dieter et al. 2007, He, Chen et al. 2008, Anwar-Mohamed, Degenhardt et al. 2011, Higgins and Hayes 2011). Various studies have also investigated the role of inflammatory cytokines on ABC transporter expression in other tissues with similar results regarding protein, mRNA and functional expression (Hartmann, Vassileva et al. 2005, Lickteig, Slitt et al. 2006, Ronaldson,

Ashraf et al. 2010). Initial characterization of primary mouse microglia demonstrated comparable basal expression of ABC transporters to BV-2 cells as well as alterations in ABC efflux transporter mRNA following activation with LPS (Chapter 3, Supplemental Fig. 3-4). **Chapter 3** is the first comprehensive study of ABC efflux transport function in the BV-2 microglial cell line and demonstrates that microglial activation can alter ABC transporter expression and function. These studies in combination with data from **Chapter 2** demonstrate that neuroinflammation may differentially regulate ABC transporters in the brain. This could have a significant influence on the ability of the BBB to limit the entry of toxic substances brain and affect removal of toxic metabolites.

Additional studies are needed to determine ABC efflux transporter functional changes in primary microglia and *in vivo*. Isolation of microglia from mice exposed to LPS would help clarify if transporter function is reduced *in vivo* in response to systemic inflammation. Relevance for human transporters could be further demonstrated by showing similar expression and functional changes induced by inflammation in human microglia. Follow up studies should aim to demonstrate that the Bay 11-7082 rescue of Mdr1 results in restoration of functional activity. Additionally, since many neurodegenerative diseases are characterized by persistent inflammation and microglial activation, future studies could follow transporter expression and function for longer durations to see if the observed down-regulation is persistent. Of greater value would be deeper investigation into the effect of microglial Mdr1 reduction on neurons. From the current studies, it is not clear if decreased microglial Mdr1 is beneficial or detrimental for neuronal health. It is possible that Mdr1 reduction in microglia is a protective mechanism to sequester neurotoxicants away from sensitive neurons. Alternatively, activated

microglia may serve as a reservoir for neurotoxicants thereby prolonging exposure to neurons. Co-culture systems with microglia and neurons could be used to help answer this question.

The final aim of this dissertation was to identify whether microglia-secreted factors can regulate Mdr1 in naïve microglia and endothelial cells, and to characterize the contribution of TNF, NF- κ B, and Nrf2 signaling pathways to the regulation of Mdr1 in microglia. Studies from **Chapter 3** initiated the investigation into ABC transporter regulation in microglia. **Chapter 4** built on those studies and focused on Mdr1 regulation during inflammation in microglia. Data from **Chapter 3** demonstrated that microglia that were activated by LPS had reduced expression of Mdr1. Initial studies also demonstrated that NF- κ B inhibition using Bay 11-7082 prevented LPS-induced Mdr1 down-regulation. However, in addition to NF- κ B inhibition, it appears that Bay 11-7082 also activates the Nrf2 pathway. This ability of Bay 11-7082 to activate Nrf2 has also been documented by other groups (Min, Lee et al. 2011). Therefore, experiments in **Chapter 4** investigated the role of the NF- κ B pathway, pro-inflammatory cytokines, and the Nrf2 pathway in the regulation of Mdr1. Initial studies used conditioned medium from activated BV-2 microglia. The conditioned medium contained soluble TNF and other mediators released by activated BV-2 microglia over a 24 h period. Conditioned medium did not induce pro-inflammatory cytokine production in naïve BV-2 microglia, however, Mdr1a and Mdr1b mRNA and protein were reduced, similar to LPS alone. These data suggest that soluble mediators released from microglia are responsible for Mdr1 regulation as opposed to signaling through LPS binding to toll-like receptors. Additional studies demonstrated that recombinant TNF also decreased Mdr1 mRNA and protein. Blockade of NF- κ B using the

peptide inhibitor, SN50, corroborated the results produced with Bay 11-7082 used in **Chapter 3**. SN50 prevented the lowered expression of Mdr1a mRNA in LPS-activated microglia. Interestingly, the Nrf2 activator, sulforaphane, also attenuated the down-regulation of Mdr1. However, it is noted that sulforaphane is also a potent inhibitor of NF- κ B, further supporting the role of this pathway in Mdr1 regulation. These data are consistent with publications suggesting that Mdr1 expression and function are influenced by inflammation and NF- κ B signaling (Goralski, Hartmann et al. 2003, Hartz 2005, Morgan, Goralski et al. 2008).

Conditioned medium from activated BV-2 microglia caused a decrease in MDR1 mRNA levels in hCMEC/D3 endothelial cells similar to that observed in naïve microglia. This was somewhat surprising since mouse TNF was apparently able to initiate signaling in a human cell line. Additional studies are needed to fully characterize this interaction. Demonstrating altered MDR1 function in hCMEC/D3 cells treated with conditioned medium would strengthen these results. Thorough characterization of the conditioned medium can help identify other cytokines or signaling molecules responsible for the observed changes in transporter expression. These mediators may be quantified by ELISA or LC/MS and pharmacological inhibitors can be used to probe their contribution to MDR1 down-regulation in hCMEC/D3 cells. Mdr1 regulation in microglia could be more specifically investigated by isolating primary microglia from Nrf2 knockout and NF- κ B knockout mice. Without NF- κ B, Mdr1 would likely not be down-regulated when NF- κ B knockout microglia are activated by LPS. Experiments may also reveal that lack of Nrf2 has little effect on Mdr1 during microglial activation but could play a role in basal expression. The data in this chapter demonstrate that important transporters in the

BBB, namely MDR1, are affected by soluble factors released during microglial activation. Also in **Chapter 4**, LPS systemically administered to mice, resulted in microglial activation and reduced Mdr1 expression in the brain.

Additional experiments characterized the regulation of other ABC transporters in microglia. The results of these experiments are outlined in **Appendix I**. Conditioned medium had no effect on Bcrp, Mdr1, or Mrp4 and elicited only a modest increase in Mrp5 expression. Additionally, conditioned medium did not appear to activate the Nrf2 pathway. TNF had variable effects on the mRNA expression of transporters including reducing Mrp1 and Mrp4, increasing Mrp5, and having no effect on Bcrp. NF- κ B inhibition also had no effect on Mrp1 or Bcrp mRNA. As expected, sulforaphane caused up-regulation of the Nrf2 responsive genes Ho-1 and Nqo-1, but had only a modest effect on Mdr1. Sulforaphane also prevented LPS-induced upregulation of Mrp5. These data suggest that Mrp5 may be regulated by TNF and/or other cytokines. Further studies are needed to understand the relationship of Nrf2 and NF- κ B on Mrp5 regulation. Interestingly, oltipraz, which is both an Nrf2 and NF- κ B activator, caused greater elevation of Mrp5 than LPS alone. An attempt was made to use pentoxifylline to block TNF which has been used in other studies (Taha, Grochot-Przeczek et al. 2009, Jain, Ratheesh et al. 2010, Luna, Santos et al. 2011). However, concentrations of pentoxifylline used in those publications to inhibit TNF were toxic to BV-2 microglia. Therefore, the lower concentrations used in these studies were not sufficient to inhibit TNF release. Future studies should use alternative TNF inhibitors, including monoclonal antibodies, to clarify the role of this cytokine in ABC transporter regulation.

Taken together, the data in this dissertation answer the hypothesis that Mdr1 efflux transporter expression in the mouse brain limits the accumulation and toxicity of paraquat and is negatively regulated in response to neuroinflammation. We demonstrated that paraquat is more neurotoxic in mice lacking Mdr1a/1b, especially after a single dose. Also, soluble mediators released by activated microglia, including TNF, can reduce the expression and function of Mdr1 in microglia and endothelial cells. These results lead to the hypothesis that a major factor in the susceptibility to neurodegeneration lies in the reduced functional capacity of the BBB to efflux toxicants. Reduced functional capacity may result from chronic microglial activation as observed in PD patients, decreased Mdr1 expression during aging, SNPs in Mdr1, or a combination of these factors. Multiple SNPs with reduced function and non-functional SNPs have been described for MDR1 in humans (Choudhuri and Klaassen 2006). Some recent studies have reported that loss-of-function polymorphisms are significantly associated with increased PD risk only in individuals exposed to pesticides (Drozdzik, Bialecka et al. 2003, Zschiedrich, Konig et al. 2009, Dutheil, Beaune et al. 2010). Together these data suggest that MDR1 may play a role in PD progression. Reduced MDR1 function at the BBB has also been described in early Alzheimer disease and PD (Kortekaas, Leenders et al. 2005, Deo, Borson et al. 2014). A common theme for many neurodegenerative diseases is the accumulation and aggregation of insoluble proteins. In some instances, such as Alzheimer disease, the aggregates are composed of proteins that are MDR1 substrates. In other instances, a link exists between aggregate formation and decreased MDR1 function but the aggregated proteins themselves are not proven substrates. This is the case for α -synuclein in PD and prions in Creutzfeldt-Jakob's disease (Rapposelli, Digiacomio et al. 2009). MDR1

dysfunction is observed in other neurodegenerative diseases including Huntington's disease and amyotrophic lateral sclerosis. Seemingly, induction of MDR1 function would be beneficial in these diseases for removing aggregates. However, many promising therapeutics for these diseases fail because they are MDR1 substrates. In these instances MDR1 inhibition may be useful if it can be achieved safely. Another common feature of many neurodegenerative diseases is persistent inflammation. As demonstrated in this dissertation, inflammation can have profound effects on MDR1 and other transporters. Whether MDR1 function is a major contributor to neurodegeneration remains to be seen. However, the link between inflammation, MDR1 function, and neurodegeneration is an important area for investigation. Deeper insight into the complex mechanisms of MDR1 regulation and function at the BBB may highlight important areas for improving therapeutic interventions for neurodegenerative diseases.

APPENDIX I

**Transcriptional Regulation of ABC Transporters by NF- κ B and Nrf2 Signaling
in Activated Microglia**

Abstract

ABC transporters are expressed on microglia and endothelial cells of the brain and are responsible for the extrusion of chemicals. Activated microglia release cytokines to coordinate inflammatory events within the brain. Here, we sought to determine whether microglia-derived secreted factors, including the cytokine TNF, could regulate ABC transporter expression in naive microglia. The contribution of NF- κ B, Nrf2, TNF, and nitric oxide synthase signaling to ABC transporter regulation were investigated. BV-2 microglia were incubated with lipopolysaccharide (LPS, E. coli, 500 ng/ml) for 12 and 24 hours with and without co-incubation with inhibitors and activators of signaling pathways.

Introduction

Neural inflammation is a common feature of many neurologic diseases including Alzheimer disease, Parkinson disease (PD), multiple sclerosis, and amyotrophic lateral sclerosis (Glass, Saijo et al. 2010). Microglia coordinate inflammatory events in the brain by producing neurotoxic factors like superoxide, nitric oxide (NO), peroxynitrite, arachidonic acid, chemokines, proinflammatory cytokines, and glutamate (Mosley, Benner et al. 2006, Cao, Li et al. 2011). The cellular pathways involved in pro-inflammatory cytokine release include pathways that are initiated through Toll-like receptors (TLRs), TNF receptors (TNFRs) and the IL-1 β receptor which result in activation of transcription factor, nuclear factor- κ B (NF- κ B) pathway and subsequent gene transcription. NF- κ B also regulates both apoptotic and anti-apoptotic genes and acts as a sensor of oxidative stress. Microglia also have the potential to cause neuronal damage through the production of ROS. Microglia, which are closely related to monocytes and macrophages, form active nicotinamide adenine dinucleotide phosphate (NADPH) oxidase during the respiratory burst creating one of the major sources of ROS (Miller, James-Kracke et al. 2009, Glass, Saijo et al. 2010). Other pathways adding to oxidative stress including inducible nitric oxide synthase (iNOS) and arachidonic acid which are active in microglia. Activated microglia exhibit reduced adenosine triphosphate (ATP)-binding cassette (ABC) transporter expression and function *in vitro* (Gibson, Hossain et al. 2012). ABC transporters are expressed in microglia and brain capillary endothelial cells and are an essential component of the BBB and function to regulate solute entry into the brain. Cellular oxidative stress mechanisms may contribute to changes in ABC transporter expression and function. The experiments in this study

were performed to characterize the signaling pathways regulating ABC transporters in microglia.

Methods and Materials

See Methods and Materials section in Chapter 4 for full details.

Cell Treatments. Test articles were chosen to block or activate specific signaling pathways as described in Table 1. The Nrf2 activators, sulforaphane and oltipraz, require extended pre-incubation prior to incubation with LPS (Table 1).

Quantification by Western Blot. Primary antibodies were diluted in 2% nonfat dry milk and incubated with membranes for 3 hours at the following concentrations: 1:1000 for Mdr1a/b (C219, Novus Biologicals, Littleton, CO), 1:2000 for Mrp1 (MRPr1, Alexis/Enzo Life Sciences, Farmingdale, NY), 1:2000 for Mrp4 (M₄I-80, Alexis/Enzo), 1:500 for Mrp5 (M₅I-10, G. Scheffer, VU Medical Center, Amsterdam, The Netherlands), 1:5000 for Bcrp (BXP-53, Alexis/Enzo), and 1:5000 for Gapdh (Millipore, Temecula, CA).

RNA Isolation and Real-Time Quantitative Polymerase Chain Reaction (PCR). Primer sequences for all target mRNAs are provided in Chapter 4.

Results

See figure legends for results

Discussion

The data from these experiments represent preliminary studies intended to investigate potential pathways involved in ABC transporter regulation in activated microglia and hCMEC/D3 cells. The involvement of multiple pathways appears to influence both basal expression and LPS-induced alterations in some ABC transporters. These experiments provide potential avenues for future investigation. Bcrp protein is significantly reduced in the mouse brain cortex following LPS administration. Bcrp expression in microglia is not significantly regulated *in vitro* by BV-2 conditioned medium, NF- κ B, or TNF. Conversely, oltipraz and aminoguanidine affect LPS-induced down-regulation and basal expression of Bcrp in microglia, respectively. Further investigation of the Nrf2 and nitric oxide synthase pathways are needed. Additionally, aminoguanidine inhibits advanced glycation end products, which are harmful endogenous compounds produced by all cells. These results are intriguing, and future studies should investigate how Bcrp and Mrp5 interact with advanced glycation end products.

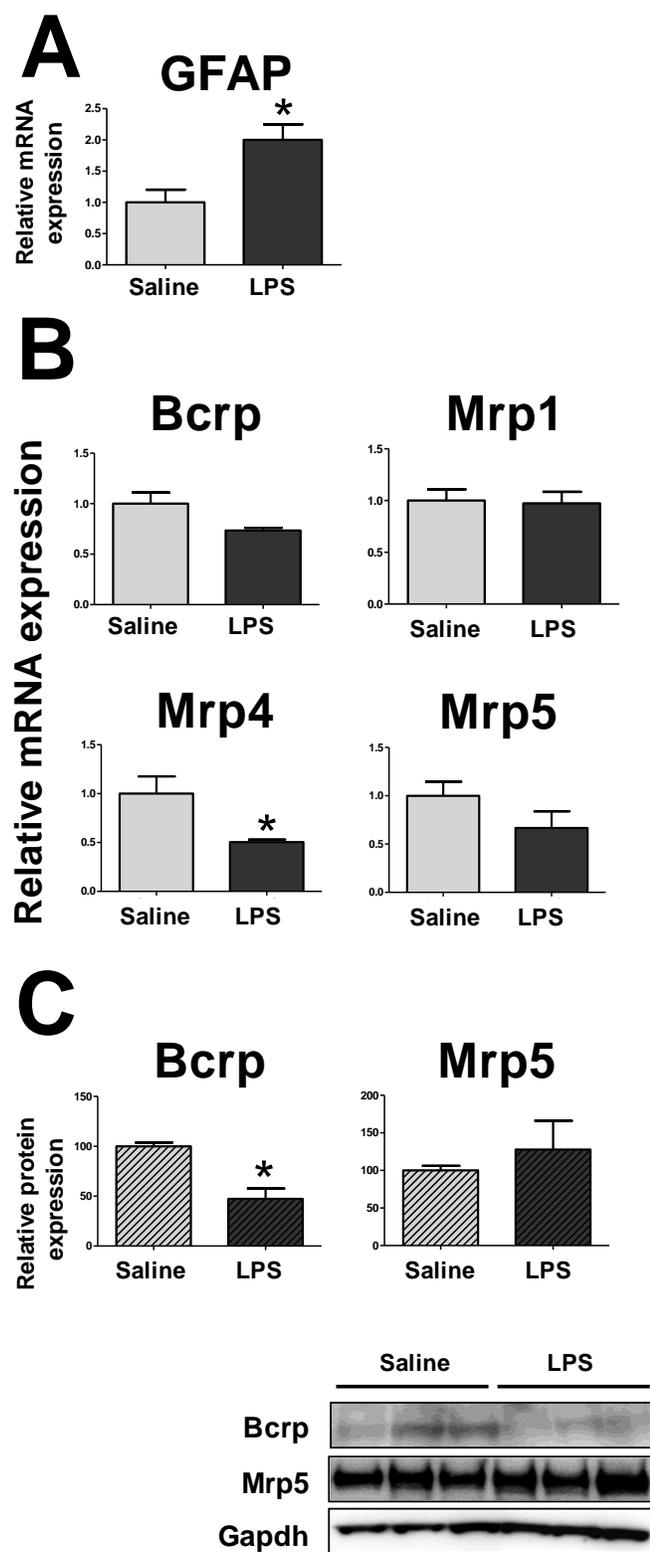


Figure A-1

Figure A-1. Brain ABC efflux transporter expression in LPS-treated mice. Mice were treated with a single dose of LPS (5 mg/kg, ip) and tissues were collected after 24 hours. Brain sections were homogenized and quantitative PCR was used to determine A) astrocyte activation (GFAP), and B) ABC efflux transporter expression. Data were normalized to Rpl13A and presented as mean relative mRNA expression \pm SE, (n = 4/group). Asterisks (*) represents statistically significant differences ($p < 0.05$) compared to saline-treated controls. C) Semi-quantitative expression of protein was determined by densitometry from western blots. Representative western blots exhibit changes in protein expression. Gapdh was used as loading control. Data are presented as mean relative protein expression \pm SE (n=3). Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control. The ABC transporter Bcrp demonstrated 25% lowered mRNA expression but was not statistically significant although protein levels were significantly lowered by 50%. Mrp1 mRNA did not change (-3%) while Mrp4 expression was reduced by 50% and Mrp5 had a trend with 30% lowered expression of mRNA. Interestingly, Mrp5 showed a trend towards elevated protein expression (30%, $p = 0.28$).

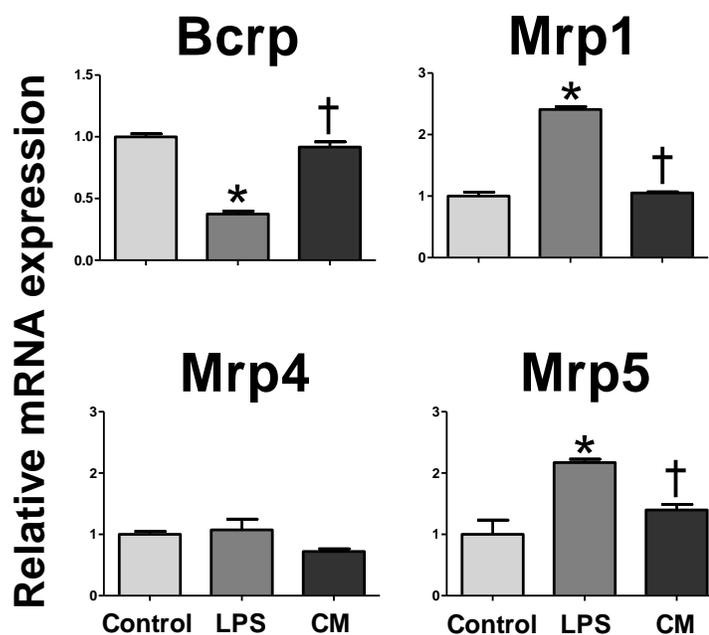


Figure A-2

Figure A-2. Effect of conditioned medium on naive BV-2 microglia. Resting microglia were treated with LPS or conditioned medium (CM) from activated microglia. Efflux transporter mRNA was determined 12 hours after incubation of BV-2 microglia with LPS or CM (Chapter 4, Fig. 1). Conditioned medium had no effect on Bcrp, Mrp1, Mrp4, or Mrp5 mRNA in BV-2 microglia. These results suggest that LPS but not soluble mediators within conditioned medium influences expression of these ABC transporters in BV-2 microglia. Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control. Dagger (†) represents statistically significant differences ($p < 0.05$) between LPS treated and CM treated.

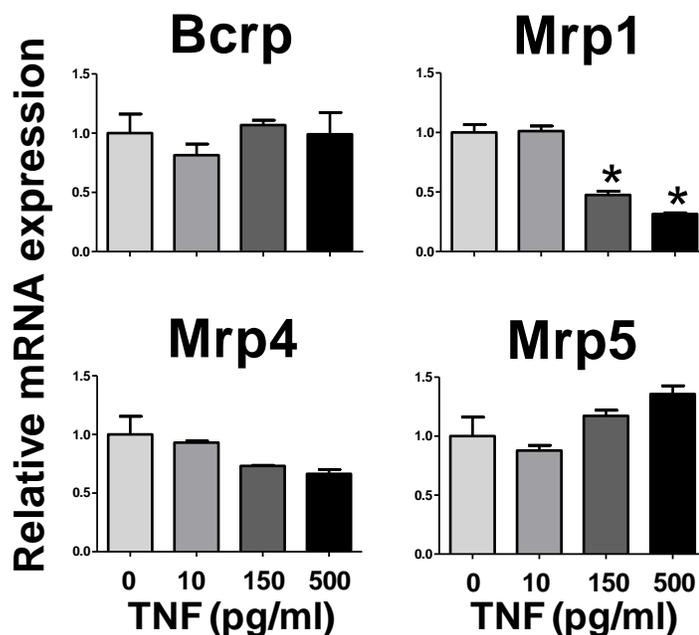


Figure A-3

Figure A-3. Recombinant TNF treatment of BV-2 microglia. The direct effect of recombinant mouse TNF (0, 10, 150, 500 pg/ml) on microglial ABC transporters was determined. ABC efflux transporter mRNA was determined after 12 hour incubation of BV-2 microglia with TNF. Data were normalized to Rpl13A and presented as mean relative mRNA expression \pm SE (n= 4-6). Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control. Bcrp exhibited no change in mRNA in response to TNF (-1%). Mrp1 was reduced by 70%, Mrp4 was lowered by 35% and Mrp5 increased 35%.

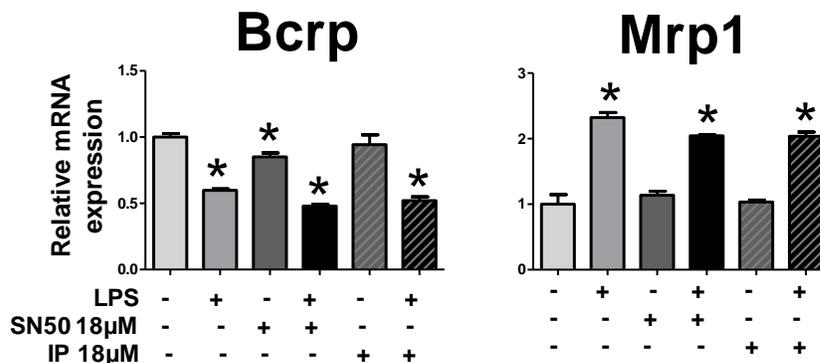


Figure A-4

Figure A-4. NF- κ B inhibition in activated BV-2 microglia. Microglia were treated with vehicle or the NF- κ B inhibitor, SN50 (18 μ M) or its inactive peptide (IP) \pm LPS. ABC efflux transporter mRNA was determined after 12 hour incubation of BV-2 microglia with SN50 \pm LPS. Data were normalized to Rpl13A and presented as mean relative mRNA expression \pm SE (n= 4-6). Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control. Single dagger (\dagger) represent statistically significant differences ($p < 0.05$) compared to LPS. Exposure to LPS reduced Bcrp mRNA by 40% and elevated Mrp1 mRNA by 130%. The NF- κ B inhibitor, SN50, had no effect alone on transporters except for a slight reduction in Bcrp mRNA (15% decrease). SN50 had no effect on LPS-induced mRNA alterations of Mrp1 or Bcrp. The negative control inactive peptide had no effect on pro-inflammatory cytokine or ABC transporter mRNA alone and did not affect BV-2 microglia response to LPS. These results demonstrate that these ABC transporters are not directly influenced by NF- κ B.

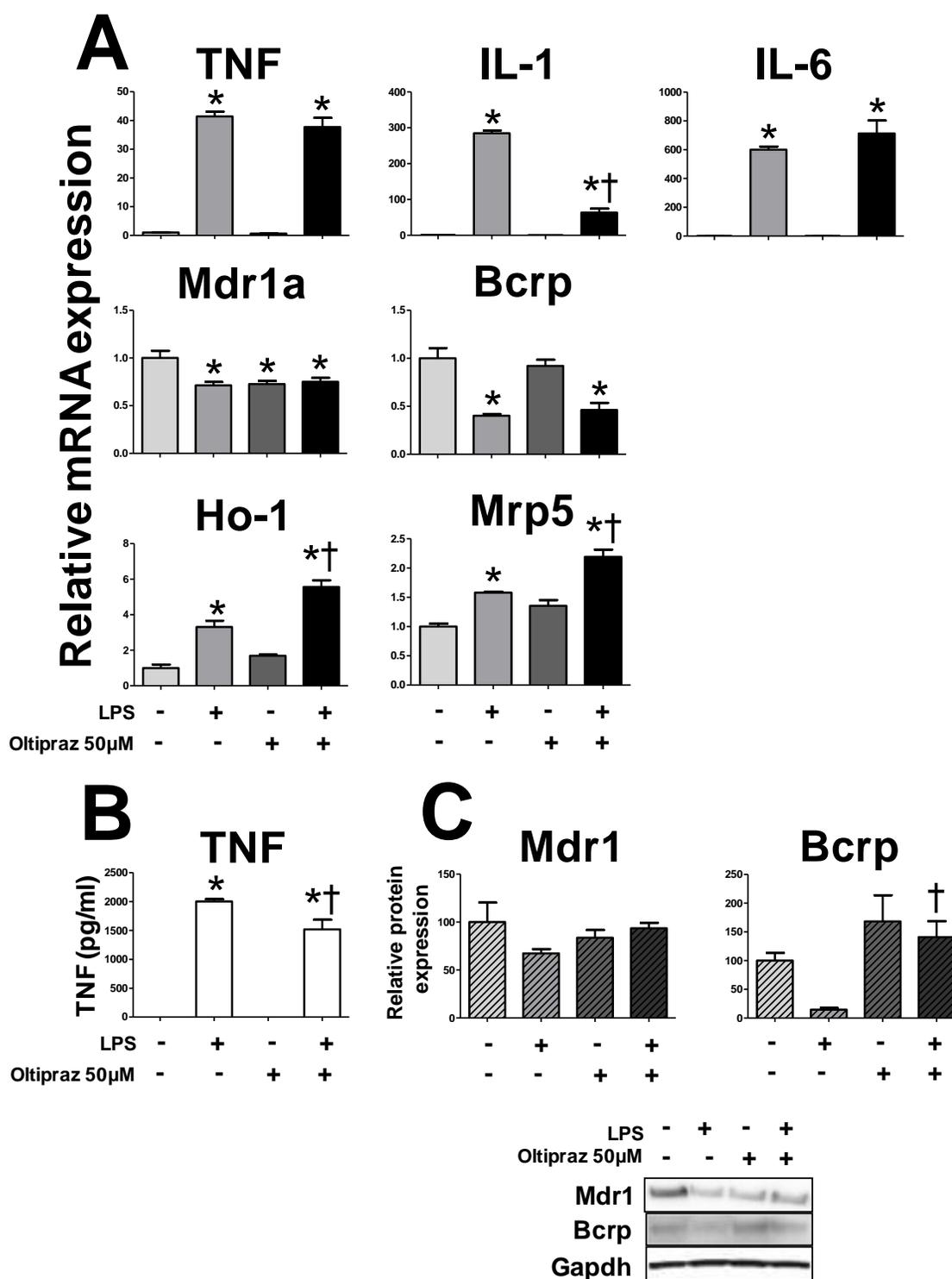


Figure A-5

Figure A-5. Oltipraz activation of Nrf2 in BV-2 microglia. BV-2 microglia were treated with vehicle or oltipraz (50 μ M) \pm LPS for 12 hours. A) Relative pro-inflammatory cytokine, ABC efflux transporter, and Nrf2 responsive gene mRNA was determined after 12 hours. Data were normalized to Rpl13A and presented as mean relative mRNA expression \pm SE (n= 4-6). Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control. Single dagger (\dagger) represent statistically significant differences ($p < 0.05$) compared to LPS. B) TNF release was determined by ELISA. Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control. Single dagger (\dagger) represent statistically significant differences ($p < 0.05$) compared to LPS. C) Relative expression of efflux transporter protein was determined after 24 hours. Semi-quantitative expression of protein was determined by densitometry from western blots. Representative western blots are provided. Gapdh was used as loading control. Data are presented as mean relative protein expression \pm SE (n=3). Single dagger (\dagger) represent statistically significant differences ($p < 0.05$) compared to LPS. Oltipraz caused a significant reduction in IL-1 β cytokine mRNA. Mdr1a mRNA was reduced with oltipraz alone. Mdr1a reduction may be due to Nrf2 activation by oltipraz or through NF- κ B activation. Induction of Mrp5 followed the same trend as for the Nrf2 responsive gene, Ho-1. Because oltipraz can activate Nrf2 or activate NF- κ B, the results demonstrated cannot be fully attributed to a particular pathway. However, pro-inflammatory cytokine expression was not increased.

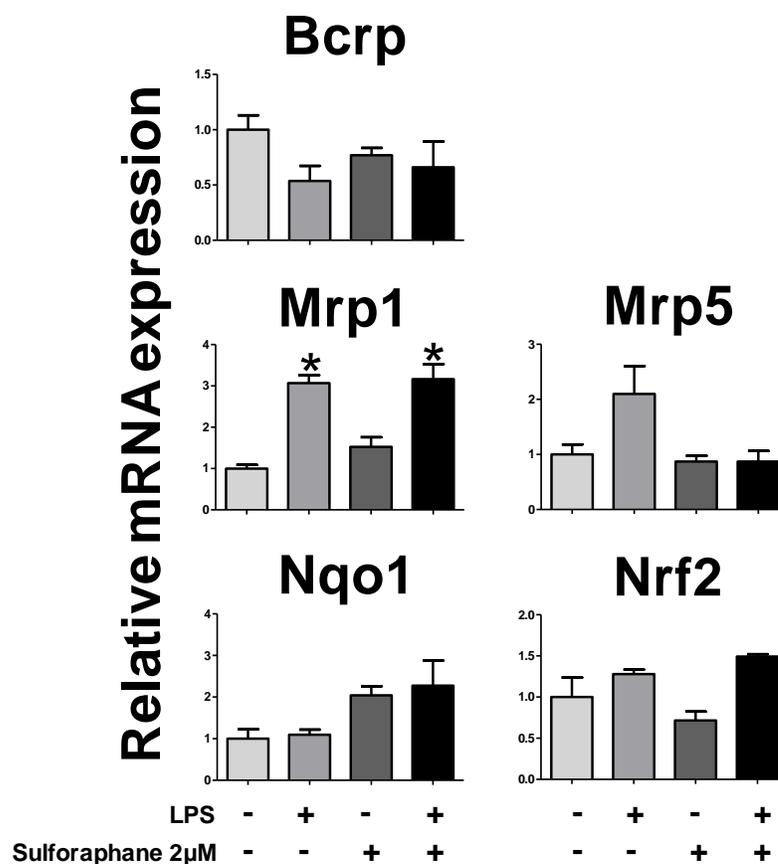


Figure A-6

Figure A-6. Sulforaphane activation of Nrf2 in LPS-treated BV-2 microglia. BV-2 microglia were treated with vehicle or sulforaphane (2µM) ± LPS for 12 hours. ABC efflux transporter mRNA was determined after 12 hour incubation of BV-2 microglia with sulforaphane ± LPS. Data were normalized to Rpl13A and presented as mean relative mRNA expression ± SE (n= 3). Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control. Sulforaphane prevented LPS-induced up-regulation of Mrp5. Since Mrp5 expression did not follow the pattern for the Nrf2 responsive genes Nqo1 or Ho-1 (see Chapter 4) a different mechanism is likely present.

Because sulforaphane also inhibits NF- κ B these data suggest that Mrp5 may be regulated by NF- κ B, TNF, or other cytokines.

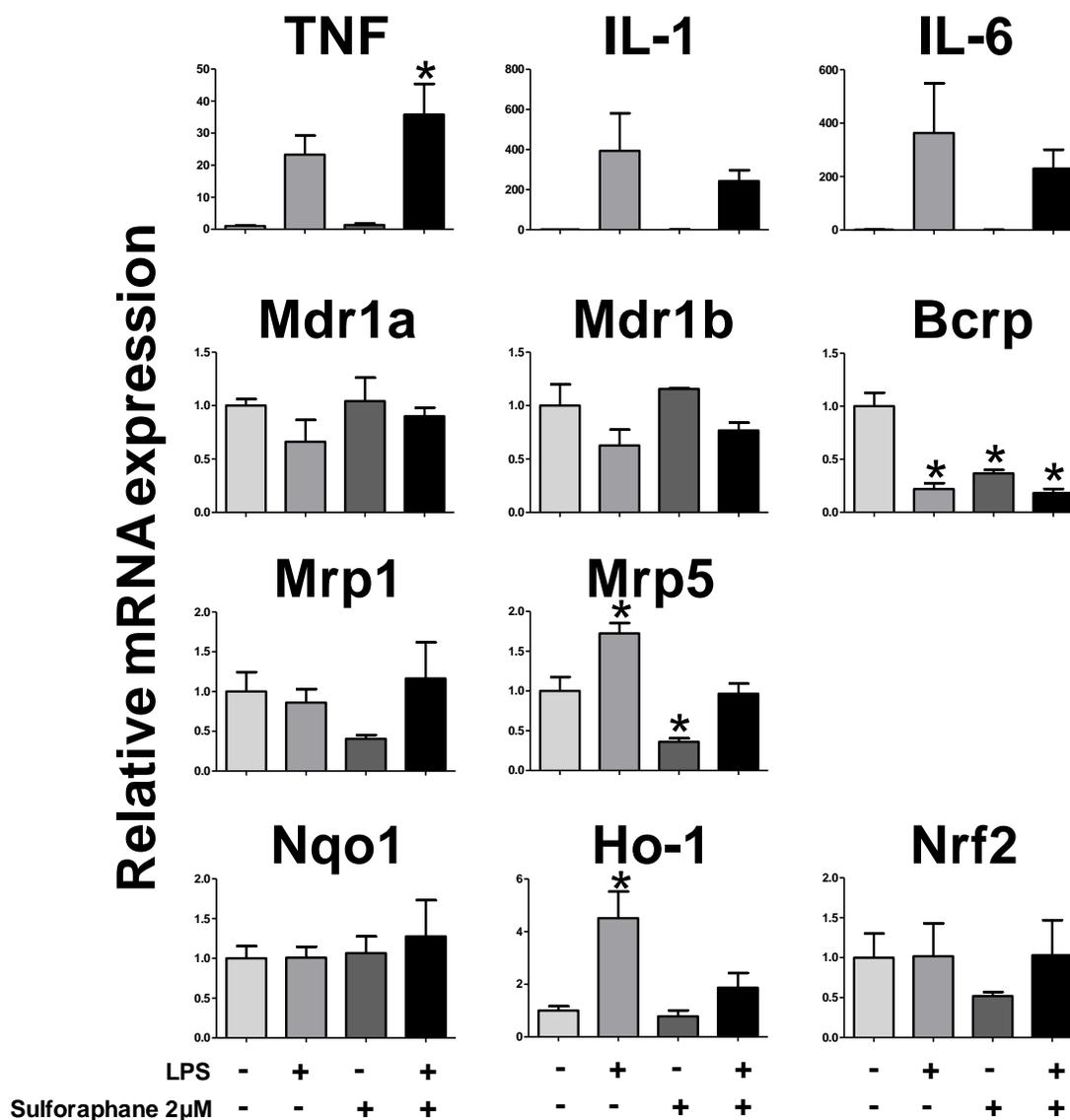


Figure A-7

Figure A-7. Aminoguanidine inhibition of NOS in LPS-treated BV-2 microglia.

BV-2 microglia were treated with vehicle or aminoguanidine (100 μ M) \pm LPS for 12

hours. Relative pro-inflammatory cytokine, ABC efflux transporter, and Nrf2 responsive gene mRNA was determined after 12 hour incubation of BV-2 microglia with aminoguanidine \pm LPS. Data were normalized to Rpl13A and presented as mean relative mRNA expression \pm SE (n= 3). Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control. Aminoguanidine alone caused significant reduction in Bcrp (-70%) and Mrp5 (-75%) mRNA and prevented LPS-induction of Mrp5. Aminoguanidine is a nitric oxide synthase inhibitor. These data suggest that nitric oxide signaling may influence basal Bcrp and Mrp5 expression.

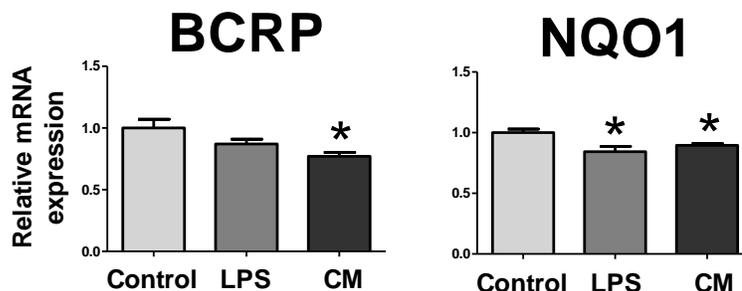


Figure A-8

Figure A-8. Effect of conditioned medium on hCMEC/D3 endothelial cells. Human microvascular endothelial cells (hCMEC/D3) were treated with conditioned medium from LPS-activated BV-2 microglial cells. BCRP efflux transporter, and Nrf2 responsive gene mRNA were determined after 12 hour incubation of hCMEC/D3 cells with conditioned medium (CM) or LPS. Data were normalized to Rpl13A and presented as mean relative mRNA expression \pm SE (n= 4-6). Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control. Single dagger (\dagger) represent statistically significant differences ($p < 0.05$) compared to LPS. Conditioned medium caused a 20% decrease in BCRP mRNA expression in hCMEC/D3 endothelial cells which was not observed with LPS treatment.

Table A-1. Inhibitors and activators of intracellular signaling pathways.

Pathway	Chemical name	Concentration	Preincubation	Mechanism	Alternate Mechanism
NF-κB	SN50	18μM	15 minutes	Inhibit NF-κB translocation	
	TNF	10 – 500pg/ml	N/A	Activate NF-κB	Activate JNK
Nrf2	Oltipraz	10μM	4 hours	Activate Nrf2	Activates NF-κB, CAR
	Sulforaphane	2μM	4 hours	Activate Nrf2	Inhibits NF-κB
NOS	Aminoguanidine	100μM	15 minutes	Inhibit iNOS	3-deoxyglucosone

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