

NOVEL PHARMACEUTICAL COMBINATION CONFERS PROTECTION
FROM DELAYED CELL DEATH FOLLOWING
TRANSIENT CEREBRAL ISCHEMIA

by

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ABSTRACT

Stroke is a leading cause of death and disability throughout the world; ischemia is the most common form of stroke. Medical procedures such as cardio-pulmonary bypass surgery can cause ischemic stroke can be caused. There are no treatments to limit neural impairment following stroke. The current research investigates neuroprotection offered by treatment with a novel drug combination consisting of SimvastatinTM, GemfibrozilTM, TroglitazoneTM, and SpironolactoneTM.

Animals were treated with the drug cocktail three weeks preceding and one week subsequent to surgery. Ischemic insult was induced by clamping the carotid arteries for 5 min. Sham subjects underwent similar surgical procedures, but the carotids were not clamped. Twenty-four hrs following the surgical procedure locomotor activity was monitored in an open field for 5 min. Seven to fourteen days following ischemia or the sham procedure animals were sacrificed and sections containing the hippocampal CA1 region were mounted on slides and stained with cresyl violet. The CA1 region was rated on a 4-point scale for level of damage.

Rodents generally show increased locomotor activity following transient global ischemia in an open field. In our study, ischemic animals that received vehicle demonstrated increased activity relative to the animals that received the drug treatment on all behavioral measures. Ischemic animals that received vehicle treatment had significantly more neural damage in the hippocampal CA1 region than ischemic animals receiving the drug. The appearance of neurons in the CA1 hippocampal regions of animals in the sham condition was not significantly different from ischemic animals in the drug treatment condition. It is concluded that the drug treatment is effective in offering neuroprotection during transient global ischemia.

The next step is to characterize the biochemical mechanisms behind the neuroprotection conferred by the drug treatment. Contrasting the protein expression levels of animals receiving the vehicle treatment with animals receiving the drug treatment following an ischemic insult will assist in elucidating these pathways. Predictions are made regarding the biochemical mechanisms affected by the drug treatment based on previous research on the biochemical pathways affected by each pharmaceutical.

INTRODUCTION

Hippocampus

The hippocampus is one of the most thoroughly studied areas of the mammalian brain for two main reasons. The first reason is that the structure has a well defined cytoarchitecture, making the structure readily identifiable and distinctive (Buhl & Dann, 1991). The hippocampus was named for its anatomical semblance to a sea horse, as in Greek, *hippo* means “horse” and *kampos* means “sea monster.” The hippocampal structure consists of the dentate gyrus, hippocampus, and subiculum, all of which are composed of a single cell layer with less-cellular or acellular layers above and below that single cell layer. Cell bodies and zones of connectivity are layered in an orderly, or laminar, manner that facilitates neuroanatomical and electrophysiological studies (Johnston & Amaral, 2004). The hippocampal formation also includes the presubiculum, parasubiculum, and entorhinal cortex surrounding the aforementioned sections. These surrounding areas consist of several cellular layers (Johnston & Amaral, 2004). A Nissl stain of a horizontal section through the hippocampal formation reveals all of its divisions.

The dentate gyrus is composed of granule cells arranged into a layer about 4-6 cells thick. Granule cells have small (~10mm diameter), spherical cell bodies, and are considered monopolar because dendrites emerge from the apical portion of the cell body (Ishizuka, 2001; Parent, 2003). The hippocampus proper consists of pyramidal glutamatergic neurons, named after their triangular shape. The hippocampus proper is subdivided into three regions, CA1, CA2, and CA3, based on the size and appearance of the neurons. Originally, Ramon y

Cajal (1911) divided the hippocampus into two major regions; he termed the large-celled region closer to the dentate gyrus (now consisting of CA3 and CA2) *regio inferio* and the smaller-celled distal region (now CA1) *regio superior*. Only the CA3 region receives input from the mossy fibers of the dentate gyrus (Johnston & Amaral, 2004).

The hippocampal cells layers include the principal layer, known as the pyramidal cell layer, containing the pyramidal cell bodies. The region external to the pyramidal cell layer is occupied by basal dendrites of the pyramidal cells and is termed the *stratum oriens*. Superficial to the pyramidal cell layer is the *stratum radiatum* and the *stratum lacunosum-moleculare*, where the apical dendrites of the pyramidal cells are located. Generally, the neurons located in layers other than the pyramidal cell layers are presumed to be interneurons (Vida *et al.*, 1998).

The second reason for the large amount of research invested in the hippocampus is the profound consequences of hippocampal pathology. While Thorndike first reported a link between the hippocampus and memory in the 1890s (Thorndike, 1898; Thorndike, 1998), the profound effects of lesion studies performed in the mid-1900s were responsible for inspiring scientific interest. In 1939, Heinrich Klüver and Paul Bucy described the behavioral symptoms resulting from temporal lobe lesions. These symptoms include visual agnosia, auditory aphasia, dietary changes and hyperorality, docility, diminished affect, hypermetamorphosis, and hypersexuality. This collection of behaviors later became known as the Klüver-Bucy syndrome and linked the hippocampus with cognitive dysfunction (Klüver & Bucy, 1939; Isaacson, 2002).

H.M. underwent bilateral removal of the hippocampus to control seizures (Scoville, 1957), as seizures can arise from and propagate in the hippocampus (Walker *et al.*, 2007).

Following the procedure, H.M. was diagnosed with anterograde amnesia along with a temporally graded retrograde amnesia. In temporally graded retrograde amnesia, memories acquired close to the time of amnesia onset are lost, whereas memories formed in the distant past are spared. The temporal gradient of H.M.'s memory loss was interpreted to show both that different neuroanatomical pathways encode for distant versus recent memories and that memories become more stable with the passage of time (Scoville & Milner, 1957; Wiig *et al.*, 1996; Woodward *et al.*, 2007). H.M. showed a distinct dissociation between types of memory as his recognition memory was greatly impaired, but his immediate working memory, recall of distant events, and skill learning were all intact following the surgery. This dissociation between impairment in memory types was interpreted as showing that specific brain areas are responsible for different types of memory.

In later years, magnetic resonance imaging (MRI) studies showed that the procedure had removed the amygdala, the medial temporal cortex, and the entire entorhinal cortex, and only portions of the hippocampus. The rhinal cortices are responsible for projecting sensory information, and other input from higher-order cortices, to the hippocampus (Wiig & Bilkey, 1995; Glenn & Mumby, 1996). Removing the entire entorhinal cortex would therefore leave the hippocampal formation devoid of its input (Corkin *et al.*, 1997; Corkin, 2002). Continued research into the result of hippocampal pathology has revealed that many of the serious amnesic symptoms actually arise from damage to the areas that project to the hippocampus as opposed to the hippocampus proper. These portions of the hippocampal formation, particularly the entorhinal cortex, appear as targets in other neurological pathologies including Alzheimer's disease (Johnston & Amaral, 2007).

Amygdalo-hippocampal lesions are a good model of medial-temporal-lobe amnesia in humans due to similar memory deficits. These deficits can be shown by delayed nonmatch to sample (DNMS) tests on memory in animals, as these tests are similar to human recognition-memory tasks by making success contingent upon differentiating between novel and previously presented items (Squire *et al.*, 2007). Animals with amygdalo-hippocampal lesions show great deficits on DNMS. The greatest deficits occur following lesions either to the perirhinal and/ or the entorhinal cortex (Mishkin, 1978). Single-trial learning, long-term storage (>24 hr) of information, and high information storage capacity are all related to activity within the perirhinal cortex (Brown & Aggleton, 2001). Greater recognition memory deficits thus appear to result from trauma to the neocortical areas projecting to the hippocampus, including the perirhinal cortex, rather than to the hippocampus proper (Squire *et al.*, 2007).

Stroke

The Tragedy

Every year about 10% of all deaths worldwide are caused by stroke. Up to 170, 000 people within the United States will die from a stroke this year (CDC, 2008). The tragedy of stroke extends far beyond the death toll, as three times more people will be left debilitated from a stroke as will die, making stroke a leading cause of long-term disability. The disabilities resulting from stroke make dealing with patients extremely costly. In combined direct and indirect costs, strokes will cost almost \$68.9 billion in 2009 (Lloyd-Jones *et al.*, 2009).

Definition of Stroke

Stroke is a cerebrovascular accident, or an acute neurological disorder, specifically caused by a disturbance in the cerebral blood supply (Ter Horst & Postigo, 1997). A stroke is further defined by the World Health Organization as a “disturbance of cerebral function, lasting more than 24h or leading to death, with the only apparent cause being vascular malfunction” (Hatano, 1976, p.541). A major risk factor for stroke-induced neurological dysfunction is a transient ischemic attack (TIA). A TIA involves a sudden focal loss of neurological function due to inadequate perfusion from the carotid and/or vertebrobasilar arteries, with complete recovery in 24 hrs (Flaherty & Brown, 2004; Muir & Santosh, 2005). TIAs can be a warning of a more serious and debilitating stroke (Lovett *et al.*, 2003).

Strokes can be classified as hemorrhagic or ischemic. A hemorrhagic stroke is caused by rupture(s) in blood vessel(s) allowing bleeding in the brain. Cerebral ischemia is a stroke caused by an interruption of blood flow to the brain of a sufficient degree to cause symptomatic disruption of normal functioning (Lee *et al.*, 2004). In Western countries, 80% to 85% of strokes among adults are ischemic, while only 55% of strokes among youth are ischemic (Roach *et al.*, 2008). Cerebral ischemia is further classified by completeness, duration, and spatial extent of blood interruption (Tamura, Kawai, & Takagi, 1997). The most common cause of focal cerebral ischemia is a thrombus (i.e. a blood clot formed in the heart or blood vessel) blocking the blood supply to the brain. Animal models of focal ischemia often involve occlusion of the internal carotid or middle carotid artery. Irreversible neuronal death occurs in the ischemic core due to a severe deficit in blood perfusion.

The penumbra is the area where neurons suffer functional but not structural injury. Peripheral blood supply continues to supply oxygen and glucose, allowing cells in the penumbra to remain viable for a longer period of time (Lee, Nassief, & Hsu, 2004). Collateral blood flow is increased by vasodilation of blood vessels adjacent to the dysfunctional vessels. Duration of ischemia increases the extent of cellular dysfunction within the ischemic penumbra. However, after a period of 1.5-4 hrs, reperfusion increases the severity of ischemic damage relative to a permanent loss of blood supply. The therapeutic window is the time where interventional treatments can be given to the ischemic brain before reperfusion increases injury level (Tamura, Kawai, & Takagi, 1997).

Global ischemia is caused by diminished blood or oxygen circulation throughout the entire brain. Heart surgery and myocardial infarctions are common causes of hypoperfusion (Cronin *et al.*, 2001). Similarly to TIAs, these events increase stroke risk and risk of death in the event of stroke. In the first month following a myocardial attack, patients have a 44-fold increased risk of ischemic stroke, and risk remains drastically increased for at least three years (Witt *et al.*, 2005; Dutta *et al.*, 2006).

Persistent cerebral impairment is often found following TIAs and ischemia (Bradvik *et al.*, 1989; Godefroy *et al.*, 1994). Patients with no initial mental impairments following stroke are still found to have significantly more neurological impairment than controls for 1 to 2 years following stroke (Bokura & Robinson, 1997). The most common neurological impairments following stroke are in motor movements and coordination, sensation, vision, language, and general cognition (Kelly-Hayes *et al.*, 1998). Changes in affect frequently follow stroke. Onset

of depression is the most common change in affect as at least one third of all stroke survivors suffer from post-stroke depression (Hackett *et al.*, 2005).

Cardioplegic arrest, associated with all the risks and damage of an ischemia-reperfusion injury, is an essential component of pulmonary bypass surgery (de Lange *et al.*, 2008). A coronary artery bypass graft is similarly linked with severe cerebral morbidity. Postoperative neuropsychological impairments have been found in 79% of patients, and in at least 35% of the patients (El-Batrawy *et al.*, 2006; Shaw *et al.*, 1986, 1987). With an effective understanding of pharmaceutical interventions during transient global ischemia, we should be able to prevent neurological damage following bypass grafts.

The Brain's Blood Supply

Four main vessels are responsible for the brain's blood supply: the paired common carotid arteries and the paired vertebral arteries. The common carotid arteries split into a pair of internal carotid arteries and a pair of external carotid arteries. The external carotid arteries supply the face and scalp with blood. The internal carotid arteries enter the skull base and then branch into many smaller arteries responsible for supplying the anterior three-fifths of the cerebrum with blood with the exceptions of the occipital lobes and parts of the temporal lobes. Decreasing blood flow through the internal carotid artery impairs brain regions responsible for motor, sensory, language, visuospatial, and cognitive/executive functions. Approximately 80% of strokes affect anterior circulation (Muir, Gamzu, & Lees, 1997).

The paired vertebral arteries unite on the ventral surface of the brainstem to form the basilar artery. Terminal branches from the basilar artery supply the brainstem, cerebellum, and the posterior two-fifths of the cerebrum with blood (Pulsinelli & Brierly, 1979; Baird *et*

al., 2004). Occlusion of the vertebral arteries results in serious consequences ranging from blindness to paralysis. Strokes affecting the brainstem or cerebellum are caused by basilar occlusion. Due to the nature of these structures, this type of stroke quickly results in very severe situations including death and paralysis (Goldberg, 1997).

The Circle of Willis is a ring of communicating arteries that connect the basilar artery to the internal carotid arteries at the base of the brain. Arteries arising from the Circle of Willis supply blood to the rest of the brain. The Circle of Willis provides a safety mechanism so the entire brain can remain perfused with blood if an artery malfunctions. In patients with a fully functional Circle of Willis, the connective vessels will dilate, preventing a drop in blood flow below 50% of regular blood flow (Powers, 1991). Patients that do not show vessel dilation and an increase in collateral blood flow during stroke show neurological damage (Hendrikse *et al.*, 2001).

Animal Models of Ischemia

Animal models are imperative to understanding and treating stroke pathophysiology due to its high complexity. More simplistic *in vitro* models were used in explicating cellular and sub-cellular pathways responsible for ischemic cell death. Glutamate, the main excitatory neurotransmitter, is used throughout the hippocampus. Ischemic cell death is glutamate-dependent, as shown in cultured groups of neurons dying during anoxic conditions following intercellular communication through glutamate release. For example, cultured neurons were used to demonstrate the Ca^{++} dependency of excitotoxicity (Choi, 1985). Cultured groups of neurons were used to show that cell dysfunction occurs through

intercellular communication pathways, with glutamate shown to be the molecule carrying the pathologic signal (Rothman, 1983). Cultures containing both neurons and glial cells were used to show that astrocytes can offer protection during anoxic conditions (Vibulsreth *et al.*, 1987). Post-ischemia cultures of entire brain slices have also been used, specifically to monitor the relationship between membrane integrity and phospholipid activity (Seren *et al.*, 1989). *In vitro* stroke models have limited utility, as they cannot be used to investigate interactions of the entire neurovascular unit following stroke or the behavioral effects of stroke.

Several animal species including primates, cats, dogs, rabbits, rats, gerbils, and mice are candidate hosts for both focal and global ischemia models. Although the basic biology of cerebral ischemia remains fairly consistent between species, there are several important species differences. Cerebral energy metabolism and cerebral blood flow in mammals is inversely related to body weight. For example, the glucose and oxygen metabolism of the rat is three times that of the human (Dirangl *et al.*, 1999). There is also interspecies variation in anastomosis. While gerbils lack communicating anastomosis between arteries, rats have more effective collaterals between large cerebral vessels than humans (Tagaya *et al.*, 1997). Nonetheless, rats are the most widely used model for stroke due to the availability of pure strains along with detailed anatomical and neurochemical data (Menzies *et al.*, 1992). Rats are also relatively inexpensive, their circulation is similar to that of humans, and they have reliable responses to ischemic insult (Coyle, 1975; Yamori *et al.*, 1976; Chen *et al.*, 1986; Tamura, Kawai, & Takagi, 1997).

Global ischemia models vary widely. Decapitation is the easiest method of inducing global cerebral ischemia. While this model is useful for studying morphological and biochemical changes during very early phases of ischemia, its utility is limited by the impossibility of reintroducing proper perfusion (Xie *et al.*, 1995). Induction of cardiac arrest is useful for evaluation of post-resuscitation encephalopathy, but the utility of this model is limited by mortality rates and difficulty of reperfusion (Kawai *et al.*, 1992). Profound systemic hypotension results in incomplete, but almost global, ischemia. This model results in variability in brain damage relative to the previous models as time required for full resuscitation is more predictable (Tamura *et al.*, 1997).

Transient global ischemia can be induced by cervical compression with a high-pressure neck cuff to increase intracranial pressure above systolic arterial pressure (Ljunggren *et al.*, 1974; Ross & Duraime, 1989). However, the effects of compressing various cervical nerves and ganglia remain unknown (Nemoto *et al.*, 1977, 1981). Surgical occlusion of a combination of major arteries causes global cerebral ischemia without myocardial injury and has been performed in many animals including rabbits, cats, dogs, and monkeys (Miller *et al.*, 1980; Kowada *et al.*, 1968; Tamura, Kawai, & Takagi, 1997).

Forebrain ischemia accurately mimics many of the deficits found in global ischemia. Meanwhile, the residual hindbrain blood flow eases resuscitation and generally does not create the respiratory and systemic circulatory encephalopathy that follows other global cerebral ischemia models. Rodent models of forebrain ischemia were used in the basic research elucidating the underlying biochemical mechanisms of cellular dysfunction during anoxia. For example, this model was used to explicate how excitatory amino acids induce

changes in signal transduction pathways and thus rapidly alter gene expression and protein synthesis (Gill *et al.*, 1987; Hara *et al.*, 1990a, b; Lee *et al.*, 1991; Nowak, 1991; Abe *et al.*, 1993; Widmann *et al.*, 1991). These models were also used to elucidate the role of free radicals and nitric oxide in modulation of cellular dysfunction (Siesjo *et al.*, 1989) along with the protective effects of neurotrophic factors (Shigeno *et al.*, 1991).

The four-vessel occlusion (4-VO) rat model of forebrain ischemia involves occlusion of both common carotid arteries following electrocoagulation of both vertebral arteries (Pulsinelli & Brierly, 1979; Pulsinelli & Buchan, 1988). Drawbacks to this model include difficulty in confirming electrocauterization of the vertebral arteries and the presence of collateral blood flow pathways that mainly arise from the anterior spinal artery (Furrow, 1982; Pulsinelli, Levy, & Duffy, 1983).

Bilateral common carotid artery occlusion with systemic hypotension is an accepted two vessel occlusion model for rats and mice (Elk f & Siesj , 1972). Blood pressure is dropped by venous exsanguination and ischemia induced with a two vessel occlusion. Researchers used this model to determine the susceptibility of brain regions to anoxic dysfunction. The dentate gyrus, CA1 hippocampal region, and the subiculum are most susceptible to ischemic damage, with disruption of cell function beginning within two minutes of ischemic onset. These areas were followed by the neocortex, with cell dysfunction showing after four min, the CA3 hippocampal region at six min, and the caudoputamen at 8-10 min (Smith *et al.*, 1984a, b; Johnston & Amaral, 2004). This ranking of brain areas has facilitated understanding of the effects of external factors such as temperature and hyperglycemia on ischemic damage (Minamisawa, Smith, & Siesj , 1990; Warner *et al.*, 1987; Smith *et al.*, 1988).

Bilateral common carotid artery occlusion alone does not consistently produce ischemic changes in normotensive rats (NTR) due to dilation of collateral vessels. However, the procedure does produce ischemic changes in the brain of the spontaneously hypertensive rat (SHR), as these rats have vascular changes similar to those that are secondary to hypertension and operative in cerebrovascular diseases (Fujishima *et al.*, 1976, Ogata *et al.*, 1976). When the carotid arteries are clamped in the NTR, blood flow drops to 50% of the normal blood flow while SHR have a much greater drop in blood flow as collateral blood vessels have a reduced diameter and are unable to dilate (Coyle, 1987; Choki *et al.*, 1977). The SHR's altered vascular state is thought to arise from an increased level of the mineralcorticoid aldosterone in circulation (Endemann *et al.*, 2004).

Levine and Payan (1966) asserted that the gerbil offers distinct advantages over other animal models in ischemic research due to its susceptibility to brain damage after a unilateral common carotid artery occlusion. An absence of anastomosis between the vertebral and internal carotid circulation in gerbils allows forebrain ischemia to be induced by occluding the common carotid arteries. The SHR and gerbil models have similar damage profiles consisting of damage to the caudoputamen and the hippocampus (Kirino, 1982).

The gerbil model is especially important in evaluating delayed neuronal death in the hippocampus (Nitatori *et al.*, 1995). Similarly to the damage seen in human global ischemia, no morphological changes are detectable in the CA1 region 1 dy following a 5 min bilateral carotid artery occlusion, but changes are evident by 3 days following ischemia. The gerbil model was also used to understand the phenomena of stroke preconditioning. A brief (2-min) induction of ischemia a few days prior to a thorough ischemic insult reduces the

severity of ischemic damage to the hippocampal CA1 neurons following the more severe ischemic insult (Kitagawa *et al.*, 1990; Kirino *et al.*, 1991).

In young gerbils communication exists between the arteries (Matsuyama, *et al.*, 1983); so to ensure a level of physical development, gerbils weighing more than 60g are generally used. There is some interanimal variability in the severity of ischemia induced by bilateral occlusion of the common carotid arteries due to some variability in the existence of carotid-basilar and anterior communicating anastomoses along with variability in the extent of collateral microcirculation (Yanagihara, 1978).

Ischemic Cell Death: Cascades and Neuroprotection

Treatment of cerebral ischemia offers a special challenge due to the extreme vulnerability of the brain to anoxic conditions. Five mins of global ischemia causes cell death within the brain while 20-40 mins of oxygen deprivation is necessary to cause cell death in cardiac myocytes or kidney cells. This vulnerability is partially due to the brain's high metabolic rate; the brain represents about 2.5% of body weight, but accounts for 25% of one's basal metabolism (Lee *et al.*, 2000). A lack of stored energy in the brain means that a constant supply of glucose and oxygen is necessary for the mitochondrial respiratory chain to form ATP via oxidative phosphorylation. ATP drives membrane ion pumps and powers homeostatic mechanisms (Ter Horst & Postigo, 1997). Neurons pump ions against the electrochemical gradient for efficient and rapid intracellular signaling; neuronal functioning thus relies on ion homeostasis and ATP-powered pumps.

In the absence of oxygen, small amounts of ATP can be produced from anaerobic glycolysis. The anaerobic glycolysis reaction produces H^+ as a side product, rapidly dropping the brain's pH. The acidic environment exacerbates brain injury. The combination of high metabolic need and a lack of energy reserves cause the intrinsic inter- and intra-cellular mechanisms to become harmful during anoxic conditions. Within secs of the onset of cerebral ischemia, a shutdown of neural activity occurs as neurons hyperpolarize first by releasing K^+ passively, followed by K^+ release through ATP-powered channels (Mourre *et al.*, 1989; Lee *et al.*, 2000). In ischemic conditions, a sustained drop in intracellular K^+ levels is associated with delayed cell death. This may, however, be a symptom of decreased function of Na^+/K^+ -ATPase (Gribkoff *et al.*, 2001; Obrenovitch, 2008).

Excitotoxicity

Within mins of ischemic onset, the membrane gradient is lost due to a lack of energy. Anoxic depolarization causes an excessive release of neurotransmitters (Dawson & Dawson, 2004; Goldberg, 1997; Crack & Taylor, 2005; Lee *et al.*, 2000). Glutamate, the primary excitatory neurotransmitter, is released during anoxic depolarization, inducing the excitation of nearby neurons (Rothman, 1985; Dessi *et al.*, 1994). Cell membranes become leaky allowing a massive influx of Na^+ and Cl^- resulting in cell swelling (Mongin, 2007; Choi, 1988a, b; Rothman, 1986). Cellular edema is an early consequence of stroke and can be visualized by MRI, allowing for clear identification of a stroked brain (Dirnagl *et al.*, 1999).

Astrocytic glutamate/cysteine transporters and the malfunctioning neuronal glutamate transporters perpetuate glutamatergic excitotoxicity. Astrocytes can produce the neuroprotective antioxidant glutathione when the glutathione precursor, cysteine, is present.

Cysteine is pumped into the cell by a glutamate/cysteine pump that exchanges intracellular glutamate for extracellular cysteine. Astrocytes increase the activity of the glutamate/cysteine pump under ischemic conditions, thus increasing extracellular glutamate and excitotoxicity (Ye *et al.*, 1999). Anoxic conditions also reduce the activity of the transporters responsible for removing glutamate from the extracellular space, further perpetuating excitotoxicity (Romera *et al.*, 2007).

High levels of extracellular glutamate cause glutamatergic ionotropic receptors, including the alpha-amino-3-hydroxy-5-methyl-isoxazolepropionic acid (AMPA)-type and the N-methyl D-aspartate (NMDA)-type receptors, to open. Treatment with certain glutamate antagonists offers neuroprotection in transient global ischemia models (Rod *et al.*, 1990; Shuaib *et al.*, 1996b; Shuaib *et al.*, 1995; Owen *et al.*, 1997). Excessive amounts of Ca^{++} enter the cell through ionotropic channels (McCulloch, 1992; Wong *et al.*, 1986). Activation of metabotropic glutamate receptors increases cytosolic $[\text{Ca}^{++}]$ by mediating release of intracellular Ca^{++} from stores within the endoplasmic reticulum. Excess extracellular glutamate thus causes a dramatic rise in cytosolic Ca^{++} levels (Mattson, 2008).

It is clear that the rapid and sustained elevation of intracellular Ca^{++} concentrations underlie many of the devastating processes of ischemic stroke by activating degradation processes that induce cell death. A variety of intracellular enzymes are activated by Ca^{++} including proteases, lipases, and nucleases. These proteins in turn cause the degradation of membranes, proteins, and nucleotide strings. Calcium activates transcription factors, including nuclear factor- κB (NF- κB), which upregulates the expression of proteins that perpetuate many of the cell's deleterious reactions to ischemic conditions (Mattson, 2008).

Kinases of interest for stroke treatment include Calcium-activated kinase (CAMK), protein kinase C (PKC) and the mitogen-activated protein kinase (MAPK) family (Van Elzen *et al.*, 2008). CAMKII increases the permeability of NMDA receptors to Ca^{++} following stroke (Xu, 1997); decreasing CAMKII activity reduces the amount of Ca^{++} that enters the cell through NMDA receptors (Xu, 2008). CAMK also phosphorylates cAMP response element binding protein (CREB) and modulates the expression of numerous genes (Lutein *et al.*, 1997). The MAPK family transduces signals to the nucleus. While activating one MAPK subtype protects the cell from death, the activation of other members causes increased expression of inflammatory cytokines that promote cell death (Van Elzen *et al.*, 2008).

NMDA receptor antagonists are effective in preventing neural damage in animal models of focal brain ischemia (Dirnagl *et al.*, 1999). Inhibition of neuronal nitric oxide synthase (nNOS), renders cultured neurons resistant to NMDA-induced death (Samdani *et al.*, 1997; Lee *et al.*, 2000). NMDA and AMPA antagonists have been shown to offer neuroprotection when applied before ischemia in models of transient global ischemia (Shuaib *et al.*, 1995; Wiard *et al.*; 1995; Block & Schwartz, 1996a, 1996b; Block, Schmidt, & Shwartz, 1996; Block, Pergande, & Schwarz, 1997; Owen *et al.*, 1997). NMDA receptor antagonists do not offer the same level of neuroprotection in animal models of transient global ischemia as offered in focal ischemic models (Mrsic-Pelcic *et al.*, 2002; Lee *et al.*, 2000).

During transient global ischemia a shift from NMDA to AMPA-facilitated cell death appears to occur (Lee *et al.*, 2000). Multiple reasons for this change have been proposed including the acidity of the brain during global ischemia, but not during focal ischemia due to the maintenance of partial perfusion. Also the makeup of AMPA channels shifts

following global ischemia onset, increasing their permeability to Ca^{++} while making them nonselective against Zn^{++} (Lee *et al.*, 2000).

A substantial pool of Zn^{++} is localized to synaptic vesicles in glutamatergic nerve terminals and is released with glutamate in depolarization. Thus, Zn^{++} becomes available in the extracellular space during anoxic depolarization. High extracellular Zn^{++} levels decrease NMDA conductance while increasing AMPA conductance (Weiss *et al.*, 1993). When taken into the cell, Zn^{++} is thought to promote neural damage by multiple mechanisms including the generation of reactive oxygen species (ROS). During ischemia, Zn^{++} thus is pathological by accelerating ion influx and neurotoxic effects upon cell entry (Sorenson *et al.*, 1998).

Astrocytes become activated during ischemia, causing them to release pro-inflammatory cytokines including tumor necrosis factor- α (TNF α) and interleukin-1 β (IL-1 β). These agents increase neuronal excitability by increasing the conductivity of AMPA and NMDA receptors (Milligan & Watkins, 2009). The presence of dopamine in the extracellular space is necessary for glutamate damage (Globus *et al.*, 1988). High extracellular dopamine levels may increase excitotoxicity by many methods including increasing oxidation damage, inhibiting sodium/potassium adenosine triphosphatase ($\text{Na}^{+}/\text{K}^{+}$ ATPase), by uncoupling glucose metabolism from cerebral blood flow, and/or by enhancing glutamate receptor currents, thus increasing excitotoxicity (Toner & Stamford, 1996; Milton & Lutz, 1998; Shih *et al.*, 2007; Zhang *et al.*, 2008). Serotonin, GABA, and adenosine, can all mitigate glutamate-induced cell death (Obrenovitch & Richards, 1995). Preventing GABA degradation has neuroprotective effects during transient global ischemia (Shuaib *et al.*, 1996a). Several drugs have been attenuate excitotoxicity indirectly by limiting neuronal depolarization by blocking

Na⁺ channels, mimicking the inhibitory/ hyperpolarizing actions of GABA, or reducing Ca⁺⁺ influx into neurons (Block, 1999).

The actions of increased intracellular Ca⁺⁺ ions are another common target for neuroprotection during an ischemic insult. Blocking Ca⁺⁺ channels protects neurons (Block *et al.*, 1990). Adenosine agonists prevent deleterious effects of Ca⁺⁺ in global ischemia models (Rudolphi *et al.*, 1992; Von Lubitz *et al.*, 1996a, 1996b). Pre-treatment with a PKC inhibitor reduces neuronal damage in the hippocampus and attenuates the impairment of working memory following global ischemia (Hara *et al.*, 1990a, b; Ohno *et al.*, 1991).

Apoptosis vs Necrosis

Cell death can be categorized by whether or not it is purposeful and programmed. Apoptosis is cell death that proceeds through a series of carefully orchestrated events involving several genes. Cells and organelles shrink, the chromosomes condense, the DNA fragments, and the cell disintegrates into membrane-bound bodies. These bodies can be phagocytosed by neighboring cells, recycling the cell's contents (Wyllie *et al.*, 1972; Walker *et al.*, 1988; Hengartner, 2000; Shi, 2001).

Necrosis is the type of cell death that occurs when cells are exposed to adverse conditions that exceed the buffering capacity of the cell's protective systems. This irreversibly compromises homeostatic mechanisms and extensively damages the cell. The morphological features of necrosis are markedly different from those of apoptosis (Kerr *et al.*, 1972; Clarke, 1990). These include swelling of the mitochondria and endoplasmic reticulum, vacuolation of the cytoplasm, loss of a chromosome pattern, karyolysis (disintegration of the nucleus), and eventual cell lysis without the formation of vesicles (Leist & Jaattela, 2001).

The release of cellular contents following necrosis often damages neighboring cells and induces inflammation (Majno & Joris, 1995).

The loss of oxygen and essential nutrients that occurs during ischemia is an example of the extreme stressors that induce necrotic cell death (Syntichaki & Tavernarakis, 2003). However, ischemia-induced cell death shows features of both apoptosis and necrosis (Martin *et al.*, 1998; Nicotera *et al.*, 1999; Nicotera, 2002). Paraptosis is a term for an intermediary cell death type similar to necrosis in that there is no chromatin condensation and there is extensive vacuolation, but paraptosis resembles apoptosis by requiring *de novo* protein synthesis (Sperandio *et al.*, 2000; Wyllie & Golstein, 2001). The method of ischemic cell death along a continuum between necrosis on one end and apoptosis appears to be determined by the intensity of the ischemic insult (Pagnussat *et al.*, 2007). The insult's intensity is defined by the extent of increase in cytosolic levels of Ca^{++} and Zn^{++} above homeostatic levels (Lobner *et al.*, 2000; Jiang *et al.*, 2001). Caspase proteins are a key feature of apoptosis, but also play a role in necrosis as excess intracellular Ca^{++} elicits mitochondrial damage, causing cytochrome *c* release, and caspase activation (Jiang *et al.*, 2001; Zhu *et al.*, 2000). Reducing Ca^{++} efflux from intracellular stores protects from necrotic cell death (Yu *et al.*, 2000).

Excitotoxic conditions increase mitochondrial production of reactive oxygen species and cytotoxic proteins, which then facilitate mitochondrial transition pore formation.

Mitochondrial transition pore opening releases apoptosis-inducing factors into the cell, including superoxide ions, Ca^{++} , and soluble proteins such as cytochrome-*c*. Mitochondrial transition pore opening and caspase activation are markers of cellular dysfunction during

ischemia and interfering with these pathways prevents cell death (Uryu *et al.*, 2002; Chan, 2001). Preconditioning experiments showed that protecting mitochondrial integrity is highly associated with cell survival following ischemia (Keller *et al.*, 1998; Racay *et al.*, 2007; Iijima *et al.*, 2008).

Apoptosis can be induced by certain proteins including Bax and Bak from the Bcl-2 family, which induce cytochrome-*c* release from mitochondria (Franke *et al.*, 2007). Bcl-2 is an antiapoptosis molecule that prevents the actions of Bax and thereby limits mitochondrial superoxide production, mitochondrial transition pore formation, and cytochrome-*c* activation of the caspase apoptosis cascade (Chan *et al.*, 2001; Franke *et al.*, 2007). Upregulation of Bcl-2 suppresses ischemic cell death via mitochondria-mediated mechanisms (Asoh *et al.*, 2002, Mattson & Kroemer, 2003). Once in the cell's cytosol, cytochrome-*c* activates caspases, a protein family key in apoptosis (Endres *et al.*, 1997). Caspase inhibitors are neuroprotective during ischemia reperfusion injury (Ko *et al.*, 2008). The binding of tumor necrosis factor α (TNF α) to TNF death receptors is an initiating factor for the caspase apoptotic pathway; interfering with TNF α binding death receptors confers neuroprotection (Ko *et al.*, 2008).

Oxidation-Induced Damage

A free radical is any chemical species that contains one or more unpaired electrons. Free radicals are oxidizing agents, meaning they cause other molecules to lose electrons, and in stealing electrons from other molecules cause cellular damage. Oxidation occurs when a molecule loses an electron. The most common cellular free radicals are hydroxyl radicals, (OH $\cdot$), superoxide radicals (O $_2^{\cdot-}$), and nitric oxide (NO $\cdot$). Other cellular oxidizing agents, such

as hydrogen peroxide (H_2O_2) and peroxynitrate (ONOO), are also harmful as they oxidize other molecules and generate free radicals in the process. Strong oxidizing agents and free radicals are classified together as reactive oxygen species (ROS) to signify their ability to promote oxidative changes within the cell (Simonian & Coyle, 1996).

Generation of ROS during cerebral ischemia/reperfusion injury induces the expression of cytokines, adhesion molecules, and enzymes involved in the inflammatory response (Dirnagl *et al.*, 2003). Postischemic reperfusion causes tissue damage in excess of that caused by ischemia alone, probably through overwhelming endogenous antioxidant defenses with excessive quantities of oxidants and reactive oxygen species (ROS). As the reoxygenation process re-introduces oxygen, this presence provides oxygen as a substrate for numerous enzymatic oxidation reactions that increase the levels of ROS and oxidative stress (Chan, 1996; Chan *et al.*, 2001)). In addition, reflow after occlusion often increases oxygen levels that which mitochondria can utilize under normal physiological conditions; excessive oxygen allow for increased oxidative stress (Nita *et al.*, 2001).

There are many sources that generate ROS in the ischemic brain including peroxidant enzymes and the mitochondria. The inactivation of detoxification systems, the consumption of previously available antioxidants, and the underproduction of new antioxidants all prevent antioxidant actions (Nita *et al.*, 2001; Chan *et al.*, 2001). Oxidative stress occurs when ROS production exceeds ROS elimination by the body's natural antioxidant defense system and results in cellular damage and apoptosis. Although the brain has high levels of endogenous antioxidant enzymes and compounds, they are inadequate to sequester the amount of oxidants generated during ischemia.

The brain's antioxidant enzymes include superoxide dismutase (SOD), catalase, and peroxidase, as well as some support enzymes. SOD is responsible for creating hydrogen peroxide (H_2O_2) from superoxide radicals and protons. Catalase breaks H_2O_2 and into the oxygen and water (Girnun *et al.*, 2002). Thus, the two enzymes work together to make inert substances from superoxide. The brain contains several hundred different antioxidants, but only a few of these molecules are synthesized by the body. The antioxidant glutathione is synthesized by astrocytes. The astrocytes' attempts to increase glutathione synthesis increases extracellular glutamate levels through the glutamate/cysteine exchange.

The brain is thought be especially prone to oxidant damage due to limited means of mitigating oxidant-induced damage, as the brain has only moderate amounts of catalase, superoxide dismutase (SOD) and glutathione peroxidase. Also certain are of the brain are rich in iron ions, which are released from injured cells or from bleeding in the reperfused area. Iron ions enhance lipid peroxidation. The brain has high concentrations of polyunsaturated fatty acid side-chains attached to membrane lipids; polyunsaturated side-chains are quickly oxidized by free radicals. Membrane alteration by ROS is thought to be partially responsible for delayed neuronal injury following global cerebral ischemia (Nita *et al.*, 2001).

The brain also contains pro-oxidant enzymes that increase oxidative stress during cerebral ischemia. These enzymes can be categorized by the oxidant they produce. Superoxide is produced by cyclooxygenase type 1 and 2 (COX-1 and COX-2), NADPH oxidase, and xanthine oxidase (Chan *et al.*, 2001). Nitric oxide (NO) is produced by the three NO synthases, neuronal nitric oxide synthase (nNOS), inducible nitric oxide synthase

(iNOS), and endothelial nitric oxide synthase (eNOS). NO produced by iNOS enhances COX-2 activity in the ischemic brain, which in turn increases COX-2 mediated production of pro-inflammatory substances and ROS (Nogawa *et al.*, 1998). Both nNOS and iNOS are harmful during ischemia, as superoxide radicals react with NO to create the strong and stable oxidant peroxynitrate. This process is linked to cellular damage, including increased DNA damage and consequential activation of the repair enzyme poly ADP-ribose polymerase (PARP), which further depletes cellular energy stores. Inhibiting iNOS decreases infarct volume when given 24 hrs following focal ischemia. A viscous cycle occurs as many of the cellular responses triggered during ischemic injury promulgate ROS formation, which then induces increased damage and increased ROS formation. An example of this occurs when Ca^{++} stimulates phospholipases that then break down lipids, creating oxidated lipoproteins, which then extend the activity of the cysteine/glutamate transporter, and further facilitating excitotoxicity-induced damage.

The angiotensin 11 receptor is a mineralcorticoid receptor (MR) that is agonized by the mineralcorticoid Angiotensin II (AtII). AtII binding with At1r increases free radical generation in the ischemic brain (Wassman *et al.*, 2001; Cimino *et al.*, 2007). At1r activation also increases the inflammatory response by recruiting immune cells to the central nervous system (CNS) through upregulating cytokines while increasing adhesion protein expression so immune cells are retained within the CNS (Ishikawa *et al.*, 2007). Astrocytes infiltrating the ischemic area upregulate At1r expression in response to cytokines, which then increases production of harmful products (Yoshida *et al.*, 2006). The blood-brain-barrier becomes leaky during ischemia, allowing increased levels of mineralcorticoids including angiotensin

following ischemia. Neuroprotection is offered by preventing the upregulation of MRs, downregulating MRs, or antagonizing MRs (Oyamada *et al.*, 2008; Rajagopalan *et al.*, 2002).

ROS affect the entire neurovascular unit. Very soon after the onset of ischemia in animals, ROS are generated at the interface between the microvessels and the brain tissues (Kontos, 1992). Treatment with free radical scavengers both preceding and following transient global ischemia limits neurological and learning deficits (Block *et al.*, 1995, Wright *et al.*, 1996; Lees *et al.*, 2006). Reducing expression and activation of receptors that activate ROS-generating enzymes reduce overall ROS levels. Increasing the expression of SOD reduced cell death in the CA1 region of the hippocampus is decreased by 50% following global ischemia (Murakami *et al.*, 1997, 1998; Chan *et al.*, 1998).

The Neurovascular Unit

For many years biomedical researchers have looked for neuroprotective agents that function on the neuron by limiting excitotoxicity, elevated intracellular Ca^{++} concentrations, free radical generation, and so forth. While many agents have been identified that decrease injury to cultured neurons or animal models of ischemia, a treatment that protects stroke patients from neural damage has still remained elusive (del Zoppo, 2006). However, neurons constitute less than five percent of the cells involved in ischemia and ischemic stroke is a vascular disorder that affects neural functioning. In July of 2001, the National Institutes of Neurological Disorders and Stroke (NINDS) convened the Stroke Program Review group to come to a consensus as to the direction research should proceed to limit damage caused by stroke. It was agreed that research should focus on the dynamic interactions between the endothelium, vascular smooth muscle, astroglia and microglia, neurons, and associated tissue

proteins (NINDS, 2002). The unit of focus for stroke treatment has thus shifted from the individual neurons to the entire neurovascular unit. It is hoped that including the entire neurovascular unit in treatment will allow for the discovery and implementation of treatments that mitigate neuronal damage caused by stroke. (Hawkins & Davis, 2005; Allan, 2006; Guo & Lo, 2009).

The devascularization that occurs during cerebral ischemia can alone induce apoptosis and cell death (Lee *et al.*, 2004). Microvessels that supply oxygen and nutrients respond as rapidly as neurons to an ischemic insult (Mabuchi *et al.*, 2005). Research regarding neuroprotection now focuses on understanding and mitigating the ischemic initiation of inflammation, the increase of microvascular permeability, the production of tissue edema, and the causes of local hemorrhage (del Zoppo, 2006).

Astrocytes undergo greater proteomic changes than neurons, greatly increasing their expression of glycolytic and pro-inflammatory proteins (Van Elzen, 2008). Activated astrocytes support tissue repair processes by removing debris and secreting a number of neurotrophic factors. Simultaneously, astrocytes can suppress axon growth and repair by releasing various cytotoxic mediators such as ROS and inflammatory cytokines, including iNOS-generated NO, TNF α , IL-1, 6, and 8 (Ho & Blum, 1997; Oyamada *et al.*, 2008). Therefore, whether astrocytes are ultimately beneficial or harmful during ischemia is unclear and may be dependent upon the current environmental signals.

The brain contains two major groups of proteases, the metallomatrix proteins (MMPs) and the plasminogen activators (PAs). Tissue plasminogen activator (tPA) is currently the only prescribable stroke treatment, and it can only lyse stroke-inducing clots by breaking

down the proteins that hold together blood clots (Newby, 2005). Under normal circumstances, the MMP and PA systems modulate the extracellular matrix to allow neurite outgrowth, neural plasticity, and cell migration. MMPs are highly expressed throughout the developing brain. In the adult brain, MMP expression is generally limited to areas important to learning and memory, including the hippocampus (Kaczmarek *et al.*, 2002; Oliveira-Silva *et al.*, 2007).

MMP expression and activation is vastly increased in activated astrocytes and microglia following ischemic injury (Rosenberg *et al.*, 2001, 2002; Kelly *et al.*, 2006). Excessive MMP activity following ischemia breaks down tight junction proteins and the intercellular matrix, thereby creating holes in the endothelium and disrupting the blood-brain barrier (Newby, 2005; Moraes *et al.*, 2006). MMP activity instigates anoikis, which is cell death due to detachment of the cellular membrane from the extracellular matrix, and is at least partially responsible for glial cell and delayed neuronal cell death found in ischemia/ reperfusion injury (Lee *et al.*, 2004; Magnoni *et al.*, 2004). MMPs may also directly; Michaluk & Kaczmarek, 2007). MMPs may also induce apoptosis by influencing cytochrome *c* release, activating cytokines, and increasing the release of pro-apoptotic ligands (Copin *et al.*, 2005; Amantea *et al.*, 2008). Holes in blood vessels from MMP activity can cause a loss of microvascular integrity and can cause hemorrhagic transformation upon reperfusion (Hamann *et al.*, 1996; Heo *et al.*, 1999).

The role of tPA in ischemic dysfunction is less clear-cut. Similar to MMPs, PA exacerbates neurotoxicity, can increase vascular permeability, and can induce hemorrhagic transformation. PA knockout mice are protected against excitotoxic injury (Yepes *et al.*,

2003). The practical use of tPA has yielded equivocal results, as only during the window of opportunity following stroke onset will the beneficial clot-lysing effects of tPA offset the tPA-modulated proteolytic actions. In the ischemic brain, tPA amplifies the MMP-mediated harm and breakdown of the blood brain barrier. Simultaneously activity of the two proteases combined can thus greatly increase neural damage (Kelly *et al.*, 2006).

Within min of stroke onset, several pro-inflammatory cascades are initiated. By 4-6 hrs following ischemic onset, circulating leukocytes are activated and adhere to vessel walls within ischemic brain tissue. Once present and activated, leukocytes release a variety of cytotoxic agents including cytokines, MMPs, NO, and ROS. These agents increase cell damage, disrupt the extracellular matrix, and intensify the migration, accumulation, and activation of an escalating amount of leukocytes (Wang *et al.*, 2007). Recruitment of circulating leukocytes is a critical first step in the brain's inflammatory process (Moraes *et al.* 2006). Neutrophils are generally the first leukocyte subtype recruited to the ischemic brain (Hallenbeck, 1996) and inhibition of neutrophil infiltration during ischemia/reperfusion injury greatly limits damage (Lenzser *et al.*, 2007; Ritter *et al.*, 2008).

There are three major steps by which leukocytes access the ischemic brain rolling along, adhesion to, and migration the endothelium (Sughrue *et al.*, 2004). The leukocyte-endothelium interaction is controlled by three groups of adhesion molecules: the selectins, the immunoglobulin superfamily (including the intercellular adhesion molecules (ICAMs) and the vascular cell adhesion molecules (VCAMs)), and the integrins (DeGraba, 1998, Emsley & Tyrell, 2002). Integrins on leukocytes bind with adhesion molecules on the endothelium; reducing expression of either of integrins or leukocytes reduces the extent of leukocyte

infiltration (Yenari *et al.*, 1998; Menge, 2005). Ischemia induces upregulation of adhesion molecules on the endothelial cell surface (Wang *et al.*, 1994; Qi *et al.*, 2009). The resulting adhesion and infiltration of neutrophils through the vascular wall of the brain parenchyma increases injury in hemorrhagic and ischemic strokes (Frijns & Kapelle, 2002). Infiltrating cells including the reactive microglia, macrophages, and leukocytes produce the majority of cytokines in the ischemic brain. Leukocyte-mediated damage is exacerbated by reperfusion due to the sudden and massive influx of ROS and leukocytes into the injured brain (Wang *et al.*, 2007).

Genes encoding for pro-inflammatory transcription factors, including the *Fos* gene, which codes for activator protein-1 (AP-1), and *Jun*, which codes for nuclear factor- κ B (NF- κ B), are upregulated within mins of occlusion. NF- κ B increases transcription of iNOS, COX-2, MMPs, ICAMs, and many pro-inflammatory cytokines, including TNF- α and IL-1 β , within 1 hr of ischemia onset (Chan, 2001; Glass & Ogawa, 2006). Activation of these mediators increases ROS production and overall harm (Barone & Feuerstein, 1999). IL-1 β levels directly increases glutamate excitotoxicity by increasing the rate of the astrocytic glutamate/cysteine transporter (Fogal *et al.*, 2007).

Under normal circumstances, NF- κ B is sequestered in the cytoplasm by the protein I κ B. TNF- α binding to the TNF-receptor acts as a pro-inflammatory signal and enhances the activity of Ca⁺⁺-mediated kinases, which phosphorylate I κ B. Subsequent to phosphorylation, I κ B is degraded, releasing NF- κ B. NF- κ B then translocates to the nucleus and upregulates pro-inflammatory factors (Thurberg *et al.*, 1998; Van Elzen *et al.*, 2008). AP-1 is another important transcription factor that responds to inflammatory stimuli. AP-1 upregulates many

of the same proteins as NF- κ B and the two transcription factors enhance one another's actions (Roger *et al.*, 1998). The AP-1 pathway, but not the NF- κ B pathway, upregulates MMP transcription (Benbow & Brinckerhoff, 1997).

Microvasculature

Endothelial dysfunction is one of the earliest manifestations of atherosclerosis and proceeds stroke in many individuals (Takemoto & Liao, 2001). Anti-inflammatory processes are disrupted within a dysfunctional endothelium, leading to increased pro-inflammatory actions and thereby increased neural damage (Laufs, 2003). While iNOS and nNOS-derived NO is harmful during cerebral ischemia, eNOS-derived NO is unequivocally beneficial by acting a vasodilator along with having antithrombotic, anti-inflammatory, and antiproliferative effects. The eNOS-derived NO inhibits leukocyte and platelet aggregation while maintaining a thromboresistant, or clear, interface between the bloodstream and vessel wall (Vaughan & Delanty, 1999). Hypoxia, ROS, cytokines, and mineralcorticoids decrease both eNOS expression and NO bioavailability in the vasculature (Vaughan & Delanty, 1999; Li *et al.*, 2008; Bauersachs & Fraccarollo, 2008).

Neurogenesis, Angiogenesis, and Synaptogenesis

Under normal circumstances, glutamate release is a sign of learning and memory and therefore increases axon growth, synaptogenesis, and synaptic plasticity. Increased levels of extracellular glutamate found during ischemic conditions can upregulate neurotrophic factors, limiting the extent of neural damage and inducing functional recovery (Mattson, 2008). Ischemia induces transcription of genes involved in neuroplasticity and neuroregeneration

including growth factors (Kawahara *et al.*, 2004). The growth factors block neuronal apoptosis while enhancing nerve fiber sprouting and synapse formation. Growth factors with increased cerebral expression following ischemic injury include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF) (Sondell *et al.*, 2000; Zhu *et al.*, 2000; Zhang *et al.*, 2005).

It has been shown that bFGF protects cultured brain neurons from hypoxia through protecting mitochondrial integrity by preventing the ischemia-induced downregulation of Bcl-2 expression (Ay *et al.*, 2001). Increasing bFGF levels also maintains $\text{Na}^+/\text{K}^+-\text{ATPase}$ activity and limits ROS accumulation (Mark *et al.*, 1997). The upregulation and release of bFGF from astrocytes following excitotoxicity enhances the sprouting of new fibers (Akaneya *et al.*, 1993; Ho & Blum, 1997; Speliotis *et al.*, 1996). The presence of bFGF also increases the number of migrating neuroblasts to an ischemic area, thus increasing neurogenesis (Kuhn *et al.*, 1997).

Factors Affecting Ischemic Results

Small differences in brain temperature have large effects on ischemic cerebral damage as hypothermia during and/or following the ischemic insult decreases cerebral damage (Busto *et al.*, 1987; Minamisawa *et al.*, 1990). Hypothermia limits leukocyte infiltration and ROS generation (Webster *et al.*, 2008). Reciprocally, elevated temperatures increase cell damage (Ginsberg *et al.*, 1992). Hypothermia is currently the most potent neuroprotective treatment

for human stroke patients as it can greatly decrease behavioral changes and learning deficits (Dietrich *et al.*, 1993; Green *et al.*, 1992, 1995).

Elevated glucose has also been reported to affect stroke outcomes bidirectionally. At times hyperglycemia has been asserted to block neural damage (Ginsberg, *et al.*, 1987; Kraft *et al.*, 1990), but it has also been associated with increased damage in animals and humans (Nedergaard & Diemer, 1987; de Courten-Meyers *et al.*, 1988; Kushner *et al.*, 1990). As previously discussed, acidity level, or the brain's pH, influences the outcome following stroke. This effect is much more pronounced during global than focal ischemia (Kraig *et al.*, 1987). Hypotension increases damage during ischemia, further decreasing blood flow. A slight elevation in blood pressure can prevent a stroke from occurring in models of focal ischemia or global ischemia including hypotension (Tamura, Kawai, & Takagi, 1997). Ischemic lesions are also thought to be negatively affected by age during insult (Matsuyama *et al.*, 1983).

While either sex animals can be used in cerebral ischemia research, the vast majority of studies utilize male animals. Males tend to have greater stroke damage than females (Payan & Conrad, 1977; Nakatomi *et al.*, 1979; Hall, Pazara, & Linesman, 1991). Nuclear hormone receptors, including testosterone and estrogen receptors, have similarly been investigated for neuroprotective effects, but most studies have yielded equivocal results. Testosterone can increase stroke damage while estrogen can ameliorate stroke damage (Hawk *et al.*, 1998). However, estrogen-mediated neuroprotection is only effective if treatment is begun at a specific life period (Rossouw *et al.*, 2002; Grodstein *et al.*, 2003). The mechanism behind the estrogen-mediated neuroprotection is also not yet well understood, as

it appears that estrogen can be neuroprotective independently of actions on the estrogen receptor (Dubal *et al.*, 2001; Sampei *et al.*, 2000).

Neuroprotection: The Drug Treatment

In order to protect the entire neurovascular unit, a wide variety of biochemical mechanisms need to be treated simultaneously. There is currently a great need for a combination of effective pharmaceutical compounds that protect the neurovascular unit (Meairs *et al.*, 2006). The drug treatment used in this study is a multipotent cocktail that fulfills the calls for treating dysfunction through a multitiered biological intervention. The treatment includes SimvastatinTM, GemfibrozilTM, TroglitazoneTM, and SpironolactoneTM, each of which has been shown to have neuroprotective effects during an ischemic insult through affecting different cellular pathways. The extent of neural protection possible with a cocktail of these four compounds has not been previously investigated.

SimvastatinTM

SimvastatinTM is of the statin drug family, whose primary function is to reduce plasma cholesterol levels by reversibly inhibiting 3-hydroxy-3-methyl coenzyme A (3-HMG-CoA) reductase, the rate-controlling enzyme in cholesterol synthesis (Reiss, 2007).

SimvastatinTM can penetrate the blood brain barrier. Preclinical experiments, observational studies, and randomized controlled trials indicate that statins can treat a wide range of neurologic diseases including prevention and protection from ischemic stroke, acute central nervous system (CNS) injury, neurodegenerative diseases, autoimmune/inflammatory CNS diseases, and neurometabolic diseases (Orr, 2008). Statins have huge preventive effects in

stroke, as demonstrated by meta-analyses predicting that in 1,000 individuals with moderate cholesterol (lower risk of stroke), 150 cardiovascular events could be prevented and at least 51 patients would be spared of their first event (Sacks *et al.*, 1996).

Statins offer neuroprotection during stroke in excess of that expected by the cholesterol lowering capabilities, as shown by comparison of statins with other LDL-reducing medications. Very low drug concentrations are necessary for pleiotropic protective effects from statins (Sacks *et al.*, 1996; Takemoto & Liao, 2001; Orr, 2008). Statins interrupt the biosynthetic pathway of two phyrophosphates, farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP), both of which serve as lipid attachments for the posttranslation modification of a variety of proteins (Takemoto & Liao, 2001). FPP and GGPP are necessary for the proper subcellular localization, intracellular trafficking, and activation of many proteins.

Through interrupting biosynthesis of FPP and GGPP, the proteins statins modulate include G proteins, heme-a, nuclear lamins, Ras (a GTP-binding protein), and the Ras-like proteins Rho, Rab, Rac, Ral, and Rap (Goldstein & Brown, 1990; Casey, 1995; Takemoto & Liao, 2001). Inhibiting Rho signaling is neuroprotective as the Rho family mediates much membrane trafficking, mRNA stability, intracellular transport, and gene transcription (Laufs & Liao, 2000). Statins further limit immune system activation and can offer neuroprotection when by minimizing reperfusion injury if given with tPA as a combined therapy (Blanco-Colio *et al.*, 2003; Zhang *et al.*, 2005).

GemfibrozilTM and TroglitazoneTM: PPAR agonists

The Peroxisome Proliferator Activated Receptors (PPARs) are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors (Lehmann *et al.*, 1995). There are three PPAR subtypes: α , β/δ , and γ (Pascual Glass, 2006). Each receptor is activated by receptor-specific ligands, many of which are fatty acids (Berger & Moller, 2002; Collino *et al.*, 2008). GemfibrozilTM is a member of the fibrate drug family, the most commonly prescribed drug class of PPAR- α agonists. TroglitazoneTM is of the thiazoladine drug family, the most commonly prescribed drug class of PPAR- γ agonists. PPARs modulate genes involved in systemic lipid metabolism and energy homeostasis (Theocharis *et al.*, 2004). Fibrates binding PPAR- α increases the expression of lipid metabolizing enzymes (Collino *et al.*, 2006), and creates a healthier lipoprotein profile (Staels *et al.*, 1998b; Rubins *et al.*, 1999).

Thiazoladines are oral antidiabetic agents that decrease insulin resistance while having non-steroidal anti-inflammatory effects (Willson *et al.*, 2001). Other non-steroidal anti-inflammatory pharmaceuticals, including ibuprofen, are also ligands for PPAR γ , but with a greatly decreased affinity relative to the thiazoladines (Jiang *et al.*, 1998). Ligand bound-PPAR- γ has many anti-inflammatory effects include opposing actions of the cytokines TNF- α and IL-2 (Theocharis *et al.*, 2004). PPAR γ can also decrease hypertension independently of insulin resistance (Walker *et al.*, 1999; Olefsky, 2000).

Ligand-binding is necessary for PPAR modulation of transcriptional activity. Ligand-bound PPARs further complex with the retinoid X receptor (RXR), which binds and regulates specific DNA sequences known as PPAR response elements (PPREs) (Schachtrup

et al., 2004). If no PPAR ligand is present, co-repressor molecules will bind the PPAR/RXR heterodimer and prevent it from binding the PPRE (Krogstad *et al.*, 2002). Ligand binding can release corepressor molecules (Burns & Vanden Heuvel, 2007).

Phosphorylation of corepressor molecules by kinases stabilizes the corepressor's presence on the PPAR/RXR heterodimer so the corepressor proteins will remain bound upon ligand binding. The Ca^{++} -dependent kinase MAPK is responsible of phosphorylation and stabilization of corepressor molecules bound to the PPARs (Juge-Aubrey *et al.*, 1999; Hu *et al.*, 1996). Ligand-bound heterodimers will retain some activity despite the attached phosphorylated corepressors, but this activity will be greatly diminished (Burns & Vanden Heuvel, 2007). Due to increased kinase activity and therefore decreased PPAR/RXR activity, in order to benefit from PPARs during ischemic conditions, increased levels of both PPARs and ligands are necessary (Hu *et al.*, 1996; Adams *et al.*, 1997; Camp *et al.*, 1999). During ischemia the cell naturally upregulates the quantity of available PPARs. However, ligand availability is greatly decreased. External ligand administration is therefore necessary to exploit benefits of PPAR-activated gene transcription (Zhang *et al.*, 1996).

Activated PPARs also repress the transcription of certain genes via multiple mechanisms (Glass & Ogawa, 2006). The PPAR/RXR heterodimer binds co-activators required by other transcriptional factors and the limited availability of co-activators reduces the expression of genes modulated by other transcription factors (Kodera *et al.*, 2000). PPAR/RXR complexes will also directly bind to transcriptional factors and prevent the other factor's actions (Glass & Ogawa, 2006). PPAR α physically interacts with a subunit of NF- κ B, preventing NF- κ B transactivation of DNA and thereby decreasing cytokine

expression. This method of transcriptional inhibition only works for some of the genes regulated by NF- κ B, as PPAR α inhibition of NF- κ B is also dependent upon the affinity of the PPAR α -ligand for a gene's promoter region (Delerive *et al.*, 1999).

PPAR- α is highly expressed within the hippocampus, especially within the CA1 and dentel gyrus areas (Moreno *et al.*, 2004). PPAR- α is involved in oxidative stress defense by increasing the expression and activity of SOD and catalase (Farioli-Vecchioli *et al.*, 2001; Moreno, *et al.*, 2004). It has also been hypothesized that PPAR- α modulates dopaminergic and glutamatergic neurotransmission (Avshalumov & Rice, 2002).

PPAR- γ is constitutively expressed in macrophages and microglial cells (Marx *et al.*, 1998; Bernardo *et al.*, 2000; Willson *et al.* 2001) and helps regulate the cell cycle of macrophages, lymphocytes, and endothelial cells (Gupta *et al.*, 2001a). PPAR γ also regulates the expression of genes involved in energy storage and utilization (Willson *et al.*, 2000). Thiazolidines, as PPAR- γ ligands, exert anti-tumoral effects and have been used in cancer treatment (Theocaris *et al.*, 2004; Gupta *et al.*, 2001b). PPAR γ also regulates TNF α . Thiazolidine-activated PPAR γ blocks the TNF α activated signaling cascade, but does not repress TNF α at a transcriptional level (Peraldi *et al.*, 1997; Willson *et al.*, 2001).

SpirolactoneTM

SpirolactoneTM is a competitive mineralcorticoid receptor (MR) antagonist in a class of pharmaceuticals termed potassium-sparing diuretics. It was first shown in the 1960s that the mineralcorticoid aldosterone increases stroke risk independently of hypertension (Conn *et al.*, 1964). Along with offsetting detrimental effects of mineralcorticoids, SpirolactoneTM has an anti-androgen effects (i.e. it limits the detrimental effects of

testosterone). Under normal conditions MR expression is only found in very specific brain areas including the hippocampus, basomedial and central nucleus of the amygdala, cortical layers II, III, and V, and the paraventricular nucleus in the hypothalamus (Oyamada *et al.*, 2008).

Spirolactone™ is being investigated for both preventative and post-stroke amelioration effects. The addition of Spirolactone™ to SHR fed a high-salt diet prevents spontaneous cerebral infarctions without reducing blood pressure (Rocha *et al.*, 1998) and limits the damage of MCA occlusions in SHR to no different from NTR (Dorrance, 2001). Spirolactone's™ success is associated with limiting eutrophic remodeling (i.e. decrease in a blood vessel's lumen size and an increase in the wall thickness), resulting in decreased of the myogenic tone (the ability of an artery to contract in response to an increase in intraluminal pressure) (Rigsby *et al.*, 2007; Dorrance, 2008).

Evaluation of Damage and Functional Recovery

The rodent global ischemia mimics the cognitive deficits and physical pathology observed in stroke patients (Ginsberg & Busto, 1989; Grotta, 1994; Ginsberg, 1996). In evaluating cognitive deficits following pathology one must ask whether behavior is influenced. If so, which tasks do and which tasks do not show the behavioral change and is the change in behavior transient or permanent? Following global ischemia, there is a long-lasting atteration in performance during tasks of learning and memory (Block, 1999). Alertness and sensorimotor capacities can also bet transiently altered following stroke, but

this lasts only for the first 24-48 hours (Capdeville *et al.*, 1986; Combs & D'Alecy, 1987; Gionet *et al.*, 1991).

Locomotor Activity

Total activity must be evaluated before testing cognitive function for if activity is too high or low, tests of cognitive levels will be ineffective. Spontaneous locomotor activity can be assessed by placing an animal into a chamber and measuring activity level (Babcock *et al.*, 1993a). Following ischemia, a transient locomotor hyperactivity has been observed that is most prominent in the first 24 hrs. Ischemia-induced increases in locomotor activity decreases with repeated testing with stroked animals showing no significant difference from controls by 5-7 days following ischemia (Wang & Corbett, 1990).

Animals will show hyperactivity if tested for the first time at 13 and 14 days post-occlusion, showing that the hyperactivity is probably demonstrating a cognitive deficit. A deficit in habituation or spatial mapping can explain the different activity levels (Babcock *et al.*, 1993a). Over a 10 min open field test, control animals will explore the new surroundings for the first 3-4 min, followed by a rapid decrease in exploratory behavior. In contrast, ischemic animals will continue to explore the area for up to the entire 10 min. Ischemic animals will show this habituation deficit for up to 180 days following ischemia. An inability to form spatial maps of the new area would explain the animal's inability to adjust (Colbourne & Corbett, 1995). Most studies have attributed the increase in locomotor activity to neuronal damage to the CA1 hippocampal region mainly because of the high correlation between the level of damage and hyperactivity (Gerhardt & Boast, 1988; Ramos-Zúñga *et al.*, 2008).

Neuroprotective treatments generally symmetrically reduce hippocampal damage and hyperactivity (Babcock *et al.*, 1993b; Janac, Selkovic, & Radenovic, 2008). However, certain interventions can mitigate behavioral changes without offering neuroprotection (Plamondon & Roberge, 2008). Another problem with this explanation is that hippocampal degeneration and locomotor hyperactivity are not temporally consistent, as cell death develops over the days following ischemia, but locomotor hyperactivity has immediate onset. To explain this temporal discrepancy, it has been proposed that locomotor hyperactivity is caused by acutely injured, but still functionally active, neurons in the CA1 region of the hippocampus (Kuroiwa, Bonnekoh, & Hossmann, 1991).

It has also been proposed that damage to the striatum could cause the hyperactivity. The striatum is involved in locomotor control. The striatum is similar to the CA1 hippocampal region in its vulnerability to ischemia and therefore often similar in their damage profiles following ischemia (Pulsinelli *et al.*, 1982a; Svensson, Carlsson & Carlsson, 1995). However, damage to the striatum following ischemia is widely variable, making it impossible to find a direct correlation between striatal damage and hyperactivity. In contrast, this correlation between damage and hyperactivity is well established within the CA1 hippocampal region (Milesen & Schwartz, 1991).

T-Maze

The most famous version of the T-maze is the “single choice” and was necessary in constructing Tolman’s influential theories about cognitive maps and learning that then helped lead American psychology away from behaviorism and back towards cognition (Tolman, 1948). The Y-maze, a modified T-maze was first used to demonstrate goal-

dependent recall of goal independent (latent) learning by showing learning of spatial orientation independently of the desire for food. Rats explored a Y-maze where the left arm holds water and the right holds food. Later, with food or water deprivation, rats were able to choose the correct arm based on their goal state, showing that rats had previously learned food and water placement independently of a goal state. Tolman did many of his critical experiments with a more complicated T-maze that looks similar to a radial arm maze. With the more complicated maze, Tolman demonstrated that rodents can choose the shortest path to the goal once the practice mechanism was blocked (Tolman *et al.*, 1946).

A significant correlation between hippocampal damage and learning impairment in a T-maze task has been shown (Ord *et al.*, 1988; Handelsmann & Olton, 1981). Also, the unilateral ischemic rat will tend to favor the arm ipsilateral to the lesion (Balduino *et al.*, 2000). One of the main ways of reviewing deficit after a stroke is evaluating spontaneous alternation, or the tendency of the animal to enter opposite goal boxes in consecutive unrewarded trials in a T-maze (Douglas, 1966). The task demands of the T-maze determine whether pre-training is needed for the ischemic animal to learn the task. Rewarding an animal for returning to the previously visited arm necessitates pre-training while rewarding the animal for alternating does not. This indicates that alternation between arms is an easier task for the animal to learn (Babcock & Graham-Goodwin, 1997).

Radial Arm Maze

The radial arm maze was first proposed in 1976 by Olsten and Samuelson as an alternative to the current tests of memory. They asserted that rats have a natural proclivity towards using “place learning” or “an innate proclivity towards distinctive exteroceptive

stimuli associated with a specific spatial location” (Olten & Samuelson, 1976, p.97). In the most basic form of the radial arm maze, a piece of food is placed at the end of each of eight arms and the rat is placed in the center. In order to get all the food as rapidly as possible, the rat should travel down each arm only once. Thus, the cognitive requirements are simple: the rat must be able to locate each of the eight arms and determine which one has already been chosen. This test exploits rodents’ natural foraging behavior and cognitive abilities to associate place with food (Babcock & Graham-Goodwin, 1997). Many modifications of the radial arm maze exist to test different cognitive abilities (Paganelli *et al.*, 2004). Before the task an animal is usually restricted to 85% of its normal caloric intake to provide the motivation to reach and consume food as quickly as possible.

The radial maze task can test both reference and working memory. A working memory error is displayed when an animal reenters a previously chosen baited arm. Reference memory can be tested by repeatedly baiting the same five out of eight arms and having the animal learn which five arms contain food relative to room cues (Davis *et al.*, 1986). Global cerebral ischemia causes a significant increase in both reference and working memory errors, though reference memory seems to have a slightly greater deficit than working memory (Kiyota *et al.*, 1991; Maurer *et al.*, 1995). Animals pretrained in the reference memory task have been comparable to controls following ischemia, showing that ischemic animals may have more trouble acquiring knowledge than retaining already learned knowledge. With 65 trials ischemic animals were able to perform similarly to controls (Davis *et al.*, 1986). Though at 26 weeks, ischemic animals still show working and reference memory deficits relative to controls (Langdon *et al.*, 2008).

A negative correlation has been shown between number of errors and number of pyramidal cells in the CA1 hippocampal region (Schwartz *et al.*, 1998; for an exception see Paganelli *et al.*, 2004). Neuroprotection studies similarly show a correlation between the ischemia-induced deficit in working memory and behavior (Grotta *et al.*, 1988; Wishart *et al.*, 1994).

SimvastatinTM's ability to decrease ischemic damage was tested using a radial arm maze with three arms baited with water. The dependent variables included time taken to visit the three baited arms, the number of working memory errors meaning reentries into already visited baited arms, and the number of reference memory errors meaning entering a nonbaited arm. Group effects were found in all three measures, with only the ischemic group, but not the SimvastatinTM + Ischemia group, significantly different from the control group (Balduino, 2001).

Water Maze

Morris developed the water maze task to test the neurobiology of spatial learning, memory, and ability to find a place in space defined only by its position relative to distal, extramaze cues (Morris, 1984). The water maze consists of a circular pool filled with water and a submerged platform onto which the rat can climb to emerge from the water. Animals do not need to be food or water deprived as escape from water is sufficient motivation. The most basic test of learning in the water maze is measuring escape latency, or acquisition of knowledge of pedestal location. If the animal does not spontaneously find the pedestal during the time, the researcher will place the animal on the pedestal for a period before starting the next trial. It should be noted that overall, ischemic and control animals swim at

the same speed (Langdon *et al.*, 2008), making escape latency and path travelled the main dependent variables to demonstrate learning and memory impairments.

Increased escape latency and swim distance suggest a disturbance of the ability to use distal, extramaze cues, as the water contains no cues regarding orientation. The water maze reveals a reliable main effect of group and a group by trial interaction, as the ischemic group's escape latency is significantly longer than the control group's latency in the first trials. With an increasing number of trials, ischemic and sham animals decrease the amount of time needed to find the platform, showing that the ischemic animals do eventually learn the pedestal's location (Hagan & Beughard, 1990). Increased escape latency reveals a deficit in spatial learning that is highly correlated with neuronal cell loss in the CA1 subfield of the hippocampus following ischemia (Block & Schwarz, 1998; Green *et al.*, 1995; Hagan & Beughard, 1990; Jaspers *et al.*, 1990; Nunn *et al.*, 1994). However, not all animals show a deficit in the simple learning task and it appears that a neuronal cell loss of at least 60% in the CA1 sector of the hippocampus for animals to display a deficit in the water maze (Auer *et al.*, 1989; Green *et al.*, 1992). Thus, cell loss in the hippocampal CA1 sector seems to be a critical factor determining the deficit in spatial learning in the water maze (Block, 1999).

Interestingly, this deficit in the simple spatial learning paradigm is model-specific. Overall, the four-vessel-occlusion model of the rat and carotid occlusion in gerbils results in a learning deficit, while the two-vessel occlusion with hypotension model in rats does not show a deficit (Nunn & Hodges, 1994; Block, 1999). The learning deficit in the gerbil is no longer present at 21 days following ischemia, showing that this deficit is specific to time following ischemia (Corbett *et al.*, 1992).

Probe trials with the pedestal removed can also show changes in memory. The amount of time spent in the area of the former platform and number of crossings over the former platform are two common dependent measures. Sham-operated animals spend about 50% of the time swimming within the quadrant of the former platform position, while ischemic animals spend significantly less time in the quadrant formerly containing the former platform. Ischemic animals seem to use a different search strategy to find the platform. Ischemic animals tend to swim a certain distance from the outer wall of the water tank in an attempt to find the platform. In contrast, control animals tend to learn the area containing the platform and mainly swim in that area (Kiyota *et al.*, 1991). This discrepancy between ischemic and sham animals is present at 26 weeks following transient global ischemia (Langdon *et al.*, 2008). Animals treated with SimvastatinTM proceeding ischemia performed significantly better than ischemic animals that did not receive treatment in both the learning and probe tasks. However, these SimvastatinTM-treated ischemic animals still demonstrated mild neurological impairment, as they performed significantly worse than sham operated animals in the tasks (Balduini, 2001).

STATEMENT OF PURPOSE

The goal of this experiment was to determine whether a drug treatment containing four prescription medications offers neuroprotection during transient global ischemia. Previous research has revealed that neuroprotection can be conferred by administration of each medication in the cocktail (SimvastatinTM, Vaughan & Delanty, 1999; GemfibrozilTM, Deplanque *et al.*, 2003; TroglitazoneTM, Sundarajan *et al.*, 2005; SpironolactoneTM, Oyamada *et al.*, 2008). Each of the medications can offer neuroprotection through modulating different biochemical pathways. This research investigates treatment with the combination of these drugs with the aim of decreasing cellular dysfunction following anoxia by intervention in a large diversity of cell signaling cascades. Establishing whether the drug offers neuroprotection will lay the foundation for further research regarding which biochemical mechanisms are responsible for preventing cellular dysfunction in anoxic conditions.

METHOD

Experimental Animals

Twenty-seven male gerbils weighing 60-80 gms were used. The animals were allowed unrestricted access to standard food and pellets. Housing consisted of separate solid-bottom cages kept on a 12 hr light/dark cycle at a temperature of at 23° C. All procedures were approved by the Montana State University Animal Care and Use Committee.

Surgery

Transient global ischemia was induced by bilateral clamping of the common carotid arteries. Animals were anesthetized with isoflurane. Body temperature was monitored with a rectal thermometer and maintained at 37° C with an infrared heating lamp and a heating pad. The common carotid arteries were exposed via a midline incision made to the ventral surface of the neck and were isolated from surrounding tissue. The arteries were clamped using 85 gm pressure aneurysm clamps for 5 min. The neck incision was closed with sutures and gerbils were returned to their cage. Sham subjects underwent an identical procedure except arteries were not clamped.

Treatment Protocol

The drug treatment consisted of cocktail of 4 drugs: Simvastatin™ (20mg/kg/day), Gemfibrozil™ (150mg/kg/day), Troglitazone™ (1.3 mg/kg/day), and Spironolactone™ (50mg/kg/day). All animals were gavaged with 1.5 µL of a suspension twice daily for 21 days

preceding and 7 days subsequent to surgery. Animals were not gavaged on day of surgery. Animals in the drug treatment group received a suspension containing all four drugs once daily and a suspension containing only Gemfibrozil™ 8-12 hrs later. The control group received a vehicle suspension at identical dosing intervals. Purified powder forms of all drugs were used in the oral suspensions. Suspensions further included an inert suspension agent and a nonreactive aldehyde resembling peanut butter. Suspensions were coded at preparation to keep investigators blind to treatment condition throughout the experiment and data analysis.

Behavioral Testing

Behavioral testing of the animals occurred 24 hrs following surgery. Subjects were placed in an open-field apparatus 77x77 cm with 15 cm-high walls. Testing lasted 5 min, during which behavioral data was collected using a digital camera (Logitech, Inc., CA) connected to a computer with tracking program (ANY-maze; Stoelting, IL).

Assessment of Hippocampal Damage

Seven days following surgery, subjects were euthanized with CO₂ and perfused transcardially with Phosphate Buffered Saline followed by 4% paraformaldehyde. The brains were post-fixed in 4% paraformaldehyde solution for at least 48 hrs prior to sectioning. 40µm vibratome sections through the hippocampal region were collected, mounted on slides, and stained with cresyl violet. Three investigators blinded to condition ranked neural damage on a four point-scale with a score of 0 (4-5 compact layers of normal neuronal

bodies), 1 (4-5 layers of cells bodies with the some altered neurons), 2 (sparse neuronal bodies with “ghost spaces” and/or glial cells between them) or 3 (intense gliosis of the CA1 subfield with normal neuronal bodies either rare or completely absent) as previously described (Coimbra & Cavalheiro, 1990).

Statistical Analysis

Statistical analyses comparing the three treatment conditions (Sham, Vehicle + Ischemia, or Drug + Ischemia) were performed using SPSS (version 17.0). The behavioral measures of distance traveled (m), speed (m/sec), and time mobile (sec) were compared between conditions with separate one-way ANOVAs to determine whether the animals exhibited similar behaviors. If the omnibus P value was statistically significant, ($P < .05$), post hoc testing for pairwise comparisons was performed using the Bonferroni correction to adjust for multiple comparisons. Kruskal-Wallis and subsequent Mann-Whitney U tests were used to evaluate histological damage rating scores.

RESULTS

Participants

One animal in the Drug + Ischemia group was excluded due to incomplete drug dose. One animal in the Vehicle + Sham group was excluded due to equipment malfunction during behavioral testing.

The Control Group

The *a priori* hypothesis that the drug treatment would not affect behavioral outcomes or neurological damage ratings was evaluated by contrasting animals in the Drug ($n = 6$) and Vehicle ($n = 5$) groups following the sham procedure. The dependent variables did not vary between animals in the separate treatment conditions ($p > .05$). The mean outcome for each dependent variable by treatment condition is shown in Table 1. Data for Sham animals was conflated into the control condition for subsequent analysis.

Table 1. Mean histological ranking and mean behavioral measures for animals that underwent the sham surgical procedure, separated by treatment condition, drug or vehicle

Treatment	Ranking [0-4] ($\pm$ SEM)	Distance Traveled ($\pm$ SEM)	Speed of Travel ($\pm$ SEM)	Time Mobile ($\pm$ SEM)
Drug ($n = 6$)	0 ($\pm$ 0.0)	42.8 ($\pm$ 6.0)	.16 ($\pm$.02)	269.1 ($\pm$ 10.6)
Vehicle ($n = 5$)	0 ($\pm$ 0.0)	42.7 ($\pm$ 4.7)	.16 ($\pm$.01)	269.2 ($\pm$ 9.5)

Mean Speed

The mean speed (m/sec) traveled during the open-field test was measured by dividing distance traveled by time mobile. There was a significant effect of treatment condition on mean speed of travel for the three conditions ($F(2, 23) = 4.29, p = .02$), as shown in Figure 1.

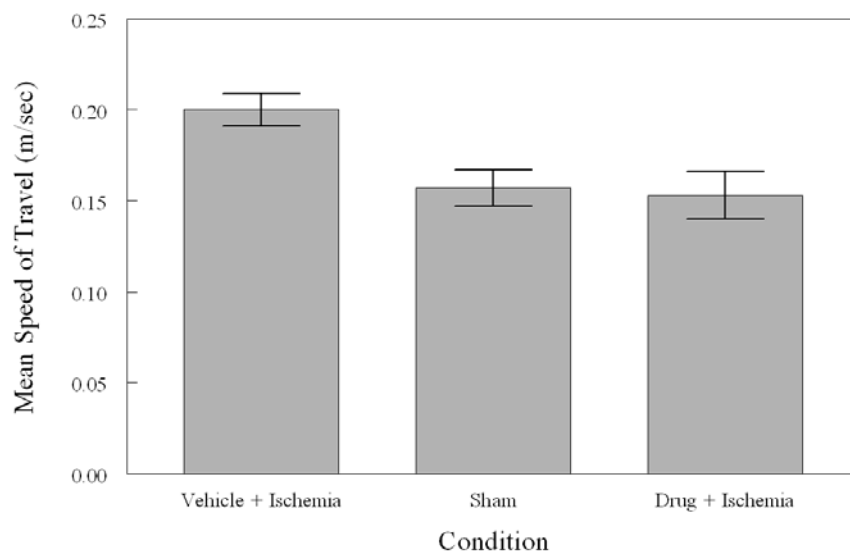


Figure 1. Mean speed of travel ($\pm SEM$) during the open-field test. Locomotor activity was recorded 24 hrs following transient global ischemia or a sham procedure. Animals received treatment with a drug cocktail or vehicle treatment for the three weeks preceding surgery. A comparison of means for Drug + Ischemia ($n = 7$), Vehicle + Ischemia ($n = 8$), and Sham ($n = 11$) conditions showed the mean speed of travel for the ischemic animals receiving drug treatment and the sham group was not significantly different ($p > .05$). Ischemic animals receiving only vehicle traveled at a significantly higher mean speed than the other treatment conditions (* $p < .05$).

The Vehicle + Ischemia condition traveled at a higher speed ($M = .2, SEM = .009$) than the Drug + Ischemia ($M = .15, SEM = .01$) and Sham conditions ($M = .16, SEM = .01$). Subsequent analysis revealed the Vehicle + Ischemia condition traveled at a significantly different mean speed than both the Drug + Ischemia group ($p < .05$) and the Sham group (p

$< .05$). The mean speeds of travel for the Drug + Ischemia and the Sham conditions did not differ ($p > .05$).

Distance

A significant difference was found between animals of the three conditions in distance traveled ($F(2, 23) = 4.4, p = .02$), as shown in Figure 2. Animals receiving only vehicle proceeding ischemia covered the greatest mean distance during the open field test ($M = 52.5, SEM = 3.3$).

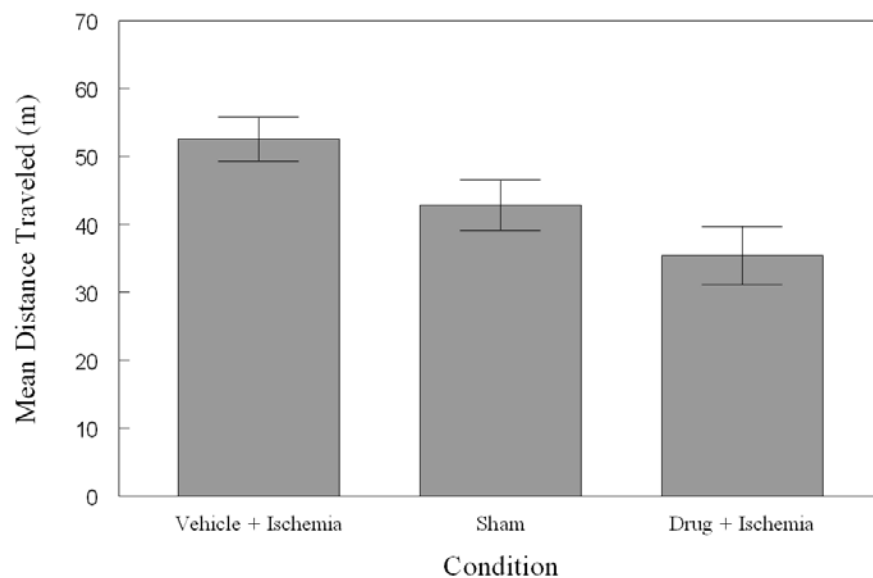


Figure 2. Mean distance of travel ($\pm SEM$) during the open-field test for Drug + Ischemia ($n = 7$), Vehicle + Ischemia ($n = 8$), and Sham ($n = 11$) groups. Locomotor activity was recorded 24 hrs following transient global ischemia or a sham procedure. Animals received either a drug cocktail or vehicle treatment for the preceding three weeks. Ischemic animals receiving only vehicle traveled further than the ischemic animals receiving the drug treatment ($* p < .05$). The Sham condition was not significantly different from either ischemic condition ($p > .05$).

The Drug + Ischemia group traveled the least distance ($M = 35.4$, $SEM = 4.3$) while the sham groups traveled a distance intermediate to the two ischemic groups ($M = 42.8$, $SEM = 3.7$). Subsequent analysis revealed that the mean distance covered by the Vehicle + Ischemia and the Drug + Ischemia conditions were significantly different ($p = .02$). The mean distance covered by the Sham group was not significantly different the mean distance covered by either the Vehicle + Ischemia or the Drug + Ischemia groups ($p > .05$).

Time Mobile

A significant difference between conditions was found for time spent mobile during the open-field test ($F(2, 23) = 5.6$, $p = .01$), as shown in Figure 3.

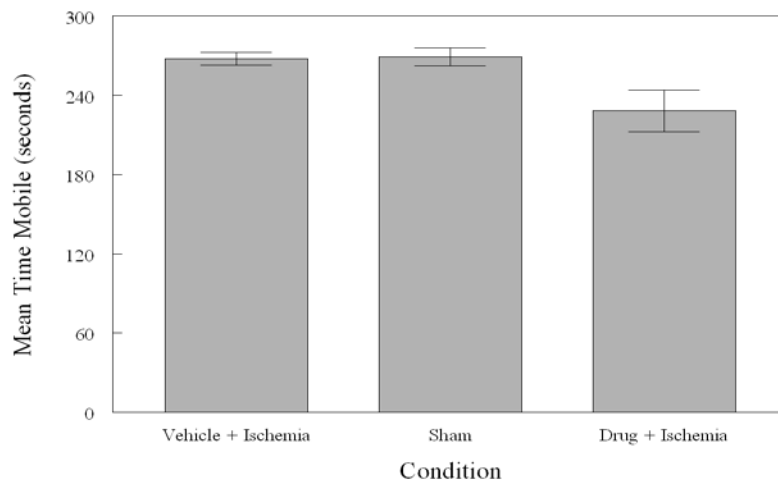


Figure 3. Mean time spent mobile ($\pm SEM$) during the open-field test for Drug + Ischemia ($n = 7$), Vehicle + Ischemia ($n = 8$), and Sham ($n = 11$) groups. Locomotor activity was recorded 24 hrs following transient global ischemia or a sham procedure. Animals received either a drug cocktail or vehicle treatment for the three weeks preceding ischemia. Ischemic animals in the drug treatment spent significantly less time mobile than both the Vehicle + Ischemia and Sham groups ($*p < .05$). The Vehicle + Ischemia and Sham conditions were not significantly different ($p > .05$).

Subsequent analysis showed the time spent mobile by the Vehicle + Ischemia condition ($M = 267.6$, $SEM = 5.2$) and the Sham condition ($M = 269.16$, $SEM = 6.85$) was not significantly different ($p > .05$). However, the Drug + Ischemia condition spent significantly less time mobile ($M = 258.2$, $SEM = 15.8$) than both the Vehicle + Ischemia ($p = .01$) and the Sham groups ($p = .03$).

Histology

Brain sections were mounted, stained with cresyl violet, and assigned a rating of 0-3 for level of neuronal cell body damage (Babcock *et al.*, 1993). Representative photomicrographs of stained sections from each condition are depicted in Figure 4.

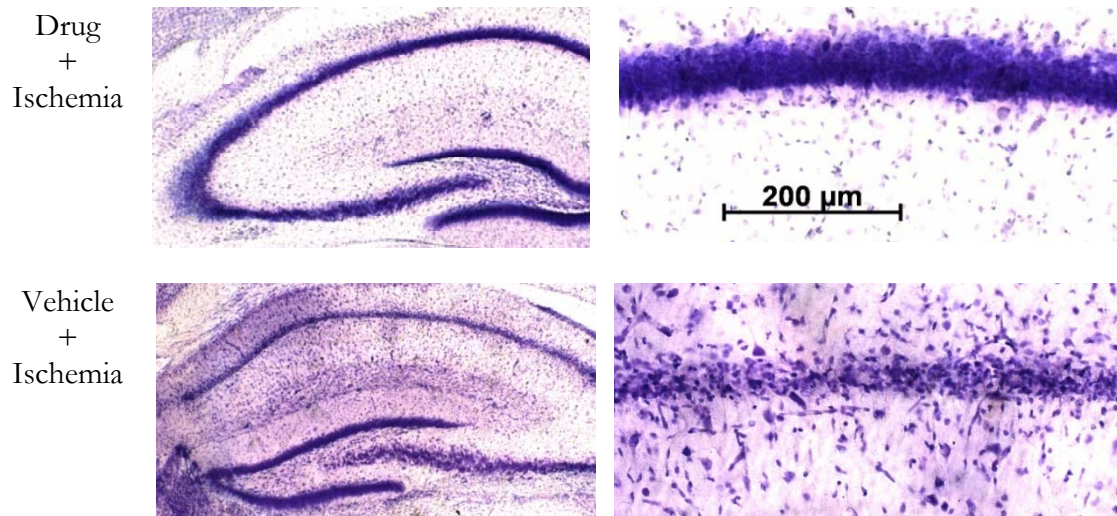


Figure 4. Photomicrographs of cresyl violet stained sections containing the hippocampal region. Tissue was collected 7 dys following surgery. The CA1 region of the hippocampus is shown for the Drug Treatment + Ischemia condition in the upper row and the Vehicle + Ischemia condition in the lower panel.

The Vehicle + Ischemia condition showed the most damage ($M = 1.6$, $SEM = .4$), the Drug + Ischemia condition showed less damage ($M = .02$, $SEM = .06$), and the Sham group showed no damage ($M = 0$, $SEM = 0$). Damage ratings are illustrated in Figure 5. A

significant difference was found between the groups ($p < .01$). The Vehicle + Ischemia group was significantly different from both the Drug + Ischemia group ($p = .02$) and the Sham group ($p < .01$). The Drug + Ischemia group and the Sham group were not significantly different ($p > .05$).

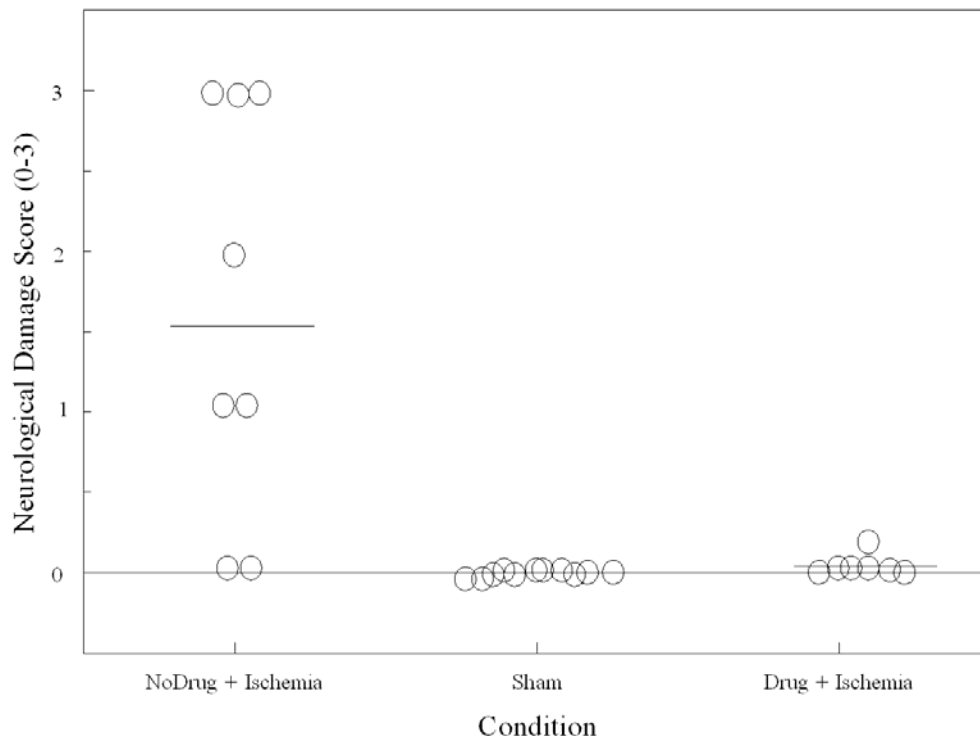


Figure 5. Ratings of neurological damage for Drug + Ischemia ($n = 7$), Sham ($n = 11$), and Vehicle + Ischemia ($n = 8$) conditions. A score of 0 (4-5 compact layers of neuronal bodies), 1 (4-5 compact layers of neuronal bodies with the presence of some altered neurons), 2 (sparse neuronal bodies with “ghost spaces” and/or glial cells between them), or 3 (complete absence or presence of only rare normal neuronal bodies with intense gliosis of the CA1 subfield) was assigned for each animal by raters blind to the conditions. The Vehicle + Ischemia condition exhibited significantly more damage than either the Drug + Ischemia or Sham conditions (* $p < .05$). The Drug + Ischemia and Sham conditions were not significantly different ($p > .05$).

DISCUSSION

This is the first investigation into the neuroprotection from ischemic dysfunction offered by treatment with the novel combination of SimvastatinTM, GemfibrozilTM, TroglitazoneTM, and SpironolactoneTM. The animals received either the drug treatment or vehicle for 21dys prior to, and 7 dys following, transient global ischemia or a sham procedure. The gerbil bilateral carotid artery occlusion model was used because it accurately replicates the brain damage found in humans following cardiac arrest or cardio-pulmonary bypass surgery (Ginsberg & Busto, 1989; Grotta, 1994; Ginsberg, 1996). Specifically, cells within the CA1 hippocampal region are particularly vulnerable to delayed cell death following global hypoperfusion.. Each of the compounds used in the drug treatment has been identified as a possible neuroprotective agent during an ischemic event

Previous research has shown that SpironolactoneTM, a MR antagonist, will not affect behavior or memory in the absence of confounding factors such as corticosteroid injections (Myers & Greenwood-Van Meerveld, 2007; Khakasari *et al.*, 2007). The only behavioral factor previously found to be affected by SpironolactoneTM treatment was reduced time immobile in the forced swim test (Stone & Lin, 2008). In contrast, TroglitazoneTM and the thiazoladine drug class was whosn to increase time immobile during the forced swim test while open-field behavior remained unchanged (Rosa *et al.*, 2008). Differences in time immobile during the forced swim test without changes in any behavioral or cognitive measures changing relative to control animals is a method of testing possible anti-depressant effects of a pharmaceutical.

Extensive testing for possible behavioral affects of SimvastatinTM treatment over a two month period showed that SimvastatinTM does not alter the daily activity or working memory of rodents (Li *et al.*, 2006; Bytan *et al.*, 2008). Similarly, treatment with PPAR- γ agonists or the thiazoladine class of pharmaceuticals has not affected locomotor activity (Pathan *et al.*, 2006; Maeda *et al.*, 2007; McTigue *et al.*, 2007). PPAR- α agnoism has been found normalize behaviors including sleep patterns, eating habits, and energy homeostasis (McCreary & Handley, 2000; Chakravarty *et al.*, Lo Verme *et al.*, 2005; 2007; Hidenori *et al.*, 2007; Lecarpentier *et al.*, 2008).

This combination of drugs has not previously been investigated. The *a priori* hypothesis that the drug treatment would not affect behavior was confirmed as the drug treatment did not affect the dependent variables of animals in the sham group. This result is consistent with the previous findings that these pharmaceuticals will not affect spontaneous locomotor activity or memory. Using a single sham group for subsequent analyses minimized animal use.

The open field test has been shown to be a sensitive indicator of an animal's ability to habituate to a new environment. Hyperactivity during an open field test correlates highly with ischemic injury to the CA1 region of the hippocampus (Colbourne & Corbett, 1995). Gerbil behavior was evaluated in an open field test 24 hrs following the ischemic event. We predicted that the animals receiving only vehicle (Vehicle + Ischemia) will be hyperactive in the open field, as shown by increased speed of travel, increased distance traveled, and decreased time immobile relative to the other two groups. It was hypothesized that animals in the drug condition would not show hyperactivity or neural damage following ischemia.

This result would demonstrate that the drug treatment protects subjects from neural damage during transient global ischemia. We further hypothesized that the drug + ischemia condition will perform similarly to sham subjects, demonstrating full protection from damage normally caused by transient global ischemia.

The Drug + Ischemia and the Vehicle + Ischemia groups were significantly different on all dependent variables. Ischemic animals receiving vehicle for treatment traveled a greater distance, with a greater average speed, and spent more time mobile than ischemic animals receiving the drug treatment. These results demonstrate that the drug treatment protected the subjects from behavioral hyperactivity following transient global ischemia.

Relative to the behavior of the sham group, subjects in the vehicle and the subjects in the drug condition had opposite responses to ischemia. Ischemia increased activity in the Vehicle + Ischemia group relative to the sham group, as shown by the mean speed of travel. However, it appears that ischemia decreased activity in the Drug + Ischemia group, as shown by the Drug + Ischemia spending significantly less time mobile relative to the Vehicle + Ischemia and the Sham conditions. There has been no discussion in the literature as to whether decreasing amount of time mobile decreases neural damage by stabilizing a lower metabolic rate and/or a lower internal body temperature has not been discussed. There is currently no clear consensus on a possible correlation between time mobile and extent of damage (Tejtlaková *et al.*, 2007; Girard *et al.*, 2009). In general, the behavioral measure of time mobile has only begun to be reported recently, probably due to the recent development of tracking programs that ease the quantification of this measure of locomotor activity.

Whether neuroprotective agents generally decrease time spent mobile following stroke a possible correlation with neuroprotection should be more thoroughly investigated.

Neurological damage scores show that drug treatment protected the CA1 region of the hippocampus from delayed cell death following an ischemic attack. Ischemic animals receiving the drug treatment was no different from sham animals with ratings showing little to no neural damage. Ischemic animals receiving only vehicle as treatment showed significantly more neural damage than both the ischemic animals that received drug treatment and the sham group.

The CA1 region of the hippocampus is extremely vulnerable to a global ischemic insult. Delayed cell death occurs in this region for up to a week following stroke. Under normal conditions, when cell death occurs in the CA1 hippocampal region or the dentate gyrus, nascent neural cells can readily replace dead cells. It is thought that the process of neurogenesis is an intrinsic part of learning and memory. Following transient global ischemia the dentate gyrus increases the rate of neurogenesis (Liu *et al.*, 1998), but the inhospitable conditions of the hippocampus cannot incorporate new neurons. Cells entering the hippocampus will therefore die (Whitney *et al.*, 2009). The lack of neural damage to the CA1 region of the hippocampus could indicate that the drug treatment prevents cell death. It could also mean that the hippocampus is able to support neurogenesis and synaptogenesis, so dying cells are rapidly replaced with viable cells. Further characterization of the biochemical actions of the drug treatment is necessary to accurately identify which processes occur.

We do not know the biochemical mechanisms behind the neuroprotection conferred by the drug treatment. However, concrete predictions can be made regarding how the drug treatment confers neuroprotection through reviewing the current research regarding how each pharmaceutical modulates the genomic and proteomic responses to ischemia. Previous research has shown that statin treatment protects against excitotoxicity by attenuating the increase in extracellular glutamate (Berger *et al.*, 2008), limiting cytosolic Ca^{++} levels (Bosel *et al.*, 2005), and preventing a second-messenger amplification of glutamate signals (Zacco *et al.*, 2003; Lee *et al.*, 2008). Ligand-bound PPAR- γ also reduces excitotoxicity during ischemia (Ou *et al.*, 2006) and can limit NMDA-mediated excitotoxicity at some point downstream of Ca^{++} influx (Uryu *et al.*, 2002). Constitutive treatment with PPAR- γ ligands increase the expression of a neuronal glutamate transporter, thereby increasing the amount of glutamate removed from the extracellular area. (Romera *et al.*, 2007). Thiazolidines, especially TroglitazoneTM, enhance the permeability of inward K^+ channels while inhibiting the actions of inward Ca^+ channels (Nomura *et al.*, 2008; Knocke *et al.*, 1999; Uryu *et al.*, 2002).

The drug treatment most likely interferes with cell death cascades. PPAR- γ agonists directly reduce apoptosis of neurons, possibly through modulation of nitric oxide production in astrocytes (Heneka *et al.*, 1999; Kim *et al.*, 2002; Victor *et al.*, 2006). SpironolactoneTM treatment reduces apoptosis within the ischemic striatum (Dorrance, 2008). The pharmaceuticals in the drug treatment inhibit pro-apoptotic factors. Statins and PPAR- γ ligands both inhibit the expression and activity pro-apoptotic factors NF- κB , Bax, and caspases (Carloni *et al.*, 2006; Collino *et al.*, 2006b, 2008; Lin *et al.*, 2006). Statins also increase the important anti-apoptotic factor Bcl-2 (Franke *et al.*, 2007) while activating the protein

kinase Akt (Endres & Laufs, 2004). Akt activation inhibits apoptosis and acutely increases NO production within the vasculature.

SimvastatinTM lowers ROS through multiple pathways (Endres & Laufs, 2004; Orr, 2008), for example by increasing the activity of several anti-oxidant enzymes. Fibrates decrease ROS production and lipid peroxidation in rats subjected to ischemia/reperfusion injury, while simultaneously offering protection against glutathione depletion (Poynter & Daynes, 1998; Collino *et al.*, 2006; Deplanque *et al.*, 2003). Both PPAR- γ agonists and SpironolactoneTM protects against oxidative stress while limiting the generation of ROS within ischemic tissue (Aoun *et al.*, 2003; Oyamada *et al.*, 2008)

Statin, thiazolidine, and fibrate treatments increase the expression and activity of the anti-oxidant enzymes including SOD and catalase (Ponter & Daynes, 1998; Adam & Laufs, 2008; Toyama *et al.*, 2004; Hwang *et al.*, 2005; Girmun *et al.*, 2002; Tureyen *et al.*, 2007; Shimazu *et al.*, 2005). Pre-ischemic thiazoladine treatment reverses glutathione depletion in the hippocampus (Collino *et al.*, 2006b). Fibrates increase the activity of enzymes involved in glutathione metabolism (Deplanque *et al.*, 2003; Poynter & Daynes, 1998).

Decreasing levels of pro-oxidant enzymes will also decrease the levels of free radicals. Fibrates & thiazoladines interfere with COX-2 proliferation when induced by pro-inflammatory signaling (Genolet *et al.*, 2004; Collino *et al.*, 2006b; Theocaris *et al.*, 2004; Kim *et al.*, 2002). Thiazoladines, fibrates, and statins downregulate iNOS while statins further inhibit the ischemia-induced activation of nNOS and iNOS (Cimino *et al.*, 2007; Heneka *et al.*, 1999; Kim *et al.*, 2002; Victor *et al.*, 2006; Sundararajan *et al.*, 2005; Kim *et al.*, 2002; Pereira *et al.*, 2005; Collino *et al.*, 2006). Both SimvastatinTM and SpironolactoneTM decrease

the levels of assembled NAD(P)H oxidase while further interfering with the MR's ability to stimulate NAD(P)H oxidase. Statins reduce the MR At1r expression by destabilizing At1r mRNA (Wassman *et al.*, 2001a, 2001b; Laufs *et al.*, 2004; Adam & Laufs, 2008; Endres & Laufs, 2004; Oyamede *et al.*, 2008). Thiazoladine treatment also decreases expression of NAD(P)H oxidase (Hwang *et al.*, 2005).

Statin, fibrate, and thiazoladine treatment preceding ischemia decrease lipid peroxidation (Collino *et al.*, 2006b). Thiazoladines further enhance cell survival via direct and rapid effects on mitochondrial respiration, including protecting electron transport and preventing superoxide anion generation (Kudin *et al.*, 2005; Brunmair *et al.*, 2004; Dello Russo *et al.*, 2003). It should be noted that PPAR γ also increases the expression of uncoupling proteins in mitochondria (Fleury *et al.*, 1997; Willson *et al.*, 2000); this limits the ATP production and the amount of ROS produced by decreasing the proton gradient (Newsholme *et al.*, 2007).

Reducing ROS levels prevents the initiation of pro-inflammatory cascades and leukocyte recruitment. Statins inhibit the activation of T-cells and leukocytes, diminish macrophage and leukocyte adhesion, destabilize pro-inflammatory cytokines including IL-1 β and TNF- α , and reduce C-reactive protein levels (Minnerup & Schabitz, 2008; Zhang *et al.*, 2005; Plenge *et al.*, 2002; Kwak *et al.*, 2000; Weitz-Schmidt *et al.*, 2001). Fibrate's antioxidant effects directly limit neutrophil infiltration, reduce production of proinflammatory cytokines (including TNF- α and IL-1 β), reduce PARP activity, and prevent the upregulation of adhesion molecules (including ICAM-1 and P-selectin) during reperfusion (Cuzzocore *et al.*, 2004; Staels *et al.*, 1998). Thiazoladine treatment curtails the expression of many pro-

inflammatory genes including IL-1 β , IL-6, macrophage inflammatory protein-1, monocyte chemoattractant protein-1, iNOS, ICAM-1, cyclooxygenase-2 (COX-2), and early growth factor (Moraes *et al.*, 2006; Tureyen *et al.*, 2007).

Statins and thiazoladines offer neuroprotection through attenuating the ischemia-induced increase of MMP expression (Crisby *et al.*, 2002; Minnerup & Schabitz, 2008; Peireira *et al.*, 2005; Luo *et al.*, 2006). Statins directly inhibit the upregulation of ICAM-1 following CNS injury (Pannu *et al.*, 2007) while further preventing t-PA induced increased expression of ICAM-1, MMP9, and leukocyte adhesion molecules (Zhang *et al.*, 2005). Thiazoladines can reduce PA expression by endothelial cells, limiting hemorrhagic transformation with reperfusion (Marx *et al.*, 1999b). Thiazoladines can also limit expression of MMPs from activated macrophages (Marx *et al.*, 1998). Fibrates prevent cerebral I/R-induced upregulation of vascular cell adhesion molecule-1 (VCAM-1) and ICAM-1, partially through direct inhibition of the transcription factor NF- κ B (Collino *et al.*, 2006, Deplanque *et al.*, 2003, Wayman *et al.*, 2002; Marx *et al.*, 1999a). Fibrates and thiazoladines reduce induced ICAM-1 expression along with repressing endothelin-1, a potent vasoconstrictor peptide (Moraes *et al.*, 2006). Decreased expression of these adhesion molecules further inhibits the infiltration of neutrophils into the brain ischemic area (Chan, 2001; Lee *et al.* 2000).

With an ischemic insult, pro-inflammatory transcription factors including activator protein-1 (AP-1) and NF- κ B promulgate damage and reducing pro-inflammatory transcription activity reduces damage to the brain (Sarraf *et al.*, 1998; Kim *et al.*, 2002). GemfibrozilTM inhibits both the AP-1 and the NF- κ B transcription of proinflammatory cytokines (Pahan *et al.*, 2002). Ligand-bound PPAR- α bound to ligands directly and indirectly

reduces the transcription of NF- κ B subunits (Genolet *et al.*, 2004). Fibrates increase I κ B expression, and I κ B sequesters NF- κ B within the cytoplasm (Delerive *et al.*, 1999, 2000, 2002; Willson *et al.*, 2000). Ligand-bound PPAR γ directly binds to and inhibits NF κ B (Genolet *et al.*, 2004). Statins also reduce the levels of molecular markers of inflammation including NF- κ B, interleukins, and the pro-inflammatory cytokines IL-6, IL-1 β , and TNF α , (Cimini *et al.*, 2007; Sironi *et al.*, 2006).

Fibrates can also limit pro-inflammatory effects of TNF α -activated macrophages by promoting apoptosis in these harmful cells (Genolet *et al.*, 2004). Ligand-bound PPAR γ inhibits TNF α expression and activity while TNF α inhibits PPAR γ expression and activity. Without added PPAR γ ligands present in the brain during the onset of ischemia, PPAR γ levels drop dramatically due to pro-inflammatory factors. Pro-inflammatory factors such as COX-2 reach their peak levels when PPAR γ is at its lowest levels (Zhao *et al.*, 2006).

Another important method of immune system modulation is influencing the direction of T-helper cell activation. The TH1 subtype has a pro-inflammatory effect while the TH2 subtype does not have detrimental effects. Fibrates and statins both direct the differentiation of activated T-cells into the TH2 subtype (Genolet *et al.*, 2004). Statin treatment further inhibits T-cell activation and proliferation. Ligand-bound PPAR γ further inhibits T-cell proliferation (Moraes *et al.*, 2006). Similarly to affects on macrophages and microphages, ligand-bound PPAR α and PPAR γ inhibit cytokine mRNA expression in activated T-cells (Marx *et al.*, 2002).

Statin treatment protects the microvasculature by increasing vasomotion, eNOS levels, and cerebral blood flow (CBF) while decreasing vasoconstriction and ROS levels

(Laufs, 2003; Orr, 2008; Wassman *et al.*, 2003; Adams & Laufs, 2008). Statins upregulate eNOS by inhibiting cytoskeleton-mediated destabilization of eNOS mRNA (Takemoto & Liao, 2001; Endres *et al.*, 2004). Statin treatment restores and increases eNOS activity during conditions that normally lead to endothelial dysfunction including hypoxia and oxidized LDL (Endres, *et al.*, 2004; Takemoto *et al.*, 2001). Constitutive statin treatment offers further neuroprotection through downregulating angiotensin I receptor (At1r) expression (Marino *et al.*, 2007).

Neurogenesis, angiogenesis, and synaptogenesis are all important mechanisms of sustained cellular presence following stroke. Administration of statins up to 24 hrs following stroke increases the number of migrating neurons along with increasing synaptogenesis and angiogenesis in the CA1 region of the hippocampus and the dentate gyrus (Lu *et al.*, 2007; Dimmerler *et al.*, 2001; Chen *et al.*, 2005). Statin treatment also increases the expression level of certain growth factors including VEGF and BDNF while increasing the proliferation, migration, and survival of endothelial progenitor cells (Maeda *et al.*, 2003; Chen *et al.*, 2005; Liao & Laufs, 2005). Unlike the CA1 region, the CA3 pyramidal region is not capable of neurogenesis, but statin treatment up to 24 hrs following stroke reduces neuronal cell loss in the CA3 region (Lu *et al.*, 2007; Zacharek *et al.*, 2009).

Spironolactone's™ main neuroprotective actions appear to be through effectively repairing the brain following stroke. Spironolactone™ treatment greatly increases the number of neuroblasts entering and remaining viable in the ischemic area. Spironolactone™ also mediates repair of the vasculature and extracellular matrices, which reciprocally allows the nascent neural cells to survive. The Spironolactone™-mediated upregulation of growth

factors for the first week following stroke has been highly correlated with increased blood flow and improved neurological outcomes (Oyamada *et al.* 2008; Dorrance, 2008).

While the combination of these drugs may be effective in protecting against damage during stroke, possible side effects need to be discussed. Side effects from statin treatment are limited, but discontinuing the drug can result in overproduction of the proteins previously suppressed by statin treatment. This means a large increase in Rho activation which consequentially downregulates eNOS levels well below baseline (Gertz *et al.*, 2004).

The side effects of fibrate treatment are unknown. The actions of PPAR α is highly variable between species, and the ligand binding domains of PPAR- α exhibit a low level of homology, probably reflecting adaptation to different dietary ligands (Willson, 2000). PPAR α was first identified due to its ability to cause proliferation of peroxisomes (Isserman, 1990), which are organelles responsible for oxidizing compounds while releasing toxic compounds in the process. Due to peroxisome proliferation, fibrates induce liver cancer or hepatotoxicity in rodents (Lambe *et al.*, 1999). However, no PPAR subtype has been shown to instigate peroxisome proliferation in a non-rodent species, including humans (Cattley *et al.*, 1998). In fact, in humans PPAR α appears to prevent peroxisome proliferation (Lambe *et al.*, 1999; Roberts *et al.*, 1998). Thus, where human PPAR α protects cell survival, rodent PPAR α will induce apoptosis. There is still an ongoing investigation into whether PPAR- α ligands could have harmful effects in humans similarly to those found in rodents. At present, no evidence for harmful effects has been found in humans (Peters *et al.*, 2005; Klaunig *et al.*, 2003).

Side effects of thiazolidines and TroglitazoneTM include the development of edema, congestive heart failure, and weight gain (Willson *et al.*, 2000). It has been asserted that PPAR γ actions on macrophage activation may facilitate the development of tumors (Lefebvre *et al.*, 1999). A second concern for PPAR γ agonists is hepatotoxicity. In a study where 2,510 patients received TroglitazoneTM altered levels of liver proteins were found to be 3x normal in 1.9% of participants. TroglitazoneTM treatment was discontinued due to these abnormalities in 20 participants (.8%), which brought these levels back to normal. Elevated liver proteins were found in .6% of the diabetic individuals receiving placebo treatment (Watkins & Whitcomb, 1998). For this reason, TroglitazoneTM treatment has been discontinued in England and is banned in North America as a unimodal treatment, though it can be prescribed legally as part of a multimodal treatment (Willson *et al.*, 2000).

As a MR antagonist, SpironolactoneTM may have detrimental effects on neurons because MR agonism in neurons is necessary for neurogenesis and survival during global ischemia while blocking MRs decreases neuronal survival (Macleod *et al.*, 2003; Fisher *et al.*, 2002). Following ischemia, MR upregulation on neurons offers neuroprotection and reduces the behavioral effects of ischemia (Lai *et al.*, 2005, 2007). A certain ratio between glucocorticoid receptor (GR) and MR activation is necessary for cell survival, and a subtle shift increasing GR activation relative to MR activation in neurons is necessary to cause apoptosis (Almeida *et al.*, 2000). It should be noted that with the increased permeability of the blood-brain barrier that occurs during transient global ischemia, aldosterone levels increase (Gomez-Sanchez *et al.*, 1990). SpironolactoneTM may not have detrimental effects

during ischemia, as it may offset the harmful results of the increased mineralcorticoid levels due to the permeability of the blood brain barrier during ischemia.

Epeleperone, a more selective MR antagonist than SpironolactoneTM, has been used in these studies on the detrimental effects of MR blockade. The use of Epeleperone may have increased the detrimental effects of MR agonism while eliminating some of the Spironolactone'sTM benefits. SpironolactoneTM may also bind receptors other than the MR including the androgen (testosterone) receptor and progesterone receptor (Dorrance, 2008). Competitive inhibition of the dihydrotestosterone receptor by SpironolactoneTM may afford neuroprotection because increased androgen levels amplify ischemic injury while diminishing the survival of neural progenitor cells (Berardesca *et al.*, 1988; Wong & Herbert, 2004; Cheng *et al.*, 2007). SpironolactoneTM also agonizes progesterone receptors and progesterone has increasingly been shown to have neuroprotective effects during cerebral ischemia (Williams *et al.*, 2006; Gibson *et al.*, 2008).

Following stroke, MRs are upregulated on both neurons and astroglia. On day one, the levels of MRs on neurons are relatively high while astrocyte levels are relatively low. On day two, however, astrocyte levels of MR are high while neuron levels are low. The beneficial effects of MR antagonism appear to result from actions on astrocytes (Oyamada *et al.*, 2008). Further tests should investigate whether optimal benefits arise from beginning SpironolactoneTM treatment following stroke. As SpironolactoneTM decreases hypertension and improves vasculature, it is important to optimize SpironolactoneTM treatment to increase vascular benefits and create the best possible myogenic tone proceeding heart surgery.

Very little research has been performed into the ability of Spironolactone™ to confer neuroprotection during ischemia/reperfusion injury. While it has been shown that Spironolactone™ decreases ROS and increases neurotrophic factors, it is unknown whether this causes a decrease in pro-inflammatory factors or infiltrating leukocytes. It has been proposed that Spironolactone™ reduces necrosis/apoptosis by increasing Bcl-2. However neither Bcl-2 levels nor a reduction of caspase activity have been experimentally demonstrated. Furthermore, MR antagonism can decrease Bcl-2 levels in neurons (McCullers & Herman, 1998). Another problem is that Spironolactone™ has only been investigated in a focal model of ischemia while our research uses and is directly applicable for a global model of ischemia.

In conclusion, treatment with the drug cocktail confers neuroprotection following transient global ischemia, as demonstrated by neuronal survival and behavioral scores. Full investigation of possible side effects, the optimization of drug treatment, and the investigation into the underlying biochemical mechanisms of the drug treatment have yet to occur. Despite these shortcomings, to rapidly translate this treatment into clinical practice will likely prevent the unnecessary decline of neurological functions in many individuals.

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