EFFECT OF CHRONIC MELATONIN PRETREATMENT ON NEURAL AND BEHAVIORAL CONSEQUENCES OF GLOBAL ISCHEMIA IN MALE GERBILS

by

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ABSTRACT

Melatonin has been shown to be an effective neuroprotectant against the neural and behavioral consequences of global ischemia in previous research; however, there is little information as to how melatonin may be used as a chronic prophylactic treatment against ischemic injury in male gerbils The current study sought to determine if chronic melatonin treatment (via a time-released subcutaneous pellet) initiated 2 weeks prior to ischemia attenuates ischemia-induced CA1 cell loss, locomotor hyperactivity, and changes in spontaneous alternation. Gerbils were divided into either a sham, placebo-treated, or melatonin-treated ischemic groups. Ischemic groups were subjected to 5-minute bilateral occlusion of the carotid arteries. Global ischemia produced significant neuronal loss in the CA1 and resulted in a significant increase in locomotor hyperactivity. Exploratory and anxiety-like behavior were not affected by global ischemia. Spontaneous alternation behavior was also unaltered. Chronic melatonin treatment did not afford neuronal protection or decrease locomotor hyperactivity. Analysis of the global ischemic injury revealed a high mortality rate and variable injury in the current sample. This is thought to be due to increasing anatomical variability in the gerbil global ischemia model. The low sample size and inconsistent ischemic injury suggest that the insignificant results of the current study are not definitive of chronic melatonin's pretreatment potential. Future research using larger sample sizes to account for the increasing variability in the gerbil model is encouraged to clarify the chronic prophylactic potential of melatonin.

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CHAPTER I

INTRODUCTION

Global cerebral ischemia occurs when the blood supply to the brain is halted or significantly impeded to the point that the metabolic demand of the central nervous system is not met. Clinically, this disruption is seen in disorders involving disruption of blood flow, arterial plaque accumulation, blood clots, compressed blood vessels, low blood pressure, congenital heart defects, sickle cell anemia, asphyxiation, and cardiac arrest. Perhaps most notably, cerebral ischemia accounts for approximately 87% of strokes in the United States. Stroke continues to be one of the most insidious and fatal neurological disorders, affecting over 795,000 people within the United States and killing almost 130,000 Americans each year (Centers for Disease Control and Prevention, 2015; Mozaffarian & Benjamin, 2015). Though an ischemic event can happen to anyone, risk factors include high blood pressure, diabetes, heart diseases, smoking, and age.

Global ischemia is a complicated disease involving a cascade of neural decay produced by excitotoxicity, free radical generation, inflammation, and apoptosis (Lipton, 1999). Development of practical, multimodal strategies is imperative to curb the detrimental neurological and behavioral effects of hypoxic events. Recently, melatonin has been demonstrated to possess anti-oxidant and neuroprotectant properties that

are surprisingly effective in combating the several pathological cascades of ischemia (Reiter et al., 2005). Due to its bioavailability, lipid- and cell-solubility, and lack of toxicity, melatonin is a prime candidate for a neuroprotective agent that may protect against ischemic insult. Though preliminary research is promising, there is a notable lack of research investigating the effectiveness of chronic melatonin pretreatment in protecting against ischemic injury. Most researchers administer melatonin either via injection or intravenously; however, this methodology is difficult to translate to clinical settings due to the narrow window of opportunity for acute treatments (Danton & Deitrich, 2004). Hence, the present study sought to determine the efficacy of chronic melatonin pretreatment through a clinically relevant model of transient global ischemia in gerbils to combat the neural and behavioral consequences of ischemic insult.

CHAPTER II

LITERATURE REVIEW

Animal Models of Global Ischemia

Over the course of research on global ischemia, numerous animal models have been developed to further understand the pathophysiology of the disease and to predict the effectiveness of therapeutic interventions. Traystman (2003) offers a brief review of the animals and types of models commonly used for global cerebral ischemia. Today, there are three main models of global ischemia utilized in rodent models: the four-vessel occlusion (4-VO) in the rat, the two-vessel occlusion plus hypertension (2-VO+hypo) model in the rat, and the transient global forebrain ischemia model in the gerbil.

Pulsinelli and Brierley (1979) first developed the 4-VO model, providing an initial example of reversible forebrain cerebral ischemia in rats, which was later refined to increase the survival rate of the animals (Pulsinelli & Buchan, 1988). This model first requires the vertebral arteries to be irreversibly occluded by electrocoagulation. The carotid arteries are also isolated to facilitate subsequent occlusion. Twenty-four hours later, carotid arteries are re-exposed and clamped using microvascular clamps for 10 minutes. Puslinelli and Buchan (1988) cite an approximate 50 to 75% success of the 4-VO technique but also acknowledge that the effects yielded by the ischemia are quite

inconsistent between different strains of rats with varying degrees of collateral circulation. Traystman (2003) offers a review of the physiological mechanisms of the ischemic insult. Within this model, blood flow to the neocortex, striatum, and hippocampus is dramatically reduced to less than 3% of control levels; subsequently, 5 to 15 minutes post-reperfusion yields considerable cerebral hyperemia. Severe cerebral hypoperfusion has been shown to persist in certain brain areas for up to 24 hours post-injury, particularly within the neocortex, striatum, and hippocampus. Striatum neurons exhibit significant damage after 30 minutes of ischemia, and hippocampal damage is observable 3 to 6 hours post-reperfusion. Damage to neocortical areas is seen 1 to 3 days post-ischemia. The 4-VO model is well described and validated within the research community; however, the difficulty in successfully cauterizing the vertebral arteries, significant variability in the ischemic results, and a potential of producing neuroprotective preconditioning due to multiple surgeries may be of concern to researchers.

Elkof and Siesjo (1972a, 1972b) first developed the 2-VO+hypo model in the rat in which a bilateral common carotid artery occlusion paired with systemic hypotension results in a forebrain ischemic event. The 2-VO occlusion is induced by surgically exposing and suturing both of the common carotid arteries (Sanderson & Wilder, 2013). Even with this occlusion, the rat's Circle of Willis allows vertebral arteries to compensate for blood flow within the brain; therefore, hypotension must be induced to reduce the amount of blood flow reaching the brain to levels that will mimic ischemic damage. While specific methods vary, this hypotension is achieved through inducing hemorrhage by withdrawing blood and thereby reducing blood pressure to 30-50mmHg for

approximately 8 to 15 minutes (Sanderson & Wilder, 2013; Traystman, 2003). Other methods include introducing various drugs into the bloodstream to elicit hypotension (Traystman, 2003). Traystman (2003) offers a review of the pathology of these methods. Typically, a reduction of cerebral blood flow to less than 5% of control is seen within the cortex during the hypertension while hypoperfusion continues post-reperfusion. The *Cornu Ammmonis* 1 (CA1) pyramidal neurons of the hippocampus, caudoputamen, and neocortex exhibit the most selective injuries with insult persisting over one week of recovery. Of particular importance for this model is the variability that may be induced through uncontrolled temperature variation during the hypotension and ischemic event (Sanderson & Wilder, 2013; Traystman, 2003). One advantage of this model in comparison to the 4-VO method is the less complicated surgical procedure. Also, reperfusion occurs more readily due to the lack of occlusion of the vertebral arteries. Clinically, this model is more suited to studies involving chronic hypoperfusion than the 4-VO model (Traystman, 2003). Sanderson & Wilder (2013) caution, however, that the administration of hypotension can elicit variable results, particularly if blood pressure is not reduced dramatically enough.

One of the most widely used animal models in global cerebral ischemia is the gerbil model. Kirino (1982) first described the method, which takes advantage of the gerbil's unique vasculature. The anastomotic system of arteries that provides blood flow throughout the brain, the Circle of Willis, is uniquely predisposed to ischemic insult in the Mongolian gerbil (*Meriones unguiculatus*). The animal lacks interconnection between the carotid and vertebrobasilar circulation due to either underdeveloped or a lack of posterior communicating arteries. Consequently, cerebral ischemia can easily be

produced by occluding the carotid arteries in the gerbil's ventral neck (Kirino, 1982). In this model, cerebral blood flow is reduced to near zero (Traystman, 2003). Bilateral occlusion of the carotid arteries can be attained through a simple incision in the gerbil's ventral neck and use of microarterial clamps. Following the ischemic event, blood flow is easily restored by removing the clamps from the carotid arteries. Using this model, occlusion produces severe damage to the CA1 hippocampal neurons and ischemic cell change in *Cornu Ammmonis* 4 (CA4) hippocampal neurons (Kirino, 1982). Many studies have noted the significant increase in spontaneous locomotor activity and latency in passive avoidance response in ischemic gerbil models, which is indicative of neuronal degradation of the CA1 region of the hippocampus (Janać et al., 2006; Mileson & Schwartz, 1991; Yamamoto et al. 1993). Yamamoto et al. (1993) have shown that this method can be used as a chronic model of cerebral ischemia with intermittent occlusions, which elicits profound behavioral changes and hippocampal degeneration. This method provides a simple procedure that eliminates the confounds of complex surgeries and extensive exposure to anesthetics and/or hypotension drugs. Additionally, the absence of communication between the carotid and vertebrobasilar arteries elicits severe reduction of cerebral blood flow, mimicking clinical models of severe ischemic events.

Mechanisms of Cell Death in Global Ischemia

The cause of neuronal injury in global ischemia involves a complex sequence of neural mechanisms that progress over time. Though there are many speculated mechanisms through which ischemic cell death occurs, research in animal models has provided three main pathways that lead to the majority of neural atrophy in transient

global ischemia: excitotoxicity, apoptosis, and free radical generation. Lipton (1999) provides an excellent review of these pathologies.

Excitotoxicity and Apoptosis

Though all cells in the body thrive off of oxygen and glucose, those within the central nervous system are noted for having a particularly high metabolic need for these substances to survive. This system is near exclusively dependent upon oxidative phosphorylation to produce energy and when it is deprived of these substrates, the sufficient amount of energy needed to polarize neuronal and glial cells is not met, sending the cells into a catastrophic state of excitotoxicity (Lipton, 1999). The depolarization initiates a sequence of cascading events during many large, rapid intracellular ion and metabolite change take place. Homeostasis is disrupted as the balanced intracellular flow of calcium (Ca^{2+}), sodium (Na^{+}), chloride (Cl^{-}), and potassium (K^{+}) dysregulates, resulting in enzymatic overstimulation and mitochondrial damage (Lipton, 1999; Szydlowska & Tymianski, 2010). Due to the rapid and concurrent nature of ion and metabolite changes, it is difficult to isolate to what extend each is individually involved in necrotic cell death; however, researchers concur that this excitotoxicity is responsible for the initial neuronal damage in ischemia (Lipton, 1999).

Though necrosis is the primary mechanism of cell death in an ischemic event, a significant amount of apoptosis occurs following the insult (Lipton, 1999). Apoptosis is a natural process through which the central nervous system programs cell death to purge cells that are no longer needed or are threatening to surrounding structures. During the cascade of events following an ischemic event, lipid peroxidation and reactive oxygen

species that emerge from free radicals induce apoptosis (Lipton, 1999; Mattson, Culmsee, & Yu, 2000). In addition to oxyradical production, disruption of the Ca^{2+} homeostasis, activation of caspases, and the dysfunction of mitochondria mediate the neuronal apoptosis following an ischemic event (Mattson, Culmsee, & Yu, 2000). Autophagocytotic cell death has been implicated within global ischemic models, particularly within gerbil models (Lipton, 1999). As the prevalence of autophagosomes increases post injury, the normally regulated process of purging dysfunctional or impertinent cells is exacerbated. Through these apoptotic mechanisms, glial cells program suicide within the surviving, damaged neurons in an attempt to rid the system of potential threat.

Free Radicals

Of particular relevance to the current study is that evidence suggests the prolonged effects of ischemia are initiated by the overrepresentation of Ca^{2+} within the intracellular space, which causes enzyme induction and free radical production (Lipton, 1999). During and after the ionic disruption of the ischemic event, oxygen free-radicals are overproduced within the central nervous system (Dugan & Choi, 1999). A free radical is a molecule that possesses an outer electron orbital with a solitary unpaired electron. Though these free radicals are naturally occurring within the metabolic and synthetic reactions of cellular homeostasis, the lack of oxygen during a hypoxic event, such as ischemia, prevents natural processes within the cell from detoxifying free radicals as they are produced, due to excessive production of reactive oxygen species and loss of antioxidant defenses (Dugan & Choi, 1999). Because brain tissue is particularly

dependent upon metabolic systems that process oxygen for energy, it is especially vulnerable to the creation of free radicals during hypoxia, lipid peroxidation, and mitochondrial damage. Additionally, the brain is made more susceptible to free radical damage due to the fact that it is rich in polyunsaturated fats that are easily oxidized, possesses elevated levels of hydroxide-increasing non-heme iron, is abundant in vitamin C levels that react with this excessive iron to form pro-oxidants, and is part of the central nervous system which has relatively little antioxidant defense systems (Reiter et al., 2005; Rodrigo et al., 2005). Numerous free radicals have been implemented in ischemic damage; however, the synthesis of peroxynitrite through elevated levels of nitric oxide (NO) reacting with superoxide is considered to be especially involved in tissue damage (Dugan & Choi, 1999; Lipton, 1999). Starved for oxygen, these free radicals seek out an electron pairing from surrounding lipids and proteins, leading to the induction of various enzymes, membrane degradation, and mitochondrial damage (Dugan & Choi, 1999; Lipton, 1999). As free radicals oxidize surrounding structures, decay is seen within lipids, proteins, and DNA sequences within the cell, culminating in necrosis. The more frequently free radicals are generated, the more endogenous scavenging systems become overwhelmed, resulting in further lipid peroxidation and membrane damage (Dugan & Choi, 1999). This system of decay revolves on a self-perpetuating cycle as free radicals damaging mitochondria yields more free radical production (Lipton, 1999). There is significant evidence to support that the prevalence of free radicals is exacerbated during ischemic events; however, much greater production of reactive oxygen intermediates recurs during the reperfusion event when oxygen is restored to the brain (Dugan & Choi, 1999). This highlights how free radical production can continue to permeate the brain for

prolonged periods following an ischemic event, resulting in global ischemia's hallmark delayed neuronal death.

Neuropathology of Global Ischemia

Within the three animal models previously described, the degradation of various neurological structures is well documented, including damage to the hippocampus, striatum, and neocortex (Kirino, 1982; Pulsinelli, Brierley, & Plum, 1982; Smith et al., 1984). The most prominent structural ischemic damage is not exhibited at the time of ischemia itself, but rather emerges as delayed neuronal death as the effects of ischemic cascade and reperfusion take place (Lipton, 1999; White et al., 1996). While most brain structures are relatively preserved after an ischemic event, selective vulnerability of pyramidal neurons within layers III and V of the cortex as well as the CA1 and CA4 sectors of the hippocampus are observed. Though notable damage may be observed within the striatum and neocortex, the neuronal death within these areas is relatively variable (Block, 1999; Lipton, 1999; White et al., 1996). As the necrotic cascade of excitotoxic events trigger within the hippocampal region, the postsynaptic neurons of the pyramidal hippocampal cells in the CA1 area of the hippocampus receive the most devastating blow (Block, 1999; Reiter et al., 2005). This sector houses pyramidal neurons that are particularly susceptible to excitotoxicity due to the abundance of glutamate at their synapses. Block (1999) offers a review of evidence supporting that neuroprotective strategies coincide with reduction in hippocampal damage and deficits in spatial learning and memory. Additionally, this review provides evidence that damage to other areas which may be implicated in learning and memory deficits, such as CA3 and

CA4 hippocampal sectors, striatum, and cortex are inconclusive in their role affecting these constructs; in contrast, CA1 hippocampal damage shows consistent correlation with memory and learning deficits across animal models (Block, 1999; Traytsman, 2003). Because other measures of hippocampal damage are inconsistent, the CA1 hippocampal sector is the standard measure for reliable histological data in global ischemia models.

Ninonenko et al. (2009) provide a review of the structural hippocampal injury present in post-ischemic models. In this, evidence surmounts to show that pyramidal neurons within the CA1 area of the hippocampus are the most susceptible to ischemic events, largely due to DNA fragmentation. Prolonged global cerebral ischemia in gerbil models show the death of up to 96% of CA1 neurons within 4 days (Colbourne, Sutherland, & Auer, 1999; Kirino, 1982). Yamamoto et al. (1986) revealed that hippocampal injury in the CA1 area can occur after 5 minutes of ischemia in global ischemia gerbil models. Later, this technique was used to demonstrate that the extent of hippocampal damage and the behavioral changes were directly related to the duration of the ischemic insult (Yamamoto et al., 1993). It is the severe and near exclusive damage to the CA1 hippocampal neurons in global ischemia gerbil models that make histological analysis concurrent with behavioral deficits a prime candidate for evaluating the effects of global ischemic damage.

Neurobehavioral Consequences of Global Ischemia

The complex process through which excitotoxicity, free radicals, and apoptosis afflict the neurons in the central nervous system emerge as neurobehavioral deficits within animal models as critical neuronal structures are damaged, which can in turn be observed

through behavioral and histological changes. In both humans and animals, ischemia affects neuronal structures that are most vulnerable to the effects of hypoxia: the hippocampus, striatum, cerebral cortex, and the cerebellum (Block, 1999; Janać et al., 2006; Lipton, 1999; Yamamoto et al., 1993). Functional changes can be directly linked to structural damage within these areas. These deficits emerge in various forms in human patients, including but not limited to deficits in in spatial orientation and decreased abilities in learning and memory (Block, 1999). Neuronal loss within the CA1 area of the hippocampus after global ischemia has been negatively correlated with errors in reference memory, working memory, spatial learning, and passive avoidance tasks (Block, 1999; Hartman et al., 2005; Yamamoto et al., 1993). In addition to deficiencies in learning and memory, global ischemic injury creates notable abnormalities in locomotor activity.

Hyperactivity

Because the neurobehavioral constructs related to hippocampal damage are not directly observable within animal models, researchers have developed behavioral measures to determine how damage within hippocampal structures affects learning and memory. Transient locomotor hyperactivity has been observed within many animal models following a global ischemic event, which has been corroborated with the extent of hippocampal damage to CA1 areas (Block, 1999). Two distinct types of locomotor hyperactivity have been described. The first is an acute phase of activity that begins approximately 1-2 hours post-ischemia and continues for 1-2 days, depending upon the extent of injury. During this time period there is a severe increase in spontaneous locomotor activity. The second is a chronic increase in locomotion beginning

approximately 2 days post-reperfusion that gradually decreases across time as compensatory, plastic neuromechanisms ameliorate the damage; however, researchers argue that this behavioral change is not limited to a brief period after occlusion and may be representative of a permanent deficit (Babcock, Baker, & Lovec, 1993; Janać et al., 2006).

One of the most common methods of measuring locomotor activity is through an open field task in which the animals are allowed to freely roam within a novel environment. Plamondon & Khan (2005) used a 4-VO rat model of global ischemia to characterize hyperactivity after injury. Rats showed a significant increase in activity on an open field test evident through 7 days post-ischemia, which was concurrent with 70-75% loss of pyramidal cells in CA1 hippocampal layers. Mileson & Schwartz (1991) used a novel open-field environment to provide evidence that chronic locomotor hyperactivity is persistent across 28-days post-ischemia in a gerbil model. These researchers also conferred that the increase of locomotor activity was consistent with extensive pyramidal cell damage in the CA1 region of the hippocampus but not with striatal or cortical damage. Babcock, Baker, & Lovec (1993) reported a significant increase in locomotor activity post-ischemia in gerbils that persisted for 16 days. These researchers confirmed previous reports that there is a dramatic increase in locomotor activity in an open field task 1 and 2 days after ischemia. Additionally, hyperactivity in gerbils was shown to persist up to 16-days after ischemic insult. Katsuta et al. (2003) applied multiple neuroprotective compounds - including NMDA modulators, glutamate release inhibitors, anti-inflammatory agents, and radical scavengers – to gerbils to determine the relation between CA1 hippocampal loss and hyperlocomotion. The study affirmed drastic increases in locomotor activity 1-4 days

after global ischemic injury, which coincided with a significant reduction in CA1 hippocampal neurons. Moreover, the more neuroprotection was offered to the CA1 pyramidal neurons of the hippocampus by the various drugs applied to the gerbil model, the greater the reduction in hyperactivity in an open field assessment of spontaneous locomotor activity. In this, the researchers indicate that post-ischemic hyperactivity is a powerful screening method in evaluating the efficacy of neuroprotective agents' preservation of the CA1 area of the hippocampus in gerbils. Ramos-Zuniga et al. (2008) affirm that locomotor activity in an open field is an observable behavioral change indicative of global ischemic injury 24 hours post-surgery. This study showed that ischemic gerbils effectively doubled locomotor activity one day after injury. Wappler et al. (2009) corroborated evidence that the open-field task is an effective measure of degradation of CA1 hippocampal neurons in the gerbil ischemic model. Animals in this study displayed massive increases in ambulation, particularly around the perimeter of the open field. Additionally, the group concluded that this measure is consistently indicative of injury regardless of age. Overall, researchers concur that the typical increase in locomotor activity post-ischemia is a reliable measure of global ischemic injury.

Exploratory Behavior

Another measure that can be assessed in the open field is exploratory behavior. Locomotor activity, duration of movement, change in activity, and rearing are behaviors that have been implemented in the assessment of exploratory behavior of rodents in the open field (Carter & Shieh, 2015; Gould, Dao, & Kovacsics, 2009). Rearing behavior, as defined by a rodent lifting both of its front paws off of the ground, is an interesting measure

because it can be easily monitored alongside a variety of other behaviors, due to the postural differences in horizontal and vertical activity. Carter and Sheih (2015) and Gould, Dao, and Kovacsics (2009) provide excellent reviews on the history of rearing behavior as a measure of exploration in rats and mice. Currently, however, there is limited and conflicting research on how this measure is affected by global ischemia in gerbils. Though Colbourne, Auer, and Sutherland (1998) found acute hyperactivity and neurobehavioral deficits in global ischemic gerbils concurrent with CA1 neuronal degradation, the researchers did not find significant differences in rearing behaviors. Similarly, Janać et al. (2006) found no changes in exploratory rearing behavior in 5-, 10-, and 15-minute occlusions of the gerbil global ischemic model either, despite noting the hallmark increase in locomotor activity. In contrast, Wappler et. al. (2009) observed that global ischemic gerbils displayed a significant reduction of rearing behaviors after injury that was consistent regardless of age in a more recent study. Because rearing behavior has been well described in other rodent models yet the current literature on how it is particularly affected in gerbil global ischemia is unclear, this measure of exploratory behavior warrants more attention in order to determine whether or not it is a reliable measure of ischemic injury.

Anxiety Behavior

In addition to hyperactivity and exploration, anxiety behavior is an additional measure that can be assessed in the open field. A common measure of anxiety in rodents is the amount of time spent within the center of an open field apparatus in which the duration of exploration of the center is inversely related to the amount of anxiety level in

animal (Bailey & Crawley, 2009; Carter & Shieh, 2015; Prut & Belzung, 2003). Though there is ample literature in how this measure related to rats and mice, there is little research in how this measure is affected by global ischemic damage in gerbils. Wappler et al. (2009) found that global ischemic gerbils did not spend significantly different amounts of time within the inner confines of an open field task regardless of age. Due to the prominent lack of research using this measure despite its use in other rodent models, more dedication is needed in determining whether or not the amount of time spent in the center of an open field is a reliable measure of global ischemic damage in gerbils.

Spontaneous Alternation Behavior

Another locomotor activity task that is closely tied to memory deficits is the Y-maze. The Y-maze is a commonly used test used to assess spontaneous alternation behavior (SAB) in animal models (Lalonde, 2002; Wappler et al., 2009). In this task, animals are placed within the center of a Y-shaped maze and allowed to freely navigate its three unbaited arms. SAB refers to an animal's natural tendency to alternate which arms it explores within the apparatus, as measured by three consecutive visits to arms unexplored just previously. This measure has been used to measure anxiety, spatial memory capacity, and locomotor activity. Historically, SAB has been shown to be significantly reduced in animals suffering from hippocampal damage, making it a classic behavioral test for evaluating hippocampal deficit (Lalonde, 2002; Wappler et al, 2009). Gyertyán, Gigler, & Simó (1999) demonstrated that the Y-maze can be used in the ischemic gerbil model to indicate CA1 hippocampal neuron loss. In this study, global ischemia created a 91-94% increase in hypermotility concurrent with severe neuronal degradation in the CA1

pyramidal neurons of the hippocampus. Additionally, the researchers showed a significant decrease of SAB in ischemic gerbils. When a neuroprotective agent was administered to gerbils, there was a significant ameliorative effect in decreasing hypermotility and increasing SAB. Wappler et al. (2009) affirmed previous research indicating a significant increase in hyperactivity in the gerbil global ischemia model. Global ischemic gerbils also exhibited a significant decrease in SAB after insult. Both of these results were associated with significant decreases in CA1 hippocampal neurons. In addition, the researchers indicated that the Y-maze provided consistent results regardless of age in this ischemic model with all exhibiting SAB above chance level. Similarly, Kim et al. (2011) found a significant reduction of SAB in ischemic gerbils indicative of severe loss of pyramidal neurons in the CA1 area of the hippocampus, which was shown to be attenuated by the administration of a neuroprotective agent. Throughout its implementation in the literature, the Y-maze task has proven to be a reliable measure of hypermotility and SAB in global ischemic models that is concurrent with loss of CA1 hippocampal neurons.

Current Treatments for Global Ischemia

Over the course of the past few decades, a plethora of research has been conducted with the intent of limiting the devastating neuropathological, behavioral, and neurobehavioral effects of global cerebral ischemia and developing neuroprotective strategies to promote recovery. This research, however, has been limited by the understanding of the neuropathways of ischemia, ethicality in human studies, and limitations of animal models. Danton and Deitrich (2004) and Woodruff et al. (2011) provide thorough reviews of current endeavors to limit global ischemic injury. With all

treatments, the temporality of therapeutics is imperative with the most effective strategies occurring either before or immediately after the insult. Animal models of global ischemia are most efficacious within 15-30 minutes post-ischemia with studies rarely showing ameliorating consequences with intervention as little as 3 hours post-onset (Woodruff et al., 2011). The most important step in limiting the extent of ischemic injury is the immediate restoration of blood flow to brain; however, even this must be done with caution due to the deleterious effects of reperfusion, which perpetuates free radical production, (Danton & Dietrich, 2004; Woodruff et al., 2011). With this in mind, it is important for researchers and clinicians to understand the complexities of interactions that create ischemic injury and to develop new therapeutic means of combating the neuropathological mechanisms of global ischemia, which often promote more damage in the long-term than the initial consequences.

Restricting Ischemic Cascade

One attempt to reduce the amount of neural damage instigated by ischemia is to restrict ionic and metabolic cascades that disrupt the neuronal homeostasis previously discussed. This has been attempted to be controlled through introducing neuroprotectant drugs to limit the imbalance of Na^+, K^+, and CA^{2+} that causes excitotoxicity (Danton & Dietrich, 2004; Woodruff et al., 2011). Drugs that are designed to block Na^+ channels, such as phenytoin, carbamazepine, lamotrigine, sipatrigine, and riluzole have shown promise in reducing ischemic insult in animal models with greater efficacy evidenced in pretreatment models that posttreatment; however, some of these Na^+ mediators have been discontinued due to toxicity and effectiveness issues (Danton & Dietrich, 2004). Ates et

al. (2013) provide the promising results of riluzole, mexiletine, and phenytoin as excitatory regulators by reducing lipid peroxidation, brain edema, and reducing neuronal damage in the hippocampal CA1 sector of a 2-VO rat model; moreover, the effect of these agents was increased by administering them prior to the onset of global ischemia.

Other interesting channel modulators are those that open K^+ channels to hyperpolarize cells as a means to prevent excitotoxicity. The research of Na^+ and K^+ regulators is promising yet still developing, and the course of such therapeutics is still unknown. There have been numerous studies involving Ca^{2+} channel blockers as a means of hindering the main pathways of excitotoxicity that initiate the cascade of ischemic cascade; however, the effects of these agents have been largely variable (Danton & Dietrich, 2004; Woodruff et al., 2011). Zhang et al. (1993) have provided one of the most promising candidates for Ca^{2+} channel blockers with the advent of dantrolene. With this agent excitotoxicity and CA1 hippocampal neuron death was significantly reduced after bilateral administration 30 minutes post-reperfusion in gerbils. Follow-up studies on the efficacy of dantrolene and other Ca^{2+} channel blockers are still in progress.

Another means of regulating excitotoxicity has been pursued through controlling the excessive glutamate activation through inhibiting receptors with competitive and noncompetitive agents (Danton & Dietrich, 2004; Woodruff et al., 2011). Both of these pathways have provided some promising results in initial trials coupled with large variability in follow-up trials that either proved inefficacious or yielded profound side effects. Up to this point research involving n-methyl-D-aspartate (NMDA) antagonists, non-NMDA antagonists, N-acetlaspartylglutamate inhibitors, and metabotropic glutamate

receptor modulators that seek to restrict the excitotoxicity of excessive glutamate influence has been largely disappointing though many have shown mild forms of neuroprotection in preliminary research (Danton & Dietrich, 2004). Magnesium is an intriguing candidate for inhibiting NMDA receptors, which is particularly appealing due to its low cost, widespread availability, and relative safety. Westermaier et al. (2013) provide a review of evidence supporting magnesium as a neuroprotective agent across various animal models of ischemia but an inability to reproduce the effect in human trials; however, research in this method is still emerging and may be subject of future experimental endeavors.

Free Radical Scavengers

As discussed above, the procreation of free radicals oxidizes lipids, proteins, and DNA in the central nervous system, leading to necrotic and apoptotic cell death (Dugan & Choi, 1999; Lipton, 1999). Consequently, some strategies for neuroprotection against ischemia focus on the development of antioxidant free radical scavengers that limit the damage of reactive oxygen species and the oxidative stress that contributes to the pathogenesis of ischemia. These free radical scavengers work by either providing an extra electron to pair with the highly reactive free radical or breaking down free radicals into harmless molecules. Enzymatic antioxidants, such as copper zinc-superoxide dismutase, manganese superoxide dismutase, thioredoxen, and peroxiredoxin, have demonstrated neural-protective effects in animal models of transient global ischemia, significantly reducing degradation of CA1 hippocampal neurons (Lee & Won, 2014). Additionally, antioxidants including uric acids and free-radical trapping agents are being

developed to combat oxidative stress; however, there is limited research in their infancy and clinical trials have produced inconsistent results (Danton & Dietrich, 2004; Woodruff et al., 2011). As knowledge of the formation and deleterious effects of oxidative stress compounds, the synthesis of novel antioxidant treatments stands out as a prominent potential neuroprotective strategy; however, research in this area is relatively new, and their efficacy is still being determined. One such novel approach is the use of antioxidant properties of melatonin as combatant traumatic central nervous system damage.

Melatonin

Melatonin, N-acetyl-5-methoxy tryptamine, is a well-described hormone that is secreted from the pineal gland and multiple extra-pineal sites, most known for its involvement in the regulation circadian rhythms and its versatile participation in numerous neuroendocrine and physiological processes (Samantaray et al., 2009). Since the identification of melatonin as a potent antioxidant and free radical scavenger less than two decades ago, a plethora of studies have been implemented to determine its neuroprotective properties (Reiter et al., 2005). One of the benefits of melatonin in these endeavors is its multimodal neuroprotective pathways, including its antioxidative, antiapoptotic, and anti-inflammatory properties, which target multiple pathologies in ischemic injury (Andrabi, Parvez, & Tabassum, 2015; Cervantes, Morali, & Letechipia-Vallejo, 2007). Though the direct antioxidant actions are the primary modality of its neuroprotection, there is increasing evidence to support melatonin's improvement of mitochondrial functioning, interference in apoptotic cell signaling, inhibiting the synthesis of inflammatory cytokines, and preserving neuro-cytoskeletal organization. In

addition, melatonin is an endogenous antioxidant which readily crosses the blood-brain barrier and has proven to have wide intracellular distribution while retaining adequate safety profiles; consequently, it is able to be safely available in higher concentrations without interfering with thrombolytic and neuroprotective actions of other drugs (Cervantes, Morali, & Letechipia-Vallejo, 2007). Because it is a bioavailable and permeable agent with relatively little toxicity, melatonin is an ideal candidate for therapeutic application. As it has been applied to global ischemia, many in vitro and in vivo models provide promising results of melatonin's efficacy (Cheung, 2003). The unexpected success of melatonin as a neuroprotectant has catalyzed researchers with the hope of developing a safe, reliable, multimodal approach to combat ischemic injury.

Neuroprotective Mechanisms of Melatonin

The main neuroprotective mechanism of melatonin against global ischemia is the reduction of oxidative damage through its antioxidant properties. Tan et al. (1993) and Reiter (1998) identified that melatonin acts as a potent antioxidant and free radical scavenger when introduced to the central nervous system. Cho et al. (1997) proposed that the significant reduction of CA1 pyramidal neurons of the hippocampus demonstrated in transient forebrain ischemia was due to the antioxidant activity of melatonin administered post-ischemia. Melatonin and its metabolites, such as N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) and N^1-acetyl-5-methoxykynuramine (AMK), have been shown to scavenge reactive oxygen species, reactive nitrogen species, and other free radicals (Andrabi, Parvez, & Tabassum, 2015; Cervantes, Morali, & Letechipia-Vallejo, 2007; Reiter et al., 2005). One of the means through which this

process occurs is by melatonin donating electrons to highly reactive oxyradicals, including the hyrdroxyl radical, peroxynitrite anion, and superoxide anion; additionally, its metabolites have shown to be effective in detoxifying free radicals (Reiter, 1998).

Not only do melatonin and its metabolites actively scavenge these free radicals, but there is increasing evidence that they also increase the expression of antioxidative enzymes, including glutathione perioxidase, superoxide dismutase, and catalase (Cervantes, Morali, & Letechipia-Vallejo, 2007; Reiter, 1998). This indirect regulation exponentially increases the amount of endogenous antioxidants in defense of free radical damage. In addition to combating existing free radicals post-ischemia, melatonin inhibits the synthesizing of NO through its interactions with Ca^{2+} during the ischemic cascade (Andari, Morali, & Letechipia-Vallejo, 2007). This NO inhibition has been demonstrated in several models of transient global ischemia in gerbils (Reiter, 1998).

Aside from the extremely effective antioxidant properties of melatonin, it has been demonstrated to exhibit antiapoptotic activity. Melatonin has been shown to promote mitochondrial homeostasis, and in animal models of global ischemia melatonin decreases the release of cytochrome c from mitochondria mediated by excessive Ca^{2+}; in turn, the ability of cytochrome c to stimulate apoptotic mechanisms in the central nervous system is limited. In addition, caspase-1 is renowned for its critical activation of apoptotic mechanisms as well, and melatonin has significantly reduced the production of this activator in ischemic models (Wang, 2009). B-cell lymphoma 2 and bax, regulators in programmed cell death, are susceptible to the influence of melatonin, leading to the inhibition of apoptosis processes (Reiter et al., 2005). By increasing the expression of

antiapoptotic proteins and decreasing apoptotic factors, melatonin significantly reduces the amount of endogenous cell death within ischemic models (Andrabi, Parvez, & Tabassum, 2007; Reiter et al., 2005; Wang, 2009).

Another mechanism of action is melatonin's reduction of inflammation in infarct regions of focal ischemic models. Its reduction of NO and malondiadehyde levels closely related to inflammatory signaling and regulation of cytokine production have evidenced melatonin to be a positive influence in reducing inflammatory illnesses (Andrabi, Parvez, & Tabassum, 2007; Esposito & Cuzzocrea, 2010). Additionally, melatonin affects binding sites located in lymphocytes and microphages, which reduces the amount of infiltration of inflammatory cells (Esposito & Cuzzrocrea, 2010). Through this process, the activation of microglia and monocytes is significantly decreased (Andrabi, Parbez, & Tabassum; Reiter et al., 2005). As these inflammatory processes are inhibited, melatonin hinders their ability to produce more neurotoxins and exacerbate neuronal damage.

Melatonin in Other Neurodegenerative Models

In addition to its application in focal and global ischemia, melatonin has been sought out as a strong potential candidate for providing neuroprotection against a range of neurodegenerative diseases due to its antioxidant, antiapoptotic, and free radical scavenging properties. Wang (2009) and Joshi et al. (2015) provide excellent reviews of melatonin's implementation across the neurodegenerative spectrum. One of the most common and widely researched of the neurodegenerative diseases, Alzheimer's Disease (AD), has been recently undertaken melatonin as therapeutic strategy. AD is defined by its accumulation of extracellular senile beta-amyloid (Aβ) plaques and neurofibrillary

tangles containing tau protein. The antioxidant and anti-amyloidogenic properties of melatonin have been reported in as making significant reductions in oxidative damage and Aβ plaque accumulation (Joshi et al., 2015; Wang, 2009). Feng et al. (2004) applied melatonin via injection at 10 mg/kg per day to an AD model in APP695 transgenic mice. Researchers found that experimental mice displayed significantly alleviated learning and memory deficits in passive avoidance tasks. Additionally, melatonin regulated apoptotic mechanisms and protected the cholinergic system, which ultimately decreased Aβ pathology. In clinical settings, several studies have indicated that melatonin regimes significantly delay the onset of AD in addition to protecting against mild cognitive impairment (Joshi et al., 2015; Wang, 2009).

Parkinson's Disease (PD) is the second-most common of the neurodegenerative diseases, identified by severe degradation of dopaminergic neurons within the substantia nigra and striatum (Joshi et al., 2015). In addition to the extensive loss of dopamine within these areas, oxidative stress, mitochondrial damage, and apoptotic pathways are thought to largely contribute to the neurodegeneration. Meta-analyses suggest that the antioxidative properties of melatonin may contribute to the regulation of 1-methyl-4-phenylpyridinium ion (MPP+) induced apoptosis (Wang, 2009). Additionally, cell culture experiments have indicated that melatonin protects against neurotoxic cell death in dopaminergic models (Joshi et al., 2015). Unfortunately, clinical applications have not indicated significant reduction of motor symptoms after melatonin administration; however, melatonin may still offer promise as a potential additive to other PD treatment due to its relatively safe biological profile (Joshi et al., 2015; Wang, 2009).

Amyotrophic Lateral Sclerosis (ALS) is a fatal disease which is characterized by the progressive deterioration of motor neurons. Oxidative stress seems to play a prime role in the cellular and extracellular alterations evidenced in the disease's pathology; thus, melatonin may be prove to be an effective candidate for hindering its progression (Joshi et al., 2015; Wang 2009). There have been a multitude of studies that speak to melatonin's efficacy in combating the motor degradation of ALS (Joshi et al., 2015). Zhang et al. (2013) for instance demonstrated in a mutant SOD1 transgenic mouse model of ALS that melatonin significantly improved motor symptoms, disease onset, and mortality across 23 weeks when administered at 30 mg/kg per day via intraperitoneal injection. Melatonin also proved to inhibit caspase-mediated cell death. Additionally, the researchers determined that ALS progression is associated with loss of melatonin and melatonin receptor 1A over time. Human clinical trials have indicated that melatonin offers significant neuroprotection against ALS through regulation of oxidative stress, exhibiting patient tolerance for high melatonin intake (up to 300 mg per day) in addition to clinical effectiveness (Joshi et al., 2015).

Efficacy of Melatonin in Ischemic Models

Melatonin has been shown to be effective in attenuating the debilitating neurological and behavioral consequences of ischemic models. Studies have shown that endogenous levels of melatonin aid in combating ischemic damage. De Butte, Fortin, & Pappas (2002) showed that a pinealectomy – a procedure which removes the largest generator of endogenous melatonin within the central nervous system – worsens the damage to CA1 and CA4 neurons within the hippocampal regions in a 2-VO rat model of

ischemia. At 122 days post-surgery, histological analyses revealed that pinealectomy alone created a 21% loss in CA1 hippocampal neurons, indicating that melatonin deficiency alone can create hippocampal damage. Furthermore, a combination of pinealectomy with 2-VO ischemia induced a 32% loss of CA1 hippocampal neurons. Pinealectomy also exacerbated disturbances in working memory performed on radial maze tasks. Across 7-weeks post-occlusion, 2-VO pinealectomized rats displayed significantly more working memory errors than those without ischemia after averaging errors across 28 training sessions. This suggests that lower levels of endogenous melatonin due to pinealectomy could not provide neuroprotection against the ischemic insult. Kilic et al. (1999) affirmed previous assertions that pinealectomized rats undergo a drastic reduction in endogenous melatonin, which leads to greater infarcts and more extensive neuronal damage following a focal ischemic event due to middle cerebral artery occlusion. Furthermore, the study administered melatonin before ischemia and reperfusion, which appeared to have ameliorated the melatonin deficiency from the pinealectomy by reducing infarct volumes up to 40% and significantly reducing neurologic deficit scores.

In an early investigation of exogenous melatonin in 4-VO transient forebrain ischemia in rats, Cho et al. (1997) increased neuronal density in CA1 hippocampal areas one week post-ischemia when 10 mg/kg was administered after 0, 2, and 6 hours after reperfusion. This provides evidence that melatonin protected against the excitotoxic ischemic cascade that promotes free radical generation, molecular damage, and necrosis when administered post-reperfusion. To directly determine melatonin's effect as a free radical scavenger, Li et al. (1997) measured its effect on OH (hydroxide) in middle

cerebral artery collusion (MCAO) rats. Melatonin was administered at 4 mg/kg at 30-minutes pre-ischemia, and researchers used a salicylate trapping method to estimate levels of OH being generated. When salicylate scavenges OH molecules, 2,3-dihydroxybenzoic acid (DHBA) is produced. Greater instances of DHBA indicate that salicylate is actively neutralizing OH, which mirrored the levels of DHBA found within ischemic control groups. When melatonin was administered, however, researchers found that DHBA levels were significantly reduced yet retained neuronal protection, indicating that melatonin scavenged OH radicals more effectively that salicylate.

Guerrero et al. (1997) successfully demonstrated free radical scavenging properties of melatonin in a gerbil ischemia model. Animals were euthanized either at 5 or 60 minutes post-reperfusion after the administration 10 mg/kg of melatonin 30 minutes prior to ischemia. Histological analyses of frontal cortices and cerebella revealed reduction of nitrate levels and cyclic guanosine monophosphate (cGMP), which is synthesized through increased levels of NO. In this, the presence of melatonin significantly deterred oxidative damage to frontal cortices and the cerebellum in a transient global ischemic model. Cuzzocrea et al. (2000) demonstrated similar levels of neuroprotection within a global ischemia gerbil model. When 10 mg/kg of melatonin was introduced at 10 minutes pre-reperfusion and 1, 2, and 6 hours post-reperfusion, the loss of CA1 hippocampal neurons was significantly reduced. Melatonin-treated gerbils also displayed no increase in locomotor activity when observed 24 and 48 hours post-ischemia in open field tasks. Additionally, these researchers investigated the role of melatonin as a free radical scavenger, noting a significant reduction in plasma levels of nitrate and nitrate formation. Lipid peroxidation was reduced in the experimental group

as evidenced by a reduction of malondialdehyde (MDA); in addition, melatonin showed to attenuate myeloperoxidase (MPO) activity, indicating a reduction of polymorphonuclear leukocytes (PMNs) within the central nervous system and subsequent reduction of inflammation. This indicates a multimodal neuroprotective effect exhibited by melatonin's ability to regulate reactive oxygen species both directly and indirectly while reducing PMNs in the inflammation site in a global ischemic model.

Ling et al. (1999) revealed anti-apoptotic properties of melatonin 24 hours after subjecting rats to a 1-hour middle cerebral artery occlusion. Immunohistological methods were used to determine the expression of B-cell lymphoma 2 (*bcl-2)* and the *bax* gene. Overexpression of the *bax* gene is known to accelerate apoptosis, and *bcl-2* expression inhibits the *bax* gene; therefore, the *bcl-2* to *bax* ratio can be analyzed in histological analyses to determine the prevalence of apoptotic cell death. In addition to reduced infarct size in rats receiving exogenously administered melatonin, there was also an increase in the ratio of *bcl-2* over *bax* within the penumbra, suggesting an inhibition of the apoptotic processes following a transient ischemic event. Furthermore, melatonin has been shown to increase the activation of the G-protein-coupled melatonin receptor, MT_2 within the CA1 hippocampus post-ischemia (Lee et al., 2010). It has been established that melatonin receptors MT_1 and MT_2 are expressed throughout various brain structures, including the hippocampus (Lee et al., 2010; Reppert, 1997; Sugden, 2000). Lee et al. (2010) found that intraperitoneal injection of melatonin prior and following global ischemia in gerbils increased MT_2 immunoreactivity and protein levels found in astrocytes in the CA1 hippocampus post-ischemia. Similarly, Chern et al. (2012) found that acute melatonin injection in rats undergoing MCAO reduced oxidative and

inflammatory stress and increased neurogenesis by activating MT_2 melatonin receptors within the hippocampus. These studies affirm the presence and activation of melatonin receptors within the hippocampus within current animal models.

Examining the long-term effects of melatonin treatment, De Butte & Pappas (2007) affirmed that pinealectomized rats display persistently degraded levels of CA1 and CA3 hippocampal neurons across a 17-month span. Treatment groups were orally administered melatonin at a dose of approximately ~0.51 mg/kg per day, raising plasma levels about 10 to 15 times normal endogenous levels. At 2 months post-surgery, melatonin showed partial protection and afforded complete neuroprotection of CA1 and CA3 cell loss for the remainder of the 17 months. Remarkably, melatonin administration actually increased the prevalence of CA1 hippocampal neurons by approximately 10% in pinealectomized rats compared to sham groups. This offered promising results affirming the potential of melatonin to supply long-term neuroprotection as chronic melatonin promoted hippocampal neurogenesis in pinealectomized rats. Letechipía-Vallejo et al. (2007) examined long-term neuroprotective effects of melatonin within a 4-VO rat model. Experimental rats were administered continuous melatonin intravenously for 6 hours at 10 mg/kg per hour 15 minutes post-occlusion. At 120 days post-ischemia, melatonin implementation preserved 78.7% of CA1 hippocampal neurons, which was vastly greater than the 23.4% of CA1 hippocampal neurons remaining in groups receiving ischemia with a continuous vehicle i.v. Additionally, melatonin combated behavioral effects of ischemia, significantly improving escape latencies in the Morris Water Maze and reducing working memory errors on the Radial Arm Maze. This study suggests that high levels of melatonin administered acutely within an ischemic model for

within a critical window post-reperfusion offered long-term neuroprotection. Following up investigations of long-term melatonin effects, one study sought to determine the effects of chronic and acute melatonin implementation in a global ischemia gerbil model (Rennie et al., 2008). Acute melatonin was administered via intraperitoneal injections of 10 mg/kg of melatonin at 0.5, 1, 2, and 6 hours after occlusion. Chronic melatonin was received orally through drinking water at concentrations necessary to achieve 0.6 mg/kg per day to achieve approximately 10-15 times normal plasma levels of endogenous melatonin. Gerbils that received chronic melatonin displayed significantly reduced postoperative locomotor activity at 3 days post-surgery, while acute melatonin did not attenuate ischemia-induced hyperactivity. This suggests that chronic melatonin implementation may be more effective in amelioration short-term behavioral effects of transient forebrain ischemia. No long-term neuroprotection effect was found for either acute or chronic melatonin groups at 20 weeks post-surgery. An assay of plasma levels of chronic experimental gerbils indicated only a 60% increase in melatonin – a much less dramatic increase than anticipated. This is speculated to have been due irregular drinking patterns of gerbils being difficult to monitor and control. Therefore, it may be that persistently high levels of melatonin may be necessary to elicit long-term neuroprotective effects. This contention is further supported by contrasting the results found by Letechipía-Vallejo et al. (2007), in which researchers found significant acute melatonin implementation effects in the long term. The difference is postulated to be that the Letechipía-Vallejo et al. (2007) study induced persistently high levels of melatonin while administering similar quantities of melatonin in four interspaced occasions leads to peaks and troughs in melatonin levels in the central nervous system.

Melatonin pretreatment has also been displayed as a valuable tool in combating excitotoxicity and free radical damage following the ischemic cascade. Pei, Ho, & Cheung (2002) demonstrated that an acute dose of melatonin 30 minutes prior to MCAO in rats displayed significant neuroprotective effects against focal ischemia. Melatonin was administered in a single dose of either 5, 15, or 50 mg/kg through an intraperitoneal injection; subsequently, infarct volume three days post-ischemia was reduced by 54.2%, 51.2%, and 44.5% respectively compared against a vehicle. Interestingly, there were no significant differences between melatonin dosages, despite a 10 fold increase in the amount of melatonin administered. Expounding upon this study, these researchers then analyzed the effect of an acute pretreatment of melatonin attenuated levels of neuronal nitric oxide synthase (nNOS), cyclooxygenase-2 (COX-2), and myeloperoxidase (MPO) (Pei & Cheung, 2004). An experimental group of rats receiving a single intraperitoneal dose of 5 mg/kg of melatonin 30 minutes prior to right-sided MCAO were compared against those receiving a vehicle substance through an identical administration. Histological analyses were performed at 24, 48, and 72 hours post-occlusion. Researchers observed a significant reduction of nNOS from a 50% increase in vehicle MCAO rats to 30% in those receiving melatonin, displaying suppression of enzyme activity. Additionally, COX-2 was significantly reduced by melatonin by approximately 70% at 24 hours post-ischemia, indicating protection against pathogenic processes that lead to inflammatory damage and oxidative stress. Finally, MPO enzyme activity seemed to be inhibited by melatonin as evidenced by an approximate 50% reduction of polymophonuclear cells within the infarct region. These findings suggest that melatonin pretreatment not only exhibits antioxidant protection against ischemia and reperfusion

injury but also partly reduces neuronal damage through inhibiting inflammatory response. Kilic et al. (2004) evaluated the prophylactic effect of melatonin when administered orally for 9 weeks at 4 mg/kg per day in mice receiving 90-minute MCAO. These mice were compared against a sham group that received a vehicle in place of melatonin as well as mice that received a single acute injection of melatonin at 4 mg/kg post-ischemia. Twenty-four hours post-reperfusion, melatonin proved to significantly reduce infarct size by approximately 30-35% in both acute administration and pretreatment. This evidences that prophylactic melatonin can attenuate focal ischemia insult 24 hours after injury to the same extent as a single post-ischemic injection. The researchers also evaluated melatonin's ability to attenuate vasoconstriction after ischemic insult. By measuring the expression of the endothelin converting enzyme-1 (ECE-1), it was identified that melatonin is a powerful inhibitor of ECE-1, which suggests protection against vascular dysfunction after ischemia.

Potential Improvements of Melatonin Treatment Models

Initial models of ischemia have provided exciting and surprisingly effective neuroprotection against hypoxia through the application of melatonin. There have been numerous studies that have found melatonin effective in reducing the neuronal and behavioral damage in animal models of ischemia (Cho et al., 1997; Cuzzocrea et al., 2000; Letechipia-Vallejo et al., 2007). Throughout the past two decades, a resounding accumulation of support has been developed to suggest that post-ischemic melatonin supplementation aids in neuroprotection; however, the large majority of these studies utilize means of administration that are impractical in a clinical setting. Most of the

administration is done either intravenously or via direct intraperitoneal injection A few of the common shortcomings of preclinical experimental trials are that therapies often focus on limited therapeutic windows, most revolve around post-ischemic actions, and implementation may not be practical in real-life scenarios (Danton & Deitrich, 2004). More research is needed in the development of techniques that lengthen the therapeutic window, develop pretreatment strategies, and integrate therapeutic models that can be effectively translated to clinical settings. Pretreatment strategies, such as those evidenced by Pei and Cheung (2004), that utilize melatonin are often done via injection into the central nervous system with relatively short temporal proximity to the ischemic invent. Developing practical pretreatment strategies that can be affectively applied to at-risk groups for ischemic events needs to be established.

Current Study

The purpose of the present study is to examine whether chronic pretreatment with melatonin would ameliorate the neurobehavioral and neuropathological consequences of global ischemia in male gerbils. It is hypothesized that chronic application of melatonin though time-released, subcutaneous pellets in the dorsal neck prior to an ischemic event would reduce the hyperactivity, errors in spontaneous alternation behavior, and neuronal death in CA1 hippocampus of global ischemic gerbils. Most research on melatonin's therapeutic potential in global ischemia focuses upon acute means. The current study seeks to identify the efficacy of continual melatonin treatment in protecting against the behavioral and neural deficits of global ischemia in gerbils. If persistently elevated levels of melatonin in the central nervous system demonstrate behavioral and neural protection,

new therapeutic treatments may be developed to deter global ischemic damage. This offers an alternative to acute means that operate within a narrow window of opportunity for neuroprotection. Additionally, there have been few efforts to investigate how melatonin may be used as a pretreatment in global ischemia. The current administration through time-released pellets is a unique strategy to examine the prophylactic effects of melatonin on gerbil global ischemia. Pretreatment strategies, such as this one, provide more opportunity to curb the deleterious effects of global ischemia by offering therapeutic intervention both prior to and during the ischemic event. If the treatment is effective, the current study will lay the foundation for developing a new prophylactic melatonin treatment to reduce the behavioral and neural deficits of global ischemia.

CHAPTER III

METHOD

Animals

All care and handling of the animals was humane and in accordance to national guidelines. Appropriate protocols were approved by the Institutional Animal Care and Use Committee prior to the experiment's onset. Thirty male Mongolian gerbils (aged 3-4 months old) were purchased from Charles River (Cambridge, MA). Gerbils were kept in a temperature-controlled environment with a 12-hour light/dark cycle with ad libitum food and water.

Melatonin Pellet Implementation

The current study used 5 milligram pellets of melatonin that are subject to a 60-day controlled release (Innovative Research of America Inc., Sarasota, FL, USA). The dose of melatonin was based on previous reports that showed this dose was effective in ameliorating the deleterious consequences of cerebral ischemia (Kilic et al., 2004; Pei, Ho, & Cheung 2002; Rennie et al., 2008). Gerbils (n = 10) were selected at random to be subcutaneously implanted with these melatonin pellets in the dorsal neck. The remaining 20 gerbils received a sham operation in which no pellet was implanted. For all surgeries,

aseptic techniques were utilized, and the gerbils were anesthetized using isoflurane (4% induction, 2% maintenance in 70% N_20 and 30% O_2 mixture).

Global Ischemia Surgery

Two weeks following the melatonin pellet implantation, gerbils were subjected to transient forebrain ischemia. The gerbils either underwent a sham surgery (n = 10) or transient forebrain ischemia surgery (n = 20). The order in which gerbils underwent these surgeries was done at random. Gerbils were divided into a control group of sham surgery with no melatonin (SHAM, n = 10), an ischemic group with no melatonin (ISX, n = 10), and an experimental group of ischemia with melatonin (ISX+M, n = 10). Gerbils were anesthetized with isoflurane (4% induction, 2% maintenance in 70% N_20 and 30% O_2 mixture). To expose the common carotid arteries bilaterally, the gerbils received a ventral mid-line neck incision. A transient global ischemic event was induced through temporary bilateral carotid artery occlusion for a duration of 5 minutes using microarterial clips. Following this hypoperfusion exposure, Marcaine was applied to the wound prior to suturing.

Behavioral Testing

Animals were first tested on the open field task 24 hours post-ischemia to examine acute hyperactivity, exploratory behavior, and anxiety behavior. One week following ischemia surgery, gerbils were then tested on a Y-maze task to assess spontaneous alternation behaviors. The experimenters were blind to animal conditions during these behavioral tests.

Open Field Task

To assess locomotive behavior post-surgery, gerbils were assessed in an open field task. Open field tasks are often used in gerbil models to assess locomotion and anxiety-related behaviors (Wappler et al., 2009). Behavioral testing for all three groups of gerbils was conducted 24 hours following either the ischemic or sham surgery. Open field testing took place in a square black box (57.6 cm × 57.6 cm) with sides 38 cm high. The floor of the box was divided into nine equal squares (6.4 cm × 6.4 cm). Gerbils were placed within the box for 6 minutes, during which time locomotor activity was monitored. Locomotor activity was assessed by monitoring the number of grid crosses, as defined by all four of the gerbil's paws crossing a grid line. Rearing – defined as lifting of the two frontal paws – was counted to measure exploratory behavior. The amount of time spent in the center – as indicated by all four paws being located within the center square – served as a measure of anxiety. A highly trained researcher with ample experience in measuring animal behavior recorded the number of grid crosses and rearing behaviors exhibited by each gerbil. Another trained researcher monitored the amount of time spent within the center square of the open field.

Y-maze

To assess working memory and locomotion through spontaneous alternation behavior, gerbils were tested on a Y-maze (Wappler et al., 2009). This occurred for all three groups at seven days following the ischemic and sham surgeries. Gerbils were placed within the center of a black Y-maze (height = 25.4 cm; arm length = 55.9 cm). Gerbils were allotted 9 minutes of free exploration within the three arms. The number of

arm entries was recorded. Spontaneous alternation was defined as the gerbil entering an arm different from the previous two visited. Same arm return was defined as the gerbil reentering the same arm most recently visited. Alternate arm return was defined as the gerbil entering an arm previously visited. A researcher trained in observing animal behavior recorded the number and type of arm entries for each animal. The number of spontaneous alternations was divided by the number of triplets and multiplied by 100 to produce a percent alternation score.

CA1 Pyramidal Cell Counts

Animals were euthanized 10 days post-surgery via transcardial perfusion using 0.9% saline followed by 4% paraformaldehyde. Brains were extracted and stored in paraformaldehyde. Coronal sections (14 μm) were cut at the level of the dorsal hippocampus and 5 sections per animal were mounted and stained with hematoxylin and eosin using a cryostat. Medial, middle, and lateral sectors from the CA1 region of the left and right hippocampus were photographed at 40× magnification. The digital images were opened and using a previously described method, non-eosinophilic pyramidal neurons were counted in section at the rostral level of the hippocampus in lateral, middle, and medial sectors 0.2mm in length of the CA1 at 1.7mm posterior to bregma (Colbourne & Corbett, 1995). All counts were performed by observers blind to group membership. Independent cell counts were performed by two researchers trained in identifying and counting surviving cells within the CA1 hippocampus of global ischemic animals. Cell counts were compared between the two researchers to create reliable measures of surviving CA1 pyramidal neurons within the gerbils. This coronal level of the CA1

sector exhibited neuroprotection and injury analogous to more posterior sections (Colbourne & Corbett, 1995).

CHAPTER IV

RESULTS

Statistical Methods

Of the 30 gerbils that entered the study, 12 animals did not complete the study. Eleven animals died as a result of the ischemia surgery due to respiratory distress and/or seizure activity. One gerbil died several days following surgery due to continuous seizure activity. The final number of animals in the 3 groups after deaths was as follows: SHAM (n = 7), ISX (n = 7), and ISX+M (n = 4). Furthermore, one SHAM and two ISX animals were excluded from cell count analysis due to technical reasons. Descriptive data for SHAM, ISX, and ISX+M groups are provided in Table 1 for behavioral and neural measures. The descriptive data for the ISX and ISX+M groups indicate relatively high degrees of variability and standard error of measure compared to SHAM. Comparisons among the three groups (SHAM, n = 7; ISX, n = 7; ISX+M, n = 4) of data collected in the open-field task, radial Y-maze, and neuronal cell counts were made using a one-way between-subjects analysis of variance (ANOVA) to identify differences among groups (SHAM * ISX * ISX+M). In some analyses, a Levene's test revealed unequal variances within groups, therefore calculations violating the assumption of equal variances were made using adjusted degrees of freedom. For analyses of variance

between groups in which equal variances could not be assumed, a Welch's F test was used. Effect sizes for Welch's F tests were made using the estimated omega squared. Post-hoc analyses for analyses of variances were made using the Games-Howell method due to violation of the assumption of equal variances. Effect sizes for Games-Howell post-hoc comparisons were made using Cohen's d.

It was hypothesized that melatonin pretreatment would significantly reduce the behavioral and neural effects of global ischemia. With the current sample, however, melatonin pretreatment did not seem to attenuate global ischemia's effect on open field, Y-maze, and CA1 cell count measures. No significant differences were found between SHAM, ISX, and ISX+M groups in these measures. It was also noted that no significant differences were found in post-hoc analyses between ISX and ISX+M groups in these measures. Furthermore, the means and standard errors of measurement for the ISX and ISX+M groups should significant variability in behavioral and neural measurements, particularly in evidence suggesting unilateral and partial preservation of CA1 hippocampal neurons. Because no significant differences were found between these two groups, the ISX and ISX+M groups were collapsed into a total ischemic group (ISX, N=11). Data collected in the open-field task, radial Y-maze, and neuronal cell counts were made between the SHAM (N=7) and all ischemic animals in the ISX group (N=11) using a t-test (SHAM*ISX). Through this comparison researchers sought to further investigate how global ischemia affected behavioral and neural measures within the current sample. For t-test comparisons between groups in which equal variances could not be assumed, degrees of freedom were adjusted. Effect sizes between particular groups were made using Hedge's g due to this violation as well. Significant differences

were evaluated at the .05 level. Post hoc power analyses were conducted using the G*Power Program (Erdfelder, Faul, & Buchner, 1996).

Locomotion but not exploration or anxiety-like behavior is significantly altered by ischemia in male gerbils

In order to test the effects of ischemia on locomotor activity, exploration, and anxiety-like behavior, gerbils were tested on the open field task 24 hours after ischemia. To determine whether ischemia or melatonin affected locomotion, the SHAM, ISX, and ISX+M groups were compared against one another on grid crosses, rearing behavior, and the time spent in the center of the open field. As illustrated in Figure 1A, there were no significant differences exhibited between groups on locomotor activity [*Welch's* $F(2,6.01) = 2.69$, $p > .05$]. Similarly, there was no significant difference detected between groups in regards to rearing behavior *[*$F(2,15) = 0.54$, $p < .05$; Figure 1B] or amount of time spent in center of the open field [*Welch's* $F(2,6.64) = 1.32$, $p > .05$; Figure 1C]. Because the placebo and melatonin treated ischemic groups did not significantly differ from one another, these groups were combined to determine whether ischemic gerbils (irrespective of treatment) differed from SHAM gerbils. As illustrated in Figure 1A, ISX animals exhibited a significant increase in grid crosses compared to SHAM [$t(12.90) = -2.51$, $p = 0.26$]. This difference indicates approximately 70% more grid crosses in the ISX ($M \pm SE = 249.64 \pm 90.89$) group than SHAM ($M \pm SE = 175.71 \pm 28.95$), revealing a large effect size ($g = 0.10$). As demonstrated in Figure 1B and C, ischemia did not seem to alter either exploratory behavior [$t(16) = -0.90$, $p > .05$] or time spent in the center of the open field [$t(15.09) = 1.689$, $p > .05$].

Table 1

Group behavioral and histological data

	SHAM (*n* = 7)	ISX (*n* = 7)	ISX+M (*n* = 4)	All ISX (*n* = 11)
Open Field				
Hyperactivity (Grid Crosses)	175.51±10.95	260.00±36.71	231.50±44.66	249.64±27.40
Exploration (Rearing)	78.86±8.08	90.29±10.12	82.00±12.46	87.27±7.59
Anxiety (Time in Center)	24.50±2.15	18.18±5.02	15.82±5.82	17.32±3.67
Y-Maze				
Spontaneous Alternation	54.00±2.13	52.70±3.31	54.18±2.33	53.23±2.11
Alternate Arm Return	34.91±1.30	38.07±3.45	40.48±2.58	38.99±2.33
Same Arm Return	11.10±2.13	9.22±3.26	5.24±0.26	7.77±2.20
CA1 Pyramidal Cell Count				
Grand Mean Sum	165.77±10.16*	57.31±23.97*	88.33±44.70*	71.10±22.86*

Values are expressed as mean ± S.E.M. Data that have different *n* are represented by *. Sample sizes for the CA1 pyramidal cell count were as follows: SHAM (*n* = 6); ISX (*n* = 5); ISX+M (*n* = 4); All ISX (*n* = 11).

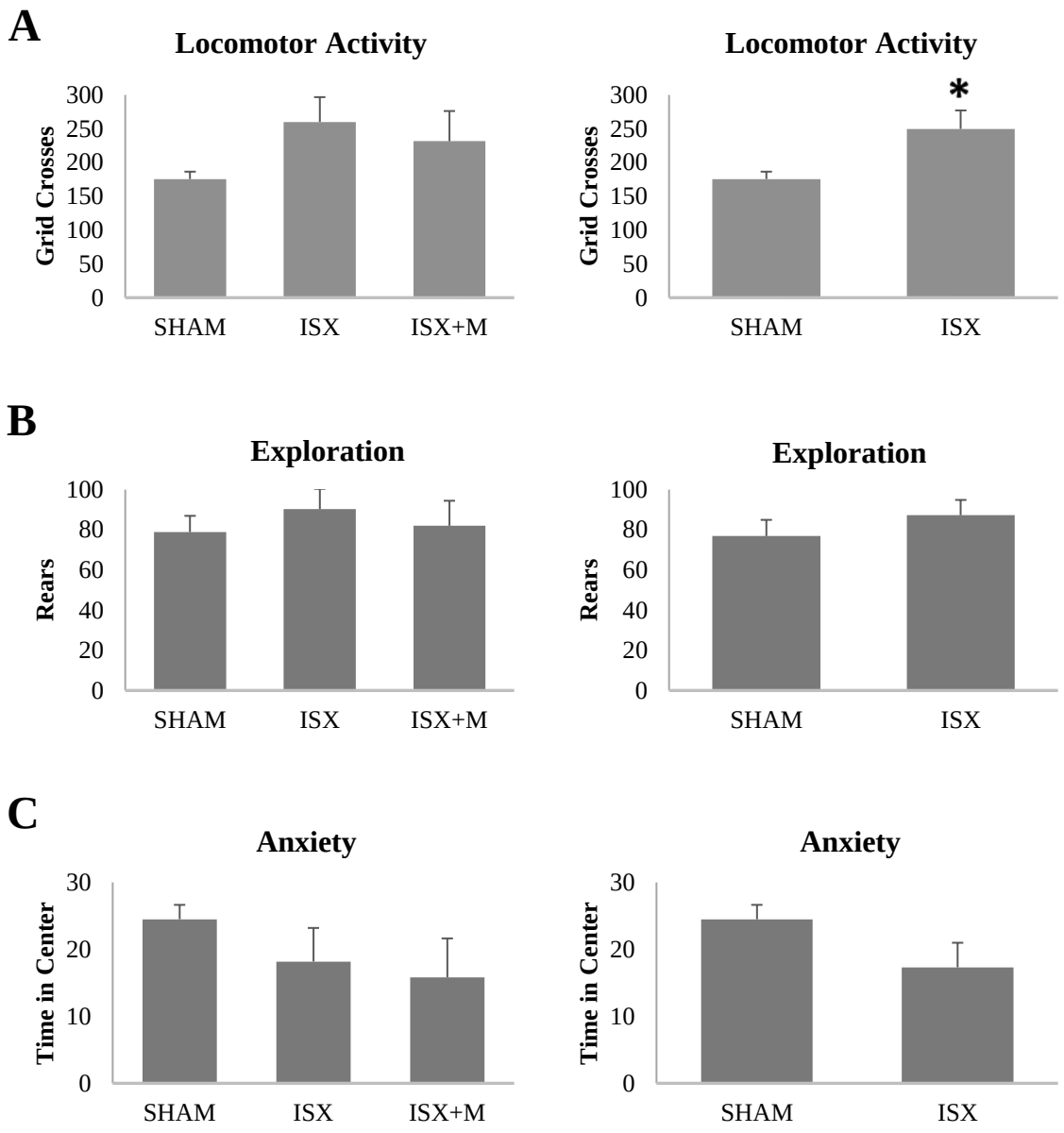

Figure 1. Locomotor activity, exploration, and anxiety did not appear to be affected by melatonin pretreatment. Global ischemia significantly increased locomotor activity, but exploratory and anxiety behaviors did not demonstrate change. (A) Locomotor activity was measured by grid crosses in the open field. Data are presented as X±S.E.M. (B) Exploration of the open field was measured as the number of rears in the open field. Data are presented as X±S.E.M. (C) Anxiety was measured by the amount of time the gerbil spent in the center square of the grid. Data are presented as X±S.E.M. Significant findings ($p<.05$) are represented by *.

Global ischemia failed to demonstrate change in spontaneous alternation behaviors

To assess working memory and locomotion, the spontaneous alternation behaviors of the animals were tested on a Y-maze. As shown in Figure 2A, all gerbils irrespective of group spontaneously alternated just above chance levels [$F(2,15) = 0.09$, $p>.05$]. Additionally, the groups did not appear to differ in regards to alternate arm return [*Welch's* $F(2,7.16) = 1.92$, $p > .05$] or same arm return behaviors [*Welch's* $F(2,8.16) = 4.10$, $p > .05$].

Because the placebo and melatonin treated ischemic groups did not significantly differ from one another, these groups were combined to determine whether ischemic gerbils (irrespective of treatment) differed from SHAM gerbils on spontaneous alternation behaviors. As illustrated in Figure 2A, no significant difference were able to be found between ISX and SHAM groups in spontaneous alternation [$t(16) = 0.24$, $p > 0.5$]. Both ISX and SHAM groups were found to exhibit spontaneous alternation behavior above the chance level of 50%. As shown in Figure 2B and C, no significant differences were reliably found between ISX and SHAM gerbils in regards to alternate arm return[$t(14.76) = 1.54$, $p > .05$] and same arm return behaviors[$t(16) = 1.06$, $p > .05$].

Global ischemia significantly decreased CA1 pyramidal neurons in gerbils which melatonin did not appear to attenuate

Due to an inability to procure the necessary materials to finish the hematoxylin and eosin staining, one SHAM and two ISX animals were excluded from the cell count analysis. Digital pictures of the pyramidal cell layer of the hippocampal CA1 region representative of each group are depicted in Figure 3. A significant main effect was

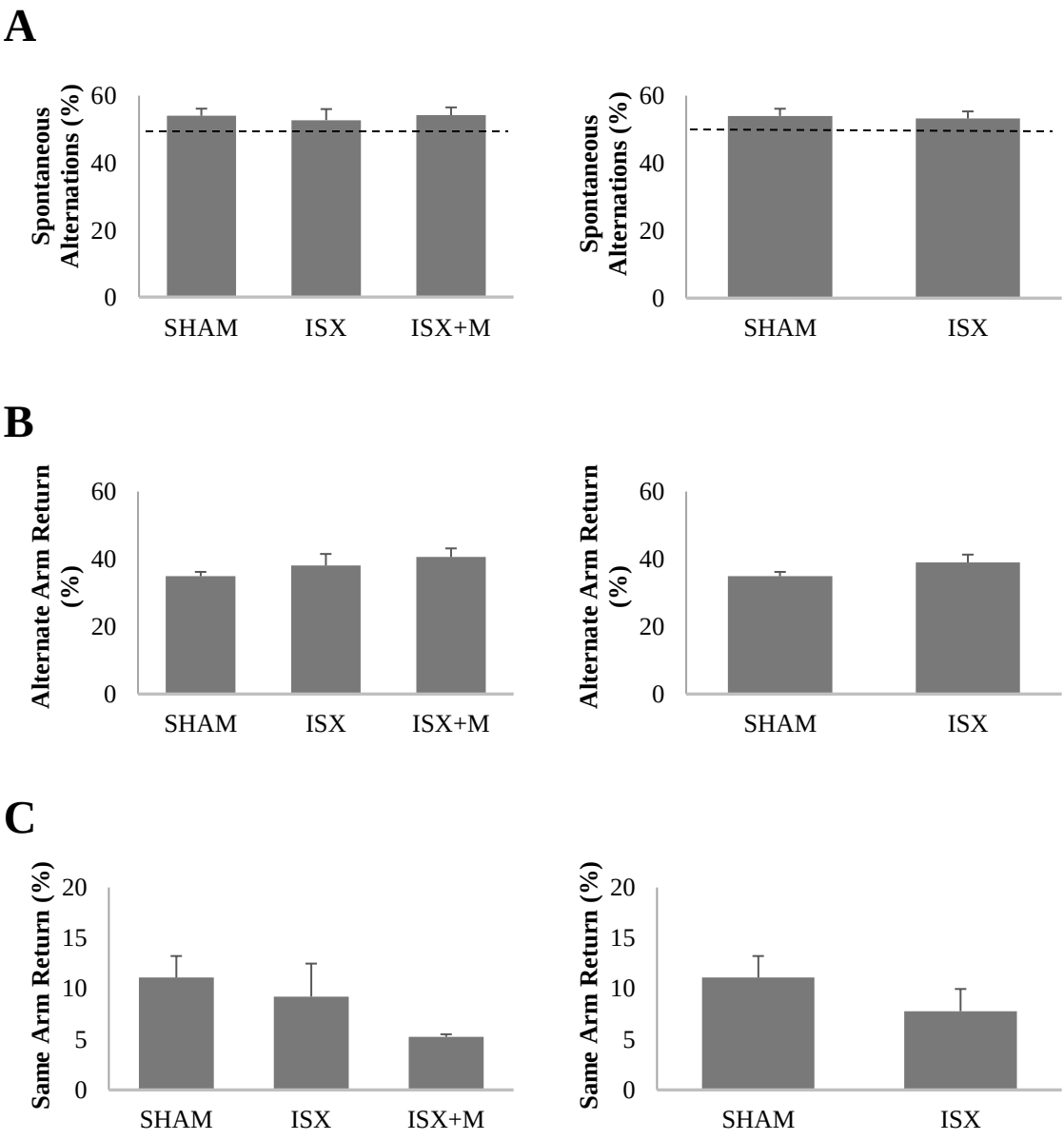

Figure 2. Spontaneous alternation behavior and errors did not appear to be affected by melatonin pretreatment or global ischemia. (A) Spontaneous Alternation Behavior was measured by the amount of times animals entered an arm different from the previous two visited. Quantities were converted to percent alternation scores by dividing total spontaneous alternations by the number of triplets multiplied by 100. Data are presented as X±S.E.M. Chance levels of 50% are represented by the dotted line. (B) Alternate Arm Return was measured by the amount of times animals entered an arm visited one entry prior. Quantities were converted to percent alternation scores by dividing total alternate returns by the number of triplets multiplied by 100. Data are presented as X±S.E.M. (C) Same Arm Return was measured by the amount of times the gerbil re-entered an arm most recently visited. Quantities were converted to percent alternation scores by dividing total same arm returns by the number of triplets multiplied by 100. Data are presented as X±S.E.M.

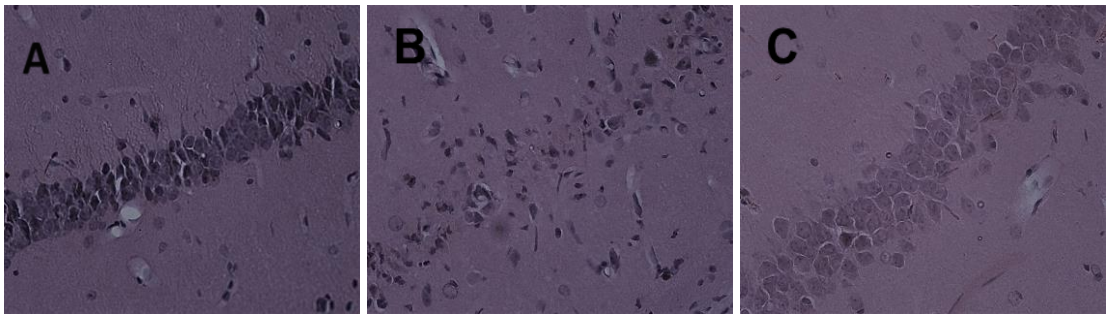

Figure 3. (A) CA1 hippocampal section from SHAM animal. (B) CA1 hippocampal section from ISX animal. (C) CA1 hippocampal section from ISX+M animal.

found between SHAM, ISX, and ISX+M groups in regards to hippocampal CA1 cell number as illustrated in Figure 4 [*Welch's F*(2,5.38) = 8.51, $p = 0.022$]. It is estimated that 50% of the total variation in CA1 hippocampal neurons is attributable to group differences, indicating a large effect ($\omega^2 = 0.50$). A Games-Howell post-hoc analysis indicated that the ISX ($M \pm SE = 57.31 \pm 23.97$) group had approximately 65% less cells than the SHAM ($M \pm SE = 165.77 \pm 10.16$), demonstrating a significant difference between groups with a large effect size ($d = 1.18$). Post-hoc analysis were unable to reveal significant differences in hippocampal cell number between SHAM and ISX +M groups nor between ISX and ISX+M.

As noted previously, the ischemia groups were analyzed statistically as separate groups, but because they did not differ the data for all ischemic animals were combined. As illustrated in Figure 4, the ISX group had significantly less CA1 cells than SHAM [$t(10.79) = 3.79$, $p = .003$]. This difference indicates that the ISX ($M \pm SE = 71.10 \pm 22.86$) group possessed approximately 57% fewer CA1 hippocampal neurons than SHAM ($M \pm SE = 165.77 \pm 10.16$), revealing a large effect size ($g = 1.69$).

Observed Power

The decrease in power for the analyses is represented in Table 3. The original sample was intended to be composed of 30 animals in the SHAM ($n = 10$), ISX ($n = 10$), and ISX+M ($n = 10$) groups. Though it is true that a sample size of approximately 52 would be necessary to detect a significant difference between groups at the suggested 80% power level, the relatively small sample size is consistent with preliminary research investigating neuroprotective agents in global ischemic models (Cohen, 1988). It is

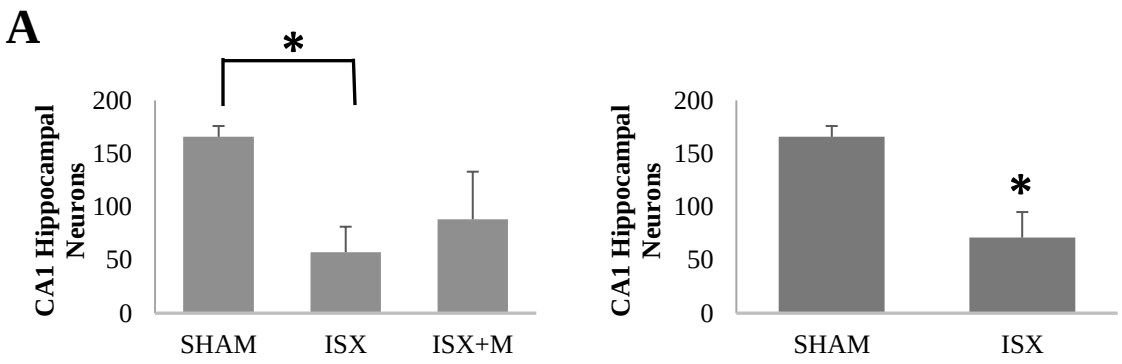

Figure 4. (A) A significant difference was found between SHAM, ISX, and ISX+M groups in the number of pyramidal hippocampal cells in the CA1 area of the hippocampus. Post-hoc analysis revealed a significant main effect between SHAM and ISX animals in CA1 hippocampal neuron counts. No significant differences were found between SHAM and ISX+M or ISX and ISX+M. Blind estimates of CA1 hippocampal neurons were performed using standard stereological techniques. Data are presented as X ±S.E.M. Significant main effects ($p>.05$) are represented by *. (B) Global ischemia significantly reduced the prevalence of pyramidal hippocampal cells in the CA1 area of the hippocampus. Blind estimates of CA1 hippocampal neurons were performed using standard stereological techniques. Data are presented as X ±S.E.M. Significant main effects ($p<.05$) are represented by *.

Table 2

Observed power for each ANOVA analysis

	Original Sample (n = 30)	Final Sample (n = 18)	Power Difference %
Open Field			
Hyperactivity (Grid Crosses)	0.38	0.57	-32.28%
Exploration (Rearing)	0.17	0.12	-26.79%
Anxiety (Time in Center)	0.35	0.20	-42.07%
Y-Maze			
Spontaneous Alternation	0.07	0.06	-11.59%
Alternate Arm Return	0.36	0.19	-45.63%
Same Arm Return	0.39	0.20	-50.25%
CA1 Pyramidal Cell Count			
Grand Mean Sum	0.97	0.73*	-24.46%

Values for Original Sample and Final Sample are expressed as Power (1 - β error probability). Power Difference % represents the percentage of difference between the original intended sample and the final sample. Data with different sample sizes are indicated by *. CA1 pyramidal cell count final sample is n = 15.

common practice for published research to utilize sample sizes of 10 or less per group when performing introductory research into novel neuroprotectant agents and methodologies in global ischemia (Gyertyán, Gigler, & Simó, 1999; Kim et al., 2011). Moreover, this practice of sample sizes of 10 or less per group has been prevalent throughout the literature documenting melatonin's effectiveness in attenuating the neural and behavioral effects of pinealectomy, focal ischemia, global ischemia, and other models of neurodegenerative diseases (Chern et al., 2012; Cho et al., 1997; Feng et al., 2004; Kilic et al., 1999; Kilic et al., 2004; Kim et al., 2011; Letechepía-Vallejo et al., 2007; Pei & Cheung, 2004). Due to mortality, the sample size within the current study was reduced to 18 for open field and Y-maze tasks with SHAM *(n* = 7), ISX *(n* = 7), and ISX+M *(n* = 4) all losing representation. The sample size for the CA1 cell counts was further reduced to 15 with SHAM *(n* = 6), ISX *(n* = 5), and ISX+M *(n* = 4) all losing representation due to technical issues. This reduction in sample size led to a subsequent loss of power for the study. Furthermore, this power loss was exacerbated by the extreme variability found within the groups. Consequently, all analyses received a significant power loss ranging from approximately 12% to 50%.

In order to account for this power loss, the nonparametric technique of bootstrapping was applied to the data; however, this transformation did not provide any new information over the current statistics. One of the assumptions upon which bootstrapping hinges is that the sample from which the artificial population is being drawn must be truly representative of the population it intends to replicate. When analyzing the raw data in Table 1, the extreme variability of injury suggests that this is not the case. This is primarily because the study's intent to use a true, complete global

ischemic event to measure the effects of the melatonin is not met within the current study due to the partial protection and unilateral damage evident in the CA1 neuronal counts. The population that would be created from bootstrapping would be representative only of this highly variable model and not the population that was intended. Secondly, the real issue with the bootstrapping method for this example can be seen within the ISX+M group. There are only 4 scores within this group with 2 scores falling on one end of the spectrum and the other 2 at the other end, creating an almost binomial distribution. One of the goals of bootstrapping is to fix skew, kurtosis, outliers, and other concerns of normality in order to create a more normal distribution to use in comparisons; however, with only 4 nearly binomial scores, resampling isn't enough to transform this effect. The effect of the binomial distribution only becomes exacerbated by amplifying it. Therefore, applying the bootstrapping technique did not prove to be of value in the current study.

CHAPTER V

DISCUSSION

The current study sought to determine how melatonin may be utilized as a chronic prophylactic treatment to attenuate the behavioral and neural consequences of gerbil global ischemia. It was suspected that chronic administration of melatonin pretreatment through time-released pellets would diminish acute hyperactivity, errors in spontaneous alternation behavior, and CA1 hippocampal necrosis in global ischemic gerbils. This novel methodology would provide the context for developing new therapeutic treatments to protect against global ischemic injury. The current study was unable to definitively determine that chronic melatonin pretreatment significantly reduced hyperactivity and CA1 neuronal loss. There was prominent variability in ischemic injury within the current sample, drawing questions as to the efficacy of the global ischemia itself. The current study failed to demonstrate a significant effect on exploratory behavior, anxiety behavior, and spontaneous alternation behaviors in gerbils due to global ischemia. The significant variability within the ISX and ISX+M groups combined with a high mortality rate suggests that the samples in current study do not exemplify the typical global ischemic model; therefore, researchers are unable to draw decisive conclusions as to the effects of global ischemia and melatonin pretreatment.

Ischemia increased locomotor activity which melatonin did not seem to attenuate

In the current study, chronic melatonin pretreatment did not significantly demonstrate reliable alteration of gerbils' performance on locomotor activity in the open-field task after global ischemia. Melatonin was unable to display changes in open field behavior. Neither ischemia nor melatonin appeared to affect exploration or anxiety-like behavior. However, when ischemic groups were combined, an ischemic-induced increase in locomotor activity was revealed. Ischemic gerbils demonstrated a 73% increase in acute hyperactivity at 24 hours post-surgery. The hyperactivity observed among ischemic gerbils is consistent with previous research (Babcock, Baker, & Lovec, 1993; Janać et al., 2006; Katsuka et al., 2003; Mileson & Schwartz, 1991; Ramos-Zuniga et al., 2008; Wappler et al., 2009).

While there is much literature providing histological evidence of melatonin's neuroprotectant properties in global ischemic gerbil models, the current study is one of few to examine the behavioral effects of melatonin in an open field task (Cuzzocrea et al., 2000; Rennie et al., 2008). The results of the current study stand in contrast to those found in other efforts examining melatonin's effect on hyperactivity in global ischemic gerbils. Cuzzocrea et al. (2000), for example, displayed that introducing acute melatonin intraperitoneally attenuated hypermobility in open field assessments. Similarly, Rennie et al. (2008) administered chronic melatonin orally through drinking water in a gerbil ischemia model and found reduced short-term hyperactivity in open field assessments; however, no long-term effects were found at 20 weeks post-ischemia. These studies suggest that acute behavioral effects of global ischemia in gerbils can be curbed via

melatonin though such an effect was not observed in the current study. Due to the severity of the injury among the ischemic animals in the current study, it could be that a larger dose of melatonin is required to attenuate this increase in locomotion. The current study is one of few paving the way to a greater understanding of melatonin's chronic melatonin's ability to curb the behavioral effects of global ischemia. Furthermore, the current study is unique in its attempt to assess the efficacy of subcutaneous melatonin pellets as means of administering chronic, prophylactic melatonin.

Ischemia did not appear to affect exploratory behavior

Global ischemia failed to demonstrate a significant difference in gerbils' exploratory behavior in the current study. Most research neglects this measure in the gerbil model, making it unclear as to how exploratory behaviors interact with global ischemic injury. Additionally, the limited supply of literature that does exist provides diverging evidence as to how global ischemia and exploration relate in the gerbil model. The current study is suggestive of the results of Janać et al. (2006). In this study, exposure to various durations of global ischemia did not elicit changes in rearing behaviors of gerbils. The current study supports these authors' contentions that exploratory rearing behavior could be insensitive to global ischemia in the gerbil model, making it an unreliable measure of ischemic injury. It is also possible that though changes in exploration exist, measuring the behavior through rearing may not be a sensitive enough measure to detect alterations. Evidence provided by Wappler et al. (2009), however, supports a contrary contention. These researchers found a significant reduction in exploratory rearing behaviors in global ischemic gerbils. This finding

suggests an inverse reaction in contrast to the current study. While the current study is important in adding to the compiling literature on exploratory behavior in the gerbil model, the contradicting evidence between research endeavors in the literature demands future examination to determine the use of rearing behavior as an effective measure of ischemic injury.

To the researchers' knowledge, the current study is the first to examine the effects of melatonin on exploratory rearing behaviors within the gerbil model. Chronic melatonin pretreatment did not elicit change in the exploratory rearing behaviors of global ischemic gerbils. It stands to reason that if ischemia was unable to create differences in the exploratory behavior then melatonin pretreatment should not either, as evidenced in the current study. This suggests that chronic melatonin pretreatment itself does not create changes in the exploratory rearing behavior of gerbils.

Ischemia was unable to significantly affect anxiety behavior

Measuring changes in anxiety behavior is also an uncommon measure of the behavioral changes in gerbil global ischemia. In the current study, global ischemia did not appear to alter the amount of time spent in the center of the open field. There is very limited research on using the time spent in the center of the open field as measure of anxiety in the gerbil model. The current study implies support for the results found by Wappler et al. (2009). These researchers found that when global ischemic gerbils were exposed to a cylindrical open field, they did not spend significantly more time within the inner divisions of the area. The current study is valuable in that it adds to the emerging evidence that this measure may be insensitive to ischemic injury in the gerbil model. It is

also possible that though changes in anxiety may exist as a result of global ischemia, using the time spent in the center of the open field may not be a sensitive enough measure to detect the effect in the gerbil model.

To the researchers' knowledge, the current study is the first to examine melatonin's effect on anxiety behavior in the gerbil model. No significant changes in anxiety behavior were able to be found as a result of the chronic melatonin pretreatment. Because no significant differences were found in anxiety behavior due to global ischemia, no changes were expected to be found when melatonin was introduced. This suggests that chronic melatonin pretreatment alone does not appear to influence the anxiety behavior in gerbils.

Ischemia did not suggest a significant change in spontaneous alternation behaviors

In the current study, global ischemia did not elicit significant change in working memory errors and locomotion as evidenced by the spontaneous alternation behaviors in gerbils. No significant differences were able to be found in spontaneous alternation behavior, alternate arm return, or same arm return. Furthermore, it is noted that all three groups displayed spontaneous alternation behavior barely above the chance level of 50%, including the sham animals. Differences between groups were unable to be found due to the sham animals not showing definitive spontaneous alternation patterns. These findings are inconsistent with those found in the literature. There have been multiple studies confirming that gerbil global ischemic models result in a significant decrease in spontaneous alternation behavior concurrent with increased alternation errors following insult, while exhibiting spontaneous alternation above chance level (Gyertyán, Gigler, &

Sumo, 1999; Kim et al., 2011; Wappler et al., 2011). Additionally, this modification of behavior has been shown to be consistent in global ischemic gerbils regardless of age (Wappler et al., 2011). Within the current study, sham gerbils exhibited a strikingly lower percentage of spontaneous alternation behavior compared against this previous research, due to inflated error scores on alternate arm return and same arm return. This abnormality could have been due to individual or strain differences in the animals of the current study. The current findings encourage further research to establish spontaneous alternation as a reliable neurobehavioral measure in the gerbil global ischemia model.

To the researchers' knowledge, the current study is the first to assess melatonin's effect on spontaneous alternation behaviors in gerbil global ischemia. No significant differences could be reliably determined in spontaneous alternation behaviors due to the introduction of melatonin pretreatment. Because no significant differences could be found in spontaneous alternation behaviors due to global ischemia, melatonin was not expected to elicit changes in this measure. This suggests that chronic melatonin pretreatment alone may not significantly impact spontaneous alternation behaviors in gerbils.

Chronic melatonin pretreatment did not seem to attenuate ischemia-induced CA1 cell loss

Chronic melatonin pretreatment was unable to reliably show significant attenuation of the death of pyramidal neurons in the CA1 hippocampi of global ischemic gerbils. A significant difference was found between sham, placebo-treated ischemic, and melatonin-treated ischemic groups. Post-hoc analysis indicated a 65% loss of CA1

neurons in the placebo-treated ischemic group compared against sham animals, but no significant difference could be noted between sham and melatonin-treated ischemic groups. A separate analysis that compared all ischemic gerbils to SHAM found a significant 57% loss of CA1 neurons among the ischemic gerbils. This finding supports the well-documented severe susceptibility of CA1 hippocampal neurons to transient global ischemia in gerbils within the literature (Cuzzocrea et al., 2000; Guerrero et al., 1997; Katsuta et al., 2003; Kirino, 1982; Mileson & Schwartz, 1991; Sanderson & Wilder, 2013; Traystman, 2003; Yamamoto, Morimoto, & Yanagihara, 1986; Yamamoto et al., 1993). Though affirming the gerbil global ischemia model's degradation of CA1 hippocampal neurons, the current study did not definitively find that chronic melatonin pretreatment significantly ameliorated this damage.

Most studies that seek to evaluate neuroprotection of melatonin pretreatment in gerbil global ischemia through acute means of administration. For example, Guerrero et al. (1997) and Cuzzocrea et al. (2000) both utilized acute melatonin 30 minutes prior to gerbil ischemia via intraperitoneal injection to reduce oxidative damage to brain structures and preserve CA1 hippocampal neurons respectively. Differences found between these studies and the current one may be explained by the different methodologies of administering melatonin. Other models have utilized acute melatonin as an acute pretreatment to significantly decrease the infarct size of focal ischemia in MCAO rat models (Kilic et al., 1999, 2004; Pei, & Cheung, 2004; Pei, Ho, & Cheung, 2002). The diverging results of these studies with the current one may be explained by the greater severity of injury in global ischemic models as opposed to focal ischemia.

Differing methodologies and species between the studies could also account for the disparity of the results.

The current study is important in adding to the literature on utilizing chronic melatonin as a therapeutic tool against the neural deficits of gerbil global ischemia. There are few studies that seek to identify the chronic neuroprotectant properties of melatonin. One such study, De Butte and Pappas (2007) found that chronic melatonin not only preserved CA1 hippocampal neurons in pinealectomized rats but also increased the density of CA1 pyramidal cells by approximately 10%. The current study adds to the limited research in evaluating chronic melatonin pretreatment's ability to prevent CA1 cell loss. In clinical settings, developing chronic avenues of treatment is preferable to acute means due to the narrow window of opportunity in which a transient global ischemic event takes place. The current study offers new insight into the potential of development of melatonin as a chronic treatment in clinical cases of global ischemia.

There is very limited research as to melatonin's chronic prophylactic effects on ischemic injury. Kilic et al. (2004) demonstrated that orally administering chronic melatonin significantly reduced infarct size of MCAO rats in a focal ischemia model. Varying models of ischemia, species, and avenues of melatonin may account for the incongruence with the current study. Additionally, Kilic et al. (2004) administered chronic melatonin for 9 weeks prior to the ischemic event in contrast to the current studies' 2-week prophylactic treatment. It could also be possible that a longer period of exposure to melatonin is necessary for chronic neuroprotection. Just as developing chronic avenues of melatonin treatment lends itself more readily to clinical application,

prophylactic treatments offer greater benefit to at-risk populations who could utilize pretreatment regimes to curb potential ischemic events. The current study is important in continuing the effort to develop safe, reliable means of preventing the deleterious effects of global ischemia in practical settings.

To the researchers' knowledge, there is only one other study that examines melatonin's pretreatment effect in a gerbil global ischemic model through a chronic avenue. The current study supports the neural investigation of Rennie et al. (2008). In this study, researchers found that oral administration of melatonin at approximately ~0.6 mg/kg per day beginning 2 weeks prior to global ischemia and persisting for 20 weeks after the insult significantly reduced acute hyperactivity but did not afford protection of CA1 hippocampal neurons in the long term. Rennie et al. (2008) speculate that irregular drinking patterns of gerbils may have created peaks and troughs in melatonin levels that averaged far below their anticipated plasma levels and led to the lack of neuroprotection. The time-released melatonin pellets in the current study were designed to plasma levels of melatonin similar to the previous efforts that provided evidence for behavioral and neural protection against ischemic injury (Kilic et al., 2004; Pei, Ho, & Cheung, 2002; Rennie et al., 2008). The dosage within the current study was selected to achieve approximately ~0.6 mg/kg per day for two weeks prior to injury. Plasma levels were not obtained in the current study, so it is possible that the melatonin concentration did not reach the hypothesized plasma levels necessary for neuroprotection, similar to the contention of Rennie et al. (2008). Additionally, it is possible that greater levels of chronic melatonin than those that were hypothesized must be present to curb the neural effects of the gerbil global ischemia model. Previous research from Kilic et al. (2004)

and Pei, Ho, and Cheung (2002) displayed significant neuroprotection using MCAO rats; thus, it may be the case that species differences require greater levels of chronic melatonin to afford defense against ischemic injury. Despite its inconclusive results, the current study provides a novel attempt to utilize time-released melatonin pellets to provide a chronic pretreatment to resist the neural consequences of gerbil global ischemia. Not only does the current study offer insight as to a new approach in utilizing melatonin but it also provides context for future research in developing melatonin treatments more suitable to clinical application.

Global ischemia elicited a high mortality rate and variability of injury in gerbils

In the current study, global ischemia resulted in a much higher mortality rate than anticipated. A total of 9 ischemic animals were lost due to severe seizure activity and respiratory issues from both ISX *(n* = 3) and ISX+M (*n* = 6) groups, resulting in a 45% mortality rate for ischemic animals. Additionally, three animals were lost from the SHAM group due to severe respiratory issues emerging during sham surgery. Altogether, 12 out of 30 animals failed to complete the study, resulting in a 40% overall mortality rate. Previous research has shown that gerbils' propensity for seizure does not influence the outcome of ischemic insult (Donadio et al., 1982; Hermann et al., 2004). In comparing the mortality rate in the current study with that of previous research, it was found that the number of studies that fail to comment on mortality is staggering. Most studies within the literature do not comment on mortality at all, despite the fact that large and often unequal sample sizes suggest that an absence of mortality is near impossible with such a severe ischemic injury. The number of deaths in the current study mimic

those found by Martínez et al. (2012) in which a 15-minute carotid occlusion elicited a 42% mortality rate in global ischemic gerbils. Lee, Yoon, and Roh (1999) also found a 41% mortality rate in gerbils receiving 5-minute bilateral occlusion. In contrast, Zapaterab, Morenoc, and Horgac (1997) lost only 1 out of 115 gerbils during 5-minute bilateral occlusion, culminating in only an approximate 12% mortality rate after 3 days. Within the rare studies that do report mortality rate using the gerbil global ischemia model, it seems there is variability in the number of animals lost due to the ischemic insult. Due to the intense injury gerbils experience post-ischemia, mortality rates should be more closely analyzed in future research.

In addition to the high mortality rate, the current study found high variability of injury within ischemic samples. Table 3 indicates the mean CA1 pyramidal neuron counts in left and right hemispheres as well as the grand mean sum of CA1 cell counts for SHAM, ISX, and ISX+M animals. The SHAM (n = 6, M = 165.77, SD = 24.88) group displayed typical CA1 pyramidal neuron counts with left and right hemispheres containing similar neuronal density. In the ISX group, 2 animals exhibited unilateral damage inflating overall CA1 cell numbers in the ISX group. One animal from the ISX+M also exhibited unilateral damage.

It has long been assumed that due to the lack of interconnection between the posterior and middle cerebral arteries, blood flow to the forebrain plummets to near zero levels when bilateral carotid arteries are obstructed in the gerbil. When the Circle of Willis is indeed incomplete, consistent selective neuronal damage occurs within the CA1 hippocampus of the gerbil (Kirino, 1982; Traystman, 2003). Recently, however, it has

Table 3

CA1 pyramidal neuron counts

Animal Number	Left Hemisphere Mean (LHM)	Right Hemisphere Mean (RHM)	Grand Mean Sum (M)
SHAM			
2	86.73±1.96	95.13±1.41	181.87±1.80
10	53.67±7.54	82.44±3.15	136.11±5.83
11	80.27±5.87	86.07±6.01	166.33±5.26
12	65.00±3.26	75.92±8.73	140.92±4.78
29	83.56±3.70	86.06±6.24	167.07±5.82
30	99.93±2.51	102.4±4.11	202.33±2.80
ISX			
16	32.60±8.19*	105.87±11.53*	138.47±17.96*
22	35.20±9.77	40.87±5.92	76.07±7.73
23	4.80±0.48	4.73±0.41	9.53±0.39
24	4.93±0.63	4.93±0.34	9.87±9.85
26	48.33±4.35*	4.27±0.36*	52.60±9.85*
ISX+M			
7	6.20±0.68	6.93±0.78	13.13±0.50
8	5.22±0.47*	156.56±4.26*	161.78±22.91*
13	83.56±5.42	86.06±4.28	169.61±4.27
14	3.73±0.50	5.07±0.56	8.80±0.54

Data are presented as mean ±S.E.M. Values indicating unilateral damage are represented by *.

been asserted that the anatomical variability within the gerbil population is greater than previously speculated, particularly within animals procured from North American vendors (Laidley, Colbourne, & Corbett, 2005; Martínez et al., 2012; Mayevsky & Breuer, 1992; Seal, Buchh, & Marks, 2006). Laidley, Colbourne, and Corbett (2005) assert that gerbils obtained from Charles River – the largest supplier in North America and the vendor from which animals were purchased for the current study as well – are no longer viable for use as a model for global ischemia due to variability in injury. These researchers noted significantly higher instances of bilateral and unilateral anastomoses of posterior communicating arteries compared to another supplier, which in turn led to significant attenuation of the hallmark CA1 pyramidal neuron loss and hyperactivity in the animals. Seal, Buchh, & Marks (2006) affirm this variability within gerbil populations from the Charles River and Harlan Sprague-Dawley suppliers – the two main suppliers of gerbils for laboratories in the United States. These researchers found that the extent of CA1 hippocampal damage in gerbils after global ischemia was directly related to presence and diameter of posterior communicating arteries. Posterior communicating arteries less than 50 μm in diameter did not affect the severity of neuronal damage while neuron counts of those greater than 50 μm did not differ from sham animals up to 14 days post-surgery. Martínez et al. (2012) induced a more severe global ischemic injury of 15-minute bilateral carotid occlusion in gerbils. Animals in this study also exhibited bilateral, unilateral, and no posterior communicating arteries of varying diameters. The degree of anastomosis negatively correlated with neuronal density of the CA1 hippocampal area as well as the neurological symptoms of the ischemic gerbils.

The current study supports the contention of these researchers that increasing variability of the cerebral blood flow in gerbil populations used in global ischemia studies severely impacts the utility and reliability of the model. Gerbils receiving ischemic injury in the current study exhibited extremely variable results. Some animals displayed severe neuronal degradation in the CA1 hippocampus that is typical of a global ischemic event while others displayed partial or complete resilience to the injury. Additionally, several animals were noted to have significant unilateral damage, further offsetting the results. It may be possible that the gerbil global ischemic model is no longer a reliable means of assessing injury and neuroprotection, particularly for gerbil strains obtained from United States vendors. However, with proper precautions the variability within the model may be controlled. Martínez et al. (2012) describe a method in which the diameters of posterior communicating arteries are prescreened for each gerbil. This data was incorporated into the experimental procedure and interpretation to produce a more reliable and severe model of global ischemia. Animals possessing posterior communicating arteries large enough to sustain blood flow during ischemia could also be excluded from analyses. Using very large sample sizes could also cancel out the underlying interference of cerebrovascular variability within gerbil populations. Due to the relative ease and practicality of this model compared to the technically complicated 4-VO and 2-VO+hypo models of global ischemia, it should not be completely abandoned, but special precautions should be taken in future endeavors to limit and account for the anatomical variability within gerbil populations.

The disproportionate mortality rate combined with the heterogeneous variability exhibited within the current sample severely impacted the power of the study that may

have played a role in limiting the significance in some analyses. For instance, a post-hoc power analysis revealed that a 24.46% loss of power from the intended sample size was observed in the CA1 hippocampal cell counts. This particular measure showed a trend of melatonin neuroprotection that did not reach significant level. A Games-Howell post-hoc analysis indicated a medium effect between ISX and ISX+M groups; however, the mean difference between these groups did not reach significance *(d* = 0.425). Based on the current sample and effect size, an *n* of approximately 18 would be needed to reach the recommended power level of 0.80 (Cohen, 1988). An even more illustrative example of the power issue within the current study is that of the hyperactivity measure. There is a notable trend of data that suggests an attenuation of locomotor activity due to chronic melatonin pretreatment that did not reach significance. A Games-Howell post-hoc analysis indicated a medium effect between ISX and ISX+M groups; however, the mean difference between these groups did not reach significance either *(d* = 0.305). With the current sample and effect size observed, an *n* of approximately 45 would be necessary to achieve statistical power at the recommended 0.80 level. The same principle holds true for the other statistical analyses of the current study. It is possible that deflated power issues within the current study led to findings of non-significance. Because it is not possible to know whether the lack of significant differences between groups were due to the absence of effect or the inability to detect the presence of effect, the current study cannot draw definitive conclusions as to the efficacy of global ischemia and chronic melatonin pretreatment on the behavioral and neural measures. This revelation encourages future research to employ larger sample sizes in future endeavors to account for power issues that may arise from mortality and variable ischemic insult.

Additionally, the interesting effect sizes and trends noted within measures that approached significance encourage more research in determining the effect of chronic melatonin pretreatment of behavioral and neural measures, particularly in hyperactivity and CA1 pyramidal cell counts.

Conclusion

The present study is valuable in determining how melatonin can be used to combat the neural and behavioral consequences of global ischemia. Particularly, thus study contributes to the emerging effort of utilizing melatonin as a chronic prophylactic treatment. The current research provides groundwork for future endeavors that seek to develop methodologies that lend themselves more readily to clinical application of populations at-risk for global ischemic events through its use of a non-invasive and non-toxic administration of a known neuroprotectant. The present study did not find that chronic melatonin pretreatment significantly diminished the hyperactivity and CA1 hippocampal degradation that is the hallmark of the gerbil global ischemia model; however, notable trends toward neuroprotection were noted, particularly in ameliorating hyperactivity and CA1 pyramidal cell loss. Additionally, the current study did not find that global ischemia elicited changes in spontaneous alternation behaviors, exploratory rearing behavior, or anxiety behaviors. Global ischemia caused a disproportional mortality rate that limited the sample size of the current study. Further analysis of the global ischemia in the current sample demonstrated a highly variable neural injury with animals exhibiting partial resilience to ischemic impairment and unilateral damage. The resultant low sample size coupled with inconsistent ischemic injury hinders the current

study's ability to draw definitive conclusions as the efficacy of the chronic melatonin pretreatment's ability to curb the deleterious neural and behavioral effects of the gerbil global ischemia model. The trends in data and effect sizes that did not reach significance noted within the current study encourage future endeavors in utilizing chronic prophylactic melatonin in global ischemic models. Researchers caution that future use of the gerbil global ischemia model warrants the use of high sample sizes and measures taken to account for the emerging anastomotic variability in the animals.

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