HYPOXIA/REOXYGENATION STRESS MODULATES ATORVASTATIN TRANSPORT AT THE BLOOD-BRAIN BARRIER: A ROLE FOR ORGANIC ANION TRANSPORTING POLYPEPTIDE

by

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DEDICATION

I dedicate this work to my mother, Patricia Thompson,
who has been my best teacher and mentor.

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ABSTRACT

Cerebral ischemia occurs when blood flow to the brain is insufficient to meet metabolic demand. This can result from cerebral artery occlusion that interrupts blood flow, limits CNS supply of oxygen and glucose, and causes an infarction/ischemic stroke. Ischemia initiates a cascade of molecular events in neurons and cerebrovascular endothelial cells including energy depletion, dissipation of ion gradients, calcium overload, excitotoxicity, oxidative stress, and accumulation of ions and fluid. Blood-brain barrier (BBB) disruption is associated with cerebral ischemia and leads to vasogenic edema, a primary cause of stroke-associated mortality. To date, only a single drug has received US Food and Drug Administration (FDA) approval for treatment of acute ischemia/reperfusion injury, recombinant tissue plasminogen activator (rt-PA). While rt-PA therapy restores perfusion to ischemic brain, considerable tissue damage occurs when cerebral blood flow is re-established. Therefore, there is a critical need for novel therapeutic approaches that can "rescue" salvageable brain tissue and/or protect BBB integrity during cerebral hypoxia and subsequent reoxygenation stress (H/R). One approach that may enable neural tissue rescue following H/R is CNS delivery of drugs with brain protective effects such as HMG-CoA reductase inhibitors (i.e., statins). Our present *in vivo* data demonstrates that atorvastatin, a commonly prescribed statin, attenuates poly (ADP-ribose) polymerase (PARP) cleavage in the brain following H/R, suggesting neuroprotective efficacy. However, atorvastatin use as a CNS therapeutic is limited by poor blood-brain barrier (BBB) penetration. Therefore, we examined regulation and functional expression of the known statin transporter Oatp1a4 at the BBB under H/R conditions. In rat brain microvessels H/R (6% O_2, 60 min followed by 21%

O_2, 10 min) increased Oatp1a4 expression. Brain uptake of taurocholate (i.e., Oap1a4 probe substrate) and atorvastatin were reduced by Oatp inhibitors (i.e., estrone-3-sulfate, fexofenadine), suggesting involvement of Oatp1a4 in brain drug delivery. Pharmacological inhibition of TGF-β/ALK5 signaling with the selective inhibitor SB431542 increased Oatp1a4 functional expression, suggesting a role for TGF-β/ALK5 signaling in Oatp1a4 regulation. Taken together, our novel data show that targeting an endogenous BBB drug uptake transporter (i.e., Oatp1a4) may be a viable approach for optimizing CNS drug delivery for treatment of diseases with an H/R component.

CHAPTER 1: Introduction and Overview

Parts of this introduction have been taken in large part from a book chapter that has been accepted for publication in Advances in Pharmacology:

Thompson BJ & Ronaldson PT. Drug delivery to the ischemic brain. In: Advances in pharmacology, Volume 71 (TP Davis, ed). Philadelphia: Elsevier Inc., 2014. In Press.

1.1 General Introduction

Pharmacological treatment of cerebral ischemia requires a detailed understanding of pathophysiological changes that occur in the brain and in the cerebral microvasculature following ischemia/reperfusion injury. Cerebral hypoxia and subsequent reoxygenation is a central component of several diseases, including traumatic brain injury, acute respiratory distress syndrome, obstructive sleep apnea, high-altitude cerebral edema and acute mountain sickness, cardiac arrest, and ischemic stroke (Ronaldson & Davis, 2013). Stroke is the fourth leading cause of death and is a major cause of long-term morbidity in the United States (Feng & Belagaje, 2013). Of all stroke cases, 87% are ischemic (Roger et al., 2011). Ischemic stroke results from restricted blood flow to a portion of the brain that causes an irreversibly damaged ischemic core and a surrounding region of potentially viable, yet functionally impaired brain tissue known as the penumbra (Astrup, Siesjo, & Symon, 1981; Liu, Levine, & Winn, 2010). A complex cascade of molecular events initiated by cerebral ischemia is responsible for the widespread necrosis observed in the ischemic core and apoptosis detected in the penumbra. Theoretically, the penumbra can be salvaged if reperfusion therapy and/or pharmacotherapy are administered early during the course of disease (Shah & Abbruscato, 2013). This therapeutic objective is underscored by challenges in delivering drugs to the ischemic brain. Here, we discuss pathological mechanisms associated with cerebral ischemia and associated hypoxia and how detailed knowledge of such processes can lead to cutting-edge approaches to deliver drugs to ischemic brain. In particular, we focus on targeting of endogenous blood-brain barrier (BBB) uptake transporters, a non-invasive chemical-based approach that appears promising for effective CNS drug delivery.

1.2 Pathophysiology of Ischemia

Physiologically, energy requirements of the CNS are met by brain uptake of glucose and oxygen, which are incorporated into metabolic pathways to enable phosphorylation of ADP to ATP. Cerebral ischemia results in reduction of molecular oxygen delivery to all CNS cell types within the core of the infarct zone. Lack of oxygen availability halts molecular shuttling of electrons in oxidative phosphorylation, which is essential for ATP generation. Most ATP generated within the brain is used for maintenance of intracellular homeostasis and transmembrane gradients for monovalent and divalent ions (i.e., Na^+, K^+, Ca^{2+}) (Adibhatla & Hatcher, 2008). Energy depletion in neuronal cells causes ion gradient failure via cessation of ATP-dependent Na^+/K^+-ATPase and Ca^{2+}-ATPase activity. When energy dependent ion extrusion is impeded, cations in extracellular fluid (i.e., Na^+) follow a strong inwardly directed electrochemical gradient and accumulate within the cell. Uptake of Na^+ is accompanied by influx of monovalent anions (i.e., Cl^-). Extracellular fluid then follows this net movement of ions resulting in cytotoxic edema. Additionally, Na^+ ion uptake causes extensive plasma membrane depolarization, leading to opening of voltage-gated cation channels and reverses the direction of the Na^+/Ca^{2+} exchanger, bringing additional Ca^{2+} into the cell (Kiedrowski, 2007; Luo et al., 2008). The widespread depolarization seen in ischemic neurons thwarts plasma membrane hyperpolarization, which is required to close and reactivate these cation channels.

Influx of Ca^{2+} prompts intracellular vesicles, containing glutamate or dopamine, to fuse with the neuronal presynaptic bouton membrane, releasing the neurotransmitters into the synapse. This uncontrolled increase in glutamate and dopamine concentrations is

neurotoxic and leads to neuronal cell death and development of an infarction (i.e., ischemic stroke) (Adibhatla et al., 2008). Glutamate excitotoxicity coupled with cellular depolarization is particularly deleterious to the CNS due to overstimulation of metabatropic glutamate receptors as well as extensive activation of AMPA and NMDA receptors, resulting in disruption of CNS calcium homeostasis (Adibhatla, Hatcher, & Dempsey, 2006; Adibhatla, Hatcher, Larsen, et al., 2006; Arai et al., 2011). Energy reserves are quickly depleted in an effort to sequester increasing intracellular Ca^{2+} concentrations (Pundik, Xu, & Sundararajan, 2012). Inadvertent activation of inositol trisphosphate and ryanodine receptors, a process linked to mitochondrial reactive oxygen species (ROS) generation, can liberate intracellular Ca^{2+} stores (Camello-Almaraz, Gomez-Pinilla, Pozo, & Camello, 2006). Calcium overload also causes excessive stimulation of Ca^{2+}/calmodulin-dependent enzymes such as nitric oxide synthase (i.e., eNOS, nNOS and mtNOS), as well as a host of Ca^{2+}-dependent enzymes such as proteases, phospholipases, and endonucleases (Fellman & Raivio, 1997). Over activation of such catalytic enzymes can cause protein degradation, phospholipid hydrolysis, and DNA damage as well as a disruption of cellular signaling and enzymatic reactions. ROS generation increases dramatically during ischemia due to high Ca^{2+}-induced mitochondria dysfunction and impairment of ROS defense enzymes, and superoxide anion is released into the cytosol in increasing amounts. Neurons in the ischemic core that have died via necrotic processes release cytotoxic elements into the interstitial space which then penetrate adjacent neurons through damaged plasma membranes caused by lipid peroxidation and the activity of phospholipases. In addition to ROS generation, cerebral

ischemia is accompanied by widespread inflammation demarcated by cytokines, adhesion molecules, and other inflammatory mediators (Iadecola & Alexander, 2001).

1.2.1 Reactive Oxygen Species Generation

Oxidative stress is observed in the central nervous system (CNS) at early time points following ischemic injury and is well known to contribute to neuronal injury and cell death in the ischemic core (Candelario-Jalil, 2009). The CNS is especially sensitive to oxidative stress because it consumes substantial amounts of oxygen, contains large amounts of polyunsaturated fatty acids, accrues redox metal ions, and possesses relative low levels of endogenous antioxidants (Aksenova, Aksenov, Mactutus, & Booze, 2005). ROS have been recognized as central mediators of neuroinflammation and cytotoxicity in ischemia/reperfusion injury (Singhal, Morris, Labhasetwar, & Ghorpade, 2013). Furthermore, evidence of improved stroke outcome following clinical trials of antioxidant therapy underscores the critical role of ROS generation and oxidative stress in CNS pathology following cerebral ischemia/hypoxia (Lutsep & Clark, 2001).

Superoxide anion is the principal ROS generated when molecular oxygen is reduced by only one electron. This reaction occurs spontaneously and non-enzymatically through activity of electron transport systems in mitochondria. Briefly, electrons are donated by NADH, the reduced form of the coenzyme essential to all living cells, initiating a shuttling of electrons involving NADH-ubiquinone oxidoreductase or complex I, succinate dehydrogenase or complex II, ubiquinol-cytochrome c oxidoreductase or complex III, and cytochrome c oxidase or complex IV, ending with electron acceptance by molecular oxygen. Small amounts of superoxide (i.e., 1-4%) are

regularly generated during this process, as the majority of reactions within the electron transport chain (ETC) involve single electron transfers (Turrens, 2003). In particular, complex I as well as both sides of complex III (i.e., Qi and Qo sites) are the most common sources of mitrochondrial superoxide (Murphy, 2009). Superoxide generated within the intermembrane space of mitochondria can reach the cytosol through voltage-dependent mitochondrial anion channels (Zhang & Gutterman, 2007). Additionally, superoxide is produced by NADPH oxidases in endothelial cells, macrophages, microglia, and granular leukocytes, as well as by cytochrome P450-dependent oxygenases and cyclooxygenases (i.e., COX-2) (Pacher, Beckman, & Liaudet, 2007; Turrens, 2003). Furthermore, epithelial and neuronal nitric oxide synthase (eNOS/nNOS) directly produce superoxide when required cofactors, such as arginine or tetrahydrobiopterin, are deficient (i.e., uncoupled NOS) (Fang, Yang, & Wu, 2002), as occurs during cerebral ischemia.

Under normal physiological conditions, superoxide is scavenged by the cellular ROS defense system. However, due to activation of degradative enzymes and proteases, ROS defense enzymes (i.e., superoxide dismutases) can become compromised and overwhelmed by high ROS concentrations. ROS induce mutations in mitochondrial DNA (mtDNA) and damage enzymes and cytochrome complexes involved in the ETC. This leads to dysfunction of oxidative phosphorylation and further generation of ROS (Schild & Reiser, 2005). Superoxide levels steadily rise during ischemia in both microvascular endothelial cells and neurons (Fabian, DeWitt, & Kent, 1995; Pacher et al., 2007). This paradoxical increase of superoxide despite low oxygen concentrations has been well-described (Guzy & Schumacker, 2006; Murphy, 2009). It is possible that physiological

levels of nitric oxide (NO) can outcompete oxygen for binding to cytochrome oxidase in the setting of cerebral ischemia/hypoxia. NO binding can cause these cytochrome complexes to facilitate production of superoxide as well as increase the apparent K_m of this enzyme for NO, events that further interfere with oxidative phosphorylation (Murphy, 2009).

Peroxynitrite ($ONOO^-$), a potent cytotoxic and proinflammatory molecule, is formed rapidly and non-enzymatically from the combination of nitric oxide with superoxide and causes extensive damage to neurons and cerebral microvessels through lipid peroxidation, consumption of endogenous antioxidants (i.e., reduced glutathione), DNA fragmentation, and induction of mitochondrial failure (Pacher et al., 2007). Figure 1 illustrates ROS generation in cerebrovascular endothelial cells during ischemia/reperfusion, including peroxynitrite formation. Peroxynitrite causes cellular damage via its ability to nitrosylate tyrosine residues, leading to functional modifications of critical proteins (Salvemini, Doyle, & Cuzzocrea, 2006). Breakdown of peroxynitrite into nitrogen dioxide and hydroxyl radicals also leads to endothelial cell dysfunction and BBB disruption during cerebral ischemia (Heo, Han, & Lee, 2005). Administration of peroxynitrite decomposition catalysts 5,10,15,20-tetrakis(N-methyl-4'-pyridyl)porphyrinato iron III (FeTMPyP) and 5,10,15,20-tetrakis(4-sulphonatophenyl)porphyrinato iron (FeTPPS) during reperfusion, or even up to six hours following reperfusion, have shown effectiveness in mitigating neuronal damage and reducing infarct size in rats subjected to transient middle cerebral artery occlusion (MCAO), an *in vivo* model of ischemic stroke. Additionally, brain edema was drastically reduced (i.e., up to 70%) in these studies, implying that peroxynitrite is a major

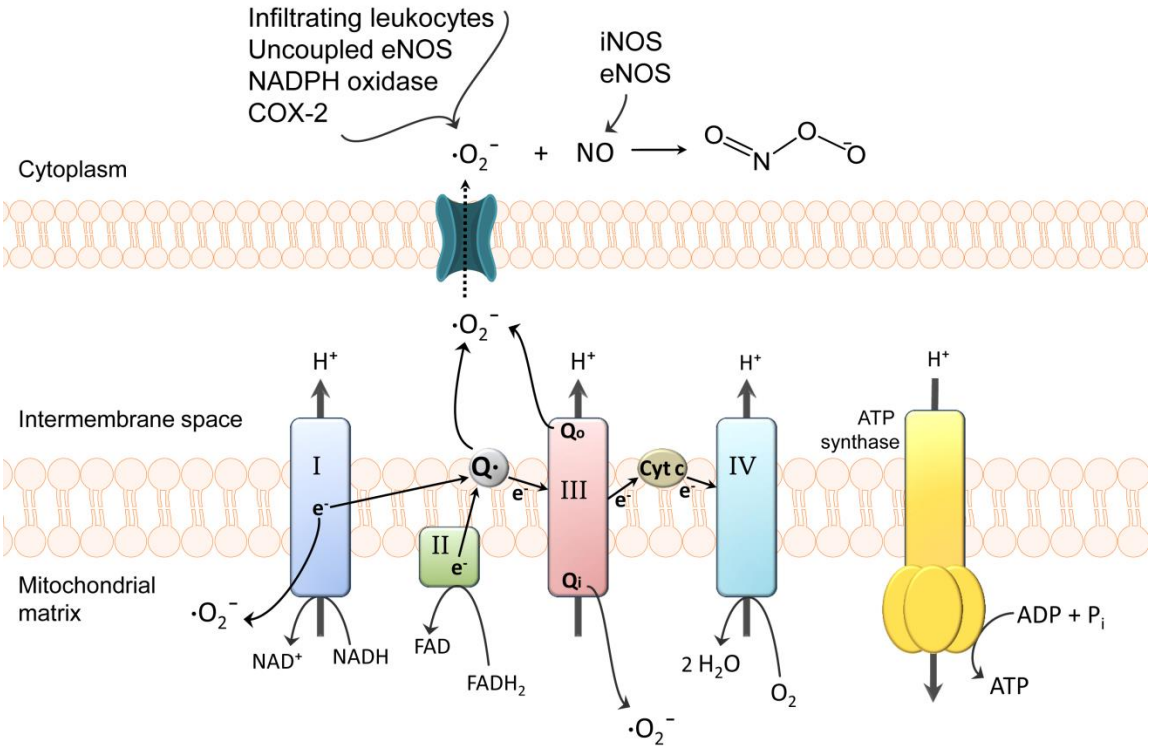

Figure 1: Generation of Mitochondrial Reactive Oxygen Species (ROS) in Cerebrovascular Endothelial Cells. During ischemia, mitochondrial superoxide levels rise through NO-inhibition of cytochrome complexes and oxidation of reducing equivalents in the electron transport chain (ETC). Complex I as well as both sides of complex III (i.e., Qi and Qo sites) are the most common sources of mitochondrial superoxide. Superoxide generated within the intermembrane space of mitochondria can reach the cytosol through voltage-dependent mitochondrial anion channels (Zhang et al., 2007). Superoxide levels further increase via cyclooxygenase-2, NADPH oxidase, uncoupled eNOS, and infiltrating leukocytes. The resulting high levels of superoxide coupled with the activation of NO-producing eNOS/iNOS, increases the likelihood of peroxynitrite formation. Peroxynitrite-induced cellular damage includes protein oxidation, tyrosine nitration, DNA damage and poly(ADP-ribose) polymerase (PARP) activation, lipid peroxidation, and mitochondrial dysfunction.

contributor to BBB breakdown in ischemic brain (Pacher et al., 2007). Peroxynitrite formation in BBB endothelial cells and neurons becomes more likely with activation of epithelial NOS (eNOS) and inducible NOS (iNOS) because NO diffuses easily through membranes and readily reacts with superoxide anion (Pacher et al., 2007).

1.2.2 Poly(ADP-ribose) Polymerase

Recent evidence suggests that deleterious effects of peroxynitrite involve direct DNA damage and subsequent activation of poly(ADP-ribose) polymerase (PARP). PARP is a family of nuclear enzymes involved in DNA repair, programmed cell death, and necrotic tissue damage. PARP-1 is the dominant member of the PARP family and is critical in detection and repair of damaged DNA. PARP-1 binding to specific DNA motifs such as single- and double-strand breaks, supercoils, cruciforms, and crossovers activates its catalytic domain. Activated PARP then utilizes NAD^+ to poly(ADP-ribosyl)ate itself, as well as other transcription-related factors (i.e., p53, nuclear factor-κB, activator protein 1, E2F-1) and DNA repair machinery (Chaitanya, Steven, & Babu, 2010; M. Y. Kim, Zhang, & Kraus, 2005). Neurons with extensive DNA damage, such as is observed during cerebral ischemia, will experience depletion of nuclear and cytosolic pools of NAD^+ due to PARP-1 overactivation (M. Y. Kim et al., 2005). To prevent energy-failure-induced necrosis, activated caspases-3 and -7 will cleave PARP between aspartic acid 214 and glycine 215, yielding protein fragments of 24 kDa and 89 kDa (Chaitanya et al., 2010). This cleavage effectively terminates PARP's ability to initiate DNA repair, an event that leads to DNA fragmentation and subsequent apoptosis. Therefore, pharmacological interventions that decrease PARP activation and cleavage in the brain are indicative of a potentially protective therapy that can attenuate neural

apoptosis. Pharmacological compounds that inhibit PARP activation (i.e., 3-aminobenzamide, INO-1001, PJ-34) bestow neuroprotection against ischemia/reperfusion injury, even if administered several hours after hypoxic insult and, therefore, are potential candidates for clinical use (Pacher et al., 2007). Indeed, genetic deletion of PARP has protected animal subjects against DNA-damage associated with pathophysiological conditions such as ischemia-reperfusion injury, neuroinflammatory stress, and glutamate excitotoxicity (M. Y. Kim et al., 2005). Significant reductions (i.e., up to 80%) in infarct volume and brain tissue damage have also been observed in PARP knockout mice following transient MCAO (Pacher et al., 2007).

1.2.3 Reperfusion & Immune Response

Reperfusion is known to cause large increases in ROS generation within the penumbra (Fabian et al., 1995; Zhao, Patzer, Herdegen, Gohlke, & Culman, 2006). Specifically, superoxide levels increase in both cerebrovascular endothelial cells and neurons during the early reoxygenation phase (Pacher et al., 2007). NO-inhibited cytochrome oxidases coupled with free radical-induced damage to the ETC machinery can further enhance production of superoxide, when molecular oxygen is reintroduced to the ischemic brain. Inhibition of ubiquinol-cytochrome *c* oxidoreductase or complex III of the ETC has been shown to reverse these reoxygenation-induced increases in superoxide *in vitro* (Therade-Matharan et al., 2005; Zhang et al., 2007). Ceramide, which has been found to increase upon the onset of hypoxia, is also positively correlated with ROS generation during reoxygenation (Therade-Matharan et al., 2005).

A major contributor to reperfusion injury is the inflammatory response initiated by the uncontrolled release of cytotoxic chemicals and cellular debris into the interstitial space within damaged brain tissue. H/R is associated with activation of hypoxia-sensitive transcription factors such as nuclear factor-κB (NF-κB), hypoxia-inducible factor-1 (HIF-1), and signal transducer and activator of transcription 3 (STAT3) (Lochhead et al., 2010; Pacher et al., 2007; Witt, Mark, Huber, & Davis, 2005). Consequently, proinflammatory molecules and cytokines such as tumor necrosis factor-α (TNFα), interferon-γ, interleukin-1β (IL-1β), IL-6, and IL-18 are produced and secreted (Pacher et al., 2007). Endothelial cell adhesion molecules like P-selectins and E-selectins and intercellular adhesion molecule-1 (ICAM-1) are activated (Pacher et al., 2007), which allow leukocytes and macrophages to flood the infarcted area upon reperfusion. TNFα has also been linked to excitotoxicity and both TNFα and interferon-γ have been shown to increase expression of inducible nitric oxide synthase (iNOS) (Pundik et al., 2012). Enzymes activated by the inflammatory response include iNOS and cyclooxygenase-2 which produce substantial quantities of NO and $\cdot O_2^-$ respectively (Pacher et al., 2007). Additionally, reactive nitrogen species (RNS) production in ischemic brain is enhanced due to activated macrophages and neutrophils releasing copious amounts of NO and $\cdot O_2^-$ in the penumbra. Nitric oxide and superoxide rapidly form peroxynitrite, an effect that escalates peroxynitrite-induced cellular damage. As reperfusion proceeds, neuroinflammation and apoptosis become more prevalent and dramatically affect viability of salvageable brain tissue within the penumbra (Candelario-Jalil, 2009).

1.2.4 ROS and Changes to the BBB

Cerebral ischemia is a complex insult that not only involves deprivation of oxygen and essential nutrient delivery (del Zoppo & Hallenbeck, 2000), but is also associated with increased microvascular permeability (Kempski, 2001; Petty & Wettstein, 2001). The BBB has developed as both a physical and metabolic barrier that is critical for survival. It is well-established that disruption of the BBB during ischemia/reperfusion leads to vasogenic brain edema, a primary cause of stroke-associated mortality (Vibbert & Mayer, 2010); however, the majority of edema formation, occurs across an intact BBB. Detectable BBB breakdown is not observed until approximately five hours after onset of ischemia in experimental stroke models (O'Donnell, Lam, Tran, Foroutan, & Anderson, 2006; Shah et al., 2013). Increased blood-to-brain net movement of Na^+ mediated by BBB Na^+ transporters appears to have a critical role in edema formation (Shah et al., 2013; Wallace, Foroutan, & O'Donnell, 2011). For example, O'Donnell and colleagues have demonstrated that increased activity of the luminal Na^+-K^+-$2Cl^-$ cotransporter in BBB endothelial cells contributes to development of cerebral edema following ischemia (O'Donnell, Tran, Lam, Liu, & Anderson, 2004). Furthermore, Na^+-K^+-Cl^- cotransporter inhibition reduces edema and infarct volume in a rat permanent MCAO model (O'Donnell et al., 2004; Wallace et al., 2011). Additionally, estradiol reduces both activity of the Na^+-K^+-Cl^- cotransporter and edema formation, suggesting that estrogens play a prominent neuroprotective role during stroke (O'Donnell et al., 2006). Integrity of BBB transport pathways during and after ischemic stroke is crucial, as perturbations in these processes can have significant effects on BBB permeability and therefore can exacerbate vasogenic edema.

BBB permeability is controlled by tight junction protein complexes localized between endothelial cells, which act to limit paracellular diffusion. Tight junctions are dynamic complexes of multiple protein constituents including junctional adhesion molecules (JAMs), occludin, claudins (i.e. claudin-1, -3, and -5), and membrane-associated guanylate kinase (MAGUK)-like proteins (i.e. ZO-1, -2 and -3) (Sanchez-Covarrubias, Slosky, Thompson, Davis, & Ronaldson, 2013). Production of ROS and subsequent oxidative stress alters expression and molecular organization of critical tight junction proteins claudin-5 and occludin at the BBB, leading to increased paracellular solute leak (Lochhead et al., 2010; Schreibelt et al., 2007). Reorganization of tight junction complexes and associated leak across the BBB following focal ischemia enables considerable movement of vascular fluid across the microvascular endothelium and development of vasogenic edema (Heo et al., 2005; Pillai et al., 2009; Sandoval & Witt, 2008). Reductions in post-ischemic edema and injury have been shown *in vivo* by vascular endothelial growth factor antagonism (van Bruggen et al., 1999), which has been identified as a possible mechanism that controls tight junction integrity (Fischer et al., 2007); these data indicate that tight junction disruption is involved in progression of ischemic brain injury.

Decreased expression of occludin is directly associated with increased BBB permeability as shown by an *in vivo* rodent model of H/R (Witt, Mark, Hom, & Davis, 2003; Witt et al., 2005). Additionally, H/R causes trafficking of occludin away from BBB tight junction protein complexes (Lochhead et al., 2010; McCaffrey et al., 2009). This occludin re-localization can be prevented *in vivo* by 4-hydoxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPOL) treatment, a superoxide scavenging antioxidant

that readily crosses the BBB (Cuzzocrea et al., 2000; Deng-Bryant, Singh, Carrico, & Hall, 2008; Kwon et al., 2003; Lochhead et al., 2012; Rak et al., 2000; Saito, Takeshita, Ueda, & Ozawa, 2003; Zhelev et al., 2009). Specifically, TEMPOL prevents breakage of disulfide bonds on occludin monomers thereby blocking disruption of occludin oligomeric assemblies and subsequent blood-to-brain leak of circulating solutes (Lochhead et al., 2010). Similarly, SOD-mimetics such as metalloporphyrin catalytic antioxidants and ceria nanoparticles have also been successful in protecting against ischemic damage to the BBB in *in vivo* rodent model systems (C. K. Kim et al., 2012; Pacher et al., 2007).

The increase in BBB permeability observed during ischemic stroke involves changes to transcellular transport pathways in addition to tight junction modifications. For example, Yeh et al. demonstrated in immortalized rat brain endothelial cells that hypoxia upregulates glucose transporters (GLUT1) (Yeh, Lin, & Fu, 2008). Functional expression of the sodium glucose cotransporter-1 (SGLT1) was also increased after ischemia/reperfusion (Elfeber et al., 2004). Vemula et al. showed that SGLT plays a significant role along with GLUT1 in glucose uptake across the BBB and cerebral edema formation during ischemia (Vemula et al., 2009). Recently, our own laboratory discovered that the endogenous transporter, Oatp1a4, also increases at the rodent BBB and is capable of promoting transcellular xenobiotic transport (Thompson et al., 2014). Specifically, Oatp1a4 contributes to blood-to brain flux of therapeutic drugs following H/R, some of which show considerable potential as neuroprotectants (Thompson et al., 2014). Increases in non-specific vesicular transport and pinocytosis within BBB

endothelial cells have also been reported (Cipolla, Crete, Vitullo, & Rix, 2004; Plateel, Teissier, & Cecchelli, 1997).

Ischemic stroke is an amalgamation of a vascular disorder and a neuronal disease. Central to the pathogenesis of ischemic damage is the neurovascular unit, a cohesive organization of endothelial cells, pericytes, neurons, and astrocytes as well as extracellular matrix. A cross section of a cerebral capillary, depicting the neurovascular unit is portrayed in Figure 2. Cell-to-cell interactions and signaling occur in a co-coordinated manner between these multiple cell types and matrix constituents, events required for physiological and pathological functioning of the BBB. For example, chemical destruction of perivascular astrocytes has been shown to cause increased BBB permeability that is characterized by decreased occludin protein expression (Willis, Leach, Clarke, Nolan, & Ray, 2004). Focal loss of astrocytes both in the necrotic core and in the apoptotic penumbra contributes to increased BBB permeability during cerebral ischemia. Similarly, pericyte association with the microvasculature endothelium is critical to vascular integrity and loss of this relationship may lead to vascular leakage and induction of edema (Bonkowski, Katyshev, Balabanov, Borisov, & Dore-Duffy, 2011). Perturbation of the neurovascular unit generally leads to compromised BBB integrity and increased permeability. Unquestionably, BBB permeabilization enables blood-borne substances that are normally restricted, such as excitatory amino acids, kinins, prostaglandins, metals, and proteins to enter the brain (Plateel et al., 1997). Pharmacological interventions aimed at preservation of the neurovascular unit and protection of neurons within the penumbra would clearly prevent exacerbation of brain tissue damage during cerebral ischemia/reperfusion.

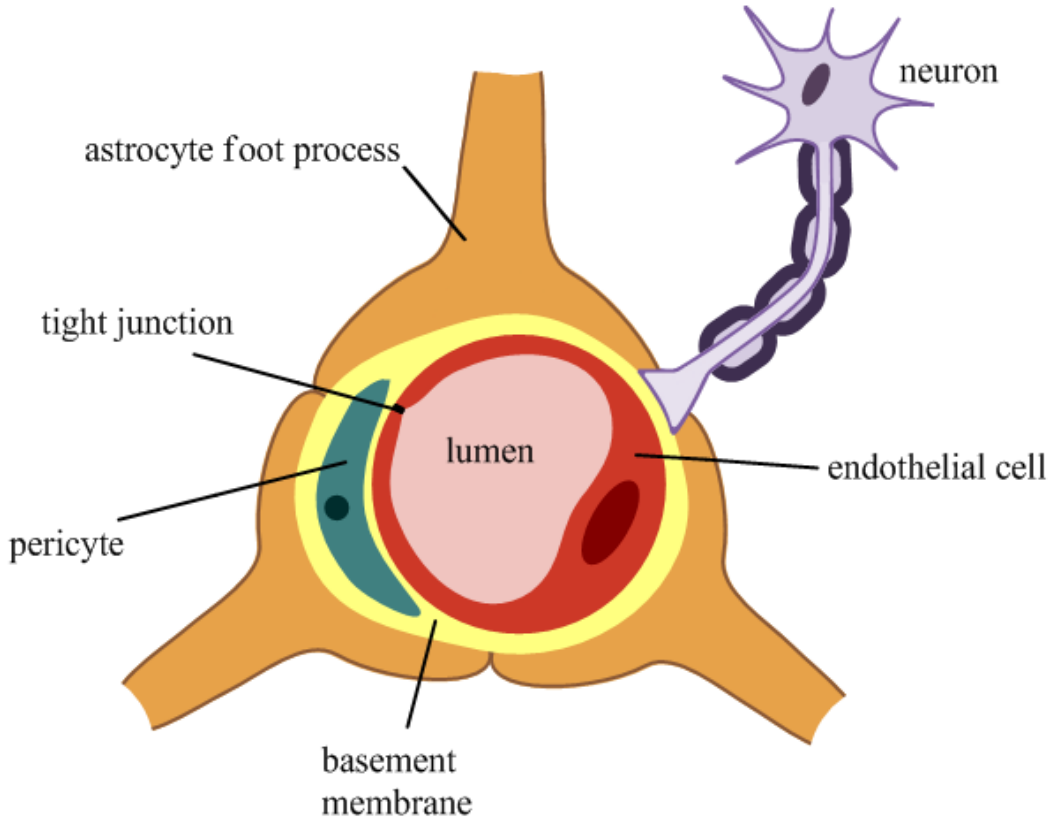

Figure 2: Cross Section of Cerebral Capillary, Illustrating Cell Types Comprising the Neurovascular Unit. From: Sanchez-Covarrubias L, Slosky LM, **Thompson BJ**, Davis TP, Ronaldson PT (2013). *Current Pharmaceutical Design*; 20(10): 1422-49.

1.3 Therapeutic Approaches for Cerebral Ischemia

Mechanisms of cell injury and/or death in the ischemic core occur extremely rapidly (i.e., within minutes), thereby rendering this region difficult to protect using traditional pharmacological approaches. In contrast, cells within the penumbra die more slowly by active cell death mechanisms (Arai et al., 2011). Residual and collateral blood flow to neurons within the penumbra allow preservation of brain tissue for up to six hours following ischemic stroke, thus rendering therapeutic interventions theoretically possible (Arai et al., 2011; Lutsep et al., 2001). The primary goal of drug therapy for acute ischemic stroke is to salvage the penumbra as much as possible and as early as possible to prevent continued growth of the ischemic core and progressively worsening neurological outcomes (Liu et al., 2010). Throughout ischemia/reperfusion brain injury, the biophysical ramifications from changes in cerebral blood flow create unique challenges to drug delivery. Differences in these changes may occur between ischemic core and penumbra, or between ipsilateral and contralateral ischemic hemispheres. For example, decreased blood flow to the penumbra will decrease drug CNS bioavailability, reducing the ability of a drug to attain efficacious concentrations at its target site. Additionally, increased BBB permeability, a key determinant of blood-to-brain drug uptake, is not a static phenomenon during cerebral ischemia/reperfusion injury. Following transient focal ischemia in experimental stroke models, enhanced BBB permeability has been observed at approximately 5 hours after ischemic insult, with a secondary increase at 72 hours (Ronaldson & Davis, 2012; Shah et al., 2013). However, in the clinic, stroke patients have been reported to experience BBB opening only during early reperfusion (Barr et al., 2010; Henning, Latour, & Warach, 2008; Kastrup et al., 2008). Focal ischemic stroke,

which causes lesions in discrete brain regions, results in regional BBB permeability differences between the ipsilateral and contralateral hemispheres (Cui et al., 2010; Hatashita & Hoff, 1990). However, within the affected hemisphere, recent studies have found no significant difference in BBB permeability between the ischemic core and the penumbra in human patients with acute hemispheric stroke as assessed by first-pass perfusion computed tomography (Dankbaar et al., 2008; Nguyen et al., 2013). Given the similarities in pathophysiological damage between the core and the penumbra, BBB permeability and therefore blood-to-brain movement of drugs and fluid is likely comparable between these two brain regions during ischemia/reperfusion injury.

Currently, there is only one therapeutic agent that has been approved by the US Food and Drug Administration for acute ischemic stroke treatment, recombinant tissue plasminogen activator (rt-PA) (Jahan & Vinuela, 2009). The objective of rt-PA therapy is to restore blood flow and oxygen supply to ischemic brain tissue. However, considerable brain cellular damage occurs when cerebral perfusion is re-established (i.e., reoxygenation). Additionally, rt-PA therapy has a narrow therapeutic window, high risk of intracerebral bleeding, and other adverse effects (Messe et al., 2012; Shah et al., 2013). Among hospitals participating in the Get With The Guidelines (GWTG)-Stroke program, only 24.7% of ischemic stroke patients that presented themselves within 3 hours were even eligible to receive rt-PA (Messe et al., 2012). Additionally, aspirin is included in the clinical standard of care for ischemic stroke because its anticoagulant properties may prevent against recurrent strokes during the high-risk period immediately after the initial ischemic insult (Chen et al., 2000). Aspirin treatment is exclusively preventative and does not confer any protection and/or rescue of ischemic brain tissue. Therefore, there is a

critical need in stroke therapy for neuroprotective and/or antioxidant drugs that can be effectively delivered to the brain for "rescue" of salvageable tissue from further damage.

Currently, there is considerable interest in neuroprotective/antioxidant properties of HMG-CoA reductase inhibitors (i.e., statins). Recent evidence suggests that statins can act as free-radical scavengers independent of their well-documented effects on cholesterol biosynthesis (Barone et al., 2011; Butterfield et al., 2012; Kassan, Montero, & Sevilla, 2010). For example, *in vivo* studies in dogs demonstrated that high-dose atorvastatin reduced markers of oxidative and nitrosative stress (i.e., protein carbonyls, 4-hydroxy-2-nonenal, 3-nitrotyrosine) and increased the ratio of GSH to reduced GSH in the brain but not in the periphery, suggesting that this drug has efficacy as a neuroprotectant and CNS antioxidant (Barone et al., 2011). Studies in an *in vivo* rodent model of subarachnoid hemorrhage showed that atorvastatin reduced brain caspase-3 activity and DNA fragmentation, implying an ability to attenuate neuronal apoptosis (Cheng, Wei, Zhi-Dan, Shi-Guang, & Xiang-Zhen, 2009; Pan et al., 2010). In addition, rosuvastatin has been shown to protect neurons from stress induced by oxygen-glucose deprivation in rat cerebrocortical neuronal cultures, perhaps by decreasing ROS levels (Domoki et al., 2009). Similarly, simvastatin, a lipophilic HMG-CoA reductase inhibitor, has shown neuroprotective effects against oxygen-glucose deprivation and subsequent reoxygenation by inhibiting production of 4-hydroxy-2E-nonenal (HNE), a cytotoxic product of lipid peroxidation, and directly reducing HNE toxicity (Lim et al., 2006). In addition to these preclinical studies, statin treatment has reduced cerebral expression of oxidative stress markers (i.e., nitrotyrosine and F2-isoprostanes) in clinical investigations (Davignon, Jacob, & Mason, 2004; Shishehbor et al., 2003).

To date, the exact mechanism for statin-induced neuroprotection has not been elucidated. It has been proposed that neuroprotective effects of atorvastatin may be due to targeting and subsequent upregulation of biliverdin reductase-A, a pleiotropic enzyme known to be involved in cellular stress responses (Barone et al., 2012). Of particular note, Barone et al. (2012) reported that increased activity of biliverdin reductase-A induced by atorvastatin was inversely correlated with indices of oxidative stress, which points towards an antioxidant mechanism for statins. It has also been suggested that statins can exert neuroprotective effects through enhancement of eNOS expression in the CNS (Endres et al., 1998; Sironi et al., 2003), thereby improving collateral blood flow to the ischemic penumbra.

CHAPTER 2: Hypoxia/Reoxygenation Stress Signals an Increase in Organic Anion Transporting Polypeptide 1a4 (Oatp1a4) at the Blood-Brain Barrier: Relevance to CNS Drug Delivery

Taken in part from the manuscript of the same title: **Thompson BJ**, Sanchez-Covarrubias L, Slosky LM, Zhang Y, Laracuente ML, Ronaldson PT (2014). *Journal of Cereberal Blood Flow & Metabolism*; 34(4): 699-707.

2.1 Introduction

Cerebral hypoxia and subsequent reoxygenation (H/R) stress is a central component of several diseases, including traumatic brain injury, high altitude cerebral edema, acute mountain sickness, acute respiratory syndrome, obstructive sleep apnea, cardiac arrest, and ischemic stroke (Ronaldson et al., 2013). H/R is directly associated with neuronal apoptosis, a proteolytic cascade characterized by cytochrome c release, caspase-3 activation and internucleosomal DNA fragmentation (Lobysheva, Tonshin, Selin, Yaguzhinsky, & Nartsissov, 2009). As neuronal cell damage transpires during H/R, neuroinflammation and apoptosis become more prevalent and dramatically affect viability of brain tissue (Ronaldson et al., 2013). Such pathophysiological processes include activation of poly(ADP-ribose) polymerase (PARP), a family of nuclear enzymes involved in DNA repair, programmed cell death, and necrotic tissue damage. *In vivo*, hypoxic-ischemic insult and/or H/R stress results in increased PARP cleavage in the brain (Martinez-Romero et al., 2009; Tu, Lu, Huang, Ho, & Chou, 2012). Indeed, genetic deletion of PARP has protected animal subjects against DNA-damage associated with pathophysiological conditions such as ischemia-reperfusion injury, neuroinflammatory stress, and glutamate excitotoxicity (M. Y. Kim et al., 2005). Taken together, these observations indicate that PARP cleavage is a reliable and sensitive early marker of CNS damage and/or neuronal stress following an H/R insult.

The critical need for novel therapeutic approaches to treat diseases with an H/R component is best illustrated by ischemic stroke. Currently, only r-tPA is approved by the US Food and Drug Administration (FDA) for acute ischemic stroke treatment (Ronaldson et al., 2013). The primary goal of r-tPA therapy is to restore blood flow and oxygen

supply to ischemic brain tissue; however, most cellular damage to the brain occurs when cerebral perfusion is re-established (i.e., reoxygenation). Therefore, there is a critical need in stroke therapy for efficacious therapeutics that can be delivered to the brain for "rescue" of salvageable neural tissue. Recent evidence suggests that statins can act as free-radical scavengers independent of their well-documented effects on cholesterol biosynthesis (Butterfield et al., 2012). The ability of statins to be effective neurotherapeutics following cerebral hypoxia requires efficient and precise CNS delivery. A recent comparative *in vitro* study that evaluated efficacy of statins as neuroprotectants by assessing their chemical structure, theoretical lipophilicity, and ability to protect against neuron cell death induced by okadaic acid, concluded that both atorvastatin and rosuvastatin were effective in mitigating neuron cell death (Sierra et al., 2011). However, both of these drugs had permeability values close to zero and BBB penetration estimates of less than 5% (Sierra et al., 2011). Such a study illustrates the critical importance of identifying and characterizing endogenous transport mechanisms that can be targeted to facilitate CNS statin delivery.

One family of transporters that may have utility in brain delivery of statins are the organic anion transporting polypeptides (OATPs in humans, Oatps in rodents). OATPs/Oatps are a group of sodium-independent transporters classified within the larger solute carrier (SLC) superfamily (Ronaldson et al., 2013). In rodent brain, expression of Oatp1a4, Oatp1c1, and Oatp2a1 have been reported in capillary enriched fractions, capillary endothelial cells, and brain microvessels (Kis et al., 2006; Ronaldson & Davis, 2011; Westholm, Stenehjem, Rumbley, Drewes, & Anderson, 2009). Oatp1c1 primarily transports thyroxine and conjugated sterols (Westholm et al., 2009) while Oatp2a1

regulates BBB transport of prostaglandins (Kis et al., 2006). In contrast, Oatp1a4 is the primary drug transporting Oatp isoform expressed at the rat BBB (Ronaldson et al., 2013). Studies in Oatp1a4(-/-) mice have demonstrated reduced blood-to-brain transport of pitavastatin and rosuvastatin as compared to wild-type controls, which indicates involvement of Oatp1a4 in statin transport across the BBB (Ose et al., 2010). The human orthologue of Oatp1a4 is OATP1A2, which exhibits an enrichment of mRNA expression in the brain as compared with other tissues including liver, kidney, and gastrointestinal tract (Kullak-Ublick et al., 1995; Steckelbroeck et al., 2004). Immunofluorescence staining of human brain frontal cortex demonstrated OATP1A2 localization at both the apical and basolateral sides of the microvascular endothelium (Gao et al., 2000). Although not directly studied at the BBB, OATP1A2 has been shown to transport rosuvastatin in isolated human hepatocytes and atorvastatin in human embryonic kidney cells (HEK293) stably transfected with OATP1A2 (Ho et al., 2006; Mandery et al., 2011). Localization and substrate profiles of OATP/Oatp isoforms known to be present at BBB are summarized in Table 1. Using our *in vivo* model of pain/inflammation, we have shown that Oatp1a4 is a BBB transporter that can be exploited to optimize CNS delivery of opioid peptide drugs (Ronaldson, Finch, Demarco, Quigley, & Davis, 2011); however, Oatp1a4-mediated delivery of statins across the brain microvascular endothelium under conditions of H/R has not been clearly elucidated.

Although pathophysiological stressors can modulate endogenous BBB transporters, such changes must be effectively controlled in order to provide optimal CNS drug delivery. For example, studies in our *in vivo* inflammatory pain model demonstrated increased BBB functional expression of Oatp1a4 only between 1 h and 6 h after induction

Table 1: Localization and Substrate Profiles of OATP/Oatp Isoforms Known to be Present at the Blood-Brain Barrier (BBB).

Human OATP Isoform	BBB expression	Rodent Ortholog	BBB expression	Potential Substrate Drugs
OATP1A2	apical & basolateral	Oatp1a4	apical & basolateral	HMG-CoA reductase inhibitors (e.g., atorvastatin, cerivastatin, fluvastatin, pitavastatin, pravastatin, rosuvastatin, simvastatin acid); opioid analgesic peptides (e.g., DPDPE)
OATP1C1	localization not confirmed	Oatp1c1	apical	thyroid hormones and conjugated sterols
OATP2A1	localization not confirmed	Oatp2a1	apical	prostaglandins

Adapted from: Ronaldson & Davis (2013). Pharmacol Rev. 65:291-314.

of pain/inflammation (Ronaldson, Finch, et al., 2011). Therefore, if Oatp1a4 is to be utilized for effective delivery of therapeutics (i.e., statins) for treatment of diseases with an H/R component, its functional expression must be controlled over a more desirable time course than is possible by only relying on pathophysiological processes. This objective can be accomplished by pharmacological targeting of signaling pathways that regulate Oatp1a4 such as the transforming growth factor-β (TGF-β) system (Ronaldson, Finch, et al., 2011). TGF-βs are cytokines that signal by binding to a heterotetrameric complex of type I and type II serine/threonine kinase receptors (Derynck & Zhang,

2003). The type I receptors, also known as activin receptor-like kinases (ALKs), propagate intracellular signals through phosphorylation of receptor-specific Smad proteins (i.e., receptor-regulated (R)-Smads). Phosphorylated (R)-Smads form complexes with the common Smad (i.e., Smad4), enabling nuclear translocation and subsequent changes in target gene transcription. At the BBB, only two ALK receptors (i.e., ALK1, ALK5) have been identified (Ronaldson, Demarco, Sanchez-Covarrubias, Solinsky, & Davis, 2009). We have previously shown that pharmacological inhibition of TGF-β/ALK5 signaling can increase Oatp1a4 functional expression (Ronaldson, Finch, et al., 2011). Therefore, targeting of TGF-β/ALK5 signaling during H/R provides an opportunity to control Oatp1a4 expression/activity at the BBB for optimization of CNS drug delivery.

In the present study, we demonstrate that the commonly prescribed statin, atorvastatin, may have utility as a therapeutic for treatment of diseases with an H/R component. We show for the first time that increased Oatp1a4 functional expression occurs following an H/R insult. Furthermore, this increase in transport activity can be used to increase brain delivery of atorvastatin. We also provide evidence indicating that pharmacological targeting of the TGF-β-ALK5 signaling pathway is a potential mechanism that can be targeted for control of CNS drug delivery in animals subjected to H/R.

2.2 Materials and Methods

Materials

[^{3}H]Taurocholic acid (4.6 Ci/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). [^{3}H]Atorvastatin (10 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Rabbit polyclonal antibodies against Oatp1a4 were purchased from Millipore (anti-Oatp2; Temecula, CA) and Santa Cruz Biotechnology, Inc. (M-50; Santa Cruz, CA), respectively. The rabbit monoclonal anti-poly (ADP-ribose) polymerase (PARP) antibody was obtained from Abcam (Cambridge, MA). Atorvastatin calcium salt trihydrate, dimethyl sulfoxide (DMSO), estrone-3-sulfate potassium salt (E3S), fexofenadine (FEX), the selective ALK5 inhibitor SB431542 and sodium pentobarbital were all purchased from Sigma-Aldrich (St. Louis, MO).

Animals and Treatments

All animal experiments were approved by the University of Arizona Institutional Animal Care and Use Committee and conform to National Institutes of Health guidelines. Female Sprague-Dawley rats (200-250 g) were purchased from Harlan Sprague-Dawley (Indianapolis, IN), housed under standard 12 h light/12 h dark conditions, and provided with food and water *ad libitum*. Animals were randomly assigned to each treatment group. Animals were subjected to hypoxic insult (i.e., 6% O_2) for 1 h as previously described (Witt et al., 2005). Rats were then subjected to reoxygenation (i.e., 21% O_2) for 10 min, 30 min, 1 h, 2 h, 6 h, or 24 h. Animals subjected to H/R were compared to animals subjected to hypoxic insult only and to normoxic controls. For assessment of atorvastatin effects on PARP cleavage, atorvastatin calcium salt trihydrate was dissolved

in 100% DMSO and was administered [20 mg/kg (1.0 mL/kg), i.p.] 1 h prior to hypoxic treatment, a time point that allowed atorvastatin to reach T_{max} immediately prior to hypoxic treatment (Reddy, Reddy, Rao, & Kumar, 2012). To determine if Oatp1a4-mediated transport is involved in atorvastatin effects on PARP cleavage, we administered the known Oatp1a4 transport inhibitor estrone-3-sulfate [2.5 mg/kg (1.0 mL/kg), dissolved in 100% DMSO, i.p.] 30 min prior to atorvastatin administration. For experiments designed to evaluate involvement of ALK5-mediated signaling on regulation of Oatp1a4 functional expression, SB431542 [1.5 mg/kg (1.0 mL/kg), i.p.], a selective ALK5 inhibitor, was dissolved in 100% DMSO and administered 30 min prior to initiation of hypoxia.

Rat Brain Microvessel Membrane Isolation

Brain microvessels were harvested as previously described by our laboratory (Ronaldson et al., 2009; Ronaldson, Finch, et al., 2011). Following anesthesia with sodium pentobarbital (64.8 mg/mL (1.0 mL/kg) i.p.), rats were decapitated and brains were removed. Meninges and choroid plexus were excised and cerebral hemispheres were homogenized in 4 mL of microvessel isolation buffer (103 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 15 mM HEPES, pH 7.4) containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). After homogenization, 8 mL of 26% dextran at 4°C was added and homogenates were vortexed. Homogenates were then centrifuged (5600 *g*; 4°C) for 10 min and the supernatant was aspirated. Pellets were resuspended in 10 mL of microvessel isolation buffer and passed through a 70 μm filter (Becton Dickinson, Franklin Lakes, NJ). Filtered homogenates were pelleted by centrifugation at 3000 *g* for 10 min. At this time, the supernatant was aspirated and the

pellet was collected for use in Western blot analyses or for preparation of plasma membrane isolates as described by our group (Slosky et al., 2013). Plasma membrane preparations were obtained by resuspending the pellet in a modified radioimmunoprecipitation buffer consisting of 50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1.0 mM EGTA, 1.0 mM sodium *o*-vanadate, 1% (v/v) Nonidet P-40, 0.25% (m/v) sodium deoxycholate, 0.1% (m/v) SDS, 200 µM phenylmethylsulfonyl fluoride, and 0.1% protease inhibitor cocktail (Sigma-Aldrich). Samples were gently rocked for 15 minutes at 4^0C to allow lysis to occur. Lysates were then centrifuged (3000 *g*; 4^0C) for 10 minutes and supernatants were collected. Denucleated supernatants were then centrifuged at 100 000 *g* (4^0C) for 60 minutes. Pellets (i.e., plasma membranes) were resuspended in phosphate-buffered saline containing protease inhibitor cocktail and frozen at -20^0C until use.

Western Blot Analysis

Rat brain microvessel samples were quantified for total protein using the bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL) and analyzed for expression of PARP or Oatp1a4. Microvessel protein samples (10 µg) were resolved on 4-12% SDS-polyacrylamide gels (Bis-Tris Criterion XT; Bio-rad, Hercules, CA) and transferred to a polyvinylidene difluoride (PVDF) membrane. PVDF membranes were incubated in Superblock (Pierce Biotechnology) containing 0.05% (v/v) Tween-20 for 1 h at room temperature. Membranes were then incubated with primary antibody directed against PARP (1:1000 dilution) or Oatp1a4 (anti-Oatp2, 1:1000 dilution; M-50, 1:2000 dilution) overnight at 4°C. The membranes were then washed in TBS-T ((15 mM Tris-HCl and 150 mM NaCl pH 7.6 containing 0.05% (v/v) Tween-20): (6 x 15 min)) and

incubated with anti-rabbit IgG conjugated to horseradish peroxidase for 1 h at room temperature. Membranes were developed using enhanced chemiluminescence (ECL, Amersham, Piscataway, NJ) and were stained for total protein with Ponceau S. The optical density of each band was normalized to total protein in each sample (i.e., loading control) according to a previously published method (Romero-Calvo et al., 2010). Ponceau S is a fast and fully reversible stain that, when applied and quantified prior to antibody staining, has been validated as an alternative means to immunoblotting of individual housekeeping/structural proteins (i.e., actin, Na^+/K^+-ATPase) in assessment of equal protein loading in Western blot analysis. Since experimental manipulations (i.e., hypoxic stress) have been shown to alter expression of housekeeping genes such as glyceraldehyde 3-phosphate dehydrogenase, beta-actin, and cyclophilin (Yamaji et al., 2003; Zhong & Simons, 1999), we chose to use total protein as our loading control. Bands were quantitated and corrected for background using ImageJ densitometric software (Wayne Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, MD). All data were normalized to normoxic control values that were matched to treated animals from the same experimental day and are reported as percent of control (%control).

In Situ Brain Perfusion

Animals were anesthetized with sodium pentobarbital (64.8 mg/mL i.p.) and heparinized (10 000 U/kg i.p.). Body temperature was maintained at 37°C using a heating pad. The common carotid arteries were cannulated with silicone tubing connected to a perfusion circuit. The perfusate was an erythrocyte-free modified mammalian Ringer's solution consisting of 117 mM NaCl, 4.7 mM KCl, 0.8 mM $MgSO_4$, 1.2 mM KH_2PO_4,

2.5 mM $CaCl_2$, 10 mM D-glucose, 3.9% (w/v) dextran (MW 60 000), and 1.0 g/L bovine serum albumin (type IV), pH 7.4, warmed to 37°C and oxygenated with 95% O_2/5% CO_2. Evan's blue dye (55 mg/L) was added to the perfusate to serve as a visual marker of BBB integrity. Perfusion pressure and flow rate were maintained at 95-105 mmHg and 3.1 mL/min respectively. Both jugular veins were severed to allow for drainage of the perfusate. Using a slow-drive syringe pump (0.5 mL/min per hemisphere; Harvard Apparatus, Holliston, MA), [^{3}H]taurocholic acid (1.0 μCi/mL; 10 mM total concentration) or [^{3}H]atorvastatin (0.5 μCi/mL; 0.013 μM total concentration) was added to the inflowing perfusate. Following perfusion, the rat was decapitated and the brain was removed. Meninges and choroid plexus were excised and cerebral hemispheres were sectioned and homogenized. At this time, TS2 tissue solubilizer (1 mL) was added and each sample was allowed to solubilize for 2 days at room temperature. To eliminate chemiluminescence, 100 μL of 30% glacial acetic acid was added, along with 2 mL Optiphase SuperMix liquid scintillation cocktail (PerkinElmer, Boston, MA). Samples were measured for radioactivity on a model 1450 liquid scintillation counter (PerkinElmer). For inhibition studies, animals were perfused with erythrocyte-free modified mammalian Ringer's solution containing a known Oatp inhibitor (i.e., 100 μM estrone-3-sulfate, 100 μM fexofenadine) for 10 min prior to perfusion with [^{3}H]taurocholic acid or [^{3}H]atorvastatin.

Results were reported as picomoles of radiolabeled drug per gram of brain tissue (C; pmol/g tissue), which is equal to the total amount of radioisotope in the brain [C_{Brain}; dpm/g tissue] divided by the amount of radioisotope in the perfusate [$C_{Perfusate}$; dpm/pmol]: $C = C_{Brain}/C_{Perfusate}$. The brain vascular volume in rats has been previously

shown to range between 6 and 9 μL/g brain tissue (Takasato, Rapoport, & Smith, 1984). Because brain tissue was processed immediately after perfusion with radiolabeled substrate, all uptake values required correction for brain vascular volume. This was accomplished by subtracting the average vascular volume (i.e., 8.0 μL/g brain tissue as calculated from data reported by Takasato and colleagues) from whole-brain uptake data obtained for [^{3}H]taurocholic acid or [^{3}H]atorvastatin.

[3H]Atorvastatin Stability Studies

Venous outflow samples were collected during in situ brain perfusion studies and were analyzed for atorvastatin content using high performance liquid chromatography (HPLC). Atorvastatin was extracted from venous outflow samples and was briefly centrifuged at 2000 *g* for 15 min (25^{0}C) to remove any precipitate. Samples (100 μL) were loaded onto a reverse phase C-18 column (5-μm, Altex Ultrasphere-I.P., 15 cm × 4.6 mm i.d.) using a Rheodyne 7125 manual injector (Rohnert Park, CA). Atorvastatin was eluted using a mobile phase composed of 0.06 mol/L KH_2PO_4, adjusted to pH 3.2 with hydrochloric acid, and acetonitrile (50:50, v/v) isocratically pumped (Perkin-Elmer series 200 LC Pump, Boston, MA) at 1.0 mL/min (37^{0}C) for 20 min. UV absorbance (246 nm) was detected by an SPD-6A UV spectrophotometric detector (Shimadzu, Kyoto, Japan) while radioactivity associated with eluted compounds was determined by an LB 509 radioflow detector (Berthold Technologies, Bad Wildbad, Germany). Peaks corresponding to atorvastatin were identified by injecting known standards of both [^{3}H]atorvastatin and non-radiolabeled atorvastatin calcium salt trihydrate. Retention times for atorvastatin were 3.9–4.2 min. All data were acquired and analyzed using PowerChrom software (eDAQ Inc., Colorado Springs, CO). [^{3}H]Atorvastatin stability

within venous outflow samples was determined by the appearance of a peak at the correct retention time, as well as its ability to co-elute at the same retention time as unlabeled atorvastatin calcium salt trihydrate (1 µg/µL) spiked with [^{3}H]atorvastatin under identical conditions.

Blood gas and electrolyte analysis

Blood was collected from the descending aorta (~700 µL aliquots) via a *safe*PICO arterial blood gas syringe (Radiometer, Copenhagen, Denmark) and immediately analyzed using an ABL 77 blood gas analyzer (Radiometer, Copenhagen, Denmark). Measurements of pH, pCO_2, pO_2, O_2 saturation, HCO_3^- concentration, anion gap, ion concentration (Na^+, K^+, Ca^{2+}, Cl^-), and hematocrit were performed in normoxic control animals, following the 1 h hypoxic exposure (Hx), or following 1 h hypoxic exposure followed by 10 min reoxygenation (H/R). Blood draws for the hypoxic (Hx) animals were done inside the hypoxic chamber to prevent inadvertent reoxygenation.

Statistical Analysis

Western blot image analysis data are reported as mean ± S.D. from at least three separate experiments where each treatment group consists of pooled microvessels or pooled whole brain lysates from three individual animals. In situ brain perfusion data are reported as mean ± S.D. from six individual animals per treatment group. Blood gas analyses data are reported as mean ± S.D. from six individual animals per treatment group. To determine statistical significance between treatment groups in Western blot experiments, Student's *t* test was used for unpaired experimental data. To determine the significance of brain [^{3}H]taurocholate or [^{3}H]atorvastatin accumulation, a repeated

measures ANOVA and post hoc multiple-comparison Bonferroni *t* test were used. To determine significance of blood gas and electrolyte measurements, a one-way ANOVA was conducted for each parameter followed by Tukey's post-hoc test when appropriate. A value of $p < 0.05$ was accepted as statistically significant.

2.3 Results

H/R Modulates Blood Gas and Circulating Electrolyte Levels

Evaluation of blood chemistries (pO_2, pCO_2, electrolyte concentrations, etc.) was conducted to validate the degree of hypoxic insult. Arterial blood samples were collected from control (i.e., normoxic) animals, animals exposed to hypoxic stress, and H/R animals that had been reoxygenated for 10 min. Analysis of blood gases (Table 2) illustrate significant decreases in pH, pCO_2, pO_2 and sO_2% in hypoxic animals. In H/R animals, these values were still suppressed but approached recovery by the 10 min reoxygenation time point. Additionally, the bicarbonate ion concentration was decreased in both hypoxic and H/R animals, causing an elevation in the anion gap. No differences in free ion concentrations (Na^+, K^+, Ca^{2+}, Cl^-) were observed. The hematocrit (i.e., erythrocyte volume fraction) of the hypoxic animals was increased compared to the normoxic and H/R animals. Taken together, these data indicate that our global model of oxygen deprivation achieved an acute state of systemic hypoxic acidosis that is recoverable, an observation that is supported by blood gas and electrolyte levels in H/R animals.

Table 2: Blood Gas and Electrolyte Measurements from Animals Subjected to Normoxia (Nx), Hypoxia (Hx; 6% O_2 for 1 h), or H/R (6% O_2 for 1 h followed by Nx for 10 min).

	Normoxic	Hypoxic	H/R
pH	7.45 ± 0.01	**7.31 ± 0.01****	**7.35 ± 0.03***
pCO_2, mmHg	37.0 ± 2.65	**29.3 ± 0.58***	39.0 ± 2.0
pO_2, mmHg	102 ± 4.6	**5.0 ± 3.0****	**115 ± 5.5***
Hct, %	40 ± 3.0	46 ± 2.0*	41 ± 1.0
Na^+, mmol/L	140 ± 0.58	138 ± 1.0	139 ± 0.58
K^+, mmol/L	3.70 ± 0.35	3.93 ± 0.40	3.57 ± 0.21
Ca^{2+} , mmol/L	1.33 ± 0.02	1.37 ± 0.03	1.38 ± 0.03
Cl^-, mmol/L	109 ± 1.5	106 ± 1.5	109 ± 1.0
HCO_3^-, mmol/L	25.1 ± 1.59	**14.3 ± 0.61****	**20.8 ± 2.08***
Anion Gap	8.90 ± 0.89	**20.9 ± 1.12****	**13.1 ± 2.06***
sO_2, %	98.1 ± 0.31	**3.60 ± 2.56****	98.2 ± 0.29

Blood was collected from the descending aorta via heparinized syringe and immediately analyzed. Blood draws for the hypoxic (Hx) animals were done from within the hypoxic chamber. Values are means ± S.D. of six animals per treatment group. * $p < 0.05$, ** $p < 0.01$.

Atorvastatin Attenuates Increases in Cleaved PARP-to-Uncleaved PARP Ratio Following H/R Stress

The ratio of cleaved-to-uncleaved PARP is an established early indicator of end-stage cell death (Chaitanya et al., 2010). PARP can be cleaved by executioner caspases (i.e., caspases-3 and -7), calpains, cathepsins, granzymes, and matrix metalloproteinases, which are all associated with various cellular death pathways (i.e., caspase-dependent apoptosis, caspase-independent apoptosis, production of pro-inflammatory mediators, energy-failure-induced necrosis) (Chaitanya et al., 2010; M. Y. Kim et al., 2005). The molecular weights of resulting PARP fragments are indicative of participation of a specific protease. Consistent with previous findings (A. Ghosh, Sarkar, Mandal, & Das, 2013), we found elevated cleaved-to-uncleaved PARP ratios in whole brain lysates prepared from animals subjected to hypoxic insult (1 h, 6% O_2) and reoxygenated for 10 min, 30 min and 1 h as compared to these same ratios in brain lysates from normoxic animals (Figure 3A, 3B). The PARP fragments detected (i.e., 89 kDa) were consistent with those produced by executioner caspases (Chaitanya et al., 2010). No significant change in cleaved PARP-to-uncleaved PARP ratios were identified in animals subjected to hypoxia only, or those subjected to H/R with reoxygenation times of 2, 6 or 24 h. Atorvastatin treatment 1 h prior to hypoxia challenge attenuated the H/R-induced increase in the cleaved-to-uncleaved PARP ratio at the 10 min, 30 min and 1 h reoxygenation time points (Figure 3A, 3B). Administration of estrone-3-sulfate reversed the atorvastatin-induced attenuation in PARP cleavage in the H/R 10 min and H/R 1 h treatment groups (Figure 3A, 3B). Estrone-3-sulfate did not cause any significant changes in PARP cleavage in normoxic control animals or animals subjected to hypoxia only

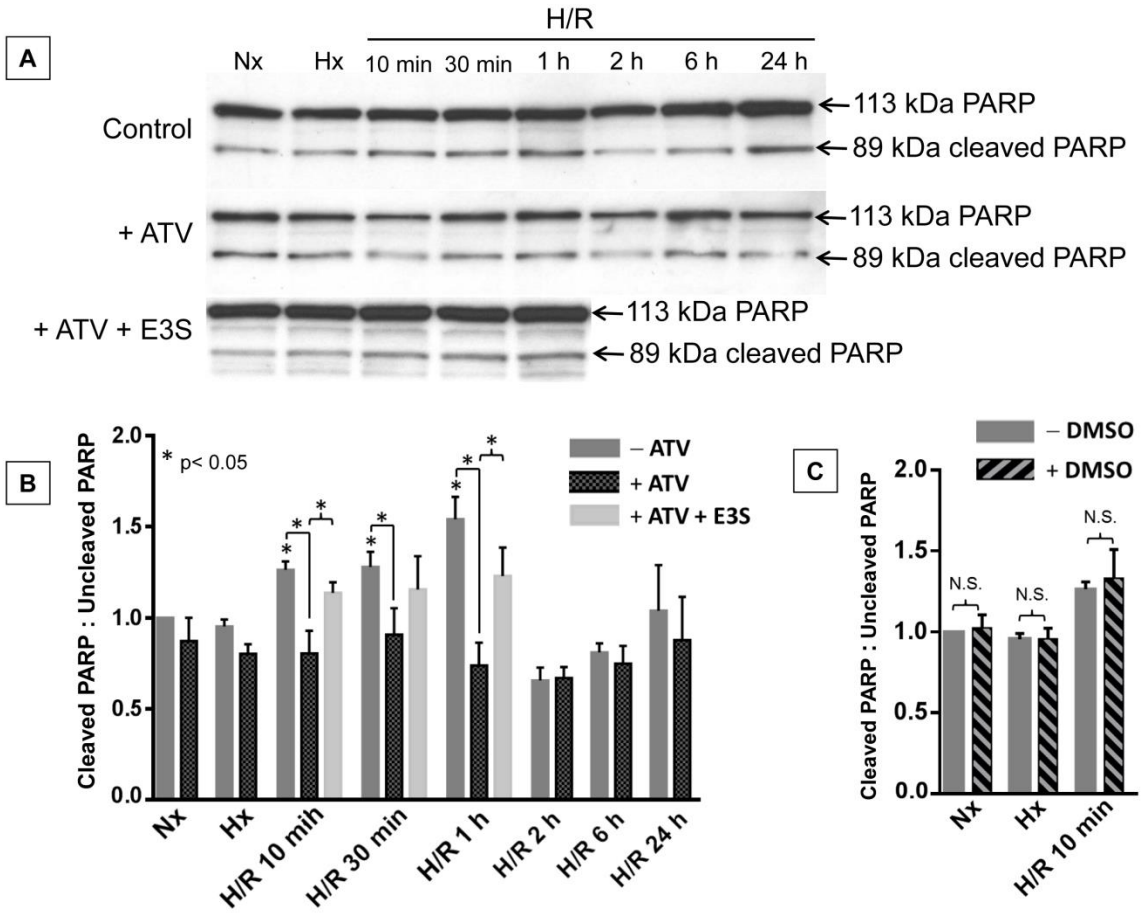

Figure 3: Atorvastatin (ATV) Attenuates H/R-Induced Increase in Ratio of Cleaved PARP-to-Uncleaved PARP Ratio. A: Western blot analysis of whole brain lysates isolated from normoxic (Nx), hypoxic (Hx), and H/R treated rats dosed with ATV (20 mg/kg). Animals receiving ATV (20 mg/kg, i.p.) were dosed 1 h prior to hypoxia treatment (1 h, 6% O_2). Animals receiving the Oatp1a4 transport inhibitor estrone-3-sulfate (E3S; 2.5 mg/kg, i.p.) were dosed 30 min prior to ATV administration. Brain samples (10 μg) were resolved on a 4-12% SDS-polyacrylamide gel, transferred to a PVDF membrane, and analyzed for expression of cleaved and uncleaved PARP. **B:** Relative cleaved-to-uncleaved PARP ratios determined by densitometric analysis and expressed as fold change over control. Results are expressed as mean ± S.D. of three separate experiments where each group consists of pooled whole brain lysates from three individual animals. Asterisks represent data points that are significantly different from control. **C:** DMSO vehicle control does not alter ratio of cleaved PARP-to-uncleaved PARP in Nx, Hx or H/R 10 min treatments (N.S.; not significant).

(Figure 3A). Since both atorvastatin and estrone-3-sulfate were dissolved in 100% DMSO, we also measured the cleaved PARP-to-uncleaved PARP ratio in animals dosed with DMSO and subjected to H/R (H = 6% O_2, 1 h; R = 21% O_2, 10 min) as well as in animals subjected to hypoxia only and in normoxic control animals. Figure 3C shows that DMSO did not cause any significant vehicle effect on the cleaved PARP-to-uncleaved PARP ratio in any of these treatment groups. Taken together, these data suggest that H/R treatment activates PARP-mediated cellular stress mechanisms, which can be attenuated by treatment with atorvastatin. These data also suggest that the observed atorvastatin-induced attenuation in PARP cleavage is dependent on Oatp1a4-mediated drug delivery to the brain.

H/R Stress Increases Oatp1a4 Membrane Expression

Use of atorvastatin as a CNS therapeutic is limited by poor BBB permeation (Sierra et al., 2011). Since our data shows that atorvastatin exhibits protective effects in the brain, we sought to study a biological mechanism that could be targeted to deliver atorvastatin to the CNS. One potential mechanism that could facilitate CNS statin delivery is Oatp1a4-mediated transport. To evaluate both BBB expression of Oatp1a4 and how this transporter is affected by H/R, we examined Oatp1a4 expression in plasma membrane fractions of rat brain microvessels using Western blot analysis. H/R animals (H = 1 h, 6% O_2; R = 10 min, 30 min, 1 h, 2 h, 6 h, or 24 h, 21% O_2) were compared to those subjected to hypoxic insult (1 h, 6% O_2) only and to normoxic control animals. Using the anti-Oatp2 antibody, Western blot analysis of rat brain microvessel membrane fractions isolated from animals subjected to H/R revealed a significant increase in

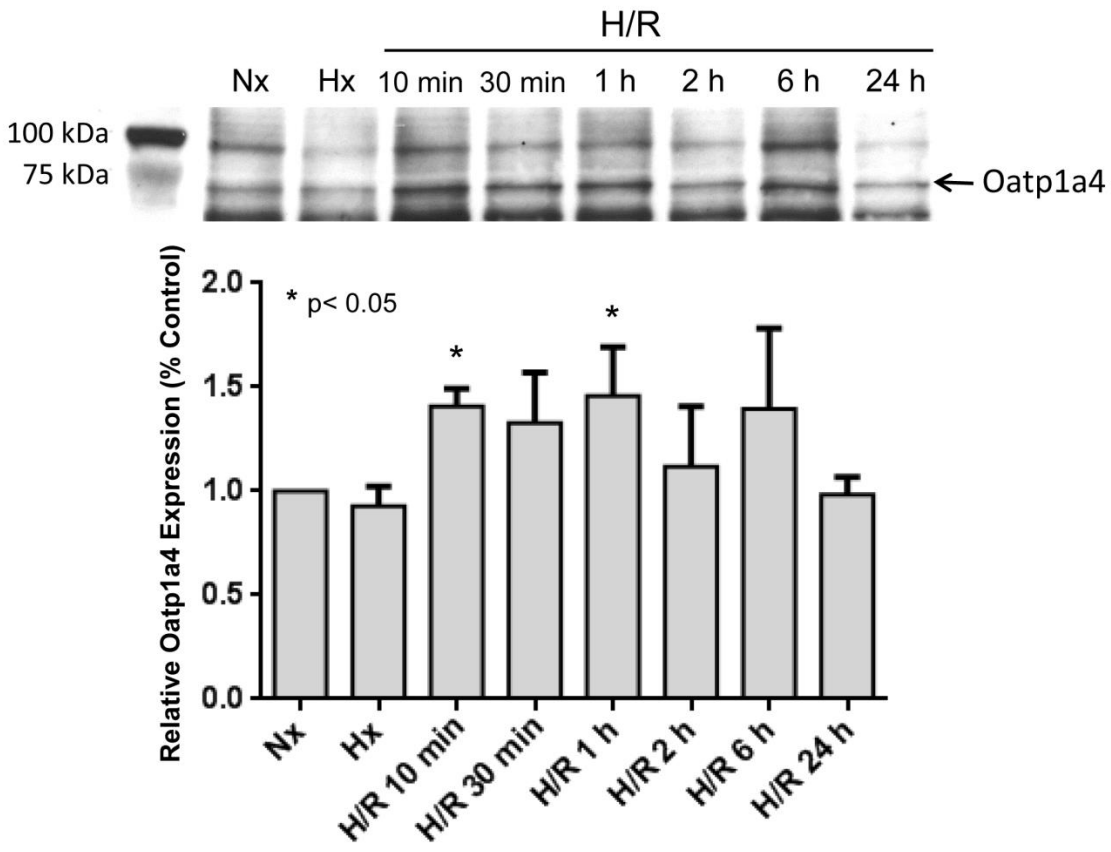

Figure 4: H/R Increases expression of Oatp1a4 in brain microvessel membranes. A: Western blot analysis of brain microvessel plasma membrane preparations isolated from Hx and H/R treated rats compared with normoxic control. Brain samples (10 µg) were resolved on a 4-12% SDS-polyacrylamide gel, transferred to a PVDF membrane, and analyzed for expression of Oatp1a4 (using the anti-Oatp2 polyclonal antibody). **B:** Relative levels of Oatp1a4 determined by densitometric analysis. Results are expressed as mean ± S.D. of three separate experiments where each group consists of pooled microvessels from three individual animals. Asterisks represent data points that are significantly different from control.

Oatp1a4 expression at reoxygenation time points of 10 min and 1 h (Figure 4). No significant alteration in Oatp1a4 expression was detected in control animals and those subjected to hypoxic insult only. Similar results on Oatp1a4 expression in rat brain microvessels following H/R, hypoxia only, and in normoxic controls were obtained using the M-50 antibody (data not shown). The anti-Oatp2 antibody recognizes a 12 amino acid epitope near the C-terminus of Oatp1a4 while the M-50 antibody recognizes a different epitope corresponding to amino acids 611-660 on the Oatp1a4 protein sequence. All subsequent hypoxia/reoxygenation experiments were performed at the 10 min time point because this was the time point where the earliest changes in Oatp1a4 expression induced by H/R were observed.

H/R Increases Oatp1a4-Mediated Taurocholate and Atorvastatin Transport

To determine whether the increase in membrane Oatp1a4 expression during H/R corresponded to altered Oatp1a4-mediated transport at the BBB, the *in situ* perfusion technique was utilized. Brain uptake of [^{3}H]taurocholate, a soluble bile salt and established probe drug for Oatp-mediated transport, was evaluated in control animals, those exposed to hypoxic insult (1 h, 6% O_2) and those exposed to H/R. Following a 10 min perfusion, [^{3}H]taurocholate (10 μM) was significantly increased (2.0-fold) in the H/R treatment group as compared to normoxic and hypoxic treatment groups. In order to confirm that observed changes in [^{3}H]taurocholate brain permeation were attributable to changes in Oatp-mediated transport, control animals and those subjected to hypoxia and H/R treatment were perfused in the presence and absence of known Oatp inhibitors (i.e., E3S, FEX) for 10 min prior to perfusion with [^{3}H]taurocholate. Both E3S and FEX significantly decreased [^{3}H]taurocholate accumulation in all treatment groups (Figure

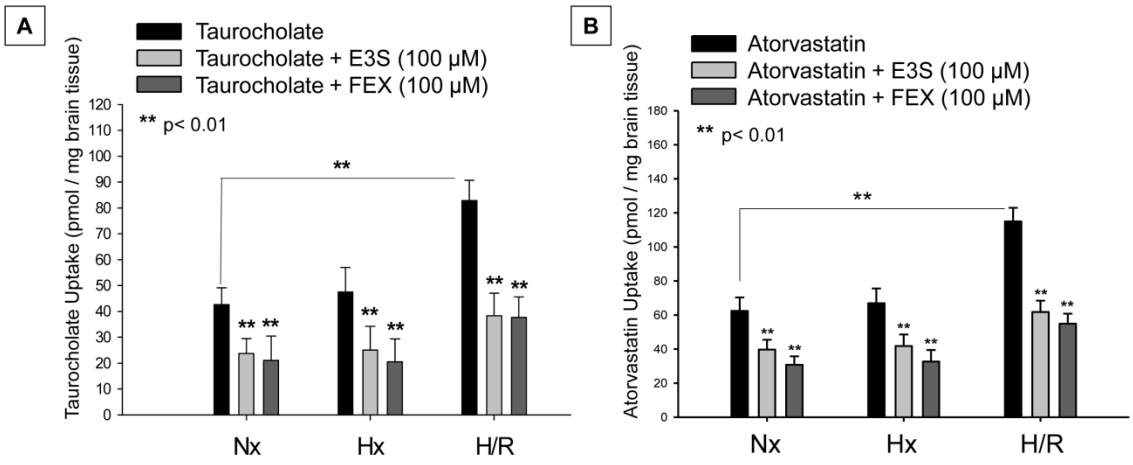

Figure 5: Increased Brain Uptake of Oatp Substrates Following H/R. Uptake of taurocholate (**A**) and atorvastatin (**B**) is increased in rat brain following hypoxia/reoxygenation (H/R) as determined by in situ brain perfusion. Animals were perfused with [^{3}H]taurocholic acid (1.0 μCi/mL) or [^{3}H]atorvastatin (0.5 uCi/mL) for 10 min in the presence and absence of Oatp inhibitors (E3S = estrone-3-sulfate; FEX = fexofenadine) (H = 6% O_2 for 1 h; R = 21% O_2 for 10 min). Perfusions including Oatp1a4 inhibitors (i.e. 100 uM E3S, 100 uM FEX) were done 10 min prior to perfusion with [^{3}H]taurocholic acid or [^{3}H]atorvastatin. Animals were then perfused with [^{3}H]taurocholic acid (1.0 μCi/mL; 10 mM total concentration) or [^{3}H]atorvastatin (0.5 μCi/mL; 0.013 μM total concentration). Nx = Normoxia; Hx = Hypoxia. Results are expressed as mean ± S.D. of six animals per treatment group. Asterisks represent data points that are significantly different from control.

5A), suggesting that H/R increases blood-to-brain transport of taurocholate via an Oatp-dependent process. Using HPLC analysis of inflow and outflow perfusate, we have previously shown that [^{3}H]taurocholate remains metabolically intact throughout our perfusion experiments (Ronaldson, Finch, et al., 2011).

To examine the relevance of this finding to delivery of a therapeutic drug, brain uptake of [^{3}H]atorvastatin was examined in control animals, those exposed to hypoxic insult (1 h, 6% O_2) and in H/R animals. Following a 10 min perfusion, [^{3}H]atorvastatin brain accumulation was significantly increased (1.7-fold) in the H/R treatment group as compared to both the normoxic and hypoxic treatment groups (Figure 5B). Similar to our data with taurocholate, perfusion in the presence of E3S or FEX reduced [^{3}H]atorvastatin uptake in all treatment groups, suggesting an Oatp1a4 contribution to atorvastatin brain uptake. HPLC analysis of inflow and outflow perfusate revealed a single peak (RT = 4 min) corresponding to atorvastatin retention, thereby confirming that atorvastatin remained metabolically intact throughout the course of our in situ perfusion experiments (data not shown). Brain accumulation of both [^{3}H]taurocholate and [^{3}H]atorvastatin in the presence of transport inhibitors E3S or FEX was slightly elevated in the H/R treatment group compared to the normoxic treatment group; however, this elevation was not statistically significant.

Effect of TGF-β/ALK5 Signaling on Oatp1a4 Functional Expression

Although the above data demonstrates that H/R alters Oatp1a4 functional expression, the biological mechanism underlying these changes in our *in vivo* H/R model

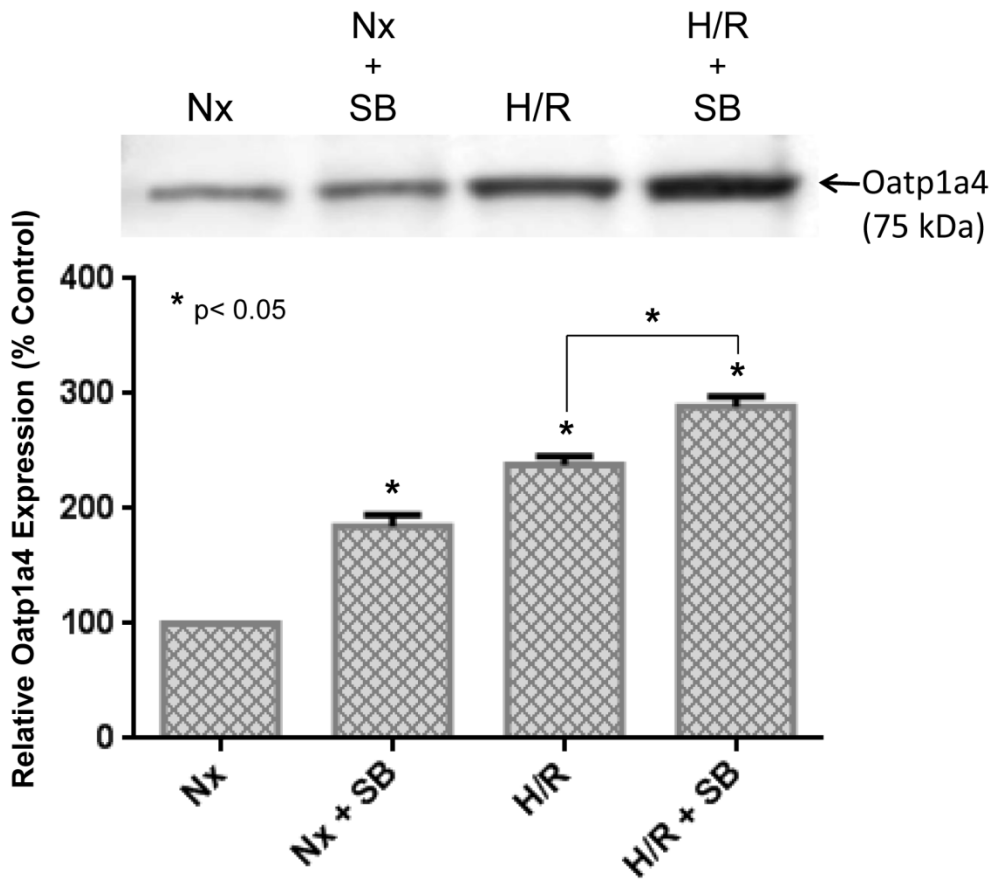

Figure 6: SB431542, a Pharmacological TGF-β/ALK5 Inhibitor, Increases Oatp1a4 Expression in Rats Subjected to H/R. Western blot analysis of rat brain microvessels isolated from H/R rats treated with SB431542 and compared to normoxic controls. Brain microvessel membrane samples (10 μg) were resolved on a 4-12% SDS-polyacrylamide gel, transferred to a PVDF membrane, and analyzed for expression of Oatp1a4 (using the M-50 polyclonal antibody). Nx = Normoxia; Hx = Hypoxia; SB = SB431542. Results are expressed as mean ± S.D. of three separate experiments where each group consists of pooled microvessels from three individual animals. Asterisks represent data points that are significantly different from control.

has not been elucidated. Our group has previously reported that Oatp1a4 is regulated by TGF-β/ALK5 signaling in an *in vivo* rodent model of peripheral inflammatory pain (Ronaldson, Finch, et al., 2011). Therefore, we hypothesized that pharmacological inhibition of TGF-β/ALK5 signaling may also control Oatp1a4 functional expression in the context of H/R. To investigate the role of TGF-β signaling in Oatp1a4 regulation following H/R, the highly specific ALK5 receptor inhibitor SB431542 (1.5 mg/kg) was given 30 min prior to hypoxia. Our group has demonstrated that this dose and dosing interval significantly blocks TGF-β/ALK5 signaling at the *in vivo* BBB.(Ronaldson et al., 2009) Administration of SB431542 prior to hypoxia treatment resulted in an increase in Oatp1a4 expression in whole rat brain microvessels as detected by the M-50 antibody (Figure 6). This increase in Oatp1a4 expression was observed in both normoxic controls and H/R animals, which further suggests that expression of the "influx" transporter Oatp1a4 at the BBB can be increased by targeting TGF-β/ALK5 signaling. Inhibition of the ALK5 receptor with SB431542 also resulted in enhanced [^{3}H]taurocholate (Figure 7A) and [^{3}H]atorvastatin (Figure 7B) brain uptake in normoxic animals, hypoxic animals and in animals subjected to H/R. Additionally, accumulation of [^{3}H]taurocholate and [^{3}H]atorvastatin in H/R rats treated with SB431542 was significantly increased as compared to normoxic animals administered SB431542 (Figure 7B), further emphasizing that TGF-β/ALK5 signaling is prominently involved in regulation of Oatp1a4 transport activity at the BBB.

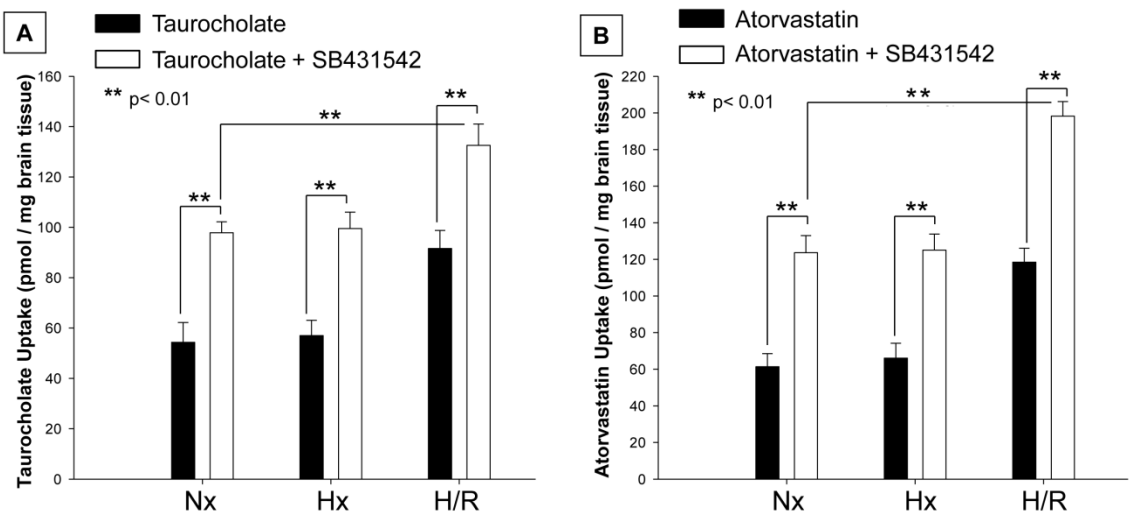

Figure 7: TGF-β/ALK5 Inhibition Increases Oatp1a4-mediated Transport in Rats Subjected to H/R. Uptake of taurocholate (**A**), a selective Oatp substrate, and atorvastatin (**B**) after H/R, in hypoxic (Hx) animals, and in normoxic (Nx) controls in the presence and absence of SB431542, a pharmacological TGF-β/ALK5 inhibitor. Results are expressed as mean ± S.D. of six animals per treatment group. Animals were perfused with [^{3}H]taurocholate (1.0 μCi/mL; 10 mM total concentration) or [^{3}H]atorvastatin (0.5 μCi/mL; 0.013 μM total concentration) for 10 min. Nx = Normoxia; Hx = Hypoxia. Results are expressed as mean ± S.D. of six animals per treatment group. Asterisks represent data points that are significantly different from control.

2.4 Discussion

Our laboratory is currently focused on characterizing regulation and functional expression of Oatp1a4 under H/R conditions and determining its role in CNS delivery of drugs. A critical factor in the translational applicability of this research objective is selection of an appropriate *in vivo* H/R model. For our studies, we have selected a non-occlusive global model of H/R where animals are exposed to an oxygen-depleted environment (i.e., 6% O_2) for 1 h, an insult that confers an acute, moderate to severe, hypoxic insult (Beck & Krieglstein, 1987). This well-established model offers several advantages including i) maintenance of interactions between BBB endothelium and other cell types/structures of the neurovascular unit (i.e., astrocytes, microglia, pericytes, neurons, extracellular matrix); ii) preservation of interactions between the BBB and circulating systemic mediators; and iii) the degree of hypoxic stress does not induce necrotic damage of the BBB endothelium, which is often associated with other *in vivo* hypoxia/ischemia models. Therefore, our H/R model permits study of a dynamically regulated and recoverable BBB. Additionally, our model reduces cerebral oxygen availability without altering cerebral blood flow (Witt et al., 2003). This allows nutrients within the systemic circulation to reach the brain and greatly reduces hydrostatic reperfusion pressures that can alter BBB integrity (Witt et al., 2003). The recoverable nature of our model is emphasized by our blood gas and electrolyte measurements (Table 1). This analysis reveals decreases in pH, pCO_2, pO_2, sO_2% and HCO_3^- levels in hypoxic animals. These parameters approach recovery by the 10 min reoxygenation time point.

The serum anion gap is used clinically as an indication of acid-base disturbances, with the normal reference range being 3 to 11 mEq/L (Winter, Pearson, Gabow, Schultz,

& Lepoff, 1990). Values above this range, such as achieved by our model, indicate a level of systemic acidosis. The anion gap is calculated as the difference between the dominant blood cations and anions [$(Na^+ + K^+) – (Cl^- + HCO_3^-)$], although in practice, potassium is often omitted as having little effect on the anion gap (Kraut & Madias, 2007). Physiologically, plasma bicarbonate acts as the first safeguard of blood pH, and therefore HCO_3^- levels decrease during hypoxia as metabolic acids are absorbed into this chemical buffer system. The observed elevation in anion gap is the result of this HCO_3^- depression. As the chemical equilibrium of blood bicarbonate shifts, CO_2 is produced in greater amounts, which triggers an increase in respiration and therefore expiration of CO_2 (Silverthorn, 2009). This hyperventilation was apparent in the hypoxic animals during treatment and is responsible for the depressed pCO_2 values observed. The hematocrit of the hypoxic animals was also increased as compared to the normoxic and reoxygenated animals, a compensatory response that typically occurs in an effort to increase oxygen carrying capacity under conditions of low blood-oxygen levels. Our global model of cerebral hypoxia has been used previously to study changes in BBB architecture and intracellular signaling following hypoxic insult (Lochhead et al., 2010; Willis, Meske, & Davis, 2010; Witt et al., 2003; Witt et al., 2005). However, it has not been used to examine regulation and functional expression of putative BBB drug transporters until the present study.

Cerebral hypoxia and subsequent reoxygenation is a central component of several CNS conditions (Ronaldson et al., 2013). There are presently very few therapeutics with clinical utility for treatment of diseases with such a cerebral hypoxia/reoxygenation component. One particularly intriguing class of drugs are the statins, which have shown

antioxidant and/or neuroprotective efficacy in several *in vitro* and *in vivo* studies (Cheng et al., 2009; Davignon et al., 2004; Pan et al., 2010; Shishehbor et al., 2003; Wood, Eckert, Igbavboa, & Muller, 2010). For example, statin treatment has reduced cerebral expression of oxidative stress markers such as nitrotyrosine and F2-isoprostanes, even in clinical investigations (Davignon et al., 2004; Shishehbor et al., 2003). Studies in an *in vivo* rodent model of subarachnoid hemorrhage showed that atorvastatin reduced brain caspase-3 activity and DNA fragmentation, suggesting an ability to attenuate neuronal apoptosis (Cheng et al., 2009; Pan et al., 2010). Statin-induced neuroprotection has been reported in a concentration range between 100 nM and 1 µM within the CNS, while neuronal toxicity has only been reported at concentrations above 1 µM (Wood et al., 2010). We therefore sought to determine whether pretreatment with the commonly prescribed statin atorvastatin could attenuate neuronal damage induced by H/R insult in our global oxygen deprivation model. We show for the first time that the ratio of cleaved PARP-to-uncleaved PARP is decreased after a single dose of atorvastatin.

Recent evidence, including our own data, suggests that PARP represents a useful biomarker for neuroprotective drug efficacy in pharmacotherapy of diseases with an H/R component. PARP is a downstream target of caspase-3 and plays a critical role in cellular stress signaling following a pathological insult such as hypoxia. *In vivo*, hypoxic-ischemic insult and/or H/R stress results in increased PARP cleavage in the brain (Martinez-Romero et al., 2009; Tu et al., 2012). The ratio of cleaved-to-uncleaved PARP is an established early indicator of end-stage cell death (Chaitanya et al., 2010; Thompson et al., 2014). Moreover, poly(ADP-ribose), the negatively charged polymer that results from PARP activity, has been shown to accumulate in neural tissue following

global cerebral hypoxia (Pacher et al., 2007). Therefore, pharmacological interventions that decrease PARP activation and cleavage in the brain are indicative of a potentially protective therapy that can attenuate neural apoptosis. Consistent with previous findings (A. Ghosh et al., 2013), our laboratory found elevated cleaved-to-uncleaved PARP ratios in whole brain lysates prepared from animals subjected to hypoxic insult (1 h, 6% O_2) and reoxygenated for 10 min, 30 min and 1 h as compared to these same ratios in brain lysates from normoxic animals. We showed that a single dose of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor atorvastatin (20 mg/kg) administered prior to hypoxic insult attenuated the H/R-induced increase in the cleaved-to-uncleaved PARP ratio at these early reoxygenation time points. Indeed, our data with atorvastatin suggests that pretreatment with statins may limit hypoxic injury as indicated by a reduction in the cleaved PARP-to-uncleaved PARP ratio. Reductions in hypoxic brain injury may reduce the incidence of associated neurological deficits, as can occur following coronary artery bypass grafting (Kuhn et al., 2013; Kulik & Ruel, 2009). Furthermore, our data emphasizes that the atorvastatin-induced neuroprotective effects are dependent on Oatp1a4-mediated drug delivery.

If atorvastatin is to act efficaciously as a neurotherapeutic, it must be able to effectively accumulate in brain parenchyma. That is, the utility of atorvastatin to treat diseases with an H/R component requires identification and characterization of a biological mechanism that can facilitate its CNS delivery. Our *in vivo* data provide evidence for increased BBB functional expression of Oatp1a4, a known transporter for atorvastatin, in response to hypoxia/reoxygenation (H/R) stress. Increased expression and/or activity of an endogenous BBB drug uptake transporter has not been reported until

the present study. This increase in Oatp1a4 expression at the plasma membrane of BBB endothelial cells may in itself be a CNS protective mechanism, allowing for enhanced blood-to-brain delivery of endogenous substances that are known Oatp1a4 substrates, such as prostaglandin E1 which has been shown to prevent neural apoptosis (Kawamura, Akira, Watanabe, & Kagitani, 1997). Previously, our group has shown *in vivo* that changes in functional expression of P-glycoprotein at the BBB may result from alterations in trafficking within cerebral microvascular endothelial cells in response to peripheral inflammatory pain (McCaffrey et al., 2012). It seems likely to us that, in a similar manner, intracellular storage pools of Oatp1a4 are rapidly trafficked to the plasma membrane of BBB endothelial cells in response to H/R stress, resulting in the observed increase in Oatp1a4 in plasma membrane fractions. In terms of pharmacotherapy, we have previously demonstrated *in vivo* that Oatp1a4 can mediate blood-to-brain drug transport and therefore be a critical determinant of CNS accumulation of the opioid peptide, DPDPE (Ronaldson, Finch, et al., 2011). Here, we expand on these critical findings by showing that Oatp1a4 is a key determinant of atorvastatin delivery to the brain, both in normoxic animals and following H/R stress. As previously described, Oatp1a4-mediated transport of statins has been shown in knock-out animals and in cell culture (Ho et al., 2006; Mandery et al., 2011; Ose et al., 2010); however, we are the first to show changes in this transporter at the BBB following a pathophysiological insult. Therefore, our present data indicate that this transport system may have considerable utility as a delivery target to treat diseases with an H/R component.

If Oatp1a4 is to be utilized for effective delivery of drugs (i.e., statins), its functional expression must be precisely controlled over a more desirable time course than

can be achieved by relying solely on pathophysiological changes. We demonstrate that TGF-β/ALK5 signaling plays a key role in the regulation of Oatp1a4. The observation that a highly selective ALK5 antagonist induces an increase in Oatp1a4 protein expression and transport activity implies that TGF-β/ALK5 signaling can be targeted for regulation of Oatp1a4 functional expression at the BBB. Our group has previously shown that SB431542 effectively blocks TGF-β/ALK5 signaling as demarcated by reduced nuclear expression of phosphorylated Smad2 and Smad3 (Ronaldson et al., 2009). Furthermore, we have demonstrated that SB431542 does not affect TGF-β/ALK1 signaling, data that emphasizes the specificity of this small molecule for the TGF-β/ALK5 signaling pathway (Ronaldson et al., 2009). Our current study also identifies TGF-β/ALK5 signaling as a target for pharmacological manipulation of brain microvascular permeability to neuroprotective and/or antioxidant drugs for treatment of diseases with an H/R component. Targeting the TGF-β signaling pathway may enable "tighter control" of Oatp1a4 functional expression, thereby enabling increased delivery of drugs to the brain. A greater understanding of this pathway under H/R conditions will enable novel therapeutic strategies for improved treatment of diseases with an H/R component such as ischemic stroke.

An important consideration in the interpretation of our drug delivery data is the influence of the efflux pump, P-gp. Atorvastatin may be a P-gp substrate (Sakaeda et al., 2002; Wu, Whitfield, & Stewart, 2000). Contradictory results have been obtained concerning P-gp transport inhibition by atorvastatin *in vitro* (Holtzman, Wiggins, & Spinler, 2006). Hochman et al. discovered that high doses of atorvastatin may inhibit P-gp transport in a concentration-dependent manner; whereas Sakaeda et al. concluded that

atorvastatin did not significantly inhibit of P-gp transport (Hochman et al., 2004; Sakaeda et al., 2006). Due to this evidence and the likelihood of P-gp involvement in atorvastatin drug delivery, we examined P-g expression at the BBB using our *in vivo* H/R model. Western blot analysis of brain microvessel plasma membranes for P-gp (data not shown) suggest that P-gp expression levels remain unaltered during the H/R time course examined in this study compared to normoxic control. Given this level of P-gp expression relative to the sharp upregulation of Oatp1a4, plus the possibility of P-gp inhibition by atorvastatin, we feel P-gp-mediated efflux of atorvastatin played a minor role in our *in situ* brain perfusion data. Arguably, P-gp inhibition may have resulted in greater levels of atorvastatin brain uptake in our drug delivery studies.

In summary, this study describes, *in vivo*, increased functional expression of Oatp1a4 during H/R stress. Our data also demonstrates that H/R can directly modulate specific BBB drug transporters and suggests involvement of TGF-β/ALK5 signaling in regulation of Oatp1a4 functional expression (Figure 8). Additionally, these observations imply that ALK5 may represent a novel target to control brain delivery of Oatp1a4 substrate drugs such as statins. Overall, these results indicate that changes in Oatp1a4 expression and/or activity can be exploited in an effort to enhance CNS delivery of statins, a class of therapeutics whose CNS utility requires effective BBB penetration.

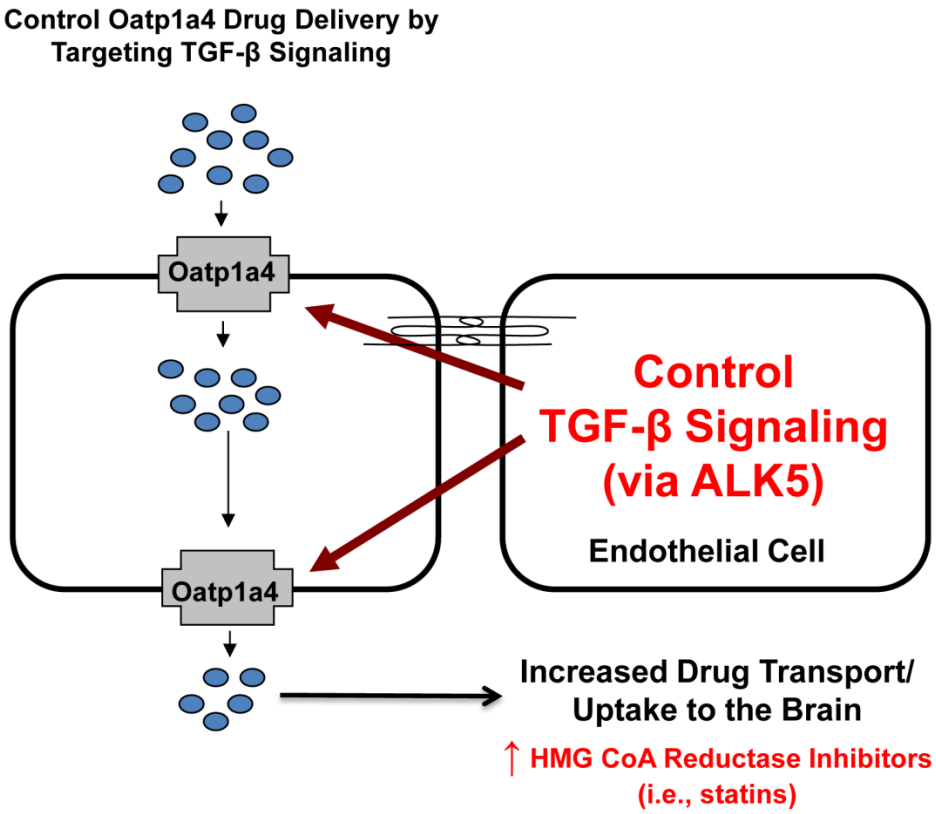

Figure 8: Enhanced Oatp1a4-mediated Statin Delivery Summary. Data from this study demonstrates that targeting Oatp transporters during pathophysiological stress may modify CNS drug delivery. We propose that Oatp1a4 facilitates brain delivery of drugs that may exhibit efficacy in treatment of peripheral inflammatory pain or cerebral hypoxia such as statins and opioid peptide analgesics. The TGF-β signaling pathway regulates Oatp1a4 functional expression via signaling mediated by TGF-β receptors (i.e., ALK5). Targeting TGF-β receptors with small molecules (i.e., SB431542) may offer an opportunity to "control" Oatp1a4 expression/activity for optimization of CNS drug delivery.

CHAPTER 3: General Discussion and Future Directions

Parts of this discussion have been taken from a book chapter that has been accepted for publication in Advances in Pharmacology:

Thompson BJ & Ronaldson PT. Drug delivery to the ischemic brain. In: Advances in pharmacology, Volume 71 (TP Davis, ed). Philadelphia: Elsevier Inc., 2014. In Press.

3.1 General Discussion

Cerebral ischemia involves a dynamic pathophysiology that comprises a multiplicity of processes, including excitotoxicity, disruption of Ca^{2+} homeostasis, ROS generation, cerebral inflammation, and neuronal apoptosis. A detailed understanding of such pathways offers immense opportunities to design novel treatment strategies for cerebral ischemia, a condition with few viable therapeutic options. Successful pharmacotherapy of cerebral ischemia requires that drugs achieve effective concentrations in the CNS, a therapeutic goal that is hindered by the BBB. It was originally believed that microvascular endothelial cells in the brain represented a static barrier to administered therapeutics and that drug delivery across the BBB largely depended on physiochemical properties of the drug (i.e., lipophilicity, charge, size) and passive diffusion. However, many lipophilic drugs have limited or no efficacy in treatment of neurological diseases due to a clear inability to cross the BBB. This observation emphasizes the involvement of ATP-binding cassette (ABC) drug efflux transporters, which are key determinants of the ability of a therapeutic agent to accumulate within the brain. Such efflux transporters are a formidable obstacle; however, recent studies have shown that small-molecule drug delivery can be facilitated by targeting endogenous uptake transporters (i.e., OATPs/Oatps) at the BBB. The hypothesis described and tested in this thesis hinges on the localization of Oatp1a4 on both sides of BBB endothelial cells. Indeed, Oatp1a4-mediated transcellular transport of atorvastatin is only possible through both luminal and abluminal localization of the bidirectional transporter Oatp1a4. Immunohistochemical and confocal microscopic analysis of rat brain microvessels has thoroughly been performed by several laboratories, visualizing the subcellular localization of Oatp1a4 to both the abluminal and luminal membrane

(Akanuma, Hirose, Tachikawa, & Hosoya, 2013; Gao, Stieger, Noe, Fritschy, & Meier, 1999; Sugiyama et al., 2003). Further existing evidence for Oatp1a4-mediated brain uptake of drugs at the *in vivo* blood-brain barrier is summarized in Figure 9.

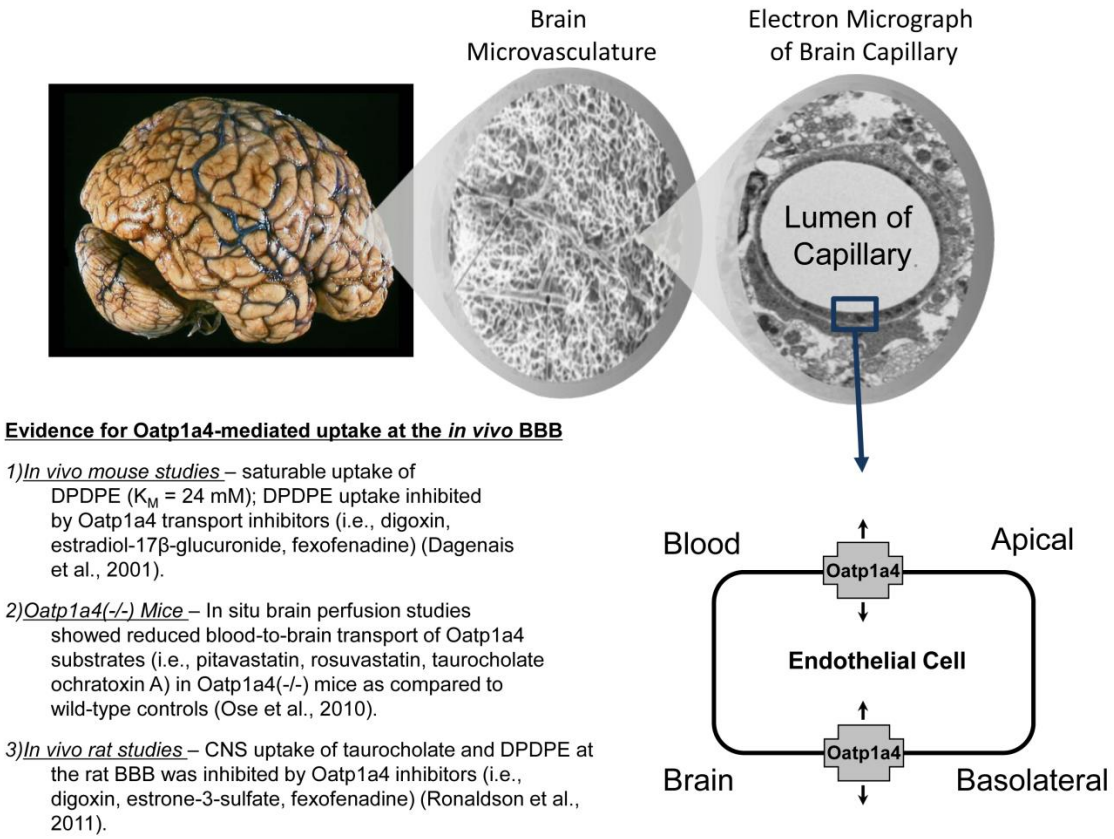

Figure 9: Evidence for Blood-to-Brain Drug Transport Mediated by Oatp1a4 at the Blood-Brain Barrier (BBB). Previous *in vivo* studies have shown that CNS uptake of drugs such as opioid peptide analgesics (i.e., DPDPE) and HMG-CoA reductase inhibitors (i.e., pitavastatin, rosuvastatin) is determined by functional expression of Oatp1a4 at the luminal and abluminal plasma membrane of the brain microvascular endothelium. Adapted from: Ronaldson & Davis (2013). Pharmacol Rev. 65:291-314.

Furthermore, our laboratory demonstrated that pharmacological inhibition of TGF-β signaling led to increased microvascular expression and activity of Oatp1a4 at the BBB (Ronaldson, Finch, et al., 2011; Thompson et al., 2014). Of particular interest was the observation that this blockade of TGF-β/ALK5 signaling using the specific ALK5 antagonist, SB431542, enhanced Oatp1a4 transport activity in saline-treated control animals as indicated by increased delivery to the brain of Oatp substrates such as taurocholate and atorvastatin (Ronaldson, Finch, et al., 2011; Thompson et al., 2014). Since TGF-β1 expression (i.e., the natural ligand for ALK5) is increased in the brain and in the periphery following cerebral hypoxia (Doyle, Cekanaviciute, Mamer, & Buckwalter, 2010), pharmacological blockade of TGF-β/ALK5 signaling may be critical in targeting Oatps for CNS drug delivery. A crucial consideration in interpretation of our data is the contribution of paracellular diffusion to brain uptake of Oatp substrate drugs. Our laboratory has previously reported that inhibition of TGF-β/ALK5 signaling with SB431542 increased paracellular BBB permeability for solutes such as sucrose by altering tight junction integrity (Ronaldson et al., 2009). The molecular weight of taurocholate (537.7 Da) and atorvastatin (558.6 Da) are greater than that of sucrose (342 Da), suggesting a lesser degree of paracellular diffusion. As our data showed no statistical difference in taurocholate or atorvastatin uptake in the presence of Oatp1a4 inhibitors in animals administered SB431542, we conclude that paracellular diffusion was not a significant factor in CNS uptake of taurocholate or atorvastatin. Nonetheless, it is critical to correct for paracellular transport in any study examining the effect of targeting TGF-β signaling for optimization of CNS drug delivery. Our work on TGF-β/ALK5 signaling highlights the potential of the TGF-β/ALK5 pathway as a

pharmacological target that can be used for optimization of drug delivery to the CNS, particularly for treatment of cerebral ischemia.

Regulation of membrane transport proteins, however, can occur by numerous mechanisms and may involve many complex signaling cascades and interactions. The notion that a transporter is only regulated by a single system, such as TGF-β signaling, is unlikely. Recognition of, and investigation into a variety of possible regulatory mechanisms is paramount to understanding transporter function. Post-translational modifications such as phosphorylation, ubiquitylation, and tyrosine nitration can occur rapidly and have drastic effects on protein activity and function. Protein phosphorylation is one of the most prevalent regulatory mechanisms in living organisms and controls limitless cellular processes, including protein activity (Newman, Zhang, & Zhu, 2014). For example, the renal Na^+-Cl^- cotransporter of the distal convoluted tubule can be activated by phosphorylation, such that increased phosphorylation of threonine/serine residues of the amino-terminal domain has been shown to increase the activity of the transporter (Gamba, 2012). Ubiquitylation has also emerged as a major player in the regulation of ion channels and transporters. Nedd4 ubiquitin ligases have been shown to negatively regulate Cl^- channels, Cl^-/H^+ antiporters, and voltage-gated cation (i.e., Na^+, K^+, Ca^{2+}) channels through channel ubiquitylation and endocytosis (Rotin & Staub, 2011). Tyrosine nitration has been shown to alter glutamate transporter activity as evidenced by a reduction in the maximum transport rate and cellular uptake of glutamate (Muscoli et al., 2013; Trotti et al., 1996). Nitrotyrosine is formed when reactive nitrogen species, such as peroxynitrite, attach an NO_2 group to the *ortho* position of tyrosine's aromatic ring (Radi, 2013). Given the pathophysiology of cerebral ischemia and the

prevalence of peroxynitrite, I believe that future studies of tyrosine nitration in the regulation of OATP/Oatp would be pertinent.

It is likely that each aforementioned type of protein modification influences transporter activity and that overall transporter regulation is a result of their relative activity levels as well as the interactions between these systems. The structure, amino acid sequence, and other biophysical parameters of a transporter can give clues into the possible involvement of certain regulatory systems. For example, the presence of nucleotide binding domains on an ion transporter would invite the possibility of regulation by pyridine nucleotides (Kilfoil, Tipparaju, Barski, & Bhatnagar, 2013). Concerning OATP/Oatp, it is clear that a great deal of work and investigation is necessary to further illuminate its regulation and mechanism of transport.

The ability of a pharmacological agent to cross the BBB endothelium and achieve efficacious concentrations within the CNS is dependent on multiple mechanisms of transport. Such mechanisms include uptake into the brain via an influx transporter and/or extrusion from the CNS mediated by an efflux transporter. For many drugs, it is this discrete balance between influx and efflux that determines whether a pharmacological agent will accumulate within the brain extracellular milieu and, therefore, elicit a therapeutic effect. The complexity of drug transporter biology at the BBB is further underscored by the observation that functional expression of such transport proteins may be dramatically altered by pathophysiological stressors (Hayashi et al., 2006; Ronaldson, Finch, et al., 2011; Seelbach, Brooks, Egleton, & Davis, 2007; Yeh et al., 2008). A thorough understanding of regulation and functional expression of endogenous BBB transporters in both health and disease is critical for optimization of pharmacotherapy.

Furthermore, such information will enable more effective targeting of transporters and/or transporter regulatory mechanisms, thus allowing endogenous BBB transport systems to be specifically exploited for improvement of CNS drug delivery.

OATP/Oatp family members are multispecific transporters capable of transporting a vast array of structurally diverse drugs, metabolites, and physiologic substrates. However, a full comprehension of how such transporters can be targeted to promote CNS delivery of therapeutics requires an appreciation that substrates transported by OATP/Oatp family members may also be transport substrates for organic anion transporters (OATs), P-gp, MRP/Mrp isoforms, and breast cancer resistance protein (BCRP in humans: Bcrp in rodents). At the BBB, organic anion transporter 3 (OAT3) (Miyajima, Kusuhara, Fujishima, Adachi, & Sugiyama, 2011; Ohtsuki et al., 2005), P-gp (Bendayan, Ronaldson, Gingras, & Bendayan, 2006; Hawkins, Sykes, & Miller, 2010; Seelbach et al., 2007), and several MRP/Mrp isoforms (Dallas, Miller, & Bendayan, 2006; Hawkins, Ocheltree, Norwood, & Egleton, 2007) are expressed and contribute to brain-to-blood (i.e., efflux) substrate transport. Many drug substrates of OATP/Oatp substrates are also transported by at least one additional transporter such as OAT1, OAT3, P-gp, MRP/Mrp isoforms, or BCRP/Bcrp. As previously described, atorvastatin is a substrate of Oatp1a4 and may also be a P-gp substrate. Additionally, DPDPE is also a substrate for both P-gp and Oatp1a4 (Ose et al., 2010; Ronaldson, Finch, et al., 2011). Rosuvastatin is a substrate for OAT3 (Windass, Lowes, Wang, & Brown, 2007), Bcrp (Huang, Wang, & Grimm, 2006), and Mrp2 (Abe, Bridges, Yue, & Brouwer, 2008). Therefore, it is highly possible that drugs that enter the brain microvascular endothelium or choroid plexus epithelium via one class of transporter may exit by another.

Understanding how changes in expression of a specific transporter might affect brain uptake of a given drug will depend upon a thorough assessment of all competing transporters.

Previous research has attempted to overcome drug efflux transport by pharmacological targeting of transporters such as P-gp. P-gp is a major obstacle to CNS delivery of therapeutics, having almost an inexhaustible substrate profile of small, lipophilic drugs and a strong presence at the BBB. Reducing P-gp activity to allow enhanced passage of therapeutics across the BBB is an attractive prospect. However, translation of direct P-gp inhibition from animal models to the clinic has been unsuccessful, due mostly to systemic toxicity of the P-gp inhibitors themselves (Cannon, Peart, Hawkins, Campos, & Miller, 2012). As an alternative, identification and targeting of molecular signaling pathways that control basal P-gp activity have recently been done by Miller et al. Sphingosine-1-phosphate (S1P), a bioactive lipid metabolite, acting through its receptor (S1PR1) was shown to rapidly and reversibly reduce P-gp transport activity in rats, thereby enhancing brain uptake of drugs such as verapamil, loperamide and paclitaxel (Cannon et al., 2012). S1P signaling was found to be a downstream link to TNFα signaling through TNFR1, endothelin, iNOS and PKCβ1, a pathway previously elucidated by Cannon and colleagues (Cannon et al., 2012). Speculation as to the mechanism of this P-gp manipulation includes covalent modification or changes to the microenvironment of the transporter. Further investigation into such a signaling pathway may lead to control of BBB efflux transport without sacrificing the neuroprotection afforded by P-gp. Theoretically, such control of P-gp-mediated transport at the molecular

level may enhance the ability of influx transporters (i.e., OATPs/Oatps) to optimally deliver drugs to the brain.

3.2 Future Directions

The success of statins in mitigating cerebral damage in both experimental stroke models and human clinical trials might be attributed to their antioxidant properties. This notion begs the question if less expensive and simpler antioxidants (i.e., vitamin E) could substitute statin therapy and achieve the same results. However, large, randomized clinical trials using vitamin E antioxidant therapies have demonstrated that vitamin E does not confer any beneficial effect in reducing cardiovascular risk in humans (Debreceni & Debreceni, 2012). Moreover, the primary purpose in prescribing statins is to lower blood cholesterol levels, which may provide the greatest benefit in reducing the occurrence of a stroke through the stabilization and/or regression of arterial plaque (Welch, 2004). Indeed, it has been well-documented by several landmark clinical trials that statins reduce the risk of stroke as a means of primary prevention in patients with existing coronary heart disease ("Prevention of cardiovascular events and death with pravastatin in patients with coronary heart disease and a broad range of initial cholesterol levels. The Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study Group," 1998; "Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S)," 1994; Sacks et al., 1996). The recent Stroke Prevention by Aggressive Reduction in Cholesterol Levels (SPARCL) study concluded that atorvastatin was effective in preventing strokes and other cardiovascular events in patients who suffered a stroke or transient ischemic attack and had no diagnosis of coronary heart disease (Amarenco et al., 2009). Therefore, the

added ‘pleiotropic’ antioxidant benefit of statins further justifies their use in both primary and secondary stroke prevention and therefore outweighs other potential antioxidants for stroke treatment.

Furthermore, statins are among the most commonly prescribed drugs in the United States. The IMS Institute for Healthcare Informatics reported rosuvastatin (brand name Crestor®) as the 2nd most prescribed and the 4th highest grossing prescription drug in the United States, totaling 22.9 million prescriptions and $5.5 billion in sales between April 2013 and March 2014 (Brooks, 2014). Given the prevalence and current widespread use, the likelihood that a patient admitted to a hospital for stroke is already taking a statin is relatively high, depending on the patient profile. This is advantageous to the patient if statins were to be given during stroke hospitalization, as it would warrant smaller, less frequent doses, if indeed necessary, for mitigation of neural damage.

Clinical evidence suggests that statin treatment before the occurrence of a stroke improves post stroke survival (Flint et al., 2012). However, little evidence exists as to the potential of statins to improve cognitive outcomes if given after an ischemic event. Therefore, as the immediate future direction for this work, I would plan to repeat the previously described atorvastatin experiments with the difference of administering the drug after the hypoxic insult. The examination of additional biomarkers not only of apoptosis, but also of redox status as well as behavior tests before and after the event would also be meaningful in these studies. Clinically, the ideal timing of statin administration for acute ischemic stroke treatment remains unclear. Studies have shown that patients who already had an active prescription for a statin when admitted to the hospital for stroke experienced improved post stroke outcomes compared to patients not

taking statins (Flint et al., 2012; Marti-Fabregas et al., 2004). Recent observational studies found that statin therapy initiated during acute hospitalization for stroke improved post stroke survival (Elkind, Flint, Sciacca, & Sacco, 2005; Flint et al., 2012). High doses (≥60 mg/day) of statins had an even greater rate of post stroke survival than medium doses (<60 mg/day) (Flint et al., 2012). Although these referenced studies were small, retrospective, and non-randomized, the outlook for statin use in the treatment of ischemic stroke looks promising.

The ability of statins to minimize hypoxia/reoxygenation damage in the brain is dependent efficient delivery across the BBB and sufficient accumulation within brain parenchyma. Consequently, development of new drug delivery technologies is an area of intense research and scientific interest. Additional future work into alternative and more efficient delivery systems may maximize the effectiveness of statin-induced neuroprotection. I recommend the exploration and utilization of nanotechnology-based delivery vehicles (i.e., nanoparticles) for this purpose. Common flaws that plague conventional drug treatments include problems with accurate dosing, rapid drug metabolism or degradation, and unwanted distribution profiles. Nanoparticle delivery vehicles have emerged as a promising solution to such drug delivery issues. Nanocarriers can give new hope to existing therapeutics that suffer from inefficient delivery or problems with drug-tissue distribution. For example, cytidine 5' diphosphocholine, found to be neuroprotective against cerebral ischemia/reperfusion, is rapidly metabolized by the liver, rendering it incapable of reaching the brain if administered via the systemic circulation (S. Ghosh, Das, Mandal, Dungdung, & Sarkar, 2010). Encapsulation of cytidine 5' diphosphocholine into nanoparticle liposomes has been shown to reduce

hepatic hydrolysis and enabled this drug to attain therapeutic concentrations in brain parenchyma (S. Ghosh et al., 2010; Pinzon-Daza et al., 2013).

The term “nanoparticle” is used broadly to describe various nanosystems including liposomes, polymeric particles, hydrogels, micelles, inorganic/solid particles, dendrimers, nanotubes, and quantum dots (Marrache et al., 2013; Singh & Lillard, 2009). Therapeutic agents are typically encapsulated, entrapped, adsorbed or chemically attached to the nanoparticle surface (Denora, Trapani, Laquintana, Lopedota, & Trapani, 2009). The most widely used and successful nanoparticle systems for delivery of bioactive compounds have been liposomes and polymeric nanoparticles or polymer-drug conjugates (Marrache et al., 2013; Singh et al., 2009). The composition of biodegradable polymeric nanoparticles along with their degradation to biocompatible components can be seen in Figure 10. Nanoparticles composed of biodegradable polymers such as poly(lactic acid-co-glycolic acid) (PLGA) are perhaps the most accepted and widely used nanoparticle material. PLGA and related polymers PGA and PLA have been approved by the FDA as biocompatible and biodegradable drug delivery systems (Marrache et al., 2013; Singhal et al., 2013). In order for nanoparticles to be therapeutically effective, they must exhibit i) prolonged circulation in the bloodstream (i.e., not immediately filtered or metabolized), ii) specificity for adequate accumulation in a target tissue, iii) selective cellular uptake by target endothelial cells, and iv) controlled release of medicinal drugs. Nanoparticles are generally defined as ranging from 10 to 1000 nm in diameter. Typically, nanoparticles >200 nm are not commonly synthesized because smaller nanoparticles are more readily taken up into cells and/or tissues (Singh et al., 2009). In comparison with microparticles of 1 μm in size, 100 nm particles showed a 2.5 fold

greater cellular uptake in Caco-2 cells (Desai, Labhasetwar, Walter, Levy, & Amidon, 1997). Due to their small size and mobility, nanoparticles are able to access a wide variety of "druggable" targets, both extracellular and intracellular. As the smallest capillaries in the body have a diameter of approximately 5-6 µm (Hans & Lowman, 2002), administration of nanoparticles through the microcirculation is a viable approach for facilitation of CNS drug delivery.

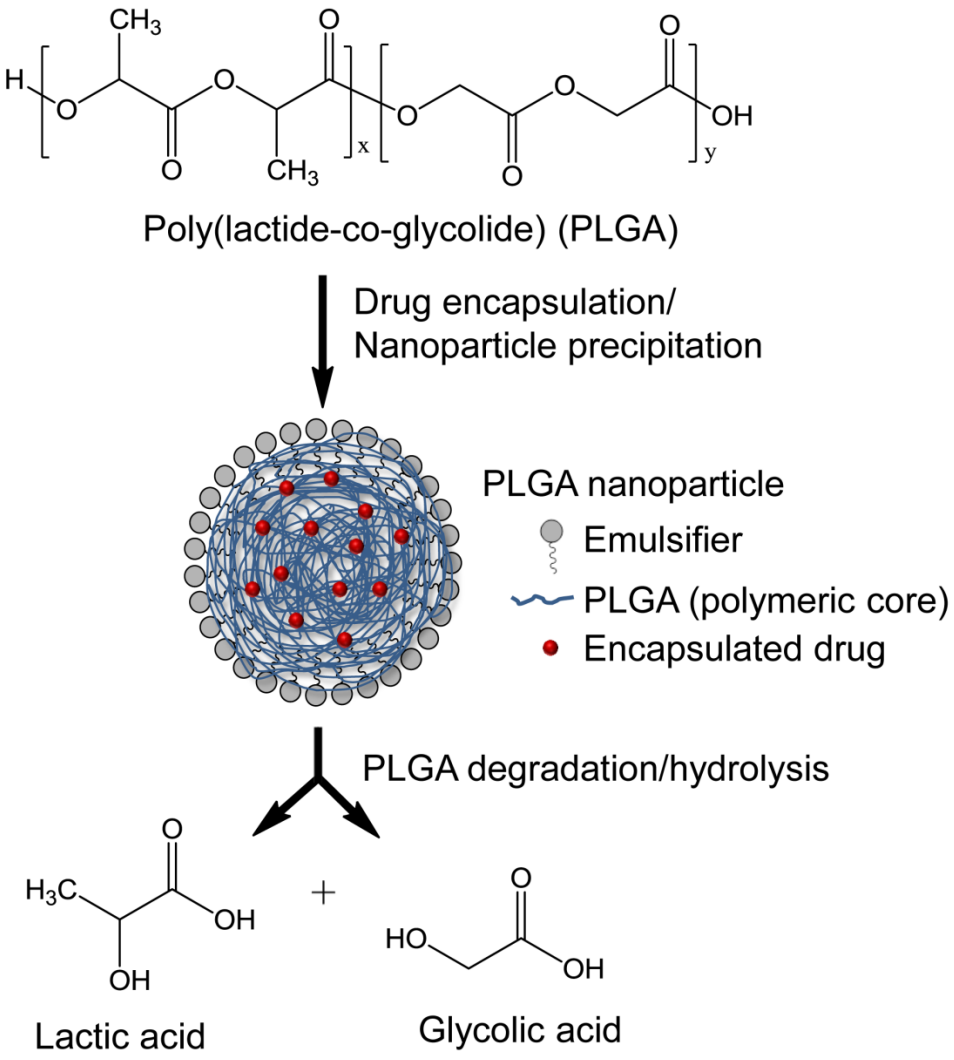

Figure 10: Schematic Representation of the Synthesis and Degradation of Poly(lactide-co-glycolide) (PLGA) Nanoparticles.

Nanoparticle size, solubility, lipophilicity, and surface charge are all critical parameters to consider for efficient CNS drug delivery. Nanoparticle surface modifications are necessary to increase biocompatibility and usually involve a coating or specific attachment of hydrophilic or amphiphilic polymers/surfactants (Pinzon-Daza et al., 2013; Singh et al., 2009). Without such surface modifications, nanoparticles introduced into systemic circulation are quickly cleared from the blood by the reticuloendothelial system (RES) at organs such as the liver, spleen, and lungs. Additionally, blood components (i.e., opsonins) bind to and mark the particle for destruction by phagocytic cells via a process known as opsonization. The degree of opsonization *in vivo* has been shown to correlate with the hydrophobicity of the nanoparticle (Singh et al., 2009). For nanoparticles to be used effectively to deliver therapeutics to the CNS, they must be retained in the systemic circulation long enough to distribute to the desired target. Polysorbate, polyethylene glycol (PEG), polyethylene oxide (PEO), poloxamine, poloxamer, and pluronic polymers are commonly used to coat the surface of nanoparticles for this purpose (Pinzon-Daza et al., 2013; Singh et al., 2009). PEGylation of drug-loaded liposomes has been particularly effective in enhancing the longevity of the particles in systemic circulation. The chemical nature of PEG allows liposomes to circulate in the bloodstream longer, avoiding collection by the reticuloendothelial system (RES) in the liver and spleen (Hans et al., 2002; Pinzon-Daza et al., 2013; Xie et al., 2012).

In addition to increasing the half-life of the encapsulated drug, nanoparticle surface coatings and covalent modifications also present an opportunity to enhance specificity, allowing nanoparticles to cross biological membranes, such as the BBB

endothelium. For many nanoparticle delivery systems, polyethylene glycol (PEG) is used for this purpose because it has minimal effects on drug-matrix interactions (Singh et al., 2009). PEG is often used as a linker, providing the chemical moieties or functional groups necessary for conjugation. Once incorporated onto the surface of the nanoparticle, the PEG "arms" of a specified length could then be conjugated with antibodies, peptide sequences, or ligands for targeting specific tissues. The adaptability of nanoparticle drug delivery systems suggests that specific transcellular transport routes can be targeted (i.e., adsorptive-mediated transcytosis, receptor-mediated transcytosis, carrier-mediated transcytosis) for CNS drug delivery (Alyautdin, Khalin, Nafeeza, Haron, & Kuznetsov, 2014). Paracellular diffusion of nanoparticles has also been achieved after administration of hyperosmotic mannitol (Avgoustakis et al., 2002), which causes "shrinkage" of microvascular endothelial cells, thereby mechanically stretching tight junction protein complexes (Denora et al., 2009; Ikeda, Bhattacharjee, Kondoh, Nagashima, & Tamaki, 2002). However, such a non-selective increase in paracellular diffusion also allows potentially neurotoxic blood-borne substances to accumulate in the brain. A more efficient approach is targeting of receptor-mediated endocytosis for delivery of drug loaded nanoparticles through the BBB (Pinzon-Daza et al., 2013). Insulin, transferrin, lactoferrin, glutathione, peptides and apolipoproteins are among the ligands successfully utilized to achieve receptor-mediated transcytosis of nanoparticles and drug delivery to the CNS (Alyautdin et al., 2014; Pinzon-Daza et al., 2013).

The biopolymer polysorbate has been especially successful in delivering therapeutics to brain parenchyma. Polysorbate coated nanoparticles are thought to permeate the BBB through receptor-mediated endocytosis by binding to low density

lipoprotein (LDL) receptors (Hans et al., 2002). Apolipoprotein E (ApoE), found in blood plasma, has been shown to adsorb on the surface of the polysorbate 20, 40, 60, or 80-coated nanoparticles and likely mimics LDL, allowing receptor binding (Kreuter, 2001). Polysorbate 80 in particular has been shown to successfully cross the BBB as demonstrated by Kreuter et al. using polybutylcyanoacrylate (PBCA) nanoparticles (Beletsi, Leontiadis, Klepetsanis, Ithakissios, & Avgoustakis, 1999; Kreuter et al., 2003; Ramge et al., 2000). PLGA-b-PEG nanoparticles loaded with atorvastatin and coated in polysorbate 80 have been characterized and shown to accumulate in the rat brain *in vivo* (Simsek, Eroglu, Kurum, & Ulubayram, 2013). Similar to polysorbate-coated particles, poly(methoxy-PEG-cyanoacrylate-co-hexadecyl cyanoacrylate) (PEG-PHDCA) nanoparticles incubated in rat serum also adsorbed ApoE and ApoB100 on their surface and were delivered across the BBB into the CNS with greater success than apolipoprotein-lacking nanoparticles (H. R. Kim et al., 2007). However, dependence on whole protein adsorption on nanoparticle surfaces may not be the most efficient method for delivery of therapeutics across the BBB. Only a fraction of the nanoparticles will have adsorbed whole protein in the correct conformation to bind to and initiate endocytosis. Additionally, maximal efficacy of receptor-mediated endocytosis has been observed when smaller, targeting peptides for a specific endogenous receptor, such as transferrin, were employed in place of the whole protein as shown by Prades et al. using gold nanoparticles (Prades et al., 2012).

Specific endogenous BBB transporters have also been targeted as means of nanoparticle drug release into the CNS. For example, Xie and colleagues synthesized liposomes modified by PEGs of varying lengths, covalently linking cholesterol with

glucose. The glucose molecule was then recognized by the GLUT1 transporter in BBB endothelial cells and the liposomes were successful in delivering their drug load into the CNS. The length of the PEG chain was also found to affect BBB crossing as PEG-modified liposomes with a relative long chain length (i.e., PEG1000) maximized drug transport across the BBB barrier and subsequent uptake into brain parenchyma. However, the PEG linker between the ligand (i.e., glucose molecule for GLUT1) and liposome can be too long (i.e., PEG>2000) and may result in self-folding and insufficient exposure of the covalently attached ligand (Xie et al., 2012).

In summary, drug-loaded nanoparticles represent a highly adaptable delivery system that offers significant advantages in drug stability and controlled release to the CNS. A better understanding of mechanisms that regulate endocytosis-mediated uptake at the BBB will allow greater control of drug permeation and/or transport across the BBB via nanoparticles. Methods of xenobiotic transport across the BBB are summarized in Figure 11. Perhaps such drug delivery approaches will prove "paradigm-shifting" for treatment of cerebral ischemia by enabling precise delivery of therapeutics with neuroprotective properties such as statins. Future work will continue to provide more insight on therapeutic targeting of the BBB via endogenous uptake transport systems and drug-loaded nanoparticles. Ultimately, data derived from these studies will allow achievement of more precise and more effective drug concentrations within the brain, thereby improving treatment of cerebral ischemia.

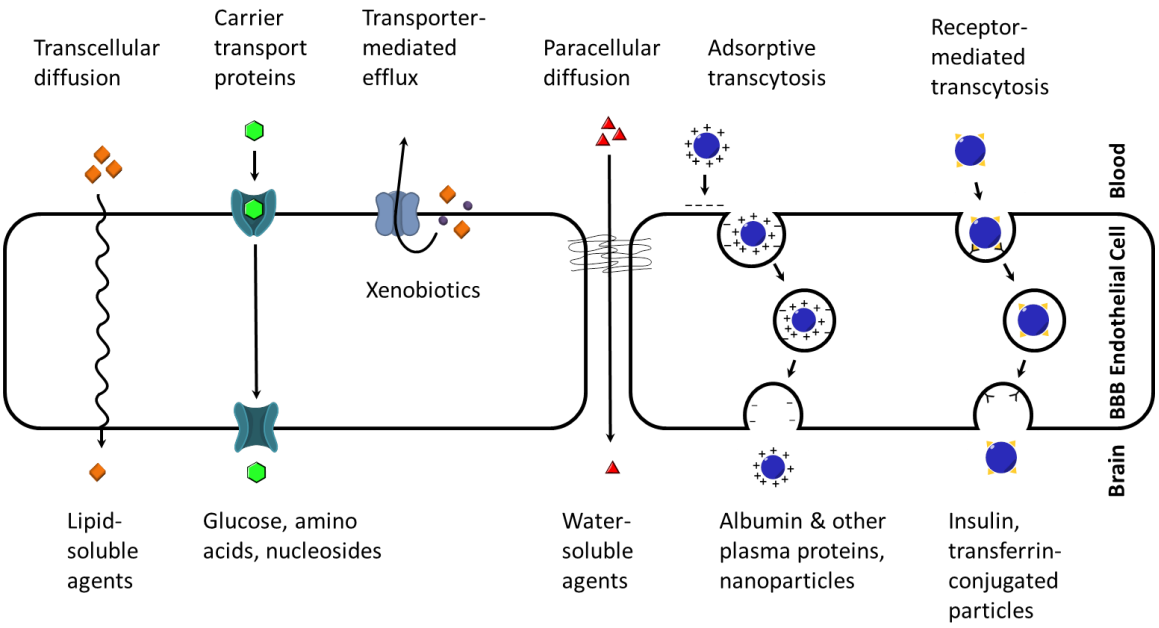

Figure 11: Methods of Drug Transport Across the BBB.

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