Abstract - INTERMITTENT HYPOXIA TRAINING TO FOSTER BRAIN RECOVERY AFTER ISCHEMIC STROKE IN RATS

Purpose: Ischemic stroke is the leading cause of disability and #5 cause of death in the US. Annually, nearly 800,000 Americans suffer an ischemic stroke, and 130,000 die. The only FDA approved treatment for stroke is recombinant tissue plasminogen activator, but this thrombolytic agent neither protects the affected tissue, nor mitigates the motor or cognitive impairments resulting from stroke.

Intermittent hypoxia training (IHT) has been shown to increase cerebral blood flow, reduce oxidative stress, mobilize cerebroprotective signaling cascades and minimize behavioral deficits in a rat model of Alzheimer's Disease. Moreover, a 20 d IHT program attenuated behavioral deficits and protected neurons in ethanol-withdrawn (EW) rats, even when EW began 35 d after IHT. Therefore, we hypothesize that IHT, initiated in rats after stroke, preserves motor and cognitive function, relative to non-IHT rats.

Methods: Ischemic stroke will be produced in rats by 90 min occlusion and abrupt reperfusion of the middle cerebral artery (MCA). Motor function and coordination will be evaluated by the rotarod test before and at 1 week intervals after MCA occlusion (MCAO). Rats must balance on a rotating cylinder that accelerates at a constant speed. High fall latency represents intact motor function. The Morris Water Maze (MWM) assesses spatial learning and memory. Rats are placed in an open, circular pool and must find a sunken platform within 90 s. 24 h after stroke, rats undergoing IHT will breathe moderately hypoxic gas (10% O_2) for 5-8 cycles, each lasting 5-10 min, with intervening 4 min room air breathing, for 20 consecutive days. These rats will be compared to an MCAO group continuously exposed to 21% O_2. At 21 d post-stroke, the brain will be harvested for analyses of infarct and neuroprotective proteins.

Results: In pre-stroke testing, the time taken to solve the MWM fell progressively over 10 days, indicating spatial learning and memory, and fall latency on the rotarod lengthened over 5 days, reflecting improved coordination and possibly a training effect. These studies have established the pre-stroke baselines for assessment of IHT's impact on post-stroke recovery.

Conclusions: We expect that IHT given after stroke will minimize motor and cognitive impairment by activating neuroprotective signaling cascades culminating in expression of anti-oxidant and anti-inflammatory proteins.

SIGNATURE PAGE

INTERMITTENT HYPOXIA TRAINING TO FOSTER BRAIN RECOVERY AFTER ISCHEMIC STROKE IN RATS

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TITLE PAGE

INTERMITTENT HYPOXIA TRAINING TO FOSTER BRAIN RECOVERY AFTER ISCHEMIC STROKE IN RATS

INTERNSHIP PRACTICUM REPORT

Presented to the Graduate Council of the Graduate School of Biomedical Sciences University of North Texas Health Science Center at Fort Worth in Partial Fulfillment of the Requirements

For the Degree of

Master of Science

By

Steven Shea Ruelas, B.A.,
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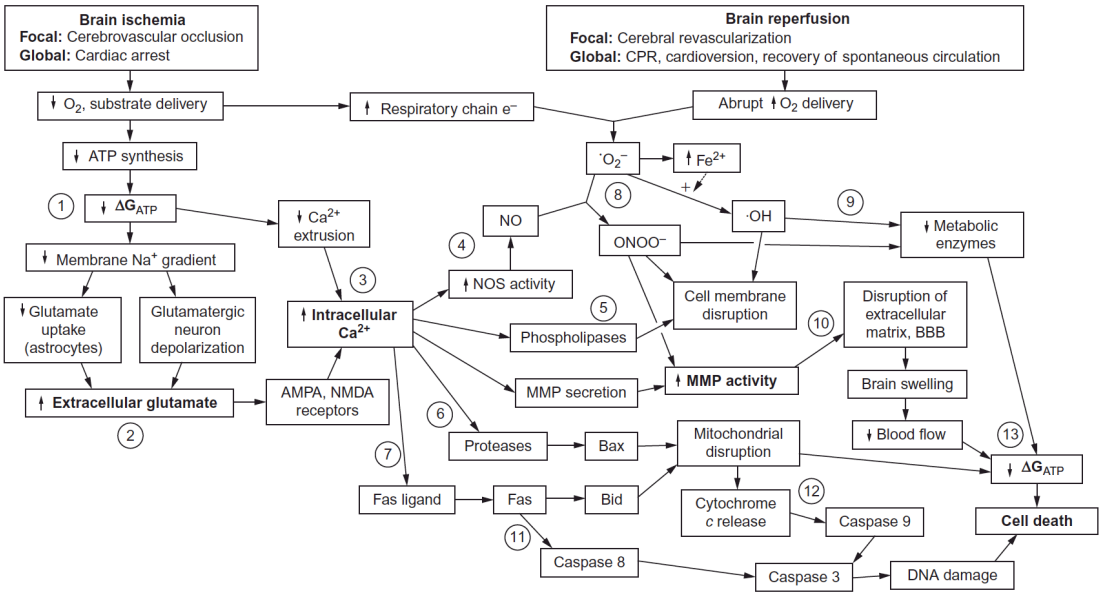


(Mallet and Ryou, 2017)

Figure 1 Middle cerebral artery occlusion (MCAO) initiates the ischemia-reperfusion-induced cascade of brain injury. Ischemia initiates a cascade of mechanisms that culminate in death of the brain parenchyma, irreversible brain damage and persistent neurocognitive, and motor impairment. Interruption of blood flow causes an abrupt decline in Gibbs-free energy of ATP hydrolysis, ΔG_{ATP} (1), which provokes glutamate secretion from depolarized excitatory neurons (2). Energy depletion and glutamate stimulation impair cellular Ca^{2+} homeostasis, thereby increasing intracellular Ca^{2+} (3). Ca^{2+}, in turn, activates nitric oxide synthase to overproduce NO (4); phospholipases that degrade membrane phospholipids (5); proteases (6) that degrade cellular proteins and activate proapoptotic Bax; and secretion of Fas ligand, which initiates the extrinsic apoptosis cascade (7). Reperfusion reintroduces O_2 to the electron (e^-)-rich environment of the ischemic cells, triggering formation of superoxide (8), a precursor of hydroxyl radical and, via biradical condensation with NO, $ONOO^-$. These compounds inactivate energy-generating and antioxidant enzymes (9) and activate MMPs (10) which degrade the extracellular matrix and blood-brain barrier (BBB), causing inflammation, edema, brain swelling, and decreased cerebral blood flow. The extrinsic (11) and intrinsic (12) apoptotic cascades activate caspase 3 to degrade cellular components including DNA. This damage, and the secondary energy depletion due to underperfusion of the edematous tissue and enzyme inactivation (13), conspires to kill neurons and other cells.

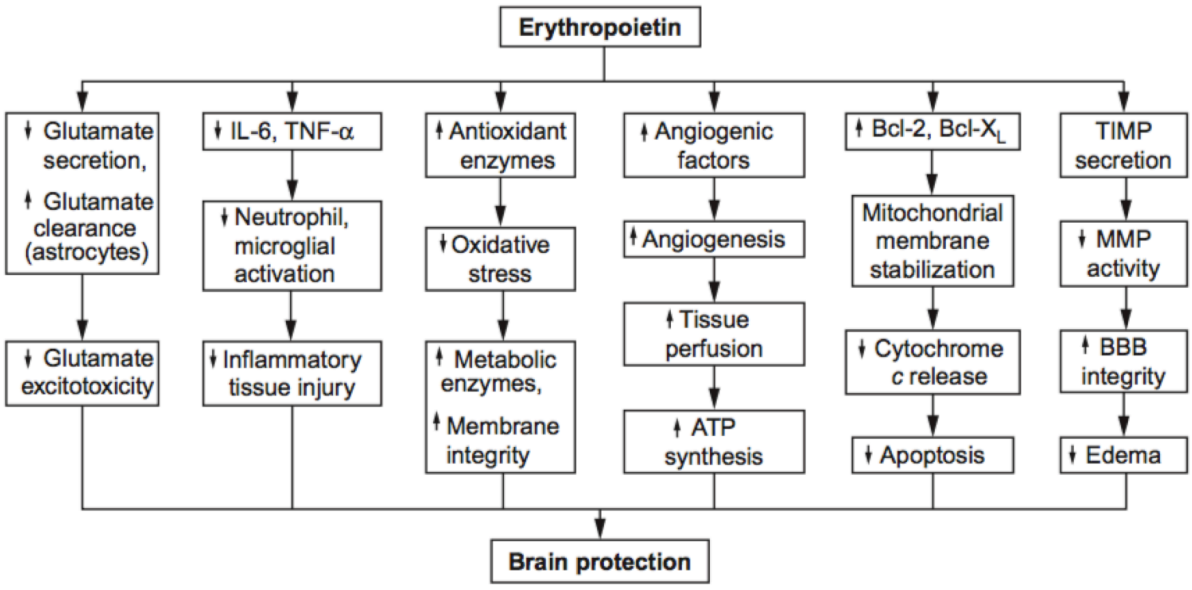
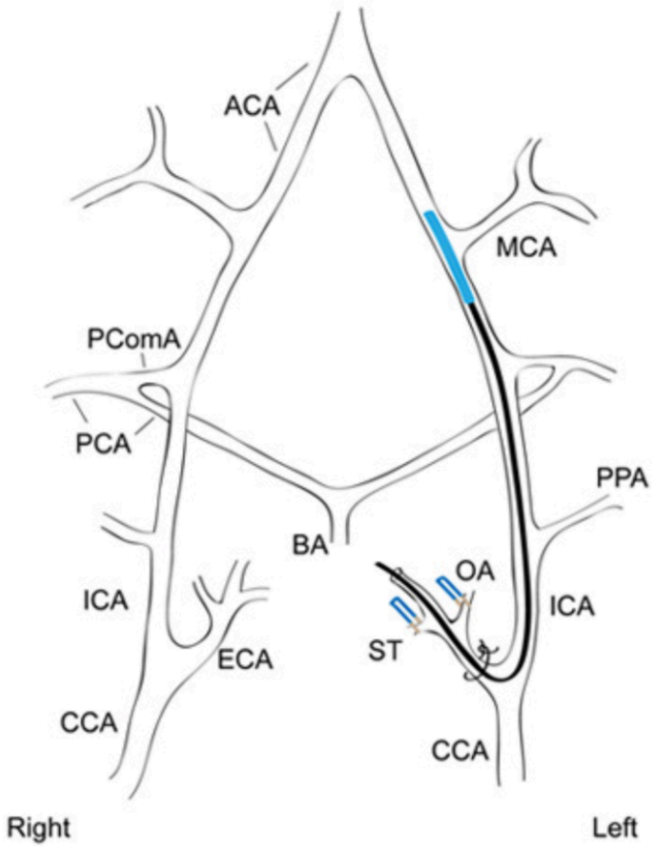


Figure 2 Erythropoietin's protective mechanisms in ischemic and postischemic brain. Erythropoietin activates several complementary mechanisms which collectively increase the brain parenchyma's resistance to ischemia-reperfusion. We expect that intermittent hypoxia training (IHT) will increase erythropoietin's activities and consequently lower pro-inflammatory cytokines and metalloproteinase activity and increase contents of antioxidant enzymes and angiogenic factors, as well as Bcl-2 and Bcl-X_L, thereby decreasing the amount of apoptosis occurring in the infarct.

(Mallet & Ryou, 2017)



igure 3 Occlusion of the middle cerebral artery by endovascular suture. A simplified diagram of the cranial circulatory system of the rat is hown with a silicon-coated intraluminal suture occluding the origin of the MCA. The OA and ST branches off of the left ECA have been ligated nd a suture tie around the ECA stump holds the intraluminal suture in place. ACA, anterior cerebral artery; BA, basilar artery; CCA, common arotid artery; ECA, external carotid artery; ICA, internal carotid artery; MCA, middle cerebral artery; OA, occipital artery; PCA, posterior cerebral rtery; PComA, posterior communicating artery; PPA, pterygopalatine artery; ST, superior thyroid artery. Figure adapted from Sasaki *et al.*[4] and ee[3].

(Uluc et al, 2011)

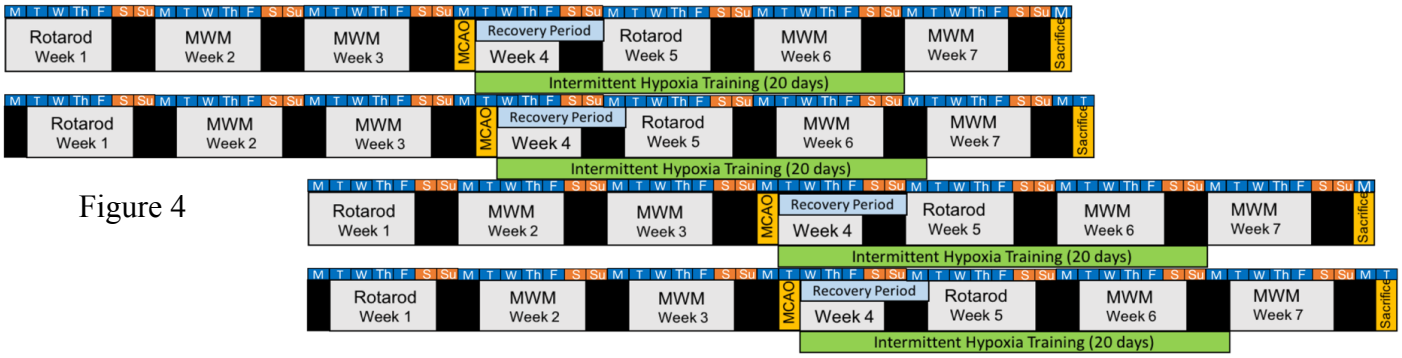
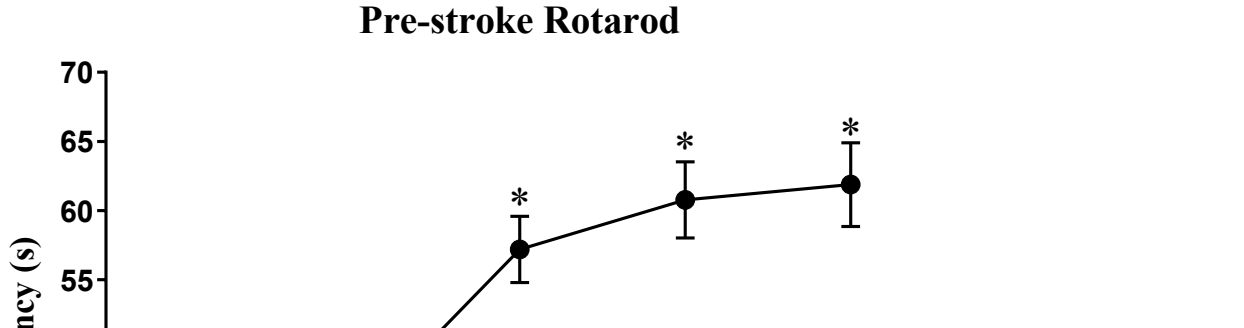
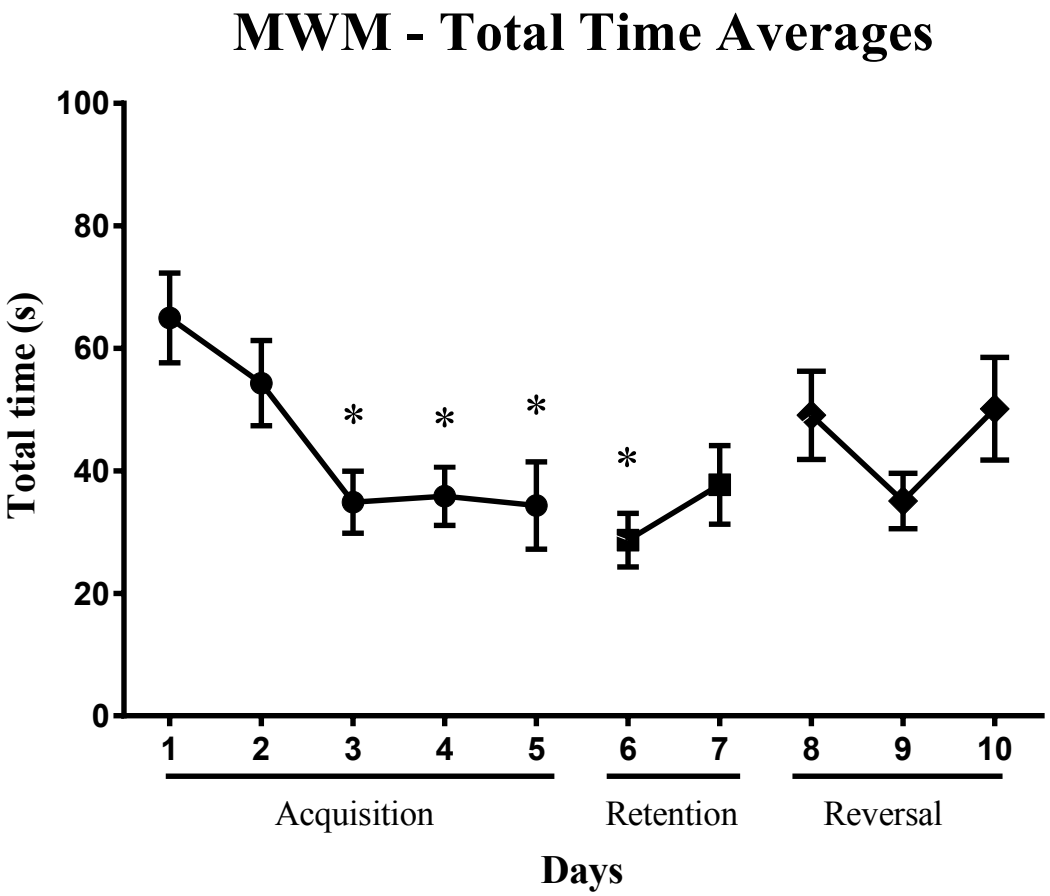


Figure 4



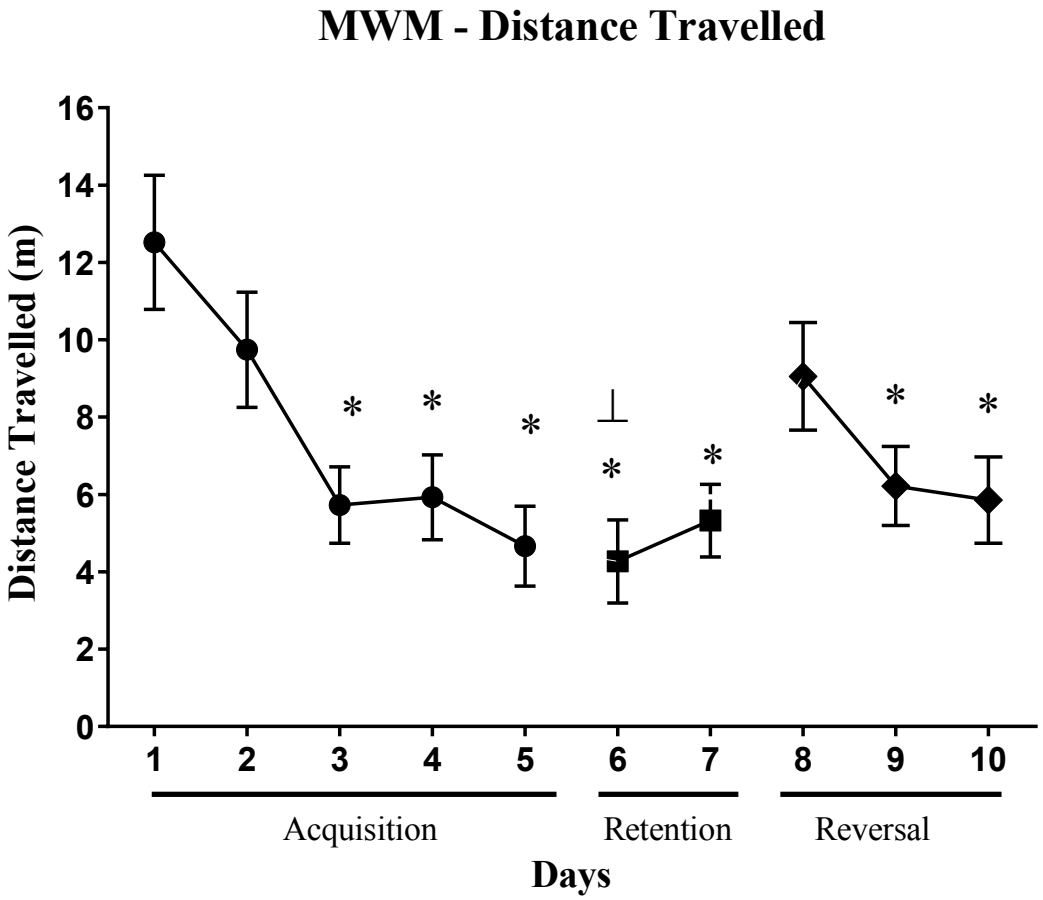
Fall latency (s) during pre-stroke Rotarod testing. Values represent mean ± SEM, n = 11. One-way ANOVA; *P<0.05 vs. days 1 and 2; *post-hoc* Tukey's test for multiple comparisons.

Figure 5



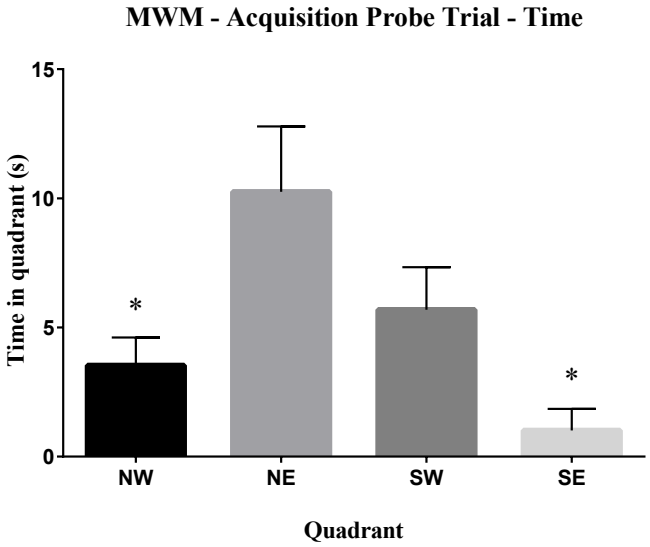
Time latency to finding the platform during the 10 day water maze protocol. Between the Acquisition and Retention stage rats had a 2 day break. The platform remained in the SW quadrant during Acquisition and Retention, but was moved to the NE quadrant during the Reversal stage. In this and subsequent figures, values are mean ± SEM, n = 11. Statistical analysis: One-way ANOVA; *Post-hoc* Tukey's test for multiple comparisons. *P<0.05 vs. day 1.

Figure 6



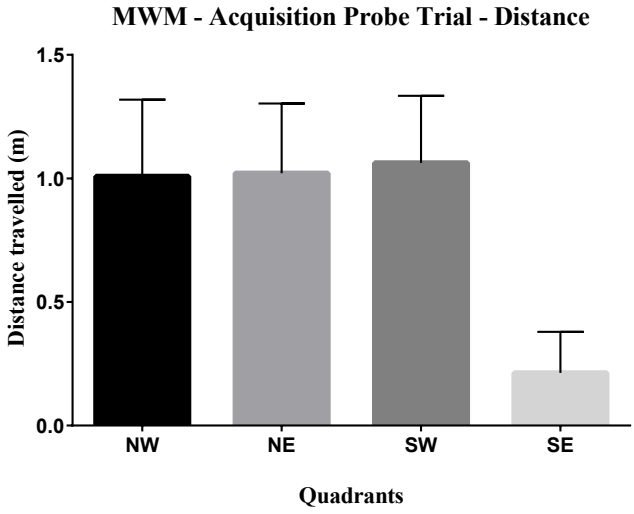
Distance travelled before finding the platform during the 10 day water maze protocol. Between the Acquisition and Retention stage rats had a 2 day break. The platform remained in the SW quadrant during Acquisition and Retention, but was moved to the NE quadrant during the Reversal stage. *P<0.05 vs. day 1; ⊥P<0.05 vs. day 2.

Figure 7



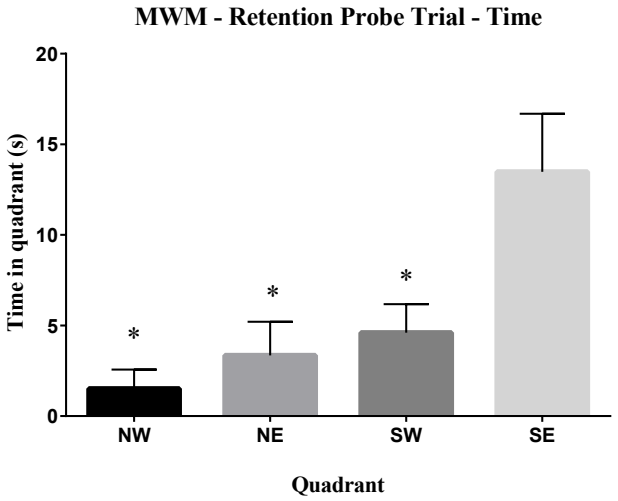
Time spent in each quadrant during Acquisition Probe Trial. Platform zone located in SW quadrant.
*P<0.05 vs. NE quadrant.

Figure 8



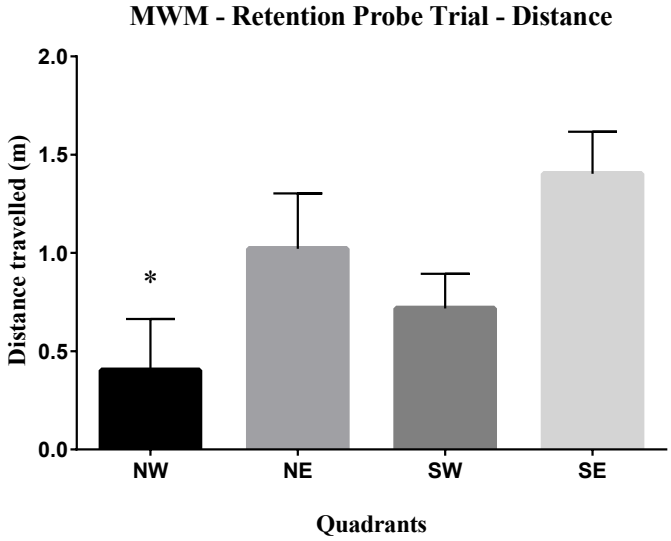
Distance travelled in each quadrant during Acquisition Probe Trial. Platform zone located in SW quadrant. No statistical significance differences among the quadrants were detected.

Figure 9



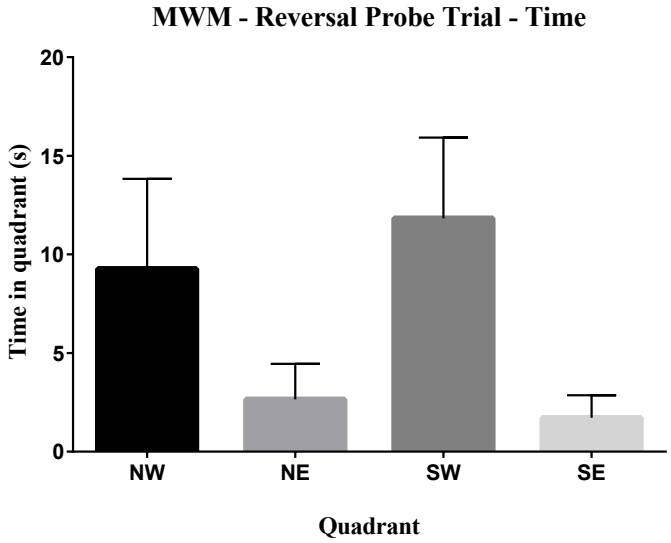
Time spent in each quadrant during Retention Probe Trial. Platform zone located in SW quadrant. Bars indicated SEM. *P<0.05 vs. SE quadrant.

Figure 10



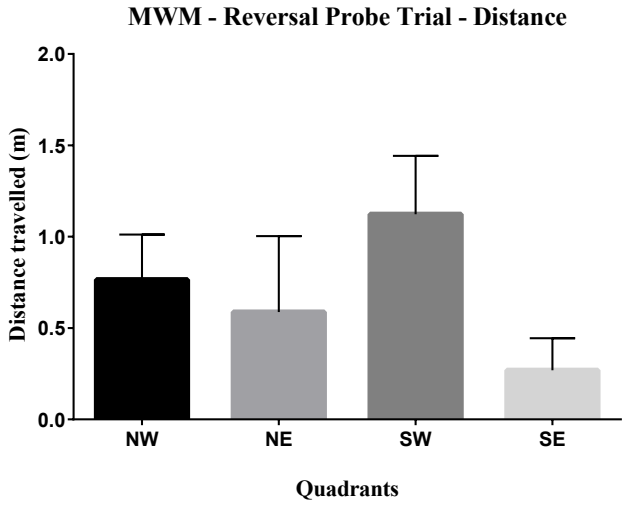
Distance travelled in each quadrant during Retention Probe Trial. Platform zone located in SW quadrant. *P<0.05 vs. SE quadrant.

Figure 11



Time spent in each quadrant during Reversal Probe Trial. Platform zone located in NE quadrant. No statistical significance differences among the quadrants were detected.

Figure 12



Distance travelled in each quadrant during Reversal Probe Trial. Platform zone located in NE quadrant. No statistical significance differences among the quadrants were detected.

Figure 13

Chapter 1: Background and Literature

Prevalence, pathogenesis, and treatment of stroke

In the United States, 795,000 people suffer strokes annually, 87% of which are a direct result of cerebral artery occlusion (Mozaffarian et al, 2016). The fifth-leading cause of death in the U.S., stroke claims 130,000 lives annually. The major causes of acute stroke are large-artery atherosclerosis, small-artery occlusion, and cardioembolic infarction, with small-artery occlusion being the most common type and cardioembolic infarction accounting for the highest mortality rate (Yip P, et al 1997). Stroke is also the leading cause of disability in adults in the U.S. and worldwide (Ovbiagele et al, 2011). Of the 7 million Americans who have suffered a stroke (Roger et al, 2012), 75% experience neurocognitive impairment that significantly impacts their quality of life (Go et al, 2013). These deficits manifest as cerebral cortical impairment, such as aphasia, neglect, apraxia, anopia and restricted motor function, as well as brainstem or cerebellar dysfunction (Yip P, et al 1997).

Risk factors for stroke include hypertension, atrial fibrillation (AF), carotid atherosclerosis, diabetes, congestive heart failure, history of smoking, age (over two thirds of strokes occur in persons >65 years), and history of stroke or transient ischemic attack (TIA) (Feigin VL, et al 2014; Becattini C, et al 2018). Interestingly, AF has shown to increase the annual risk for stroke by fivefold and accounts for 15% of all strokes (Stewart S, et al 2002). Currently the only FDA approved medication for the treatment of stroke is recombinant tissue plasminogen activator (rtPA), which only works to dissolve the thrombi and restore normal blood flow. However, the therapeutic window of rtPA is less than 4.5 hours after ischemic onset and, consequently, less than 4% of stroke patients have received rtPA (Hacke W, et al 2008; Green AR., 2008).

Cascade of brain ischemia injury and cell death

The human brain requires an enormous amount of chemical energy to function. It utilizes 15-20% of the total resting O_2 consumption in adults to replenish ATP as it is consumed (Clark & Sokoloff, 1999). Consequently, the brain's high oxygen demand makes it intolerant to interruptions in oxygen delivery. Ischemia causes a cascade of neurodegeneration, primarily caused by formation of ROS species in the mitochondria and initiation of apoptosis in affected neurons (Begley & Brightman, 2003).

When O_2 delivery to the brain is interrupted, it ignites a chain of reactions that culminate in cell death (Figure 2). Firstly, when the normal supply of O_2 is not available to the mitochondria, ATP synthesis falls and the lack of O_2 causes electrons to accumulate in the respiratory chain. This buildup of electrons leads to the partial (univalent) reduction of residual oxygen, thereby generating oxyradicals. Without ATP hydrolysis to power the membrane ion transporters, the neurons cannot repolarize and Ca^{2+} accumulates inside the cell (Kimura et al., 2002). Glutamate, the principal excitatory neurotransmitter of the brain, is then released by these depolarized neurons, producing excitotoxicity which exacerbates brain injury (Hazell, 2007). Excessive neuronal glutamate release causes hyperexcitation of postsynaptic neurons by activating α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors which causes Ca^{2+} influx and intracellular Ca^{2+} accumulation (Abramov & Duchen, 2008; Prentice et al, 2015). The Ca^{2+} buildup inside the neurons activates proteases and phospholipases, which in turn degrade structural proteins and membranes (Konstady, 2012; White et al., 2000), and activates the Ca^{2+}-sensitive nitric oxide synthase (NOS) isoenzymes (Manukhina et al, 2006; Prentice et al., 2015).

Excessive NOS activity overproduces NO, which undergoes biradical condensation with superoxide ($^{\bullet}O_2^-$), generating the cytotoxic peroxynitrite ($ONOO^-$) (Beckman & Koppenol, 1996; Ischiropoulos, 1998). $ONOO^-$, in turn, recruits proapoptotic proteins Bax and Bad and incorporates them into the inner mitochondrial membrane, consequently depolarizing the membrane and disrupting ATP synthesis. These two proteins promote the intrinsic apoptotic mechanism by facilitating depolarization of the mitochondrial membrane further disrupting ATP production (Prentice et al., 2015). Bax and Bad promote cytochrome c release into the cytosol, which under normal conditions is suppressed by overexpression of Bcl-2 (Ferrer & Planas, 2003). In the extrinsic apoptosis mechanis, Fas ligand (secreted by neurons, astroglia, other leukocytes) binds to its membrane receptor and activates caspase 8 and caspase 3. These caspases cleave the Bcl family protein Bid, allowing Bid to combine with Bad, forming a mitochondrial membrane channel through which cytochrome c is released (Broughton et al., 2009). Once cytochrome c is in the cytosol, by either the intrinsic or extrinsic mechanism, it will bind with other apoptotic proteins to create an apoptosome that leads to further cell function disruption and, eventually, cell death (Mallet and Ryou, 2017).

During a stroke, the cells within the region of most intense ischemia, i.e. the core of the

ischemic territory, are the first to die via necrosis and generally cannot be saved by conventional stroke treatments (Rami et al, 2008). However, in the surrounding penumbra the ischemia is not as pronounced due to collateral supply from other blood vessels and the apoptosis-mediated cell death in this region is more gradual (Kato & Kogure, 1999; Zheng, Zhao, Steinberg, & Yenari, 2003). Thus, timely reperfusion of the penumbra, e.g. by rtPA administration, may salvage the tissue (Paciaroni et al, 2009). Moreover, since the apoptosis cascade proceeds at a measured pace there is a chance for therapeutic intervention to save these neurons subjected to the less intense ischemia of the penumbra (Maiese, 2001).

Ischemia-reperfusion injury

One of the major limitations of rtPA as a treatment for stroke is that even though reperfusion is critical to preserve the brain tissue, it unavoidably causes an ischemia-reperfusion injury in the brain due to the abrupt reintroduction of blood-borne O_2 upon revascularization of the ischemic area. The resultant intense burst of reactive oxygen species formation inflicts further damage on the cells, and activates metalloproteinases (MMPs) that degrade the blood-brain barrier (BBB).

As discussed earlier, an interruption in the oxygen supply causes a build-up of electrons in the mitochondrial respiratory complexes. When O_2 is re-introduced into these electron-rich environments it causes a massive overproduction of superoxide ($^{\bullet}O_2^-$) in the brain (Selivanov et al., 2011; Kalogeris et al., 2014; Olmez & Ozyurt, 2012; Kimura et al, 2000). $^{\bullet}O_2^-$ undergoes additional single-electron reductions to sequentially generate hydrogen peroxide (H_2O_2) and then the aggressive cytotoxic $^{\bullet}OH$. $^{\bullet}OH$ then undergoes biradical condensation with NO to yield $ONOO^-$ which targets phospholipids and proteins, and also contributes to the production of other reactive nitrogen and oxygen species (Manukhina et al., 2006). These reactive compounds modify and disable crucial enzymes such as pyruvate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, aconitase, and creatine kinase (Robertson et al, 2009; Eaton et al, 2002; Hausladen & Fridovich, 1994; Reddy et al, 2000). $ONOO^-$ elicits membrane phospholipid peroxidation and nitrosylates tyrosyl and cysteinyl residues in proteins (Ferdinandy & Schulz, 2003; Manukhina et al., 2006).

Ischemia-reperfusion also activates MMPs such as MMP-2, -3, and -9 (Jin, Yang, & Li, 2010) found in neurons, glia, and leukocytes (Chaturvedi & Kaczmarek, 2014). MMPs are zinc

proteases which contribute to normal modeling of tissue through controlled degradation of extracellular matrix proteins, and when hyperactivated these proteases can cause brain damage and inflammation (Mallet and Ryou, 2017). The potential targets of MMPs are integral proteins of endothelial tight junctions of the BBB such as claudin, occludin, and junctional adhesion molecules (Abbott et al, 2010). Following stroke, MMPs degrade these proteins, which disrupts the BBB (Chang et al, 2014; Turner & Sharp, 2016). These actioins weaken the cerebrovascular endothelium, increasing the risk of hemorrhagic transformation (Turner & Sharp, 2016; Chaturvedi & Kaczmarek, 2014; Jickling et al, 2014) and facilitating the migration of inflammatory neutrophils into the penumbra, which together can expand the infarction (Kogure & Kogure, 1997; Vafadari et al, 2016).

Endogenous protection against ischemia

There are several endogenous proteins that serve to protect the brain against ischemia. These proteins are activated under hypoxic conditions and in turn activate neuroprotective signaling pathways that help the ischemic tissue cope with the hypoxic insults. Understanding the mechanisms, inducers and downstream effectors of these cerebroprotective cascades can foster development of innovative and novel treatments against many different ischemic diseases, such as myocardial infarction and stroke. Although some of these proteins are better understood than others, it is becoming increasingly evident that all of these ischemia-protective proteins, including those described below, influence each other and have a dynamic relationship.

Hypoxia inducible factor-1 alpha (HIF-1α)

The transcription factor HIF-1α is a master regulator of the expression of genes that orchestrate responses to hypoxia in many mammalian species (Semenza GL, 1999). From erythropoietin, the first identified product of HIF-1's gene program, the list has grown to over 100 genes whose expression are mediated by this transcription factor (Loor & Schumacker, 2008). Hypoxia Inducible Factor (HIF) is a heterodimer consisting of an α subunit and a ß subunit. HIF-ß is normally expressed but HIF-α is activated by hypoxia. Under normoxic conditions, HIF-1's α subunit is hydroxylated and subsequently degraded by proteasomes, thereby suppressing HIF-1-driven gene expression (Kumar and Choi, 2014). Even modest reductions in oxygen increase the stability of HIF-1α which then combines with the constitutively expressed ß subunit to form the

active HIF-1 dimer (Kallio P. J., et al, 1998). This HIF-1 complex binds to the hypoxia responsive element (HRE) enhancer domain of target genes, such as erythropoietin, heme oxygenase-1, vascular endothelial growth factor (VEGF), and glucose transporters (GLUT-1 and GLUT-4) to induce expression (Pugh CW, et al 1997). HIF-1a also serves to upregulate glycolytic enzymes and glucose transporters to help the cell make more ATP through glycolysis (Fukuda R, et al 2007). In astrocytes, hypoxia stabilizes HIF-1α and activates HIF-1-driven expression and release of erythropoietin, which in turn acts on erythropoietin receptors (EPOR) on nearby neurons, initiating signaling mechanisms that culminate in suppression of neuronal apoptosis (Ruscher et al., 2002).

Another inducer of HIF-1α is the mammalian target of rapamycin (mTOR), a protein kinase which responds to stress by regulating angiogenesis, cell growth, apoptosis and autophagy via HIF-1 and its downstream effector VEGF (Nakamura et al., 2006; Kang et al., 2008; Gunn et al., 2008; Shackelford et al., 2009; Miyazawa et al., 2010). Rapamycin is an inhibitor of mTOR and has been shown to induce apoptosis when administered to tumor and cyst cells (Kotulska K et al., 2009; Spirli C et al., 2010). When rats were treated with rapamycin after hypoxia-ischemia injury, p-mTOR, HIF-1α and VEGF expression were suppressed (Chen et al., 2012). The century old drug methylene blue (MB) has also been shown to activate HIF-1α. In a cell model of oxygen and glucose deprivation (OGD)-reoxygenation, MB increased O_2 concentration in the cells, ATP content, glucose uptake, and activities of hexokinase and the antioxidant enzyme glucose 6-phosphate dehydrogenase. Interestingly, MB activated the erythropoietin signaling cascade that subsequently raised HIF-1α expression (Ryou MG et al, 2015), demonstrating erythropoietin to be both inducer and product of HIF-1 activated gene expression.

Erythropoietin

The natural hematopoietic hormone erythropoietin (EPO) exhibits many diverse neuroprotective mechanisms capable of interrupting the brain injury cascade (Mallet & Ryou, 2017; Fig 2). Astrocytes are the major producers of erythropoietin in the brain (Swanson, Ying, & Kauppinen, 2004). Hypoxia activates HIF-1-mediated erythropoietin production in astrocytes, and erythropoietin protects neurons by interacting with its EPORs expressed on neurons, astrocytes and microglia (Brines et al., 2000; Ruscher et al., 2002).

Erythropoietin can ameliorate several diverse aspects of the injury cascade including production of reactive oxygen and nitrogen species, inflammation, BBB degradation, apoptosis,

excitotoxicity, and angiogenesis (Mallet & Ryou, 2017; Fig. 2). In mice, exogenous erythropoietin reactivated the antioxidant enzyme glutathione peroxidase after administration of the neurotoxin 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (Genc et al, 2002). Similarly, erythropoietin administration after bilateral carotid artery occlusion in gerbils suppressed formation of $ONOO^-$ (Calapai et al., 2000). Erythropoietin exerts its anti-inflammatory protection on all brain cells that express the EPOR (Merelli et al, 2015). After MCAO in rats, EPO was shown to suppress astrocyte activation and migration of leukocytes and microglia into the infarct area, and decreased buildup of proinflammatory cytokines such as interleukin-6, TNF-α, and monocyte chemoattractant protein-1 by more than 50% (Villa et al., 2003).

Pro-apoptotic Bax, Bak, and truncated Bid form channels in the inner mitochondrial membrane and cause the release of cytochrome c. Anti-apoptotic Bcl-X_L and Bcl-2 sequester Bax and Bak to prevent formation of these channels (Broughton et al., 2009; Sims et al, 2010). EPO exerts its anti-apoptotic effects by increasing expression of Bcl-X_L and Bcl-2 and decreasing Bax, Bak, and Bid expression (Mallet & Ryou, 2017). This mechanism was demonstrated in hypoxic hippocampal neurons (Wen et al., 2002), permanently ischemic neonatal rat brains (Sola et al., 2005), and in brains of gerbils subjected to cerebrovascular occlusion (Wen et al., 2002). EPO also suppressed glutamate excitotoxicity by preventing NMDA receptor-mediated, glutamate-induced neuronal apoptosis, and by maintaining the number of synapses in the hippocampus (Sakanaka et al., 1998). In cultured primary rat astroglia, erythropoietin increased glutamate uptake by 60% and increased glutamine synthase activity, which converts glutamate to the non-excitatory molecule glutamine (Lourhmati et al., 2013). Erythropoietin's protection of the BBB was evident in a rat model of traumatic brain injury and in rats subjected to MCAO, where erythropoietin administration after trauma or MCAO minimized BBB disruption, brain edema, lesion volume, and neurobehavioral impairment (Grasso et al., 2007; Wang et al., 2015). Wang et al. 2015, also showed that erythropoietin prevented degradation of the BBB by suppressing activation of MMPs -2 and -9 (Wang et al., 2015). Activating angiogenesis is also an effector function of erythropoietin. After permanent MCAO, EPO decreased infarct volume, increase angiogenesis, and normalize local blood flow in the penumbra, while increasing synthesis of the angiogenic factors VEGF, Tie-2, and agiopoietin-2 (Li et al, 2007).

Preclinical evidence suggests EPO can be used for treatment of stroke, traumatic brain injury, intracerebral hemorrhage, neonatal hypoxia-ischemia, and even spinal cord injury

(Rangarajan & Juul, 2014). In several studies utilizing the MCAO rat model of stroke, erythropoietin iv infusion or ip injection decreased infarct volume and minimized neurobehavioral deficits (Zhao et al., 2015), and increased differentiation of progenitor cells into neurons within the lesion (Gonzalez et al., 2007). EPO administration 10 min before ischemia decreased neurological deficits, brain edema, and circulating activities of neuron specific enolase, a brain injury marker (Ratilal et al., 2014). When infused directly into the reperfused MCA EPO decreased neurological deficit, infarct volume and apoptosis in the penumbra (Dang et al., 2011). Even when administered 6 hours after the ischemic period erythropoietin was able to cross the BBB and decrease the infarct volume by 50%-75% in rat MCAO models (Brines et al., 2000). Another study reports 70-75% reductions in penumbral apoptosis when EPO was administered 24 hours before, during, or 3 hours after MCA occlusion in rats (Brines, 2002). In a rat model of traumatic brain injury EPO, injected intraperitoneally on days 1, 2, and 3 post-injury, decreased cell loss in the hippocampus, increased angiogenesis and neurogenesis in the cerebral cortex and hippocampus, and improved sensorimotor function (Zhang et al, 2009). Collectively, these studies identify EPO as a likely mediator of hypoxia induced brain protection.

Nuclear factor erythroid 2-related factor 2 (Nrf2)

Nuclear factor erythroid 2-related factor 2 (Nrf2) is another transcription factor that activates expression of cerebroprotective genes, in this case, genes that encode antioxidant and anti-inflammatory proteins. Nrf2-driven gene expression increases cellular resistance to ROS and carbonyl stress, although its mechanisms are still not fully understood (Jung & Mallet, 2017). Under normal conditions, Nrf2 is held in the cytosol in an inactive form, where it is tethered to the Kelch-like ECH-associated protein 1 (KEAP1), an interaction that targets Nrf2 for rapid proteasomal degradation. During oxidative stress or under the influence of Nrf2 inducers, Nrf2 is freed from KEAP1 and translocates to the nucleus (Niture et al., 2014; Vargas et al., 2009); there, Nrf2 acting in collaboration with co-transcription factors, binds to antioxidant response element (ARE) promoter regions to activate expression of antioxidant proteins such as heme oxygenase-1 (HO-1), Nrf2 istelf, and two enzymes that catalyze the synthesis of the principal cellular antioxidant glutathione (GSH), namely, glutamate cysteine ligase (GCL) and GSH synthetase (GSH-Syn) (Niture SK et al., 2014; Vargas MR et al., 2009; Ishii T et al., 2004; Wakabayashi N et al., 2010). HO-1 defends the brain against oxidative stress during ischemia and inflammation

(Kim YM et al., 2011; Innamorato NG et al., 2008) and GSH is a major antioxidant as well as a substrate or cofactor for many enzymes cellular detoxification (Deponte M, 2013).

Brain-derived neurotrophic factor (BDNF)

BDNF is another potential mediator of neuroprotection during ischemia. Neurotrophic factors are similar to growth factors in that they promote the survival, differentiation, and function of neurons via activation of tyrosine kinase receptors A, B, and C (TrkA, TrkB, TrkC) (Smeyne RJ et al., 1994). BDNF functions as an endothelial cell survival factor (Donavan MJ et al., 2000) which stimulates angiogenesis by increasing the content of VEGF's membrane receptor in endothelial cells expressing TrkB (Kermani P et al, 2005; Kim H, et al., 2004). In neuroblastoma cells, BDNF increased production of VEGF by activating expression of HIF-1α (Nakamura K et al., 2006). Intermittent hypoxia is known to induce VEGF's transcription factor, BDNF (Baker-Herman et al., 2004), and its activation of important neuroprotective mechanisms makes BDNF a potential target for treatment of brain ischemia, including stroke.

Intermittent hypoxia training as a potential treatment for ischemic stroke

Intermittent hypoxia training (IHT) offers a possible solution for treating ischemic stroke without the risk of inducing hemorrhage, an important limitation of rtPA. IHT has been demonstrated to induce neuroprotective mechanisms in different disease models of rats. IHT has been shown to increase cerebral blood flow, improve endothelial production of NO, and reduce oxidative stress in rats with experimental Alzheimer's Disease (Manhukhina et al, 2016). In rats experiencing ethanol withdrawal, IHT prevented behavioral deficits at 24 h EW, decreased oxyradical and protein carbonyl formation in several brain regions, and suppressed the cytotoxic signaling cascade after excess release of glutamate (Jung and Mallet, 2017). In another study assessing IHT in myocardial infarction in canines, IHT dramatically lowered myocardial infarctions and ventricular tachyarrhythmias during ischemia and reperfusion (Zong et al, 2004).

These studies lead us to hypothesize that IHT will exert neuroprotective effects in rats post-MCAO. The importance of testing the effects of IHT after stroke, however, is due to the fact that it is essentially impossible to predict when one will suffer a stroke. Thus, a practical treatment for stroke must exert its protective effects even when applied long after the onset of cerebrovascular blockage and symptoms. This is crucial because many patients do not realize they have suffered a

stroke until several hours after the fact, due to the subtlety of their initial symptoms. Therefore, IHT could potentially provide a non-invasive, painless treatment for coping with ischemic injury, even when administered after the ischemic insult.

The Body's Physiological Response to Hypoxia

The physiological effects of intermittent hypoxia on the cardiopulmonary system depend on several factors: the intensity of hypoxia, duration of each hypoxic exposure and the intervening reoxygenation periods, total duration of the IH application, and the presence of concomitant stumuli during the cyclic hypoxia sessions (eg, exercise, hypo- or hypercapnia) (Faulhaber et al, 2010 and 2015). The human body's natural response to hypoxia is a chemoreflex-mediated increase in sympathetic nerve activity (SNA) that is directly related to the duration of hypoxia and the magnitude of O_2 desaturation. This increase in SNA leads to an increase in systemic arterial pressure, which can exceed 50 mmHg or more during severe hypoxia, and which subsequently returns to baseline after normoxic ventilation resumes (Serebrovskaya et al, 2008). The heart rate response varies; voluntary breathing of hypoxic gas increases heart rate due to vagal withdrawal (Lucy et al, 2000; Bernardi et al, 2001; Buchheit et al, 2004), whereas prolonged apneas causes increased vagal activation and bradycardia (Goksor et al, 2002). This chemoreflex is also known to increase cerebral blood flow in order to maintain adequate oxygen delivery to the brain (Young et al, 1997). However, hypoxia also increases ventilation via the peripheral cheoreceptor reflex which produces acute hypocapnia (Fletcher et al, 1997) and a respiratory alkalosis (Young et al, 1997). Since CO_2 stimulates cerebral vasodilation, acute hypocapnia can cause cerebral vasoconstriction that will counteract the hypoxia-increased cerebrovascular conductance (Serebrovskaya et al, 2008). This dynamic relationship between hypocapnia and hypoxemia may explain the conflicting reports that cerebral blood flow during acute, variable bouts of hypoxia remains constant (Lavie et al, 2000), decreased (Parish et al, 2004), or increased (Leuenberger et al, 2005).

Chronic vs Therapeutic Hypoxia and Blood Pressure

One of the most prevalent causes of clinical hypoxia is obstructive sleep apnea (OSA), characterized by recurrent episodes of arterial hypoxia and hypercapnia due to brief asphyxiation from airway collapse while sleeping (Serebrovskaya et al, 2008). These episodes can occur 30-60

times an hour, a rate associated with significant cardiovascular morbidity and mortality (Marin et al., 2005). This chronic intermittent hypoxia puts patients at risk of stroke and coronary artery disease, but most notably results in chronically elevated blood pressure that persists after apneic stimulation (Shepard JW Jr. et al, 1985; White SG et al, 1995). In contrast, therapeutic intermittent hypoxia, i.e. IHT, has been shown to decrease blood pressure in hypertensive patients and is not associated with the detrimental effects of OSA (Lyamina et al, 2011). These striking differences are due to the fact that while OSA includes short but frequent episodes of hypoxia, therapeutic intermittent hypoxia training consists of longer hypoxia phases (3-6 min) alternated with room air exposure for a limited number of daily cycles over a period of several days to weeks.

In rats, a chronic intermittent hypoxia program that mimicked the conditions seen in sleep apnea patients increased mean arterial pressure by 10-14 mmHg compared to a sham control group, and this elevated blood pressure persisted for several weeks (Fletcher et al, 1995; 1996, 2000; 2001). In contrast, in human subjects a therapeutic intermittent hypoxia session consisting of 5 bouts of 6-min ventilation of 10% O_2 interspersed with 4 min of room air ventilation did not significantly increase blood pressure or mean arterial pressure, as shown in the figures below (Liu et al., 2017). Another study assessed heart rate and blood pressure during a 3-week intermittent hypoxia program in COPD patients showed that during the hypoxic phases blood pressure was not affected appreciably, but heart rate increased significantly, to an extent dependent on the intensity of hypoxia (Faulhaber et al, 2015). Lyamina et al. showed that IHT lowered blood pressure in patients with stage 1 arterial hypertension by increasing nitric oxide production. In this study, after 37 participants completed a 20-day program consisting of alternating 3 min episodes of 10% O_2 and room air for 4-10 daily cycles, all hypertensive participants experienced a 15 – 27% reduction in blood pressure, and 85% of the formerly hypertensive participants remained normotensive without medications 3 months later (Lyamina et al, 2011).

Sex Differences to Hypoxia

While the impact of sex on the effects of therapeutic intermittent hypoxia require more investigation, chronic intermittent hypoxia, such as obstructive sleep apnea (OSA), has a higher prevalence in men compared to women (Bixler et al., 2001; Franklin and Lindberg, 2015; Mokhlesi et al., 2016; Valipour, 2012; Young et al., 1993). The prevalence of OSA is higher in men than in premenoupausal women, with a ratio of 2:1 to 3:1, while OSA incidence increases 3-4 fold in

women postmenopausal vs. premenopausal (Bixler et al., 2001; Redline et al., 1994, Young et al., 2003). In regards to this disparity, sex hormones such as progesterone and estradiol are thought to be the mediators in the oxidative stress protection that chronic hypoxia induces (Boukari et al., 2017). This idea is supported by the increased incidence of OSA in postmenopausal women, vs. the reduced prevalence of OSA in post-menopausal women receiving hormone replacement therapy (Bixler et al., 2001). Estradiol and progesterone are well known antioxidants that exert their effects by regulating the activity of NADPH oxidase, increasing mitochondrial respiration and reducing ROS production, and increasing the activities of superoxide dismutates and glutathione peroxidase (Borras et al., 2010; Moorthy et al., 2005; Pajovic and Saicic, 2008). Due to their inherent protection against oxidative stress, it is likely that premenopausal females will be able tolerate ischemic preconditioning and the therapeutic benefits may be more pronounced in females vs. males. In that case, hormone replacement therapy may enhance the beneficial effects of IHT in postmenopausal women.

Chapter 2: Research Project

I. Specific Aims

This study aims to assess the effects of IHT on motor function and spatial memory of rats when administered after MCAO and cerebrovascular reperfusion in rats. We hypothesize that rats given IHT after stroke will 1) show a decrease in motor dysfunction and spatial memory deficits compared to rats receiving no post-stroke hypoxia treatment and 2) have an increased expression of specific neuroprotective peptides such as Erythropoietin, Nrf2, and BDNF. The findings of this study may demonstrate that IHT is an effective, non-invasive treatment that preserves brain tissue after initiation of the injury cascade due to ischemic events.

II. Significance and Innovation

Stroke is the leading cause of disability in adults worldwide and in the U.S. it is the fifth-leading cause of death (Ovbiagele et al, 2011). Around 800,000 Americans suffer from strokes annually (Mozaffarian et al, 2016), and of the 7 million Americans who have suffered a stroke (Roger et al, 2012), 75% live with mental deficits that significantly impair their quality of life (Go

et al, 2013). These deficits manifest as cerebral cortical impairment, such as aphasia, neglect, apraxia, anopia and restricted motor function, as well as brainstem or cerebellar dysfunction (Yip et al, 1997) Currently the only FDA approved medication for the treatment of stroke is recombinant tissue plasminogen activator (rtPA), an anti-thrombotic agent which dissolves intravascular thrombi in order to restore normal blood flow to the ischemic territory. However, the therapeutic window of rtPA is less than 4.5 hours after ischemic onset; consequently, less than 4% of stroke patients have received rtPA (Hacke W, et al 2008; Green AR., 2008). Moreover, rtPA treatment carries a risk of intracerebral hemorrhage, which can be lethal.

IHT offers a possible solution for treating ischemic stroke without the risk of inducing hemorrhage. In previous studies, IHT has shown to induce neuroprotective mechanisms in different disease models of rats by inducing expression of antioxidant species and upregulating expression and activity of a host of neuroprotective peptides. IHT has been shown to improve cerebral blood flow, improve endothelial production of NO, and reduce oxidative stress in rats with experimental Alzheimer's Disease (Manhukhina et al, 2016). In rats experiencing EW, IHT prevented behavioral deficits at 24 h EW, decreased oxyradical and protein carbonyl formation in several brain major brain areas, and suppressed the cytotoxic signaling cascade after excess release of glutamate (Jung and Mallet, 2017). In canines subjected to coronary artery occlusion-reperfusion, IHT almost completely prevented myocardial infarctions and reperfusion ventricular tachyarrhythmias (Zong et al, 2004).

These studies lead us to hypothesize that IHT will exert neuroprotective effects in rats post-MCAO. The importance of testing the effects of IHT after stroke, however, is due to the fact that strokes are generally unpredictable, so IHT before stroke is not feasible. Thus, stroke treatments must be able to exert their effects after the onset of cerebrovascular blockage and symptoms. This is crucial because many patients do not realize they are having a stroke until several hours after the onset, due to the subtlety of their initial symptoms. Therefore, the possibility that IHT could provide a non-invasive, painless treatment for coping with ischemic injury, even when administered after the ischemic insult, merits investigation.

III. Materials and Methods

Animals

All animal use in this investigation was approved by the UNTHSC animal care and use committee (protocols # 2014 15-14-AOS and IACUC-2017-0055) and was conducted according to the Guide to the Care and Use of Laboratory Animals (U.S. National Research Council publication 85-23, revised 2011). 24 Sprague-Dawley rats were used in this study. All of them completed neurobehavioral and spatial memory tests to establish their pre-stroke cognitive and motor function baselines. Two groups of rats then underwent MCAO; one group received IHT and a control group underwent stroke without receiving IHT. A third group underwent a sham surgery procedure and sham hypoxia conditioning to control for the effects of anesthesia, surgical trauma and other factors aside from ischemia-reperfusion. Rats will be sacrificed humanely and in accordance with the IACUC protocol.

Neurobehavioral and memory testing

The Rotarod tests utilized the AccuRotor 4-Channel RotaRod. The Morris Water Maze (MWM) apparatus consists of a large pool filled with water which is made opaque by dyeing it blue with powdered tempera paint. A small camera was installed above the pool to track animal movements. Data from the camera was compared on a desktop computer equipped with ANY-maze software.

The Rotarod test

The Rotarod test assesses motor coordination, strength, and stamina in rodents (Scholz et al). We used this test to establish a baseline of motor function before stroke and to detect differences in motor function among treatment groups after stroke. The rats are placed on a rotating drum that gradually accelerates at a constant rate until the rat cannot keep up and falls down below into a plastic rectangular enclosure. The fall is high enough to cause slight pain without injury, and this positive punishment encourages the rats to stay on the cylinder for as long as possible. The floor of the machine acts as a lever which stops the timer when the rat lands on it, accurately recording fall latency. Healthy rats typically learn this test within 1-2 days and perform better over the first few days before reaching their inherent baseline as indicated in our pre-stroke rotarod data

(Shiotsuki et al; Figure 5). It is anticipated that stroke will adversely impact motor function and the rats are expected to have a difficult time staying on the cylinder, and maintaining balance and coordination.

Rotarod testing was done for 10 days, 3 trials per day with 20 minutes of rest between trials. The rotating drum accelerated at a constant rate (3 rpm/s) to 720 rpm over the course of 240 seconds, thereby gradually increasing the difficulty in order to accurately assess the rat's capabilities. If a rat falls within the first 15 seconds, usually this is due to improper placement or bad footing on the cylinder, they must repeat the trial. Some rats appeared to intentionally jump off, but this occurred beyond the 15 sec mark and the results of those tests were included in the data set. The jumping behavior was random amongst the rats and did not affect the overall baseline. Preliminary data is shown below (Figure 5).

Morris Water Maze

The MWM assesses spatial learning and memory. An important strength of this test is its relative immunity to motivational differences across a range of experimental treatment effects (genetic, pharmacological, nutritional, toxicological and lesion) that are secondary to the main focus of the experiment. For example, lesions that induce hyperactivity or treatments that cause hypoactivity can both be dissociated from learning deficits in the MWM. This is because land-based locomotor reductions do not affect swimming speed, therefore learning impairments are independent of locomotor effects (Vorhees and Williams, 2006). Thus, the MWM is appropriate for this study because it enables spatial learning and memory deficits to be differentiated before and after stroke.

Despite its name, the MWM is not a labyrinth, but rather an open, circular pool of water with a platform submerged just under the water surface. We used powdered paint to turn the water dark blue in order to obscure the platform from the swimming animal's sight. The goal of the MWM is for the animal navigate the pool and locate the platform within the allotted time. The pool is divided into four quadrants with the axes representing the four cardinal points: North (N), South (S), East (E), West (W). The platform is positioned in the middle of one of the quadrants. This is perhaps the most important detail of the MWM that the animals must learn. Instead of following the walls of the pool for a way out, they must remember that the platform is in the interior

of the pool and navigate accordingly. The rats are randomly placed in one of the 3 quadrants that do not contain the platform. The animals are given three trials each day and have 90 seconds to reach the platform before they are placed on the platform to re-orient themselves and then "rescued" from the maze. A camera positioned above the maze tracks and records the animal's movements through during the trial, assessing the path taken, swimming speed, time spent in each quadrant, and then the trial data is analyzed by the computer program ANY-Maze.

There were 4 different types of trials used during the two week MWM testing. The first is spatial acquisition, where the animals use visual cues in the room outside of the pool and memory from previous trials to find the platform. Probe trials assess the strength of the animals' memory of the platform position. In a probe trial the platform is sunk so that even when the animals swim over the appropriate area they cannot stand up – the trial is considered successful if the animal swims over the platform in under 30 seconds. Reversal trials assess the persistence of learned behavior and ability to adapt. In these trials the platform is moved to the opposite quadrant; the animals usually will start looking in the old quadrant first before proceeding to search the other quadrants. Usually over the next few trials the animals will spend less time in the old quadrant and more time finding the platform in the new quadrant.

It is our expectation that stroked rats will have a considerably more difficult time learning the MWM and navigating the maze through the various trials than non-stroked control rats. Thigomotactic swimming is expected as well – swimming in one direction, following the edge of the pool (Bingham et al, 2012). Swimming speed is not expected to be affected by the stroke.

Middle Cerebral Artery Occlusion

The middle cerebral artery (MCA) is the artery most commonly affected in stroke syndromes in humans. Due to the similarity in cranial circulation between rats and humans the MCA occlusion method in rats mimics the clinical syndrome. The MCA has no collateral blood supply, making it an ideal target for producing an infarct. In the rat, the MCA supplies the frontal, sensorimotor, auditory and occipital cortices and striatum. Blood supply to the hippocampus however is not derived from the MCA. The goal of the MCAO procedure is to create ischemic conditions in the brain to produce an ischemic territory that is similar in size and severity for all rats undergoing the procedure. To accomplish MCAO, a silicone-coated nylon monofilament is advanced into the internal carotid artery to block the MCA for a specified duration, and then

removed to allow reperfusion -mimicking stroke and thrombolysis in humans. The MCAO procedure was performed as previously described (Uluc et al, 2011) using sterilized surgical instruments and technique, and is summarized as follows.

Rats will be anesthetized with isoflurane, and the throat and neck shaved and prepped for a septic surgery with betadine and alcohol. An incision is made at the midline from the base of the neck down to the upper margin of the collar bone. The neck tissue is dissected until the left common carotid artery (CCA) is isolated up to the bifurcation point of the internal carotid artery (ICA) and external carotid artery (ECA). First the CCA is ligated with a temporary suture. The ECA is then permanently ligated as distally as possible to the bifurcation. Then, the occipital artery (OA) and the superior thyroid artery (STA), two branches off of the ECA close to the bifurcation, are cauterized. Next, the ICA is clamped with an atraumatic clip, and a small incision of the ECA is made between the distal ligation and the bifurcation point. The silicone coated filament is then inserted into the incision and advanced into the CCA. After the filament is secure in the vessel another suture is placed around the ECA and filament, to provide leverage while manipulating the filament. The ECA is then transected, just proximal to the permanent suture on the ECA and distal to the filament insertion point. With the ECA now separated from the cervical tissue it can be pulled down to create a straight path for advancing the filament into the ICA. Once the filament is in the ICA the vasoclamp is removed and the filament is carefully advanced approximately 2 cm, so that it covers the origin of the MCA. Once the filament has been advanced it is then firmly secured to keep it in place for 90 min. After the allotted time the filament is removed and the rat is allowed to recover. Signs of stroke should be apparent after the rat regains consciousness. Figure 2 (above) diagrams the vasculature and filament insertion point.

Intermittent hypoxia training regimen

24 hours after stroke, the rats designated for IHT will be placed in a special chamber that controls the inspired O_2 fraction, i.e. FIO_2, to which the rats are exposed. The rats will breathe low oxygen gas (10% O_2) for 5-8 brief periods each lasting 5-10 minutes, with intervening 4 min exposures to room air, each session lasting 4-9 min, for 20 consecutive days. These rats will be compared to a sham group of rats exposed to normoxic air (21% O_2).

Experiment Timeline

An overview of the experiment schedule is shown in figure X. Rats were trained in Rotarod and MWM before stroke. The rats underwent Rotarod training for 5 consecutive days with 3 trials per day and then given 2 days rest, followed by MWM. MWM consisted of 5 consecutive days of training in the Acquisition stage, with 3 trials per day, followed by 2 days rest and then a second 5 days of MWM consisting of two days of Retention and 3 days of Reversal. Two weeks after completion of neurobehavioral testing MCAO or sham surgeries were performed on all rats. 24 h post-MCAO rats commenced either hypoxia or sham conditioning treatments daily for 20 days. 7 days post-MCAO rats underwent post-stroke neurobehavioral testing, with the same schedule as before MCAO. 24 h after the final MWM trial the rats were sacrificed. This schedule was staggered by 2 weeks between two groups of 12 rats (figure 4).

Data Analysis

When comparing post-stroke recovery in the non-IHT vs. IHT rats at different time points we will utilize a single-factor ANOVA, where the independent factor could be time, infarct size, or performance in the neurobehavioral tests. A *post-hoc* Tukey's test will be applied when anova reveals the presence of statistically significant findings.

Limitations of the MCAO model

The MCAO protocol closely mimics in rodents clinical stroke syndromes due to the fact that the MCA is the most commonly affected vessel in humans. However, this protocol does not account for the high variability in severity between stroke cases. Some patients may experience brief durations of ischemia or long durations, they may experience partial instead of complete occlusion, and many stroke patients have other pre-existing medical problems that impact the severity, progression and outcome of a stroke. Additionally, because of its invasive nature, surgical trauma to the neck tissue and blood vessels during MCAO could possibly worsen symptoms of stroke, beyond that produced by a thrombus or other endogenous blockage.

IV. Results

Rotarod Test

Pre-stroke rotarod data (figure 5) showed a training effect, manifest as an increase in fall latency across the 5 days of training. Although post-stroke rotarod data has not yet been collected for all rats, we expect that stroked rats will show decreased fall latency across the 5 days with minimal day to day improvement.

Morris Water Maze Performance

Pre-stroke water maze data (Figures 6-13) shows a collective trend of decreased time needed to find the platform and distance travelled over the course of the 10 days of training. Between days 5 and 6, the end of the Acquisition stage and beginning of the Retention stage, the rats rested 2 days, which challenged the rats' memory regarding how the test works and where to find the platform. Memory appeared to be retained due to the decrease in time from day 5 to day 6. During the Acquisition stage the distance travelled for the rats considerably decreased over the first 5 days and remained relatively low throughout the remainder of the training. Even during the Reversal stage, the rats showed adaptation in learning the new location of the platform as evident by a decreasing trend in distance travelled.

The pre-stroke probe trials show how much time the rats spent in each quadrant and distance travelled in each quadrant. For the probe trials, the quadrant the rats spent the most time in was the quadrant in which they started, and the quadrant they spent the second most time in was the quadrant containing the platform. During the Reversal stage the platform was moved from the SW to the NE quadrant. During the Reversal probe trial, rats spent the most time in the SW quadrant and also travelled the most distance in the SW quadrant. This outcome shows that the strength of their memory of the platform location persisted even after being trained on a new platform location.

Although post-stroke water maze data currently is being collected, we expect that stroked rats will have a much more difficult time finding the platform and memorizing its location from day to day. We also predict that stroked rats will have a decreased ability to adapt when the platform's location is changed in the Reversal stage.

MCAO mortality rate and stroke signs

Due to the highly invasive nature of the MCAO surgery some mortality due to the procedure is expected. The mortality rate during these surgeries was much higher than expected 54.17% (13 deaths out of 24 surgeries). Six of these rats died at least 1 h after reperfusion, two rats died before the end of the 90 min MCAO, three rats died within 24 h after MCAO, and one rat died during the procedure due to a hemorrhage. These deaths may be due to internal hemorrhaging in the brain from filament insertion, although it is possible that the procedure produced a massive stroke that caused death. Although strokes can cause death in humans, rats undergoing MCAO are expected to have a much higher survival rate. The cause of our low survival rate is most likely due to initial inexperience in performing this complex procedure.

Rats subjected to MCAO all showed stroke signs immediately after recovery from anesthesia. This manifested as one or more of the following: facial droop, weak grip with one arm, turning in only one direction, and tail curl to one side. These symptoms persisted for several days, yet in some rats the stroke signs disappeared completely. This recovery could be due to the robust nature of Sprague-Dawley rats or an indication that the MCAO surgery did not produce an effective stroke. Assessment of infarct size by tissue staining and biochemical analysis is needed to ascertain why these stroke signs subsided.

Biochemical Analysis

Biochemical data has not been collected because the experiment is currently in progress. However, we will be measuring contents of EPO, Nrf2, and BDNF in rat cortex, sub cortex, hippocampus, and cerebellum.

V. Discussion

The goal of this project was to determine the efficacy of IHT in protecting the brain and reducing the behavioral deficits following stroke in rats. However, because our study is still in progress we are not yet able to draw conclusions about the effects of therapeutic hypoxia intervention during the injury cascade following ischemia.

During pre-stroke neurobehavioral training the rats demonstrated a learning curve was seen in both the Rotarod and MWM tests. During the first two days of Rotarod, a significant increase in fall latency was observed as the rats learned to stay on the cylinder (figure 5). For the rats

to perform better each day their balance and coordination had to improve in order to keep up with the constant acceleration. We expect that this kind of improvement will not be seen post-stroke, especially if one side of the body is significantly weaker than the other. By the end of the 5 days training, fall latency plateaued at 60-65 s, establishing a baseline for the rats' inherent motor function.

A similar progressive improvement was seen in the pre-stroke MWM. During the acquisition stage a significant drop in time and distance travelled was observed over the 5 days (Figures 6, 7). This improvement indicates that as the rats learned 1) the platform is beneath the water, 2) always in the same spot, and 3) located in the interior of the pool, their memory persisted over the course of the MWM trials. The retention stage tested the rats' memory after 2 d of rest and the results demonstrated memory retention. The reversal stage challenged the rats to learn a new platform position while assessing the strength of their memory of the first location. Progressive learning during the reversal stage was indicated by decreasing time and distance travelled for days 8-10. The probe trials test the searching prioritization when the platform is fully immersed, so that rats cannot rest on it, during a 30 s trail, indicating the strength of the learning (figures 8-13). During probe trials the rats spent the most time in the quadrant they started in and the second most time in the quadrant containing the platform, meaning the rats had knowledge of where to look for the platform even after starting in a random location. The reversal probe trials also showed that even while learning the new platform location the rats still spent the most time and travelled the most distance in the quadrant where the platform was originally located. We expect that after a stroke rats will require more time and travel greater distances during all MWM stages, but more importantly we expect that memory consolidation will be impaired, especially during the reversal stage.

VI. Summary and Conclusions

In this experiment we investigated the neuroprotective effects of IHT when initiated 24 h after 90 min MCAO in rats. Neurobehavioral function was evaluated by the Rotarod test, which tests motor function and coordination, and by the MWM, which assesses spatial memory and learning. The neurobehavioral baselines have been recorded for all rats, and post-stroke

neurobehavioral testing is in process. As such, we cannot yet draw firm conclusions about the efficacy of IHT to promote recovery from ischemic stroke.

During this past year I was able to learn and practice many of the essential skills needed in research, but more than that I came to understand that good research requires strategy, patience, and diligence. Before starting on this project I had no research experience. I quickly became familiar with learning how to navigate the peer-reviewed literature and how to find articles pertinent to my project, a skill that has become an important factor to my growth as a scientist. To execute this experiment successfully I had to carefully plan out every detail and organize my schedule efficiently. The training I received from the experts in neurobehavioral testing and rodent surgeries had to be coordinated around their schedule's availability, my class schedule, and the experiment timeline. Additionally, all practice surgeries had to be planned in advance with the goal to reach the level of proficiency needed to conduct all the surgeries on my own successfully. Strategizing how to best achieve our goals was challenging, but this long-term thinking made me appreciate just how much work goes into experimental design and why it is so necessary to finalize these details before the experiment begins. I also had to learn patience during this experience because we could not begin the experiment until I became proficient in all of the procedures being implemented in this study. Learning how to perform the Rotarod and MWM tests, conduct the MCAO surgery, acquire and analyze data with the ANY-Maze and GraphPad Prism programs, and collecting tissue for analysis were skills that took a considerable amount of time to learn and refine. However, preparing for the experiment was time and effort well spent. Despite the IHT phase of the study beginning later than originally anticipated, it has been progressing and I am excited to see the results at its conclusion.

In addition to learning how to conduct a research study, I have learned how to analyze and present scientific data in a coherent way. By preparing my research proposal and this Final Report I have had a taste of the hard work that goes into writing a peer-reviewed article or research manuscript. It has truly made me further appreciate the dedicated scientists and their novel discoveries, with the ultimate goal of treating disease more effectively and promoting health. Following my first year in the Medical Sciences program this research experience has truly elevated my knowledge of physiological concepts from a theoretical to a practical understanding and will serve to give me a better perspective on medicine as I continue my journey towards medical school.

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