THE EFFECT OF MELATONIN IN PROTECTING AGAINST THE BEHAVIORAL CONSEQUENCES OF CHRONIC HYPOPERFUSION IN MIDDLE-AGED FEMALE RATS

by

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ABSTRACT

Mild cognitive impairment (MCI) is a growing disorder among the elderly and can often go unnoticed for a significant portion of time. MCI is often preceded by vascular dysfunction and has the potential to cause irreversible damage to one's cognitive ability. The neurocognitive consequences of MCI can be imitated through the use of the 2-vessel occlusion (2VO) procedure in rats, which limits cerebral blood flow (CBF) through bilateral arterial ligation. Therefore, the current study investigated whether chronic melatonin would attenuate 2VO induced behavioral deficits in middle-aged female rats. Thirty 9- to 11-month-old female Sprague-Dawley rats were randomly assigned to one of three groups: sham, 2VO and 2VO animals that received melatonin. Melatonin was administered 2 weeks prior to the 2VO surgery as a pretreatment. The 2VO rats showed a significant increase in locomotor activity which was attenuated by melatonin treatment. 2VO rats also exhibited a significant increase in exploratory behavior that was also reduced by melatonin treatment. Melatonin treated rats also exhibited higher spontaneous alternation compared to 2VO and sham rats. Neither 2VO nor melatonin exhibited altered performance on visual and object recognition tasks. Similarly, no spatial learning deficits were observed using the Morris Water Maze task. These findings indicate that melatonin pretreatment is capable of reversing ischemia-induced hyperactivity suggesting that it had a neuroprotective role. 2VO did not cause

significant deficits in learning and memory however middle-aged rats may have subtle age-related impairments in these tasks hence making it more difficult to detect a 2VO induced deficit.

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TABLE OF CONTENTS

LIST OF TABLES

Tables Page

LIST OF FIGURES

Figures Page

CHAPTER I

INTRODUCTION

Though the boundaries between normal cognitive aging and MCI are blurred, MCI is defined as a stage in human cognition where memory is impaired, but dementia is not present (Zhang, Wei, Li, & Wang, 2011). Attention is being increasingly drawn towards the pathophysiology of amnestic MCI as well as its potential role as a predictor and precursor of Alzheimer's Disease (AD) (Nobili et al., 2008; Petersen, 2004). Palmer, Fratiglioni, and Winblad (2003) maintain that individuals with MCI are much more likely to transition into dementia within as little as 3 years and provides evidence for higher mortality rates in those with established cognitive impairment. This association demonstrates the necessity of developing treatments to slow or stop this deleterious progression, yet according to Farlow (2009), there are currently no FDA approved symptomatic treatments for the disorder.

One of the major contributors to the development of MCI is chronic cerebral hypoperfusion (CCH) or reduction in cerebral blood flow (CBF). CBF reduction seen in CCH is highly correlated with the progressive decline seen in MCI lending importance to the two-vessel occlusion (2VO) rat model of reducing CBF (Gorelick et al., 2011). De Bortoli et al. (2005), among others, believe that the 2VO rat model replicates

impairments often seen in humans with MCI, which allows for the study of behavioral changes associated with CCH (Murakami, Ikenoya, Matsumoto, Li, & Watanabe, 2000). This controlled induction of MCI also allows for the development and testing of medical interventions.

In studies performed by Cervantes, Morali, and Letechipia-Vallejo (2007) as well as Zhang et al. (2011), many neuroprotective properties of melatonin have been discovered. However, much of research has focused on the restorative effects of melatonin rather than its ability to curtail damage prior to the onset of MCI (Al-Ama et al., 2010; Furio, Brusco, & Cardinali, 2007; Cardinali, Furio, & Brusco, 2010; Letechipıa-Vallejo et al., 2007; Xu, Warrington, Beiber, & Rodriguez, 2011).

Additionally, research in this area predominantly utilizes male subjects (Cechetti et al., 2012; de Bortoli, Zangrossi, de Aguiar Correa, Almeida, & de Olivera, 2005; Letechipıa-Vallejo et al., 2007). Research indicates that females more commonly develop MCI and have a greater likelihood of progressing into more devastating neurodegenerative diseases such as AD (Azad, Al Bugami, & Loy-English, 2007; Ryan, Scali, Carriere, Ritchie, & Ancelin, 2008). According to Azad et al. (2007), this is a serious and largely unrecognized gap in research. To better understand the implications of MCI in females, the current study investigated whether melatonin as a pretreatment attenuates behavioral consequences of 2VO in middle-aged female rats.

CHAPTER II

LITERATURE REVIEW

Clinical Features of MCI

Across research studies and diagnostic manuals, criteria for MCI vary. However, most definitions describe MCI as a period where daily functions are not significantly disrupted yet cognitive function is below normal age-related decline (Petersen, 2004; Portet et al., 2006; Zhang, Wei, Li, & Wang, 2010). MCI may include impediments of working memory, response time, and strategic search strategies (Harel et al., 2011). However, diagnosis of both MCI and AD presents many challenges as poorly refined diagnostic criteria and a lack of both measurable and reliable biological determinants leave much to be desired (Growdon, 1999). According to Arsenaulte-Lapierre et al. (2011), current literature may underestimate the capabilities of those with MCI and overestimate the effects of aging leading to an overabundance of false negatives within clinical diagnosis of MCI and denial of treatment for those in need.

Janoutová, Šerý, Hosák, and Janout (2015) divided MCI into two categories pivoting on the presence of memory impairment. Amnestic MCI (aMCI) includes a significant memory deficit without meeting criteria for dementia and is most likely to progress into AD, while nonamnestic (naMCI) holds no impairments in memory and is

most often tied to non-AD dementias such as Parkinson's disease (Janoutová et al., 2015; Petersen, 2004; Winblad et al., 2004). However, Wahlund, Pihlstrand, & Eriksdotter Jonhagen (2003) suggest that another category of MCI should be created for impairments due to somatic illness, stress, or other dementias outside of AD.

While an interruption of daily living activities is not required for MCI diagnosis, deficits in short-term memory, response monitoring, and ability to employ strategic search strategies are commonly observed in MCI patients (Harel et al., 2011). This has prompted an argument presented by Nygard (2003) stating that diagnostic criteria should be augmented to include changes noticed by individuals that are too subtle to detect using current methods of assessment.

Epidemiology of MCI

The prevalence of MCI in persons older than 65 ranges from 10-20% with a yearly incidence rate of 1-4% (Mielke, Vemuri, & Rocca, 2014). Marked sex differences are present throughout the progression and development of MCI, and women are typically seen to progress through MCI into dementia at a faster rate than men (Lin et al., 2015). As the female body approaches menopause, levels of neuroprotective hormones such as estrogen and melatonin are seen to decrease (Bellipanni, Bianchi, Pierpaoli, Bulian, & Ilyia, 2001). Studies have revealed a higher prevalence of AD and dementia disorders among females (Azad et al., 2007; Ryan et al., 2008). Azad et al. (2007) attribute this principally to longer female life expectancy in conjunction with decline in hormone levels throughout aging (Ryan et al., 2008). However, when examining literature related to animal models of MCI induction and treatment, most of the research

regarding MCI treatment is performed on males (Cechetti et al., 2012; De Bortoli et al., 2005; Letechipıa-Vallejo et al., 2007).

Cerebral Hypoperfusion and MCI

According to Liu and Zhang (2012), Alzheimer's Disease (AD) was thought to be solely neurologically based until research within the most recent decades discovered strong evidence implicating vascular factors within the disease's progression. As a result, these risk factors have become a topic of major interest in MCI and dementia research. Among numerous other vascular factors, researchers agree that CCH is highly co-occurrent in those suffering from MCI (de la Torre, 2010; Dirnagl, Iadecola, & Moskowitz, 1999; Farkas, Luiten, & Bari, 2007; Liu & Zhang, 2012; Vicente et al., 2008; Zhang, Wei, Li, & Wang, 2011).

Luckhaus et al. (2008) report a significant decrease of CBF in MCI patients specifically within the mesial temporal lobe, anterior cingular cortices, and the amygdala. In a longitudinal study conducted by Huang et al. (2007), 39 MCI patients were observed over nineteen months. This study found that the rCBF of those progressing into AD was lower than that of those whose MCI was stable, thus demonstrating the predictive value of low CBF. Additional research conducted by Osawa and colleagues (2004), found that performance on the Mini-Mental State Examination, an assessment utilized in determining cognitive impairment, to be correlated with global CBF reductions. Similarly, Cantin et al. (2011) through the use of a functional MRI observed impaired cerebral vasoreactivity throughout the brain in those diagnosed with MCI. Similar

findings can be found within the work of de Bortoli et al. (2005) and van Exel et al. (2002).

These findings while informative, are inconclusive. Thus, highlighting the importance of animal models in order to better understand intricacies regarding the development, progression, and treatment of this condition.

Two-Vessel Occlusion (2VO)

To reproduce CCH often seen in aged humans with MCI, many animal models have been created (Liu & Zhang, 2012). However, permanent bilateral ligation/occlusion of the common carotid arteries (2VO) in rats is widely accepted as an ideal model of CCH (Liu & Zhang, 2012). When 2VO is performed, a mild and prolonged reduction of cortical and cerebral blood flow occurs (Farkas et al, 2007). This reduction ranges from 70-80% and is analogous to CCH (Farkas et al, 2007; Ritchie, De Butte, & Pappas, 2004; Sopala & Danysz, 2001). This reduction offers minimal risk of severe acute brain damage and does not allow for CBF to return to normal levels immediately (Liu & Zhang, 2012).

The reduction in CBF created by 2VO allows for the replication of many neurological and behavioral symptoms observed within humans with MCI (Gorelick et al., 2011). As humans begin to age, cardiovascular issues often arise such as atherosclerosis (de Bortoli et al., 2005). These issues often result in a reduction in CBF potentially leading to the development of MCI (Gorelick et al., 2011).

In studies by Choy et al. (2006), Ohta, Nishikawa, Kimura, Anayama, and Miyamoto (1997) and Otori et al. (2003) rats were subjected to the 2VO procedure and tested at various times in order to determine CBF levels. Two days following the 2VO

procedure, CBF in the cortex and hippocampus was markedly decreased (Otori et al., 2003). After 10 days, CBF began to gradually recover, and between 8 weeks to 3 months, CBF closely resembled normal levels (Ohta et al., 1997; Otori et al., 2003). Finally, after 6 months Choy et al., (2006) found CBF levels of 2VO rats to be indistinguishable from that of the control. Liu and Zhang (2012) believe these studies to provide an overall understanding that the pathological abnormalities of CCH occur between 2 days and 3 months following the injury. According to research, this period of disruption is believed to best mimic the pathological process of CCH and MCI seen in human aging (Farkas et al, 2007; Liu & Zhang, 2012). However, the neuropathological consequences of 2VO offer a deeper understanding of the interplay between 2VO and MCI.

Neuropathological Consequences of 2VO

Although damage to the hippocampal CA1 region neuron has been the most widely studied following CCH, areas such as the striatum and cerebral cortex are seen to exhibit damage as well (Block, 1999; Lipton, 1999). It is yet to be determined whether the predominant type of cell death in CCH is apoptotic or necrotic however, both may occur over the course of a reduction in blood flow (Farkas et al., 2007). Blood provides glucose and oxygen to the brain, and its disruption proves devastating for an area nearly exclusively dependent on oxidative phosphorylation for energy production (Dirnagl et al., 1999; Traystman, 2003). Void of energy causes membrane potential to be lost and excessive depolarization of neurons and glial cells to occur (Dirnagl et al., 1999). A reduction in adequate energy availability also leads to damage and death of neurons

through increases in the presence of free radicals, excitotoxins, ionic pump failure, mitochondrial injury, and increased concentrations of NA^{+}, CL^{-}, and CA^{2+} ions.

2VO results in a 35-45% and 60% reduction of CBF in the cortices and hippocampus respectively (Otori et al., 2003; Tsuchiya, Sako, Yura, & Yonemasu, 1992). CCH has varying impact throughout the brain with areas of the hippocampus, frontal lobe, striatum, and neocortex sustaining the most notable damage. (Block, 1999; Lipton, 1999; Pulsinelli & Brierley, 1979; Smith, Auer, & Siesjo, 1984). Nonetheless, the cornus ammonis 1 (CA1) region of the hippocampus is seen to be the most vulnerable to ischemia-induced degeneration and is believed to be predominantly responsible for functional disturbances in spatial learning and memory. Considering that CA1 hippocampal damage is consistently associated with deficits in learning and memory, it is often the primary focus of histological analysis (Block, 1999; Traystman, 2003).

Free Radical Involvement in Ischemia-Related Cellular Damage

Of importance to the current study is evidence of increased free radical production in response to an overabundance of CA^{2+} during CCH (Lipton, 1999). A free radical is defined as any molecule that possesses one or two unpaired electrons in its outermost orbital. Due to their structure, free radicals are highly reactive and in high concentrations can initiate harmful oxidative chain reactions posing a serious threat to the easily oxidized polyunsaturated fats which compose neuronal membranes (Dugan & Choi, 1999). During normal cellular function, a small number of free radicals are produced and eradicated. However, during an ischemic event, hypoxic metabolism leads to ionic disruption and causes free radicals to be produced in excess of the ability of the central

nervous system's (CNS) natural detoxification processes (Dugan & Choi, 1999; Schmidley, 1990).

During oxygen deprivation within the CNS, nitric oxide (NO) levels are increased initiating a chain reaction of volatile free radical production (Beckman, Beckman, Chen, Marshall, & Freeman, 1989). Although NO is a stabilized free radical, peroxynitrite is typically synthesized from the overabundance of NO. Peroxynitrite freely reacts with superoxide, a free radical commonly synthesized during ischemia which is especially damaging to neural tissues (Dugan & Choi, 1999; Lipton, 1999; Rodrigo et al., 2005). In the presence of superoxide during reperfusion, NO can lead to the formation of hydroxyl radical reactive species (Traystman, Kirsch, & Koehler, 1991). Hydroxyl radicals are more volatile than superoxide radicals, yet superoxide has a much longer half-life requiring longer for the body to rid itself of the radical (Chan, 1996).

The lack of oxygen leads free radicals to seek out an electron pair triggering enzymatic induction, membrane degradation and mitochondrial damage (Dugan & Choi, 1999; Lipton, 1999). As these free radicals begin to oxidize surrounding cells, the integrity of the system is compromised through the decay of lipids, proteins and DNA sequences within the cell (Dugan & Choi, 1999). This destruction often causes mitochondrial malfunction leading to further free radical production and ultimately to necrosis (Lipton, 1999). However, in still functioning neurons in which mitochondria have been compromised, free radicals may continue to be produced long after the ischemic event has concluded increasing the need for apoptotic processes (Dugan & Choi, 1999).

Behavioral Consequences of 2VO

Structural damage from reduced CBF often produces behavioral impairments. Some such deficits are seen in spatial orientation and in decreased learning and memory capabilities (Block, 1999). Additionally, the amount of damage seen in hippocampal areas is seen to be inversely related to performance in reference memory, working memory, spatial learning, and passive avoidance tasks (Block, 1999; Grotta et al., 1988).

Spatial reference memory impairment has been demonstrated in male rats on the Morris Water Maze (MWM) and the 8-arm radial maze (Sopala & Danysz, 2001; Vicente et al., 2008). In a study performed by Vicente et al. (2008), adult male rats demonstrated an impairment in MWM performance observed within as little as 3 days following 2VO surgery demonstrating impairment in both reference and working spatial memory (Vicente et al., 2008). Additionally, Sopala and Danysz (2001) repeatedly tested male rats (N = 27) on the 8-arm radial maze task at 1 week, 3, 10 and 16 months following 2VO revealing short-term deficits in reference and working memory.

A significant body of literature has demonstrated longer escape latencies among 2VO rats in the MWM (Liu, Zhang, Zheng, & Zhang, 2005; Xu et al., 2010; Zheng, Liu, Xu, & Zhang, 2008). In the study performed by Liu and colleagues (2005), rats were subjected to 2VO and later tested on the MWM. Rats subjected to 2VO showed a significant disruption in spatial reference memory as evidenced by longer escape latencies on the MWM. Interestingly, impaired performance on the MWM can be observed prior to any apparent damage to the hippocampal CA1 region (Pappas, de laTorre, Davidson, Keyes, & Fortin, 1996).

After prolonged CCH in rats, deficits in spontaneous alternation using both a Y-maze and T-maze have been demonstrated. In a study performed by Jaspers, Block, Heim, and Sontag (1990), rats were subjected to transient 2VO occlusion for 24 min and were tested on the MWM between 6 and 9 days following occlusion. This study found that rats' ability to use distal, extramaze stimuli was partially impaired whereas their ability to utilize proximal, intramaze stimuli was not affected.

Impairments of spatial memory has been found following 2VO (Zhang, 2011). In this study, heterochronic bilateral common carotid artery occlusion (hBCCAO), a model analogous to 2VO, was used to restrict blood flow in middle-aged male Wistar rats. The rats were studied 20, 40, and 60 days postocclusion to determine differences between groups in areas of sensorimotor function, gait, and memory. They found a significant reduction in spatial memory performance 40 days following CCH. Additionally, histological analysis showed a reduction in hippocampal CA1 neurons after prolonged restriction of blood flow beginning at approximately 33%.

Sarti, Pantoni, Bartolini, and Inzitari (2002) tested rats' visual memory using an object recognition task. Eighteen male Wistar rats were subjected to the 2VO procedure while 13 received sham operations. Rats were placed in an open field box and introduced to two identical objects for 2 min. The rat was then removed and one of the objects was replaced with another. After a 60-min latency, the rat was placed back into the box and the time spent exploring the new and old object was measured. A preference score was measured separately for each object by dividing the amount of time exploring one object by the total time spent exploring both objects. In those subjected to 2VO, significant and

progressively worsening deficiencies in visual memory were observed as the measure was repeated 30, 60 and 90 days following the procedure. Sarti et al. (2002) believe that this disturbance is due to injury in the frontal lobe rather than hippocampal impairment. Thus, they conclude that the damage caused by occluding a rat's carotid arteries is not localized to the hippocampus but spread among other areas of the brain.

Using an 8-arm radial maze, Murakami et al. (2000) found that rats subjected to 2VO employed a higher incidence of baited arm re-entry in comparison to those of the control group. This was demonstrated again in research conducted by Sopala and Danysz (2001) in which 27 male Sprague-Dawley rats were tested at 1 week, 3, 10 and 16 months after 2VO to investigate the long-term effects on spatial memory. They discovered that the 2VO rats committed significantly more reference and working memory errors as compared to the control group (Pappas et al., 1996; Sopala & Danysz, 2001).

A study by Ni et al. (1994) was also found to implicate short-term memory dysfunction in 2VO rats using a radial arm maze. This was indicated by failure to retain memory of previous arm entries after a 3-min delay period and was accompanied by hippocampal CA1 pyramidal cell loss. This finding is in accordance with that of Pappas et al. (1996), in which re-entry errors were exhibited between 4^{th} and 5^{th} arm choices in rats subjected to 2VO. Due to this delay in short-term memory, Pappas believes 2VO rats to be less able to establish reference memory in connection with extramaze cues.

Animals subjected to 2VO have been found to perform normally on open field tasks, demonstrating that global behavior is not impaired as a result of CCH (Farkas et al, 2007; Jaspers et al., 1990; Hattori et al., 2000). However, studies conducted by Babcock,

Baker, and Lovec (1993) as well as de Bortoli et al. (2005) indicated that rats subjected to CCH tend to have a higher number of grid crosses an indicator of hyperactivity, as well as an increase in exploratory behaviors and a decrease in anxiety-related behaviors.

Just as a delay in cell death is seen following 2VO, impairments in behavioral ability can present days after the injury (Block, 1999). Behavioral deficits are often a manifestation of neuromorphological changes. Therefore, neuroprotective strategies are gaining attention and have been found to lessen the effects of CCH on behavioral performance (Farkas et al, 2007). The progressive destruction caused by free radicals during CCH is dangerous to the cellular integrity of the central nervous system and causes continuous damage (Dugan & Choi, 1999). However, endogenous hormones such as melatonin have been shown to aid in the antioxidative restoration of homeostasis as well as recovery of function (Alonso-Alconada, Alvarez, Martinez-Ibarguen, & Hilario, 2013; Andrabi, Parvez, & Tabassum, 2015; Letechipia-Vallejo et al., 2007).

Melatonin and its Role in Reducing Oxidative Stress

Melatonin, n-acetyl-5methoxytryptamine, is an endogenous hormone secreted largely by the pineal gland in mammals (Kostoglou-Athanassiou, 2013). Melatonin is synthesized from serotonin and participates in many functions such as circadian rhythm modulation, sleep induction, and immunoregulation (Pandi-Perumal et al., 2012). Additionally, melatonin has been found to be a powerful antioxidant while scavenging a wide variety of free radicals leading to an intensified study of melatonin as a neuroprotective agent (Alonso-Alconada et al., 2013; Pandi-Perumal et al., 2012; Reiter, Tian, Leon, Kilic, & Kilic, 2005; Samantaray et al., 2009).

Melatonin has been found to offer a multimodal neuroprotective effect through its antioxidative, antiapoptotic and anti-inflammatory properties (Andrabi et al., 2015; Antunes et al., 1999; Cervantes et al., 2007; Pandi-Perumal et al., 2012). It has also been shown to improve mitochondrial function, inhibit the synthesis of inflammatory cytokines, and preserve neuro-cytoskeletal organization, while the direct antioxidative properties of melatonin serve as the primary course of neuroprotection (Cervantes et al., 2007). As outlined previously, these are major contributory factors in neurological damage caused during CCH. Additionally, melatonin's endogenous presence, relative ease in crossing the blood-brain barrier, and high safety profile may allow for its use in therapeutic application.

A study by Tan, Chen, Poeggeler, Manchester, and Reiter (1993) established melatonin as a potent free radical scavenger. In this study, the hydroxyl radical was exposed to two well-known free radical scavengers, mannitol and glutathione, in addition to the newly discovered scavenger melatonin. Glutathione and melatonin are both endogenous free radical scavengers, however, melatonin was found to eradicate free radicals much more effectively than either of the previously known scavengers. Additionally, Tan et al. (1998) observed melatonin's free radical scavenging properties through radiation-induced hydroxyl radical generation. They observed direct reductions in tissue loss in rats highly saturated with melatonin when exposed to high free radicals concentrations leading them to the conclusion that melatonin may be one of the first lines of defense against highly damaging oxidants.

Melatonin's preeminent neuroprotective mechanism is accomplished in the reduction of oxidative damage as a result of its antioxidative and free radical scavenging properties (Antunes et al., 1999; Reiter et al., 2005). Additionally, significant reduction in oxidative damage to hippocampal CA1 pyramidal neurons has been observed with administration of melatonin during blood flow reduction (Cho, Jon, Baik, Dibinis, & Volpe, 1997).

Many have found melatonin and its metabolites, N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) and N^1-acetyl-5-methoxykynuramine (AMK), to aggressively scavenge many types of free radicals including reactive oxygen species and reactive nitrogen species (Andrabi et al., 2015; Cervantes et al., 2007; Reiter et al., 2005). This process of scavenging and detoxification occurs when melatonin donates one of its electrons to these highly reactive oxyradicals consequentially neutralizing their degenerative effect on proximal susceptible cells (Reiter et al., 2005).

Not only does melatonin directly neutralize free radicals, but it has an indirect role in free radical elimination through an increase in the expression of powerful antioxidative enzymes such as glutathione peroxidase and superoxide dismutase (Pandi-Perumal et al., 2012). This further decreases damage caused by reduced blood flow allowing melatonin to demonstrate an indirect effect on apoptotic activity (Cervantes et al., 2007; Ling, Zhang, Lu, Li, & Sun, 1999; Reiter et al., 2005). Melatonin has also been seen to inhibit NO synthesis through its interactions with CA^{2+} and inhibition of peroxynitrite during an ischemic event while increasing the efficiency of mitochondrial cells resulting in lessened free radical production (Cervantes et al., 2007; Cuzzocrea et al., 2000).

In regard to melatonin's receptor mediated intervention in ischemia, activation of QR2 receptors by melatonin has shown an increase in antioxidant production (Boutin, 2007; Fu, Buryanovskyy, & Zhang, 2008). Similarly, melatonin's MT1 receptors, associated with modulation of neuronal firing and increased cell proliferation, have been associated with reductions in cerebral infarct volumes following ischemia (Dubocovich & Markowska, 2006; Kondoh, Uneyama, Nishino, & Torii, 2002). Although improvements in sleep quality are not seen as a result of melatonin (Malhotra, Sawhney, & Pandhi, 2004), the compounding neuroprotective effects allow melatonin to be a powerful neuroprotectant against many of the harmful effects of both acute and chronic CBF reduction.

Melatonin Protects Against Cerebral Ischemia

In more neuropathologically destructive models such as 4VO, high levels of melatonin continuously administered directly following occlusion has been shown to preserve 78.7% of CA1 hippocampal neurons (Letechipia-Vallejo et al., 2007). Groups of eight rats were used with a control group, sham group, 4VO with vehicle only at 3 mL • kg • hr for a 6-hr period 30 min following carotid clamp removal, and 4VO with melatonin at 10 mg • kg • hr following the same timeline as above. Treatment with melatonin was shown to reduce MWM escape time and reduce the number of errors made by the rats on the radial arm maze thus combatting some of the behavioral effects seen following 4VO. This is indicative of melatonin's ability to reduce both lipid peroxidation and apoptosis (Cuzzocrea et al., 2000; Ling et al., 1999).

In a study performed by Kilic et al. (2004), melatonin (4mg • kg • day) was administered orally for 9 weeks prior to occlusion in order to observe its effect after 90 min of middle cerebral artery occlusion. This study observed an effect of melatonin on endothelin converting enzyme-1 (ECE-1), which has been powerfully implicated in vascular disease in the elderly. Melatonin was found to markedly reduce ECE-1 levels in both acute and chronic implementations, thus leading to the belief that melatonin may be an aid for those suffering from advanced vascular disease.

Lee, Park, Ahn and Won (2016) conducted a study in which 48 male Sprague-Dawley rats were used to test whether melatonin possessed mediatory effects on the behavioral deficiencies accompanied by 2VO induced CCH. The animals were evenly split into three groups: control, 2VO, and 2VO + melatonin. In the animals receiving melatonin, administration was accomplished through intraperitoneal injections of 10 mg • kg • day for 28 days following the surgery. They examined spatial learning and memory using the MWM and found that escape latencies for rats in the 2VO group were significantly longer when compared to the other test groups, demonstrating a reduction in CCH-induced impairment. Additionally, melatonin treatment was found to significantly reduce the amount of neuronal death seen in 2VO induced CCH in the hippocampal CA1 region.

In contrast, there is substantial evidence supporting that a lack of or reduction in melatonin can increase the effect of insult during reduced CBF (De Butte, Fortin, & Pappas, 2002; Kilic, Ouzdemir, Bolay, & Dalkara, 1999). A study performed by De Butte et al. (2002) showed that pinealectomy – a procedure in which a gland largely responsible

for production of melatonin is removed – worsens the effect of 2VO on CA1 and CA4 hippocampal neurons. Thus, lower levels of endogenous melatonin may lead to a greater severity of neuronal damage. In a study by Kilic, Ouzdemir, Bolay, and Dalkar (1999), 25 pinealectomized rats and 14 sham-operated rats were subjected to middle cerebral artery (MCA) occlusion. The pinealectomized rats were evenly split into five groups: one group was injected with a vehicle solution, melatonin was administered before ischemia (4mg/kg, $N = 4$) or before reperfusion (4 or 8mg/kg, $N = 5$ and $N = 4$ respectively), or administered before both ischemia and reperfusion (4 + 4mg/kg, $N = 7$). This reduction was found to lead to greater infarct volume following an ischemic event. However, this study found a 40% reduction in infarct volume when melatonin was administered directly before ischemia further substantiating the neuroprotective effects of melatonin.

Pinealectomy alone has been shown to cause cell loss within CA1 and CA3 neurons. However, in a study in which pinealectomized rats were given supplemental melatonin, no loss of CA1 and CA3 neurons was observed (DeButte & Pappas, 2002). Remarkably, the prevalence of CA1 neurons increased by 10% as compared to sham groups. These findings indicate not only the necessity of melatonin for maintaining healthy brain function but also the neurogenerative ability of melatonin in instigating neuronal growth.

Although many positive effects of melatonin have been observed, improvements could be made in its implementation. Oral administration via drinking water is easily the most common method of administration, however, when placed in water, the amounts of melatonin administered can vary. Additionally, the animal's digestive system will

inevitably break down a percentage of the melatonin rendering challenges in dosage monitoring. Melatonin injection is also quite common; however, it is only an acute means of administration. A more consistent method of administration may lie within a chronic prophylactic approach through the utilization of subcutaneously implanted time-release pellets.

Current Study

Due to the lack of research in this domain, vascularly induced MCI is of increased clinical concern in the aging female population (Lin et al., 2015). Yet, Azad et al. (2007) claim that a large majority of research regarding 2VO induced cognitive impairment is performed using young male rats. In addition, a large majority of the available research regarding melatonin in reduced CBF administered treatment post-trauma or directly prior to the injury, leaving a gap in understanding how chronic melatonin pretreatment may affect the outcomes of 2VO. To address these concerns, the current study utilized a chronic pretreatment of melatonin to examine the behavioral implications of melatonin on middle-age female rats subjected to 2VO.

Although neurological damage caused by 2VO is nigh impossible to reverse, evidence has been found for mediatory treatments administered prior to the insult (Farkas et al, 2007). The highest amount of cell death is observed 3 days after CBF reduction (Block, 1999) allowing time for effective treatment to be administered before irreversible damage occurs (Alonso-Alconada et al., 2013). Although previous studies have typically administered melatonin immediately preceding or following injury, the current study administered melatonin 2 weeks prior to the injury. To date, no research exists regarding

whether melatonin would be an effective prophylactic treatment in older female rodents subjected to 2VO. Preventative treatments allow for the retention of normal cellular function rather than anticipating that normal function will be regained. This is especially critical as neurological function is exceptionally difficult to recover after damage has occurred (Goh & Park, 2012).

This study used middle-aged peri-menopausal female rats. Retired breeders were chosen for their transition into a perimenopausal state. This age group is seen to undergo a steady decline in melatonin levels leaving them vulnerable to developing MCI (DeButte & Pappas, 2002; Lin et al., 2015; Okatani, Morioka, & Hayashi, 1999). As MCI is a major precursor to dementia, the lack of research using female rats leaves our current literature limited in its scope with respect to the overall understanding of the development and progression of MCI.

Lastly, the present study utilized subcutaneously implanted time-release melatonin pellets allowing for the highest degree of control in melatonin administration. Typical administration strategies are not the most clinically appropriate, as they require timely administration in response to injury or consistent daily administration via injection. Therefore, the current study hypothesized that melatonin chronically administered prior to 2VO would mitigate the behavioral consequences seen following 2VO in middle-aged female rats.

CHAPTER III

METHOD

Animals

This research was approved by the Animal Care and Use Committee at West Texas A&M University and care of animals was in accordance with the National Institute of Health guidelines. Thirty, female Sprague Dawley rats (retired breeders, 9 to 11 months of age) were purchased from Charles River and housed three to a cage in a 12-hr light/dark cycle with food and water provided id libitum. Retired breeders were used as the majority of middle-aged women have had at least one pregnancy (Muller, Chiou, Carey, & Wang, 2002); therefore, the use of retired breeders is more clinically relevant. A total of 30 rats entered the study. The chosen sample size was considered to be adequate among animal research within the field (Charan & Kantharia, 2013).

Melatonin Pellet Implantation

Following a 1-week adjustment period, rats were randomly assigned to either a melatonin pellet implantation group (N = 10) or a no pellet group (N = 20). Rats in the melatonin supplementation group were subcutaneously implanted with a 5-mg, 60-day, time-released, melatonin pellet (0.08mg/day; Innovative Research of America Inc.). Rats were anesthetized using isoflurane (4% induction, 2% maintenance in 70% N20 and 30%

O2 mixture). Pellets were implanted dorso-laterally between the skin and muscle of the neck between the rat's ear and shoulder. The incision was closed with 5-0 nylon suture. Sham rats received the same procedure except no pellet was implanted.

2VO Surgery

Two weeks following pellet implantation, rats were subjected to 2VO (N = 20) or sham surgery (N = 10). Rats were first anesthetized using isoflurane (4% induction, 2% maintenance in 70% N_20 and 30% O_2 mixture). Rats received a ventral midline incision to expose the carotid arteries. The arteries were then separated from the carotid sheath and vagus nerve and permanently ligated with 4-0 silk suture. Sham animals underwent the same procedure with omission of the ligation procedure. Behavioral testing commenced after a 2-week recovery period.

Two rats died as a result of the 2VO surgery: one from the 2VO group and one from the 2VO+M group. As a result, one sham animal was subjected to 2VO to replace the animal that died (sham = 9; 2VO = 10; 2VO+M = 9).

Behavioral Testing

Two weeks following 2VO or sham surgery, animals commenced behavioral testing in an open field, spatial recognition, object recognition, MWM and Y-maze task. The experimenter was blind to animal conditions throughout behavioral testing. All mazes excluding the MWM were cleaned with 70% ethanol between each rat.

Open field task.

The open field task was used to measure overall global functioning and potential sickness behavior (Seibenhener & Wootne, 2015; Walsh & Cummins, 1972). Open field

testing was performed in a square black box (57.6 cm × 57.6 cm) with sides 38 cm high and the floor divided into nine equally sized squares (6.4 cm × 6.4 cm). Animals were placed in the open field for 6 min, during which time they were assessed for locomotor activity, exploration, and the amount of time spent in the center square. Locomotor activity was indicated by the number of grid crossings, defined as all four paws crossing a grid line. Rearing was defined as lifting the upper body and forepaws from the ground and was used as a measure of exploration. Anxiety-like behavior was measured as the amount of time (in seconds) spent with all four paws in the center square.

Spatial recognition task.

Twenty-four hours following testing in the open field, rats were tested on the spatial recognition task. Animals explored two identical objects for 3 min in the open field. Exploration was defined as rearing towards an object, touching the object with their paws, and sniffing/looking at the object from less than 2 cm away. Following a retention interval of 30 min, rats were placed back into the open field with the same two objects except one of the objects was relocated to another area of the box, and rats were given an additional 3 min to explore the objects. A preference score was calculated by dividing the time spent exploring the relocated object by the total exploration time, then multiplied by 100. A preference score of 50% represents chance, and a higher score has been found to indicate intact spatial recognition (Dere, Huston, & Silva, 2007).

Object recognition task.

The object recognition task is used to assess short-term memory for visual learning (Atunes & Biala, 2012). The day following the spatial recognition task, animals

were allowed 3 min to explore two identical novel objects placed in the open field box. Following a retention interval of 1 hr, rats explored a familiar and new object for 3 min. Exploration was defined in the same way as for the spatial recognition task, and preference scores were similarly calculated.

Morris water maze.

Following testing on the recognition tasks, the MWM was used to assess spatial memory (Morris, Garrud, Rawlins, & O'Keefe, 1982). This task relies on intact hippocampal function (D'Hooge & De Deyn, 2001; Morris et al., 1982). A blue circular pool, 182.88 cm in diameter and 76.2 cm in height, was filled with water (21 ± 1^{o} C). Submerged 2 cm under the water was a clear Plexiglass platform which remained in the northeast quadrant for the duration of testing. For four consecutive days, rats were given four trials in which they attempted to find the hidden platform. Animals were randomly placed among the four equally sized quadrants designated as north, south, east, and west. Rats were given 90 s to locate the concealed platform. If unsuccessful, the rat was guided to the platform and allotted a 15-s rest period. The number of seconds it took to reach the platform was recorded and averaged across sessions.

Y-maze.

Animals performance on the Y-maze was assessed following MWM testing to assess spontaneous alternation behaviors. Rats were placed on a black Y-maze (height: 25.4 cm; length of arms: 55.9 cm), and each animal was given 10 min to freely explore the three arms. The order and number of arms entered by the rats was recorded. Unimpaired animals typically alternate entry into the arms of the maze successfully

utilizing their short-term memory (Dudchenko, 2004). However, animals with frontal cortical impairments often re-enter the arm they exited, referred to as a same arm return error. Exploring two arms then returning to the first arm was recorded as an alternate arm return. For each consecutive triplet arm entry, it was determined whether the rat demonstrated a spontaneous alternation pattern, alternate arm return pattern, or a same arm return pattern. A percent score for each behavioral pattern was computed by dividing the number of spontaneous alternations made by each rat by the total number of triplets then multiplying the quotient by 100.

Statistical Analyses

Results are reported as $\bar{X} \pm SEM$. Data analysis was performed using the statistical software SPSS 24.0 for Windows. Normality and homogeneity of variance were first determined. Open field data was analyzed using a Multivariate Analysis of Variance (MANOVA). Recognition tasks were analyzed using a one-way Analysis of Variance (ANOVA). MWM data were analyzed using a mixed factorial ANOVA with groups (sham, 2VO, and 2VO+M) as the between-subject factor and day as the repeated measures factor. The Y-maze data were analyzed using a Kruskal-Wallis test as there was a violation of homogeneity of variance. Post hoc analyses were conducted using Fisher's LSD for ANOVA analyses. All descriptive statistics are reported in Table 1 of the appendix.

CHAPTER IV

RESULTS

Open Field Task

To test whether 2VO and/or melatonin treatment produces global behavioral deficits, locomotor activity, exploration, and anxiety-like behavior were assessed in the open field. In order to avoid inflating alpha by running multiple ANOVAs, a MANOVA was used to compare number of grid crosses, rears, and time spent in the center between groups. Analysis first revealed a significant difference between groups in locomotor activity, $F(2,27) = 4.53$, $p = .021$, Wilk's $\Lambda = 0.57$, partial $\eta^2 = 0.27$, $R^2 = .21$. Because Fisher's LSD adequately controls familywise error for 3 or fewer groups, post hoc analysis using Fisher's LSD was conducted. Fisher's LSD revealed that 2VO rats exhibited an increase in grid crosses compared to sham rats ($p = .016$; Figure 1A), indicating hyperactive tendencies in the 2VO rats. Melatonin treated rats demonstrated a reduction of 2VO induced hyperactivity as they differed significantly from nontreated 2VO rats ($p = .02$) but not sham rats ($p = 1.00$).

Analysis of exploration (rears) of sham, 2VO, and 2VO+M groups revealed a significant difference between groups, $F(2,27) = 8.03$, $p = .002$, partial $\eta^2 = 0.39$. As illustrated in Figure 1B, 2VO rats showed a significant increase in the number of rears

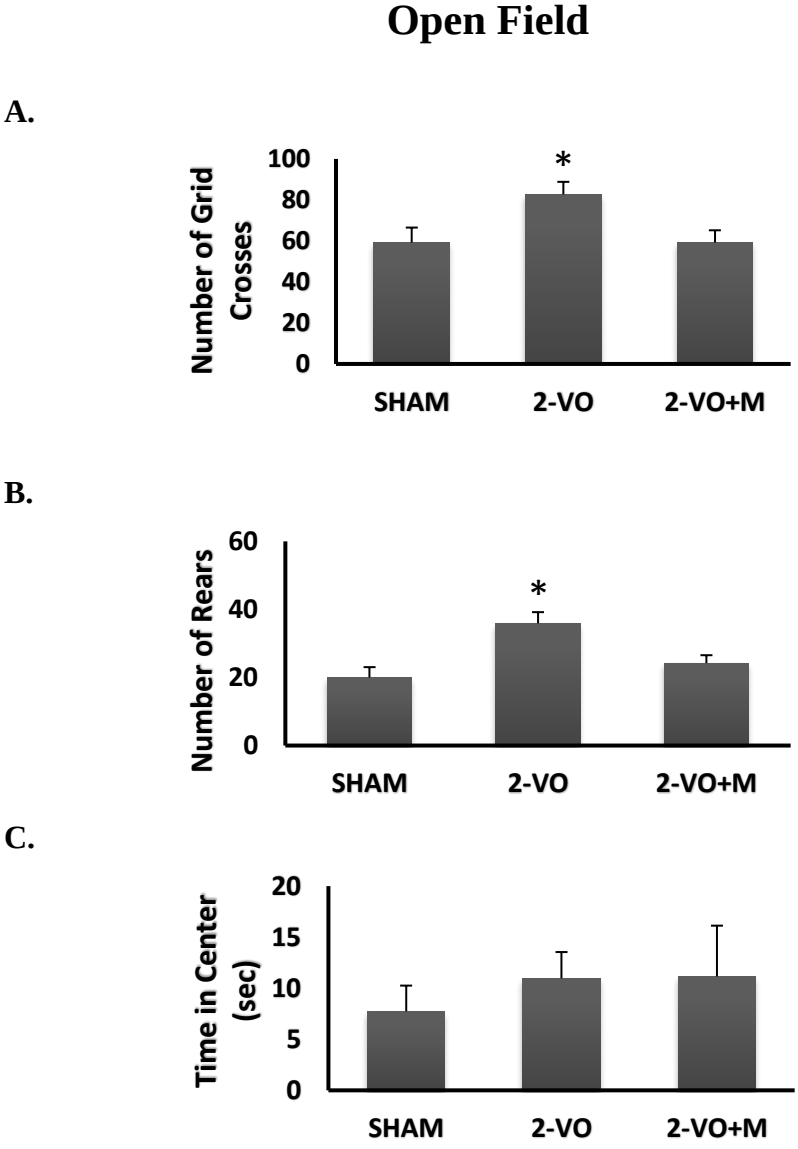


Figure 1. Locomotor activity and exploration were affected by 2VO and this was attenuated by the administration of melatonin. (A) Locomotor activity was assessed as the number of grid crosses in the open field. 2VO increased locomotion and this was attenuated following treatment with melatonin. Data are presented as $\bar{X} \pm SEM$ *$p <$ .05. (B) Exploration was assessed as the number of rears in the open field. Data are expressed as $\bar{X} \pm SEM$ *$p <$ .05. (C) Anxiety-like behavior was measured as the time spent in the center square of the open field (in seconds). No difference was found between groups. Data are expressed as $\bar{X} \pm SEM$.

compared to sham rats using Fisher's LSD (p = .001). Thus, melatonin administration led to a decrease in exploration brought about by 2VO. No significant difference was found between groups on the amount of time spent in the center, $F(2,27) = 0.30$, p = .75, partial $\eta^2 = 0.02$, Figure 1C.

Spatial Recognition Task

To test whether 2VO and/or melatonin affects spatial memory, a spatial recognition task was utilized. To control alpha across multiple comparisons, a one-way ANOVA was used to compare the behavioral effect of 2VO and melatonin on the animals' performance on the spatial recognition task. Analysis revealed that neither 2VO nor melatonin affected the animals' spatial recognition performance, $F(2,25) = 1.80$, p = .19, partial $\eta^2 = 0.14$, Figure 2. Two rats did not meet exploration criteria and were excluded from analysis.

Object Recognition Task

To determine whether 2VO and/or melatonin treatment affects visual memory, rats were tested on the object recognition task. A one-way ANOVA was used to compare the behavioral effect of 2VO and melatonin on short-term visual memory. Analysis revealed that neither 2VO nor melatonin affected visual memory, $F(2,27) = 1.89$, p = .17, partial $\eta^2 = 0.14$. As illustrated in Figure 3, all groups exhibited a preference for the novel object as evidenced by above chance preference scores.

Morris Water Maze

To assess whether 2VO and/or melatonin treatment affects hippocampal-

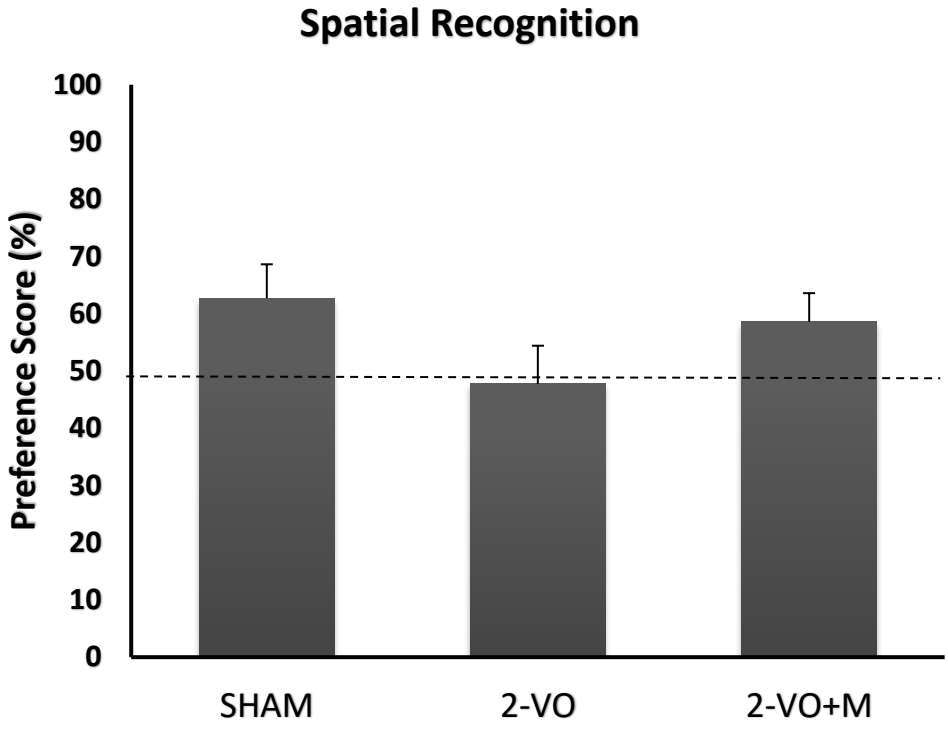


Figure 2. Neither 2VO nor melatonin was found to affect spatial recognition when compared to sham. Data are reported as mean preference scores across groups [(novel object exploration/total object exploration) X 100, $\bar{X} \pm SEM$]. The darkened line at 50% represents equal exploration of both objects (chance performance).

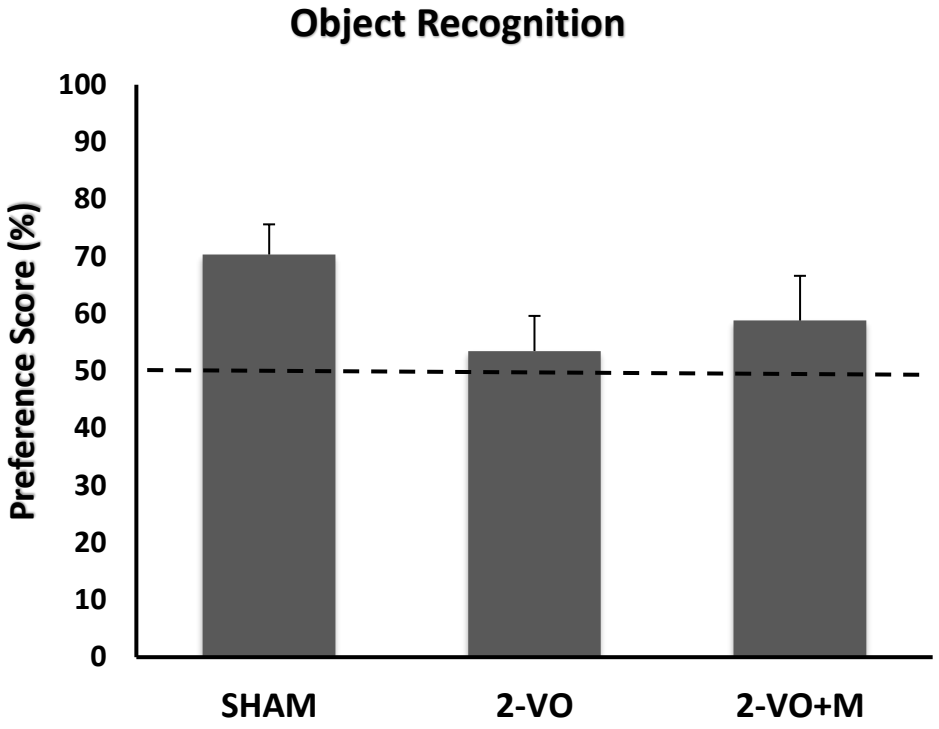


Figure 3: Neither 2VO nor melatonin was found to affect visual memory performance when compared to sham. Data are reported as mean preference scores across groups [(novel object exploration/total object exploration) X 100, $\bar{X} \pm SEM$]. The darkened line at 50% represents equal exploration of both objects (chance performance).

dependent spatial memory, groups were tested in the MWM. To compare between- and within-subject groups, a mixed factorial ANOVA was conducted (sham, 2VO, and 2VO+M) as the between-subjects factor and Day (1-4) as the within-subjects factor. Analysis revealed that neither 2VO nor melatonin affected the amount of time taken to locate the hidden platform, $F(2,25) = 0.95$, $p = .46$, partial $\eta^2 = 0.07$.

As illustrated in Figure 4, a significant effect of Day revealed that all rats exhibited shorter latencies across days, $F(3,75) = 27.49$, $p < .001$, partial $\eta^2 = 0.52$. Because identifying subsequent learning across days was desired, post hoc analyses using two-tailed paired samples t test was conducted and adjusted using Sidak alpha adjustment for multiple comparisons in order to control for familywise error. Post hoc analyses revealed that all groups spent significantly more time searching for the platform on Day 1 compared to Day 2, 3, and 4, $t(75) = 2.31$ ($p = .03$), $t(75) = 6.46$ ($p < .001$), and $t(75) = 6.90$ ($p < .001$) respectively. Additionally, all rats exhibited longer latencies on Day 2 compared to Day 3, $t(75) = 5.01$ ($p < .001$) and 4, $t(85) = 5.31$ ($p < .001$). No difference was found between latency times on Day 3 and Day 4, $t(75) = 1.55$ ($p = .132$). No interaction was found between group and day, $F(6,75) = 0.95$, $p = .570$.

Y-maze

After an initial one-way ANOVA reported violations of homogeneity of variance and normality, a Kruskal-Wallis nonparametric test for multiple comparisons was used. Kruskal-Wallis allows for non-normality in the event that each distribution has comparable kurtosis. Because the present data met these criteria, Kruskal-Wallis was used to asses for differences between groups in spontaneous alternation pattern (SAP),

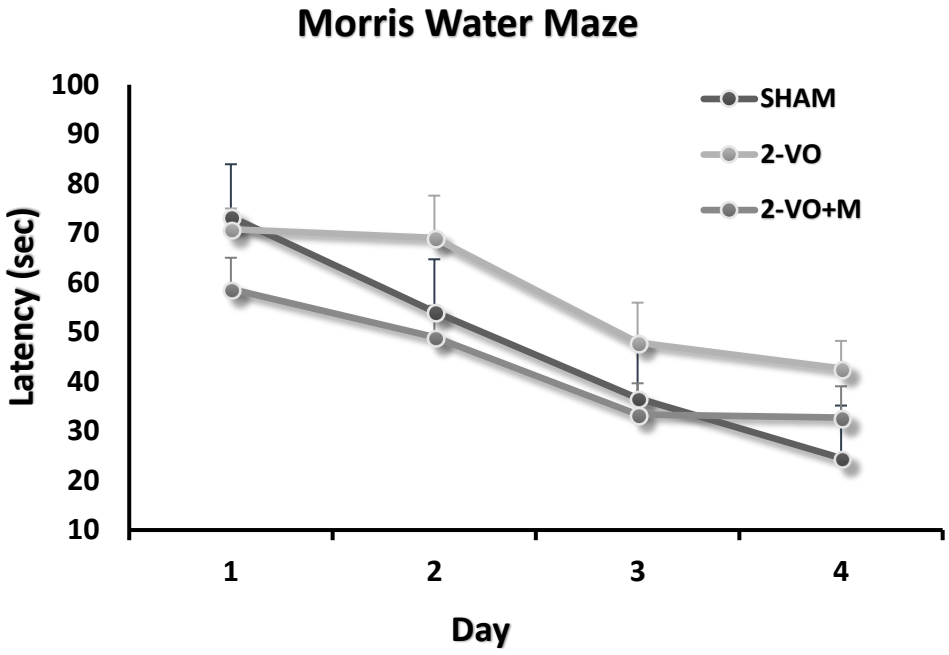


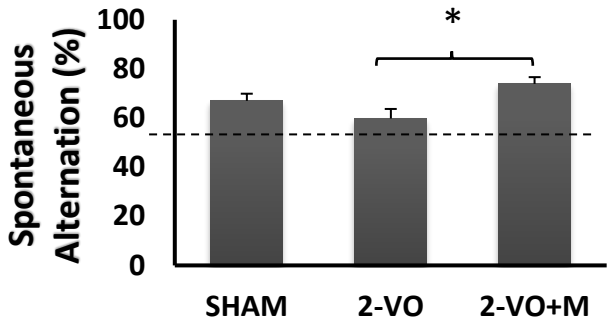
Figure 4: Neither 2VO nor melatonin was found to create a significant difference between groups on spatial memory. These data represent mean escape latency times exhibited across groups with *SEM*.

alternate arm return (AAR) and same arm return (SAR). As illustrated in Figure 5, a significant difference was detected between groups on SAP behavior, $H(2) = 7.40$, $p = .03$, partial $\eta^2 = 0.27$. However, no differences were found between groups on AAR, $H(2) = 5.95$, $p = .51$, partial $\eta^2 = 0.22$, or on SAR behavior, $H(2) = 1.94$, $p = .38$, partial $\eta^2 = 0.07$.

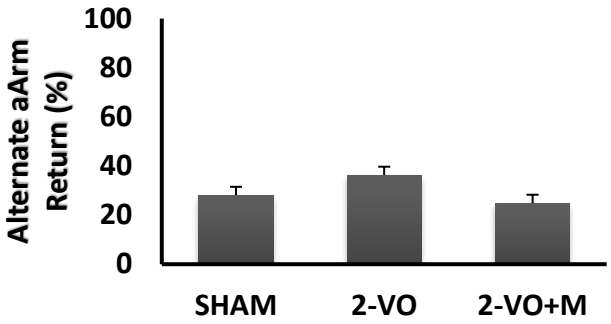
A moderately conservative post hoc using Tukey HSD revealed that the melatonin treated 2VO rats exhibited higher spontaneous alternation compared to the 2VO group ($p = .01$). However, no significant differences between 2VO and sham ($p = .28$) or between 2VO+M and sham ($p = .33$) were identified.

Y-Maze

A.



B.



C.

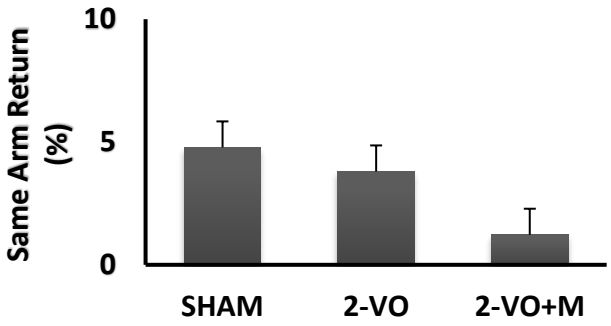


Figure 5: Melatonin was found to increase the number of SAP behaviors as compared to the 2VO group. Melatonin nor 2VO were found to influence AAR or SAR behaviors. Data are reported as percentages of spontaneous alternation pattern (SAP) [A], alternate arm return (AAR) [B], and same arm return (SAR) [C]. Data represent the number SAP, AAR, or SAR behaviors divided by the total number of behaviors and multiplied by 100 with error bars representing $\bar{X} \pm SEM$ *$p < .05$.

CHAPTER V

DISCUSSION

Melatonin Mediated 2VO Induced Hyperactivity

Statistical analysis of performance within the open field task indicated higher levels of locomotor activity among 2VO rats as compared to sham and 2VO+M groups. An increase in locomotor activity is a common finding following ischemic events (Katsuta, Umemura, Ueyama, & Matsuoka, 2003; Liu et al., 2014; Sagvolden, Hendley, & Knardahl, 1992), and this hyperactivity was ameliorated by treatment with melatonin. Similarly, an increase in rears was observed within the 2VO group which was not mirrored by rats in the sham or 2VO+M groups. This increase in exploratory activity is also commonly found among rats with 2VO induced hippocampal damage (Cechetti et al., 2012; Katsuta et al., 2003; Kilic et al., 2008; Liu et al., 2014; Milot & Plamondon, 2008; Wang & Corbett, 1990).

Though the current study did not analyze neuropathological consequences of 2VO and melatonin administration, the mediation of hyperactivity within the 2VO+M group may be due to a reduction in hippocampal damage as suggested in previous research (Block, 1999; Douglas & Isaacson, 1964; Katsuta et al., 2003; Kilic et al., 2008; Liu et al., 2014; Traystman, 2003; Wang & Corbett, 1990). Wang and Corbett (1990) observed

an increase in locomotor activity in female rats exposed to 2VO with histological analysis revealing localized damage to the CA1 hippocampal region. As the hippocampus is an integral part of spatial learning and memory, Wang and Corbett (1990) believed the increase in locomotor activity to be attributed to an impairment in the rat's spatial mapping ability.

Katsuta and colleagues (2003) believe hypermotility to be a reliable predictor of CA1 damage as demonstrated in their study in which gerbils subjected to 2VO were found to exhibit hyperlocomotion in conjunction with severe loss of CA1 hippocampal neurons. Similarly, Liu et al. (2014) examined the protective effects of a traditional Japanese medicine, yokukansan, on gerbils undergoing 2VO and observed significant increases in locomotor activity within those gerbils not receiving treatment. Again, Milot and Plamondon (2008) tested a group of 2VO male Wistar rats (N = 59) on global exploratory behavior under varying lighting conditions. Rats with 2VO were found to have exhibited an increase in exploratory behavior including locomotion and rearing when compared to sham animals.

Regarding melatonin-specific treatment, a study by Kilic et al. (2008) utilized oral administration of melatonin (4mg • kg • day dissolved in water) as a treatment in adult male mice (N = 21) 24 hr after undergoing transient ischemia. Six days after the ischemic event, increases in locomotor activity were observed in the open field task in 2VO mice. However, mice treated with melatonin expressed no such increases in activity. Kilic and colleagues (2008) reported that melatonin and/or its metabolites were found to stimulate

neurogenesis and cell proliferation within the brain leading to the mediation of hyperactivity seen to accompany 2VO.

As demonstrated, research has correlated an increase in exploratory behavior to ischemia in rodents (Katsuta et al, 2003; Liu et al., 2014; Milot & Plamondon, 2008). Additionally, treatment models which have mediated hippocampal damage have been seen to mediate increases in exploratory behavior associated with 2VO within the open field task (Kilic et al., 2008; Liu et al., 2014). These findings align with the current study. This study observed sham rats and 2VO+M rats performing at comparable levels of activity while the 2VO group exhibited significant increases in both rears and locomotion. Thus, while this demonstrates melatonin's protective effect at the behavioral level, until neurological analysis has been completed, it is impossible to state that CA1 damage was ameliorated as a result of chronic melatonin pretreatment. However, due to hyperlocomotion being used as a strong predictor of CA1 damage in rodents (Katsuta et al., 2003; Milot & Plamondon, 2008) and previous research identifying the neuroprotective effects of melatonin and its metabolites (Kilic et al., 2008), it is probable that melatonin treatment reduced 2VO generated hippocampal damage. Brains from the animals used in the current study were stored for future analyses in order to evaluate this hypothesis.

Neither 2VO nor Melatonin were Found to Affect Spatial Recognition

The spatial recognition task, used to estimate spatial memory performance, relies heavily on a rat's natural tendency to explore novel object placement as well as the proper function of the hippocampus, medial temporal lobe and adjacent cortices

(Broadbent, Squire, & Clark, 2004; Hartman, Lee, Zipfel, & Wozniak, 2005; Liune, Wallace, & Frankfurt, 2011). While it appeared that the sham and 2VO+M groups possessed an overall higher preference score as compared to 2VO, no statistically significant differences were determined between groups. Although no definitive statements can be made about group differences, the trend in data may warrant additional future analysis.

Age is heavily connected with performance on the object placement task as demonstrated in studies by Frick, Baxter, Markowska, Olton, and Price (1995) and Paris, Walf, and Frye (2011). The study by Frick et al. (1995) implemented a spatial working memory task on varying age groups of male Fisher-344 rats (4, 11, 17, and 24 months old). When comparing the 11-month-old rats to the 4-month-old rats a disruption in spatial recognition was observed leading Frick et al. to believe that age is indeed correlated with performance in the object recognition task. Furthermore, as the age gap increased between groups of rats, impairments on the task increased.

Additionally, Paris et al. (2011) conducted a study in which 12-month-old female rats were tested on an object placement task. They concluded that as the female rat ages, a decline in performance on the object placement task follows. Paris et al. (2011) believe that female rats that preserve reproductive function longer are better able to perform on this task. However, even rats not experiencing reproductive decline showed deficits when compared to their younger counterparts.

Further studies have been conducted regarding age and spatial recognition performance. However, a vast majority of research in the area utilize stark age

differences rather than investigating the subtle differences accumulating throughout the lifespan of the rat (Liune et al., 2011; Paris et al., 2011). Liune et al. (2011) performed a study in which ten 21-month-old virgin female rats and eight 4-month-old virgin female rats were compared on object placement task performance. After a 1.5-hr delay, only the young rats were able to discriminate between the objects. They believe this finding to be an age dependent impairment correlated, in part, to the 16% degradation of dendritic spine density within the CA1 hippocampal area of the aged rats. Liune et al. (2011) state that dendritic spine density relates to a neuron's ability to properly receive information therefore muddling signals between neurons and hindering learning. While Liune et al. (2011) utilized rats far older than those in the current study, it lends further credit to the idea that aging does impact performance within the object placement task.

The findings within the aforementioned studies, suggest that an age effect may underlie the findings of the current study. As a rat ages, the ability to discriminate between objects in the object placement task is seen to deteriorate (Frick et al., 1995; Liune et al., 2011; Paris et al., 2011). Rats at 12 months of age area already seen to have declining spatial recognition memory. Therefore, it is possible that the current study was unable to locate a strong difference between groups due to a lowered threshold for performance. This lowered capacity for performance would make it more difficult to detect differences between groups as the ceiling for performance was lowered by aging (Frick et al., 1995; Liune et al., 2011; Paris et al., 2011).

Neither 2VO nor Melatonin Treatment Affected Visual Memory

The object recognition task is used as a measure of visual memory, which relies heavily on a rat's natural preference for novel objects (Broadbent et al., 2004; Dere, Huston, & Silva, 2007). To understand why this study did not locate a difference in behavior between any of the groups, the neuropathological progression of 2VO should be considered alongside methodological differences among research.

Progressive damage within the hippocampus has been found in rats who have undergone 2VO (Block, 1999). Interestingly, posterior parietal cortices, highly implicated in visual working memory, are not seen to sustain significant damage or volume reduction (Vicente et al., 2008). Nonetheless, 2VO has been shown to lead to a reduction of approximately 6-10% in hippocampal volume (DeButte, Fortin, & Pappas, 2002; Farkas, Institoris, Domoki, Mihaly, & Bari, 2006; Ritchie et al., 2004), which has been correlated with impairments in object recognition tasks (Broadbent et al., 2004; Cohen et al., 2013).

In contrast with the findings of the current study, many studies have observed deficits in visual memory following 2VO induced CCH (Cechetti et al., 2012; Shu et al., 2013; Zhao, Murakami, Tohda, Obi, & Shimada, 2007). As the length of time between injury and testing increases, CCH progressively impairs visual memory performance. Cechetti et al. (2012) tested 37 young male rats on an object recognition task 7 days, 3 months and 6 months after 2VO. No impairments were found at day 7, but when tested at 3 and 6 months, progressively worsening impairments in visual memory were identified. Zhao and colleagues (2007) performed an object recognition task on 170 male mice 2 weeks following the 2VO procedure and again identified a deficit in object recognition.

Shu et al. (2013) conducted a study in which 24 adult male rats were tested on object recognition 1, 4 and 8 weeks following 2VO during which time the deficits were seen to increase as the amount of time since surgery increased. These articles, in conjunction with others (Farkas et al, 2007; Sarti et al., 2002), indicate that behavioral deficits on object recognition tasks often appear 1-2 weeks following 2VO and persist up to 6 months (Cechetti et al., 2012; Shu et al., 2013; Zhao et al., 2007).

According to Ohtaki et al. (2006), this timeline correlates with damage to the hippocampus. Ohtaki and colleagues observed cell death 2 weeks following 2VO, but over time, rats were seen to compensate for reductions in CBF through increased vascular dilation. Nonetheless, because the neurological damage is largely irreversible, impairments within memory tasks are seen to persist even after CBF has returned to normal levels (Farkas et al, 2007; Ohtaki et al., 2006; Shu et al., 2013).

The current study tested performance on the object recognition task at 15 days following 2VO. The timeline may help to account for indiscernible differences between groups. While some studies have found behavioral deficits within as little as 1 week following surgery (Shu et al., 2013), other studies have reported significant differences in behavior 2 weeks following reduced CBF but more reliably following 4 to 8 weeks of hypoperfusion (Cechetti et al., 2012; Ohtaki et al., 2006; Shu et al., 2013; Zhao et al., 2007). Hence, it is plausible that rats in the current study may have shown greater impairment at a longer delay between 2VO and testing.

Sex differences may also play a role in explaining the results of the current study. Langdon et al. (2014) acknowledge a vast underrepresentation of female rodents in the

2VO literature. In an experiment by Langdon and colleagues (2014), forty 6-month-old ovariectomized female rats were used to test the hypothesis that physical exercise and maze learning was protective against 2VO. According to Langdon et al. (2014) this form of treatment exhibits a positive effect on recovery for male rats, however, when attempted with female rats, the same treatment was found to be ineffective. Langdon et al. (2014) believe that this finding lends credence to the idea that what is observed as a behavioral deficit of 2VO in male rats may not directly correspond to a behavioral deficit of 2VO in female rats. Langdon et al. (2014) revealed no effect of 2VO on visual memory in female rats. This was explained in part by the reliance of visual memory more heavily on areas not largely affected by 2VO such as hippocampal CA3 region and the dentate gyrus.

However, in a study by Plamondon, Morin and Charron (2006), visual memory was affected by transient global ischemia in female rats. This study utilized 32 ovariectomized female Sprague Dawley rats and tested them on the object placement task. Although the Plamondon study did reveal impairments in visual memory, the situational factors were quite different from those of the current study. Transient global ischemia typically yields more intensive behavioral deficits in comparison to 2VO (Block, 1999). This in conjunction with ovariectomization would have placed these rats in a vulnerable state as ovariectomy alone has been shown to lead to impaired performance on the object recognition task (Wallace, Liune, Arellanos, & Frankfurt, 2016). In contrast with Plamondon et al. (2006), the current study utilized female rats

with intact reproductive hormone production and a mild CCH. These differences may help to explain why such a stark difference in results was displayed.

In summary, sex differences, degree of injury, and amount of time between injury and behavioral testing may have contributed to the lack of observable performance detriment on the object recognition task. Currently, the mechanisms of impairments of CCH as related to structural damage is not understood. Furthermore, sex differences have yet to be identified as demonstrated by Plamondon et al. (2006). Due to the lack of identified sex differences in the effects of 2VO on visual memory performance as well as the short latency period between surgery and testing, the current study in itself cannot rule out melatonin as a potential neuroprotectant against CCH. However, the current study may conclude that an observable difference in visual memory performance among experimental and control groups was not observed in middle-aged female rats.

2VO and Melatonin not Found to Affect Performance on the MWM

The MWM is a test of spatial reference memory reliant on the rat's perception and utilization of extramaze cues (Hok, Poucet, Duvelle, Save, & Sargolini, 2016). In the current study, mean escape latencies decreased across testing days for all groups indicating that each group was able to learn the task. No group differences were found. Thus, neither 2VO nor melatonin treatment was found to affect the rats' spatial reference memory performance.

Because 2VO is seen to significantly impair hippocampal function associated with the MWM, it was predicted that impairment would not be seen within the control group. However, age and spatial memory performance are negatively correlated. In a study

conducted by Bizon et al. (2010), male rats aged 6, 12 and 22 months were compared to each other on the MWM in order to assess spatial reference memory. Analysis revealed that the middle-aged and aged rats performed significantly worse than their younger counterparts. Similarly, Frick et al. (1995) compared performance on the MWM between male Fisher-344 rats of 4, 11, 17, and 24 months old through which they observed spatial reference memory deficits in rats as young as 11 months.

The animals selected for the current study were female retired breeders aged 9-11 months. As spatial reference memory is seen to decline with age, it is to be expected that performance on the MWM would ebb as well. Spatial reference memory requires a sophisticated processing relying on both intramaze and extramaze cues over the span of 5 days (Frick et al., 1995). Research has observed that while a female rat ages, endogenous protective hormone levels and cognitive function diminish in tandem (Paris et al., 2011). This is to say that the ability of the subjects in the current study to perform maximally on this task may have already been compromised thus lessening the observed effect of 2VO and treatment.

Melatonin Increases Spontaneous Alternation

Y-maze tasks are often used to observe SAP in rats (Lennartz, 2008; Sarter, Bodewitz, & Stephens, 1988). Because SAP is a rat's natural exploratory tendency, the y-maze is used to determine the ratio of normal to abnormal exploratory behavior among rats through comparing the number of SAP behaviors in relation to AAR and SAR behaviors (Bak, Pyeon, Seok, & Choi, 2017; Hughes, 2004). The current study did observe an increase in SAP behavior within the 2VO+M group as compared to the 2VO

group. Thus, indicating an increase in natural exploratory behavior in 2VO rats treated with melatonin as compared to rats with 2VO (Xu et al., 2012).

Additionally, according to an extensive review by Lalonde (2002), there is an effect of age on spontaneous alternation. One such study examining this possibility was conducted by Lamberty and Gower (1992). This study utilized groups of virgin female mice aged 3, 11, 17, and 22 months and examined SAP behavior using the Y-maze task. When comparing performance, 3-month-old mice were found to spontaneously alternate significantly more than their 11-, 17- and 22-month-old counterparts. Furthermore, Xu et al. (2012) observed a decline in SAP behavior in 22-month-old rats when compared to 3-month-old rats under normal conditions.

According to Okatani, Morioka, and Hayashi (1999), as a rat ages, melatonin levels are seen to decline, and this decline is seen to correspond to behavioral deficits. Although baseline behavior melatonin levels were not determined within this study, it may be that an increase in melatonin levels allowed for an increase in normal exploratory behavior within the 2VO+M group. However, looking back at melatonin levels prior to administration would have allowed for a stronger claim towards this possibility. To date, published studies have not yet examined the effect of melatonin on Y-maze exploratory behavior.

The current study did not indicate a difference between sham and 2VO groups. This finding is consistent with research performed by Ni, Ohta, Matsumoto and Watanabe (1994) who found little to no deficits in 7-week to 9-month-old male rats when tested on the Y-maze task 1 month after 2VO. Similarly, Pappas et al. (1996) do not

report a deficiency in SAP behavior among 10-month-old male Sprague Dawley rats who have undergone 2VO.

Conclusion

With MCI and CCH being closely related, research within the area is crucial to furthering the understanding MCI's progression and treatment (Palmer et al., 2003). MCI is quickly becoming one of the most common clinical diagnoses in the elderly population (Mielke et al., 2014). More specifically, older females are diagnosed with MCI more often than males (Lin et al., 2015), yet literature searches yield animal studies focusing almost exclusively on male rats (Cechetti et al., 2012; de Bortoli et al., 2005; Letechipıa-Vallejo et al., 2007). This leaves a large hole in the understanding of MCI and its implications within the female population and leaves a sizeable opening for further research. For this reason, the current study utilized middle-aged female rats in order to broaden the current understanding of the interplay of vascularly induced MCI and sex. Yet, while pathological and neurological aspects of MCI are being investigated, treatments are scarce and largely ineffective (Mufson et al., 2012).

The current study was the first to utilize a chronic subcutaneous melatonin pretreatment for the 2VO model and was successful in mitigating some of the behavioral consequences of CCH. This study demonstrated the ability of chronic low-dose melatonin to decrease hyperactivity and increase normative exploratory behavior in rats when administered as a pretreatment for 2VO. This suggests that melatonin may possess neuroprotective qualities leading to the prevention or reduction of behavioral differences that follow vascular MCI (Block, 1999; Katsuta et al., 2003; Kilic et al., 2008).

This method of treatment addressed two main areas of concern within the literature. Firstly, according to Xu et al. (2011) a massive limitation regarding MCI is likely due to the focus of treatment being restorative rather than preventative. Due to the delicate nature of the CNS, restorative treatments are limited in their ability and face more difficult obstacles as compared to attempts to prevent neuronal loss and degradation (Xu et al., 2011). Thus, Xu et al. (2011) calls for the development of treatments which work to prevent neurological and cognitive damage, therefore minimizing the need for procedures with heightened risk and lowered effectivity. In order to address this, the current study administered melatonin 2 weeks prior to 2VO administration. This allowed for elevated melatonin levels prior to the onset of reduced CBF and permitted melatonin to undertake a preventative role.

Additionally, much of the available literature administers treatment through injection or oral administration (Bassani et al., 2014; Kilic, Kilik, Reiter, Bassetti, & Hermann, 2004; Lee et al., 2016; Pei, Ho, & Cheung, 2002). Melatonin posesses a relatively short half-life of 59-65 min causing these methods of administration to be acute in nature, leading to fluctuations in drug blood level (Gooneratne et al., 2012). To address this, the time-release subcutaneous pellet was utilized in the current study. This administration is constant and reduces fluctuation in blood drug levels thereby ensuring that each rat receiving treatment was constantly permeated with a steady flow of melatonin (Andrade, 2015). Thus, melatonin was able to reach its peak level and persist throughout the duration of the study (Gooneratne et al., 2012).

This study also demonstrated the ability of chronic low-dose melatonin to decrease hyperactivity and increase normal exploratory behavior in rats when administered as a pretreatment for 2VO. While considering that low-dose melatonin revealed an effect, a larger dose of melatonin may serve as a stronger neuroprotective supplement to mitigate damage inflicted by CCH (Kilic, Kilik, Reiter, Bassetti, & Hermann, 2004; Lee et al., 2016; Pei et al., 2002). Therefore, future studies may consider utilizing a larger dose while employing subcutaneous pellet administration.

Upon examining the graphically represented data in the results section, a consistent pattern emerges in which the 2VO group performs differently from the sham and 2VO+M groups. This pattern yielded significance only within the open field task and the Y-maze. However, this pattern was also visible within the spatial recognition task, object recognition task and the MWM. In this study, attrition accounted for 10% of the sham and 2VO+M groups. If initial sample size was maintained, the power of the present study to determine a difference between groups on the additional tasks may have been sensitive enough to locate differences between groups on the other tasks selected. Consequently, subsequent studies may consider accommodating for potentially high attrition rates and aptly increase their sample sizes.

It is worth mentioning that this study took a cross-sectional approach to understanding the acute behavioral effect of melatonin on 2VO as opposed to the chronic effect. This type of analysis examines aggregate change and does not focus on change at an individual level. Because select tasks within the battery implemented are sensitive to habituation and practice effects, baseline measures prior to injury and testing were not

acquired (Anderson, Moenk, Barbaro, Clarke, & Matuszewich, 2013; Walsh & Cummins, 1972). In lieu of baseline measures, a control group which underwent sham surgeries was used. However, future studies are encouraged to gather and provide baseline measures to assess longitudinal change where possible.

Animal models such as the one implemented in the current study are important for gaining initial knowledge in the utilization of various treatments, yet the ultimate end to this study is to instigate further research which can then generalize to the human condition. While the exact mechanisms of protection may not yet be fully understood, the relationship between melatonin pretreatment and reduced behavioral impairment is apparent and warrants further investigation in both animal and human trials. To better understand and treat this growing disorder, further research must be directed towards areas of need such as preventative treatments and vulnerable populations.

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APPENDIX

Table 1

Descriptive Statistics Summary

Task/Measures	Group	Mean	SD	SEM
Open Field Task				
Grid Crosses	Sham (*N* = 9)	59.22	21.88	7.29
	2VO (*N* = 10)	82.70	19.40	6.13
	2VO+M (*N* = 9)	59.22	17.91	5.97
Number of Rears	Sham (*N* = 9)	20.00	9.11	3.04
	2VO (*N* = 10)	35.90	10.42	3.30
	2VO+M (*N* = 9)	24.22	6.96	2.32
Time in Center	Sham (*N* = 9)	7.74	7.55	2.52
	2VO (*N* = 10)	10.97	8.14	2.57
	2VO+M (*N* = 9)	11.16	14.92	4.97
Spatial Recognition	Sham (*N* = 8)	62.71	16.74	5.92
	2VO (*N* = 10)	47.73	20.04	6.68
	2VO+M (*N* = 8)	58.57	14.20	5.02
Object Recognition	Sham (*N* = 9)	53.51	13.72	4.57
	2VO (*N* = 10)	56.27	7.00	2.21
	2VO+M (*N* = 9)	62.95	19.20	6.40
Morris Water Maze				
Day 1	Sham (*N* = 9)	75.16	12.43	4.14
	2VO (*N* = 10)	72.31	19.57	6.19
	2VO+M (*N* = 9)	58.70	19.81	6.60
Day 2	Sham (*N* = 9)	58.09	25.86	8.62
	2VO (*N* = 10)	71.13	16.55	5.23
	2VO+M (*N* = 9)	49.01	23.56	7.85
Day 3	Sham (*N* = 9)	42.59	27.71	9.24
	2VO (*N* = 10)	52.13	17.32	5.48
	2VO+M (*N* = 9)	33.33	17.96	5.99

Table 1 (continued)

Task/Measures	Group	Mean	SD	SEM
Day 4	Sham (*N* = 9)	31.81	26.33	8.78
	2VO (*N* = 10)	47.39	22.01	6.96
	2VO+M (*N* = 9)	32.72	23.02	7.67
Y-Maze				
Spontaneous Alternation	Sham (*N* = 9)	67.00	8.70	2.90
	2VO (*N* = 10)	59.80	12.34	3.90
	2VO+M (*N* = 9)	73.89	8.28	2.76
Alternate Arm Return	Sham (*N* = 9)	28.11	11.75	3.92
	2VO (*N* = 10)	36.30	8.93	2.82
	2VO+M (*N* = 9)	24.89	8.12	2.71
Same Arm Return	Sham (*N* = 9)	4.78	7.28	2.43
	2VO (*N* = 10)	3.80	5.09	1.61
	2VO+M (*N* = 9)	1.22	3.67	1.22

Note. Descriptive statistics for each task conducted