CHARACTERIZING THE MELATONERGIC SYSTEM AFTER BRAIN INJURY

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Submitted to the Graduate Faculty of

School of Nursing in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy (PhD) in Nursing

University of Pittsburgh

2016

UNIVERSITY OF PITTSBURGH

SCHOOL OF NURSING

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July 12, 2016

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Traumatic brain injury (TBI) affects individuals of all ages, races, and geographies and is often associated with expensive clinical management. Melatonin (MEL) has been trialed as a TBI therapeutic with mixed results, due in part to a lack of understanding of the mechanism. This study characterizes endogenous changes in melatonergic receptors (MT1 and MT2) after experimental TBI induced using pneumatic CCI (2.8 mm depth) in young adult Sprague Dawley rats. Half the rats were exposed to sham surgery to control for the effects of anesthesia and craniectomy. In total, 25 rats were enrolled in the study, with 6-7 rats per group. Test animals were sacrificed at 2 post-surgery time points, either 6 hours (hr) or 24 hr post-surgery. Following sacrifice, the test animal's brain was harvested, dissected, and flash frozen until analysis. Whole cell lysates were prepared, aliquoted, and used for western blot analysis, probing for cytochrome C (to validate injury severity), MT1, MT2, and beta actin (to control for protein loading). ImageJ and Image Lab software were used to quantify protein data; SPSS software was used to run t-tests to compare group means on a single variable and correlation testing was used to explore the relationship between outcomes of interest. Melatonin receptors were down-regulated in a brain region- and time point- dependent manner. MT1 was downregulated in the frontal cortex at 24 hr and in the hippocampus at both 6 hr and 24 hr post-TBI. Similarly, MT2 was downregulated in the frontal cortex at 24 hr and in the hippocampus at both 6 and 24 hr post-TBI. This is the first study to report downregulation of MT1 and MT2 after neurotrauma; receptor downregulation may affect the efficacy of MEL therapy. Additional research to characterize these changes after TBI are necessary

including efforts to establish the time course and regional patterns, replication in more diverse samples, as well as inclusion of additional cellular, histological, and behavioral endpoints. TBI in rats modeled using CCI results in acute downregulation of MEL-specific receptors (subtypes MT1 and MT2); replication of these findings is necessary as are evaluations of the implications of lower receptor levels.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

My life has been dedicated to the quest for knowledge and I have found great joy in pursuing my education. While this ETD is by far my most ambitious achievement to-date, I know it represents the start of what I anticipate to be a long and productive scientific career. While there are many individuals from whom I have drawn strength and received support (including those I thank below and countless others), I want to first acknowledge my own incredible effort, dedication to my education, and perseverance. I have not always valued or even acknowledged the personality characteristics I possess that have led to-date to my successes. Throughout the pursuit of my doctoral degree I have come to appreciate and be proud of my work ethic, fastidiousness, the tremendous amount of time and effort I have expended, and growth I have attained. I also know that this work could not have been completed without countless others.

First and foremost, my pursuit of knowledge has been inspired and facilitated by the brave and unrelenting feminists of past and present generations who fought, and continue to fight, tirelessly for equal rights and opportunities for women. Not only have they paved the way for my own formal education, they have proven to me that women and girls are a source of seemingly limitless untapped potential. They have inspired me to contribute my voice to conversations in the scientific community and beyond, and to have the resolve and tenacity needed to pursue my dreams in spite of the challenges and doubts I have faced. While the number of noteworthy feminists is

long, I want to acknowledge a few: Victoria Woodhull, Elizabeth Cady Stanton, Alice Paul, Rosalind Franklin, Frederick Douglas, Mae Jameson, Marie Curie, and Diane Fossey.

I also want to acknowledge my family, some of whom I have succeeded because of and others in spite of—both groups have been motivators in their own right. I want to especially recognize 5 particularly supportive individuals: 1) my grandmother, who always believed in me and stimulated a love of learning by an early age; 2) my father, who taught me to always see the humor in life and took me to so many educational places as a young child that I bored the interviewer who was conducting cognitive testing on me with my excessive knowledge of the Wright Brothers; 3) my aunt who taught me that some women thrive without children, self-expression and creativity make life worth living, and that sometimes you have to take risks to follow your dreams; 4) my mother-in-law, who has edited nearly every single paper, poster, and slideshow I have generated during my doctoral studies and pushed me to be produce the highest quality work I was capable of; and 5) especially my husband and who has shown me unwavering support and a seemingly limitless amount of love in the near-decade we've been together. He has made many accommodations so that I could pursue my career dreams and for that I am forever grateful. With a smile on his face he has done nearly all of the cooking and cleaning, helped me generate novel figures to communicate my work, been my videographer, the list goes on and on—I could never fully express my gratitude.

Next, I want to acknowledge several notable individuals at the University of Pittsburgh without whom this work would not have been possible. First, my primary academic advisor & dissertation chair, Sheila Alexander, who has made tremendous efforts to support my professional development and helped me to navigate the ups and downs while being true to myself in the process. Second, the sponsor on my F31 and principal investigator in my lab: C. Edward Dixon,

who has given me a training experience beyond what most people dream of and kept me calm and upbeat during trials and tribulations. Third, Yvette Conley, the director of the T32 that brought me to Pittsburgh, who further inspired my love of genomics and taught me how invaluable it is to just take time to think. Fourth, Dianxu Ren, who has been an invaluable source of statistical support and also helped me realize my self-agency in analysis. Fifth, Ava Puccio, who has facilitated a rich training experience and become a role model for me, as a nurse scientists working in a transdisciplinary environment. Sixth, Shaun Carlson, the post-doctoral fellow in my lab, who trained me with incredible patience, helped me trouble-shoot, and provided a number of restaurant recommendations. Seventh, my peers in the School of Nursing who helped me navigate the program and make memories along the way (especially: Meghan Mattos, Taya Irizarry, Bethany Nugent, and Kelley Martin-Baumgartel). Last, but not least I want to acknowledge select graduate (Lacey Wright) and undergraduate mentees (Amanda Savarese, Lan Pham, Bunny Pugh, Sarah Kettell); these individuals supported all aspects of my work, helped me to hone my teaching and mentorship skills, and were an absolute pleasure to work with. It truly took a village to actualize this dissertation and I am so fortunate to have had the support of so many amazing people.

Finally, I want to acknowledge the funding sources which have supported my doctoral education and the research project. Specifically, I want to thank my mentors for contributing funding, resources, and personnel. I also want to thank the School of Nursing for the tuition and travel support I have received. Most importantly, I want to thank the following funding sources:

1) The National Institutes of Health (F31NR014957 & T32NR009759)
2) The Copeland Award of the Pittsburgh Foundation (awarded 2014 and 2015)
3) Sigma Theta Tau International-- Eta Chapter
4) American Association of Neuroscience Nursing & Neuroscience Nursing Foundation

5) International Society for Nurses in Genetics

6) Margaret Wilkes Award

1.0 INTRODUCTION TO THE STUDY AS ORIGINALLY PLANNED

Traumatic brain injury (TBI), is a significant public health problem affecting millions of Americans (Faul, Xu, Wald, & Coronado, 2010). Approximately 2.5 million of Americans were reported to have sustained a TBI in 2010 alone (Centers for Disease Control and Prevention, 2015). TBI results in distressing and life-altering symptoms, including functional deficits such as memory, learning, and motor problems (Anderson, Parmenter, & Mok, 2002; Corrigan, Selassie, & Orman, 2010; Ergh, Rapport, Coleman, & Hanks, 2002; McKinlay, Brooks, Bond, Martinage, & Marshall, 1981; Schalen, Hansson, Nordstrom, & Nordstrom, 1994). At the cellular level**,** apoptosis begins rapidly after TBI (Barth, Schilling, & Schmiedek, 2000; Bayir et al., 2007; Conti, Raghupathi, Trojanowski, & McIntosh, 1998) and culminates in loss of brain cells which has been associated with functional neurological symptoms (Miñambres et al., 2008; Piao et al., 2012; Yakovlev et al., 1997). Mortality following a severe TBI has decreased over the years; however, outcomes among TBI survivors are highly variable, ranging from full recovery to chronic disability necessitating long-term care provided by family caregivers or healthcare professionals. Differences in TBI functional outcomes have been linked to the heterogeneity of TBI, with initial brain insult morphology being highly variable with subsequent symptomatology; long-term outcomes are also associated with underlying genetic (Garringer et al., 2013; Jordan, 2007), hormonal (O'Connor, Cernak, Johnson, & Vink, 2007; Olivecrona, Dahlqvist, & Koskinen, 2013) and environmental factors such as diet (McIntosh, Faden, Yamakami, & Vink, 1988; Wu, Ying,

& Gomez-Pinilla, 2004) and supplement use (Burd, Breen, Friedman, Chai, & Elovitz, 2010; Conte et al., 2004; Ozdemir et al., 2005). Available evidence suggests that personalized medicine and tailored therapeutic regimens are worth pursuing to promote the best individualized outcomes for TBI patients.

Clinical trials in TBI have failed through the years, most likely due to the heterogeneity of the injury and subsequent rehabilitation efforts. Few new treatments have proven effective at improving clinically relevant outcomes of TBI over the current, evidence based, standard of care regimens. Current clinical management strategies for TBI primarily focus on supporting hemodynamic stability, maximizing oxygenation and cerebral perfusion and preventing secondary injury. In preclinical studies of TBI and other central nervous system (CNS) disorders (e.g. stroke; spinal cord injury), melatonin (MEL) has been found to have promising neuroprotective properties including preservation of healthy tissue and improvement in symptom profiles. Conventionally, the beneficial effects of MEL within the CNS are attributed to its anti-oxidant capacity (Dikmenoglu, Ileri, Seringec, & Ercil, 2008; Hashimoto et al., 2012; Onur, Semerciöz, Orhan, & Yekeler, 2004; Tamura et al., 2013; Tan et al., 2002; Taysi et al., 2008). The anti-apoptotic effects of MEL have been documented within the CNS (Alonso-Alconada, Alvarez, Lacalle, & Hilario, 2012; Bavithra, Selvakumar, Krishnamoorthy, Venkataraman, & Arunakaran, 2013; Bruce-Keller et al., 2007; Kireev, Vara, & Tresguerres, 2013; Ma et al., 2013; Olcese et al., 2009; Ozyener et al., 2012; Reiter, Tan, Leon, Kilic, & Kilic, 2005; Samantaray et al., 2008, 2009; Suwanjang, Abramov, Govitrapong, & Chetsawang, 2013; Wang et al., 2011; Zhang et al., 2013) with only limited evidence available from pre-clinical TBI studies (Campolo et al., 2013; Jadhav et al., 2009; Kelso, Scheff, Scheff, & Pauly, 2011; Mésenge et al., 1998; Ozdemir et al., 2005). The lack of a

well-tested conceptual framework surrounding the neuroprotective actions of MEL affects the ability of researchers to ask the proper questions when evaluating MEL as a post-TBI therapy.

In mammals, there are two MEL-specific receptors, melatonin receptor subtype 1A (MT1) and melatonin receptor subtype 1B (MT2); this study focuses on MT1 which has been implicated in outcomes of CNS disorders. MT1 is a MEL-specific receptor found throughout the mammalian brain (Mazzucchelli et al., 1996; Naji, Carrillo-Vico, Guerrero, & Calvo, 2004). High MT1 receptor densities have been reported in the suprachiasmatic nucleus (C. Liu et al., 1997). MT1 has also been detected in the cerebral cortex, hippocampus, thalamus, cerebellum, and retina (Dubocovich, Rivera-Bermudez, Gerdin, & Masana, 2003) using reverse-transcriptase polymerase chain reaction (RT-PCR). Outside of the brain, MT1 and MT2 have been detected in the cardiovascular system (Masana et al., 2002), reproductive system (Clemens, Jarzynka, & Witt-Enderby, 2001; Frungieri et al., 2005), gastrointestinal system (Naji et al., 2004), and immune system (Dubocovich & Markowska, 2005; Guerrero & Reiter, 2002; Skwarlo-Sonta, Majewski, Markowska, Oblap, & Olszanska, 2003). It is also worth noting that MEL-specific receptors (both MT1 and MT2) localize to both cellular membranes and the nucleus adding to their widespread effects (Naji et al., 2004). Moreover, recent evidence from a mouse model of Huntington's disease suggests that the anti-apoptotic effects of MEL are MT1 receptor-dependent (Wang et al., 2011). The role of MT1 remains unclear in the context of TBI and may be relevant to personalized medicine since functional polymorphisms in MT1 have been identified in humans (Barrett et al., 1997; Natarajan et al., 2012), including a missense mutation in rs1800885 (Choudhury et al., 2014). MT2 is also characterized by genetic variation, but is less well-characterized and well-studied than MT1.

The extremely low toxicity of MEL (Jahnke et al., 1999; Seabra, Bignotto, Pinto, & Tufik, 2000; Wiechmann, Chignell, & Roberts, 2008), high numbers of MEL-specific receptors (both MT1 and MT2) in the CNS (Laudon, Nir, & Zisapel, 1988; Mazzucchelli et al., 1996; Naji et al., 2004; Weaver, Rivkees, & Reppert, 1989), and ability of MEL to cross the blood-brain-barrier (Di Bella & Gualano, 2006; Le Bars et al., 1991; Reiter et al., 2007), suggest it could be administered via multiple routes and exert neuroprotective effects within the brain. Despite these desirable properties and substantial evidence that MEL moderates cell death within the CNS (Alonso-Alconada et al., 2012; Bavithra et al., 2013; Kireev et al., 2013; Ma et al., 2013; Olcese et al., 2009; Ozyener et al., 2012; Reiter et al., 2005; Samantaray et al., 2008, 2009; Suwanjang et al., 2013; Wang et al., 2011; Wang, 2009; Zhang et al., 2013), including after TBI (Campolo et al., 2013; Jadhav et al., 2009; Kelso et al., 2011; Mésenge et al., 1998; Ozdemir et al., 2005) it is not routinely used in clinical practice. Enhanced understanding of the mechanism of action is necessary before MEL therapy could be safely and effectively applied to TBI clinical care. Known genetic variation in MEL-specific receptors (MT1 and MT2) may modify the functioning of the receptors, reduce the ability of MEL to bind, and/or otherwise affect MEL signal transduction; addressing this gap could lead to the identification of patients likely to benefit from MEL therapy. This innovative study is the first to evaluate if the anti-apoptotic effect of MEL following TBI is MT1 receptor-dependent. The study uses a 2x2x2 (8 group) design, that controls for injury exposure (TBI vs. sham), therapy (MEL vs. vehicle control) and MT1 presence [MT1 knockout (KO) vs. wild-type (WT)]. *The overall study hypothesis is that the beneficial effects of MEL therapy are, at least partially, dependent on the MT1 receptor.* This early study will build the evidence base that may lead to the translation of MEL as an effective therapeutic agent following

TBI. This research has the potential to contribute to the development of personalized medicine approaches and the design of tailored treatment plans for TBI patients.

1.1 PURPOSE AND SPECIFIC AIMS

The aims of this pre-clinical study as originally planned and presented at the time of the candidate's Comprehensive Examination & Overview were to:

1) Determine if the effects of post-TBI MEL therapy (vs. vehicle control) on levels of apoptotic proteins (caspase-3 & cleaved caspase-3) are MT1 receptor-dependent.
2) Determine if the effects of post-TBI MEL therapy (vs. vehicle) on functional outcomes are MT1 receptor-dependent, by comparing results of behavioral tests in MT1 KO and WT mice, specifically, assessments of learning/memory via the Morris Water Maze (MWM), novel object recognition (NOR), and motor function via Beam Balance Test (BBT).

The doctoral candidate's long-term research interest surround further categorization of genetic and environmental factors that explain variability in outcomes of TBI by conducting additional preclinical studies and clinical trials. This research trajectory has the potential to improve lives of TBI survivors, a group for whom there are currently limited treatment options. Results of the proposed dissertation may contribute to the mechanistic understanding of the role of the melatonergic system in TBI recovery.

1.2 BACKGROUND AND SIGNIFICANCE

Each year in the United States there are approximately 275,000 inpatient hospitalizations and 52,000 deaths due to TBI; although just over 3% of TBIs are fatal, these cases account for nearly a third (30.5%) of all injury-related deaths (Faul et al., 2010). Healthcare costs for TBI survivors are high, totaling $76.5 billion in 2010 alone (Coronado, McGuire, Faul, Sugarman, & Pearson, 2012). The impact of TBI extends beyond the acute injury period and often persists for years or decades after injury. A recent estimate found 3.2 million Americans living with one or more TBI-related disabilities (Corrigan et al., 2010). Of the functional impairments known to occur following TBI, memory-, learning-, and motor- deficits are commonly reported and particularly distressing to TBI patients and their families (Anderson et al., 2002; Ergh et al., 2002; McKinlay et al., 1981; Schalen et al., 1994), resulting in life-changing reductions in independence and quality-of-life (Anderson et al., 2002; Corrigan et al., 2010; Ergh et al., 2002; McKinlay et al., 1981; Schalen et al., 1994; Tate & Broe, 1999). Unfortunately, current treatment options are not particularly effective and no new therapies have been successfully translated to TBI care; thus further research is needed (Cernak, 2006; Ghajar, 2009). Apoptosis, genetically programmed cellular death, is a promising target for drug therapy because it begins rapidly after TBI (Barth et al., 2000; Bayir et al., 2007; Conti et al., 1998), results in decreased numbers of viable brain cells, and correlates with lesion size and functional status (Jain, 2008; Reilly, 2001; Werner & Engelhard, 2007).

MEL is an endogenously produced substance, generated naturally in response to darkness and implicated in sleep; through its actions and regulation of other hormones, MEL plays a critical role in maintaining the circadian rhythm (Klein et al., 1997; Pandi-Perumal et al., 2006; Reiter,

1993). Notably, sleep disruption is common after TBI (Duclos, Beauregard, Bottari, Ouellet, & Gosselin, 2015; Lucke-Wold et al., 2015; Singh, Morse, Tkachenko, & Kothare, 2016; Theadom et al., 2015); reported sleep-related symptoms may be due to alterations in MEL signaling in response to injury (Grima, Ponsford, St Hilaire, Mansfield, & Rajaratnam, 2016; Paparrigopoulos et al., 2006; Seifman et al., 2008; Shekleton et al., 2010), though this relationship remains to be further clarified. MEL biosynthesis involves acetylation of serotonin and further methylation to form the final hormone (Bernard et al., 1999; Klein et al., 1997). The presence of MEL has been reported in the brain (Reiter, Richardson, Johnson, Ferguson, & Dinh, 1980; Rollag, Panke, & Reiter, 1980), retina (Zawilska & Nowak, 1991), gastrointestinal tract (Bubenik, 2002), lymphocytes (Antonio Carrillo-Vico et al., 2004), and testes (Tijmes, Pedraza, & Valladares, 1996). MEL is available exogenously both as a medication and over-the-counter supplement with a known low toxicity profile (Jahnke et al., 1999; Seabra et al., 2000; Wiechmann et al., 2008). MEL's ability to freely cross morpho-physiological barriers including the blood-brain-barrier suggests it could be delivered via multiple routes and exert protective effects in the brain (Di Bella & Gualano, 2006; Le Bars et al., 1991; Reiter et al., 2007). Anti-apoptotic effects of MEL therapy have been demonstrated in various contexts (Celik & Nazıroğlu, 2012; Mukherjee et al., 2012; Patschan et al., 2012; Sokolovic et al., 2013), including the brain (Alonso-Alconada et al., 2012; Bavithra et al., 2013; Kireev et al., 2013; Ma et al., 2013; Ozyener et al., 2012; Samantaray et al., 2008; Suwanjang et al., 2013). Pre-clinical evidence from models of TBI demonstrate neuroprotective/anti-apoptotic effects of MEL (Campolo et al., 2013; Jadhav et al., 2009; Kelso et al., 2011; Mésenge et al., 1998; Ozdemir et al., 2005).

Moreover, analysis of TBI and non-TBI patients found that naturally occurring MEL levels are altered in the cerebrospinal fluid (CSF), blood, and saliva during the acute post-TBI period

(Paparrigopoulos et al., 2006; Seifman et al., 2008; Shekleton et al., 2010). One study (Seifman et al., 2008) collected CSF and serum out to 13 days post-TBI and compared MEL levels to that in controls who had a CSF sample collected during surgery for a condition distinct from TBI. In CSF, MEL showed a biphasic pattern: increasing until day 2 post-TBI, decreasing to a minimum on day 5, and increasing to a maximum on day 8; serum MEL levels also increased from admission to day 2 post-TBI and then reached a minimum level on day 5 (Seifman et al., 2008). A second study (Paparrigopoulos et al., 2006) examined MEL levels in the blood 8 times daily for 2 days after TBI but lacked a control group for comparison; serum MEL levels were lower than established clinical means and patients with lower Glasgow Coma Scale (GCS) score showed disrupted diurnal variation in MEL levels compared to those with a higher GCS (Paparrigopoulos et al., 2006). A third study (Shekleton et al., 2010) examined long-term effects of TBI on MEL levels, examining salivary levels in TBI survivors 6 months after the initial insult and comparing levels to non-injured controls. In this study, there was no significant difference in dim light MEL onset between TBI patients and controls; however, controls had significantly higher MEL production ($p=0.031$) than their TBI counterparts (Shekleton et al., 2010). Overall, the evidence suggests MEL levels increase and then decrease in the acute period and may remain depressed chronically; it is plausible that MEL release is a response to TBI but may be inadequate for post-TBI neuroprotection (Paparrigopoulos et al., 2006; Seifman et al., 2008; Shekleton et al., 2010), a possible target for exogenous MEL.

In addition to anti-apoptotic properties of MEL, there are antioxidant and free radical scavenging properties of MEL. Unfortunately, this research has not led to successful translation of MEL to the bedside (Dikmenoglu et al., 2008; Hashimoto et al., 2012; Onur et al., 2004; Tamura et al., 2013; Tan et al., 2002; Taysi et al., 2008). Three MEL receptor subtypes have been

identified, two of which (MT1 and MT2) are detectable in the brains of mammals, (Mazzucchelli et al., 1996; Naji et al., 2004) suggesting a role for MEL and its receptor within the brain and a target for MEL therapy (Hardeland, 2010; Pandi-Perumal et al., 2008; Reiter et al., 2005; Reiter & Tan, 2002; Skinner & Malpaux, 1999). Evidence from animal models of neurodegenerative disorders show that MEL's anti-apoptotic effects depend on the MT1 receptor; this evidence validates the focus on MT1 in this study (Wang et al., 2011; Zhang et al., 2013).

1.2.1 Rationale for studying traumatic brain injury

Traumatic brain injury (TBI), a type of acquired injury affecting the brain, represents a significant public health problem that affects 2.5 million Americans each year (Centers for Disease Control and Prevention, 2015), with a total of $76.5 billion spent on TBI healthcare in 2010 (Coronado et al., 2012). TBI is recognized as a worldwide problem that has been afflicting humans for thousands of years, as supported by a wealth of written and fossil evidence. Contributing to the high incidence of TBI are the multiple mechanisms by which injury can occur, including assaults, falls, motor vehicle accidents, gunshots, and non-penetrating blast injuries. Thus, individuals across the lifespan and in various environment are at risk. Although there are some occupations or activities that are known risk factors for brain injury and may warrant use of protective equipment (e.g. helmet), TBI is often unexpected and unavoidable. This dissertation project and the candidate's emerging program of research are focused on identifying factors that moderate TBI recovery, in hopes to ameliorate the individual and public health burden of this common, devastating, and costly acquired injury.

1.2.2 Rationale for using a pre-clinical (animal) model of brain injury

The use of animal models plays a major role in health science research and has added approximately 15 years to the human life expectancy since 1940 (Oregon Health & Science University, n.d.). It is also worth noting that the role of pre-clinical research within nursing research is being increasingly appreciated (Holtzclaw & Hanneman, 2002; Rodgers, Anderko, Witek-Janusek, & Page, 2004; Rowsey, 2015). Ethical and legal considerations prohibit the use of randomized experimental designs using human test subjects, and necessitate ongoing pre-clinical research. Indeed, of the 45 phase II and III clinical trials of TBI being conducted, many have failed and none have resulted in new FDA-approved therapies (Gold et al., 2013). Fortunately, over the last century, several models have been proposed and refined (Denny-Brown & Russell, 1941; Kramer, 1896; Lindgren & Rinder, 1965). Rodent models of brain injury have been especially well characterized in recent years particularly the controlled cortical impact (CCI) model, described elsewhere in this document (see Sections 1.4.4, 2.1.2, and 3.3.3).

For this study specifically, the state-of-the-science specific to understanding the role of MT1 after TBI is also limited to animal models, thus this study is preclinical in nature. In this study the least sentient animal (mice) expected to be appropriate to meet the experimental goals was chosen, as part of the effort to reduce animal suffering. Laboratory mice (*Mus musculus*) were chosen over rats because of the widespread availability of genetically modified mice, specifically knockouts; the study as originally designed, seeks to compare melatonin 1A (MT1) receptor knockout (KO) mice to wild-type (WT) mice of the same background strain. According to the National Human Genome Research Institute (NHGRI), mice and humans share 85 percent of their genomes (National Human Genome Research Institute, n.d.), making them an appropriate model

in many contexts including this study, as evidenced by a high-degree of homology between humans and mice on the receptor of interest.

1.2.3 Rationale for using a molecular genetic approach

Human genetic variability is increasingly being recognized as a factor that affects overall health status and ability to recover from disease and injury. Moreover, molecular genetic research techniques are increasingly being recognized as important methodologies in the nursing research portfolio (Baumgartel et al., 2011; Rudy, Grady, & Bingham, 2005). Indeed, this candidate transferred to the University of Pittsburgh to complete her doctoral degree because of the unique training opportunity afforded through a T32 specific to applying molecular genetic research techniques to nursing research (T32NR009759).

Genetically modified animal models offer a unique opportunity to explore the role of melatonin receptors in the context of brain injury recovery. While other techniques (discussed in detail in Section 1.5) could be used to examine the role of MT1, genetically modified animals offer the highest degree of certainty in the underlying mechanism by ensuring absence (or over-abundance) of the receptors at the genetic level and verified through phenotyping. Moreover, commercially available transgenic strains derived from the C57BL6/J mouse have well-categorized phenotypes known to lack any biological or behavioral characteristics likely to confound this study. The C57BL/6 strain and sub-strains derived from it have been used in CCI research for over 20 years (Smith et al., 1995; The Jackson Laboratory, 2013a, 2013b).

1.2.4 Rationale for examining the melatonergic system

MEL is an extremely low toxicity compound (Jahnke et al., 1999; Seabra et al., 2000; Wiechmann et al., 2008) for which an LD50 in animals could not be determined. It is produced endogenously in the pineal gland and is known to have actions within the brain. There are high numbers of MEL-specific receptors throughout the CNS and periphery (Laudon et al., 1988; Mazzucchelli et al., 1996; Morgan, Barrett, Edward Howell, & Helliwell, 1994; Naji et al., 2004; Weaver et al., 1989). Differences in the nature and location of melatonin receptors have been noted across species (Morgan et al., 1994), with MT1 and MT2 receptors found in mammals (Reppert et al., 1995; Reppert, Weaver, & Godson, 1996), whereas MT3 is exclusively found in amphibians and birds (Morgan et al., 1994). Moreover, MEL is able to rapidly cross the blood-brain-barrier (Di Bella & Gualano, 2006; Le Bars et al., 1991; Reiter et al., 2007), suggesting activity in the brain is possible after a peripherally administered dose. MEL is most well-known for its role in sleep, which is often disrupted after TBI (Ouellet, Beaulieu-Bonneau, & Morin, 2006). Another major role of MEL is neuroprotection, and the compound has been found to prevent apoptotic cell death within the CNS (Alonso-Alconada et al., 2012; Bavithra et al., 2013; Kireev et al., 2013; Ma et al., 2013; Olcese et al., 2009; Ozyener et al., 2012; Reiter et al., 2005; Samantaray et al., 2008, 2009; Suwanjang et al., 2013; Wang et al., 2011; Wang, 2009; Zhang et al., 2013), including after TBI (Campolo et al., 2013; Jadhav et al., 2009; Kelso et al., 2011; Mésenge et al., 1998; Ozdemir et al., 2005).

Before MEL therapy can be extended to TBI clinical care, enhanced understanding of the mechanism of action is necessary. The lack of a well-tested conceptual framework surrounding the mechanism of MEL after TBI affects the ability of researchers to ask the proper questions when evaluating MEL as a post-TBI therapy. MEL is a known antioxidant, which sometimes contributes to oxidation (Dikmenoglu et al., 2008; Hashimoto et al., 2012; Onur et al., 2004; Tamura et al.,

2013; Tan et al., 2002; Taysi et al., 2008). Anti-apoptotic effects of MEL have also been documented in pre-clinical TBI studies (Campolo et al., 2013; Jadhav et al., 2009; Kelso et al., 2011; Mésenge et al., 1998; Ozdemir et al., 2005) and in the context of other CNS conditions (Alonso-Alconada et al., 2012; Bavithra et al., 2013; Bruce-Keller et al., 2007; Kireev et al., 2013; Ma et al., 2013; Olcese et al., 2009; Ozyener et al., 2012; Reiter et al., 2005; Samantaray et al., 2008, 2009; Suwanjang et al., 2013; Wang et al., 2011; Zhang et al., 2013). In Huntington's disease, the anti-apoptotic effects of MEL are MT1 receptor-dependent (Wang et al., 2011); these receptors are found throughout the mammalian brain (Mazzucchelli et al., 1996; Naji et al., 2004) and are known to be affected by genetic variation in MT1-encoding genes (Barrett et al., 1997; Natarajan et al., 2012), including a missense mutation in rs1800885 (Choudhury et al., 2014).

1.2.5 Rationale for examining apoptosis as a cellular level outcome

The term apoptosis was first coined by Kerr et al. (1972) to describe cells exhibiting a constellation of morphological characteristics associated with cell death including, but not limited to: karyorrhexis (i.e. nuclear fragmentation), pyknosis (i.e. chromatin condensation), overall cellular shrinkage, blebbing, and phagocytic engulfment (Kerr, Wyllie, & Currie, 1972). Other ways cells can die after TBI include necrosis, necroptosis, and autophagy. The earliest observation of apoptosis after TBI comes from a CCI study, where apoptotic cell death was detected using silver impregnation and cresyl violet staining in the hippocampal CA1 and CA3 out to 2 weeks following injury (Colicos, Dixon, & Dash, 1996). Another study found increased levels of the apoptotic executioner protein, cleaved caspase 3, at 2 weeks post injury, which was reduced in Gliberclamide treated animals compared to their vehicle-treated counterparts (Patel, Gerzanich, Geng, & Simard, 2010). It is worth noting that while a handful of CCI studies assessed apoptotic outcomes at or

beyond two weeks, the majority of studies evaluating apoptosis do so in the acute (hours-to-days) post-injury period.

Overall, apoptosis is known to begin rapidly after TBI (Barth et al., 2000; Bayir et al., 2007; Conti et al., 1998); the resulting reduction in viable cells within the central nervous system (CNS) is associated with negative outcomes (Miñambres et al., 2008; Piao et al., 2012; Yakovlev et al., 1997). Apoptosis has been reported in several brain regions after TBI, and the hippocampus is particularly vulnerable, contributing to post-TBI problems including attentional processing problems, distraction, and memory impairment (Draper & Ponsford, 2008; Gentilini, Barbieri, De Renzi, & Faglioni, 1989).

1.2.6 Rationale for functional outcomes: learning, memory, and motor

Cognitive symptoms after TBI are among the most common and distressing symptoms reported by survivors of TBI (Binder, 1986). The brain regions of interest in this project (frontal cortex; hippocampus) are known to affect multiple aspects of cognitive function (Baron et al., 1985; Chudasama & Robbins, 2006; McDonald, Flashman, & Saykin, 2002; Seeman et al., 1978); moreover, these regions are also known to be impaired after TBI resulting in reduced quality of life (Binder, 1987; Levin, 1990; Levin et al., 1990; McDonald et al., 2002; McDowell, Whyte, & D'Esposito, 1997; Millis et al., 2001). Thus, these outcomes are clinically relevant.

In the context of animal models, cognitive dysfunction is readily assessed using established behavioral outcome testing. The Beam Balance Test (BBT) is often used to measure gross motor skills. Spatial memory and learning is most commonly assessed using the MWM, whereas reference memory is often evaluated via the MWM probe trial (Hamm, Lyeth, Jenkins, O'Dell, &

Pike, 1993). Recognition memory is frequently measured using the Novel Object Recognition (NOR) task. These outcome measures are well-established as sensitive enough to reliably detect deficits after experimental TBI in both rats and mice. The timeline of the assessments for this study are as follows: BBT day 0-5; NOR day 11-12; MWM hidden platform task day 14-18; MWM visible platform task day 19-20; MWM probe trial day 20. The above mentioned testing schedule is commonly used in the University of Pittsburgh laboratory and has been validated by cellular measures after experimental brain injury (Singleton, Yan, Fellows-Mayle, & Dixon, 2010; A. Wagner et al., 2007). Overall, a review of the literature supports the importance of memory, learning, and motor function and the specific measures chosen as clinically relevant following TBI.

1.2.7 Conceptual framework

The literature lacks a conceptual framework regarding the neuroprotective role of MEL and underlying mechanism of action within the context of TBI. To date, most research is based on an assumption that MEL-induced benefits are related to anti-oxidant activity (Dikmenoglu et al., 2008; Hashimoto et al., 2012; Tamura et al., 2013). In Huntington's disease and amyotrophic lateral sclerosis, MEL-induced neuroprotection is MT1 receptor-dependent (Wang et al., 2011; Zhang et al., 2013). This work informed the proposed conceptual model, which guided this study (Figure 1).

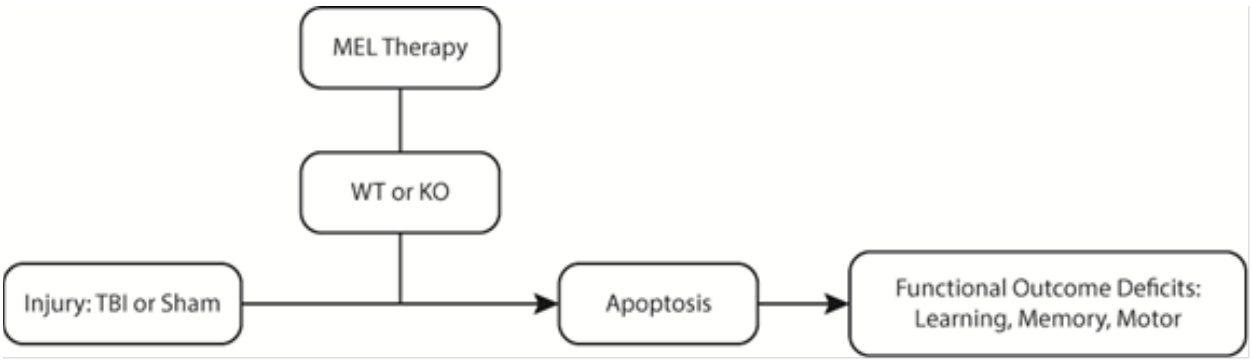

Figure 1: Conceptual framework

It is worth acknowledging that this study is molecular genetic in nature and examines programmed cell death occurring via a well-established apoptotic pathway. Thus, for the protein markers included in this study, selection was made based on well-established biological pathways. Cleaved caspase-3 is a known executioner of apoptosis occurring via both the intrinsic and extrinsic pathways, which are the biological pathways of interest in this study (Figure 2).

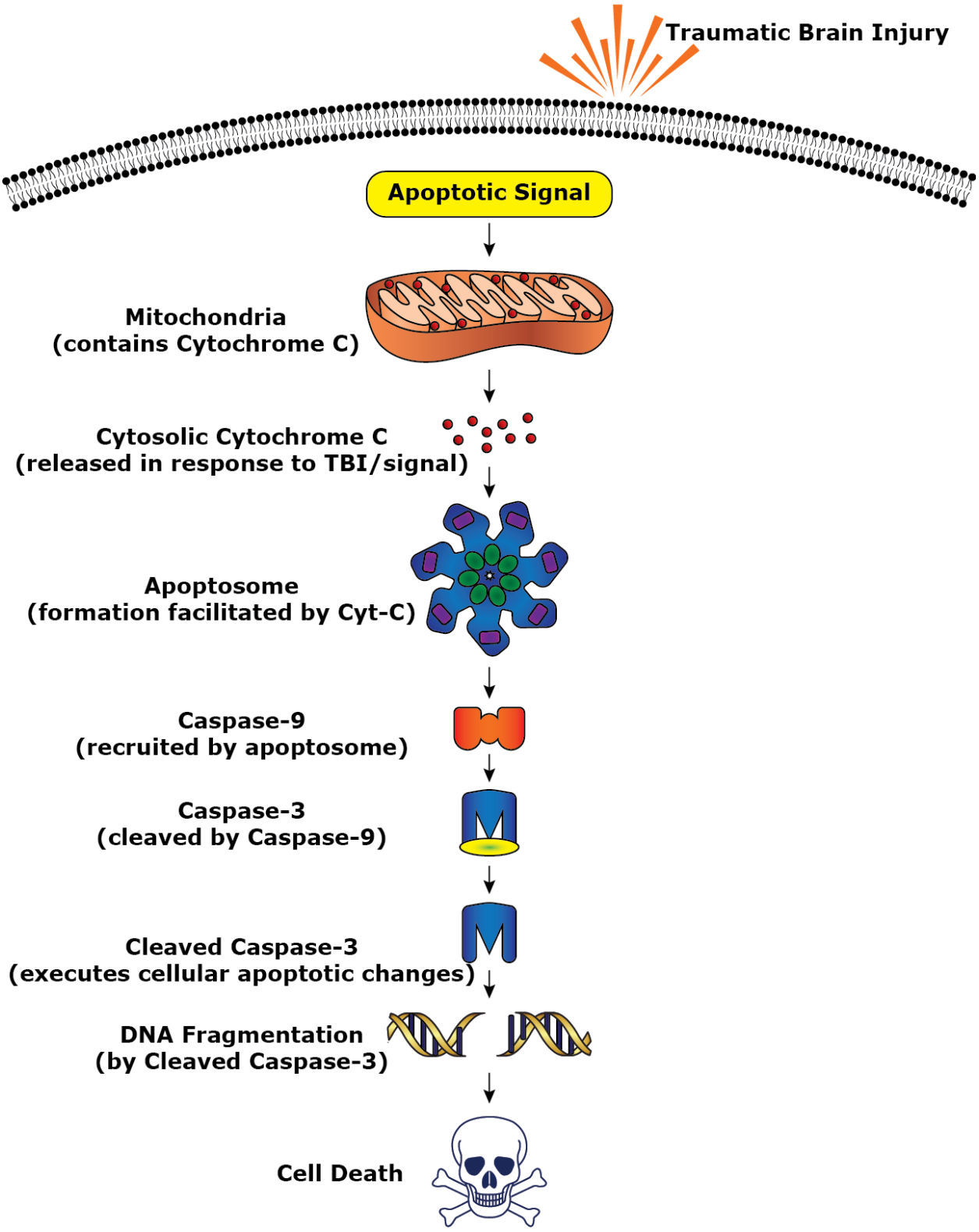

Figure 2: Apoptosis pathway after brain injury

1.2.8 Innovation

The originally planned study, presented at the Comprehensive Examination and Overview, is innovative in that it is the first to explore if the neuroprotective effects of MEL therapy following TBI are receptor-dependent. Although MT1 KO mice are commonly used in other research areas (Baba et al., 2009; Comai, Ochoa-Sanchez, & Gobbi, 2013; Contreras-Alcantara, Baba, & Tosini, 2010; Dubocovich, Hudson, Sumaya, Masana, & Manna, 2005; Hutchinson, Hudson, & Dubocovich, 2012; Imbesi et al., 2009; Imbesi, Uz, & Manev, 2008; Kilic et al., 2012; Mühlbauer, Albrecht, Bazwinsky-Wutschke, & Peschke, 2012; Pfeffer, Rauch, Korf, & von Gall, 2012; Unfried, Ansari, Yasuo, Korf, & von Gall, 2009; Weil, Hotchkiss, Gatien, Pieke-Dahl, & Nelson, 2006; Yasuo, Yoshimura, Ebihara, & Korf, 2009), no published articles were identified where a MT1 KO was used in a TBI experiment. Moreover, many of the studies conducted to date which explore the potential for MEL to improve TBI outcomes are limited in that they partially or exclusively used pre-injury treatment (Jadhav et al., 2009; Kerman et al., 2005; Mésenge et al., 1998). While studies exploring the benefits of pre-injury MEL therapy may be useful for individuals at high risk for TBI (e.g. soldiers; boxers; non-emergency neurosurgery patients), post-injury treatment has more therapeutic significance for the general TBI population. MEL has been found to have low toxicity at a range of doses in both humans (Erman, Seiden, Zammit, Sainati, & Zhang, 2006; Seabra et al., 2000; Zammit, Wang-Weigand, Rosenthal, & Peng, 2009) and animals (Jahnke et al., 1999; Wiechmann et al., 2008), with no lethal dose identified. In rare cases, hypotension, nightmares, itching, and gastrointestinal pain has been reported (Garfinkel, Laudon, Nof, & Zisapel, 1995; Guardiola-Lemaître, 1997); notably, risk of toxicity is likely even lower with short-term administration.

The proposed study addresses a gap in the knowledge surrounding the mechanism of MEL-induced neuroprotection after TBI, based on evidence from other neurodegenerative conditions. Human variation in genes encoding MEL-specific receptors have been identified (Barrett et al., 1997; Natarajan et al., 2012), suggesting patients may not uniformly respond to MEL therapy. Additional research correlating MEL-specific genotypic and/or epigenetic data to outcomes of TBI could facilitate personalized medicine approaches and assist in the identification of patients most likely to benefit from MEL therapy.

1.2.9 Summary

Despite evidence that TBI may alter endogenous MEL levels and be responsive to therapeutic MEL, efforts to better understand these relationships have yielded conflicting evidence. Moreover, the mechanism underlying these relationships remains poorly understood and the role of MT1 has never been tested in a pre-clinical TBI study. The original study, as proposed in the candidate's Comprehensive Examination and Overview challenges the long-held assumption that MEL acts via anti-oxidative effects, and rather focuses on its anti-apoptotic properties. The role of MT1 on apoptotic outcomes of TBI has never been studied. Since genetic variation in human MEL receptors have been documented, the knowledge gained from this project has the potential to lay the groundwork for efforts to translate MEL therapy to the bedside after TBI. Overall, this line of research has the potential to reduce brain injury complications and improve quality of life for survivors.

1.3 PRELIMINARY STUDIES

The following table (Table 1) lists several milestones that have been achieved since entrance into the BSN to PhD program in August of 2011. All milestones listed support the feasibility and scientific merit of the proposed dissertation research project titled “Characterizing the Melatonergic System after Brain Injury.”

Table 1: Milestones in doctoral study

Milestone	Date(s)
Nurses in Genomics (T32NR009759), University of Pittsburgh Fellow: Targeted Research & Academic Training Program for School of Nursing	August 2011 through August 2014
Preliminary Exam Completion, University of Pittsburgh School of Nursing	July 2013
Recipient: Margaret E. Wilkes Scholarship, University of Pittsburgh School of Nursing	September 2012 & September 2013
University of Pittsburgh Institutional Animal Care & Use Committee (IACUC) Approval for pilot study: "Efficacy and dosing of Melatonin Supplementation after severe TBI in mice" (Protocol ID: 13072038)	July 2013
University of Pittsburgh IACUC Approval for dissertation study: "Mechanism of Melatonin-Induced Neuroprotection in Traumatic Brain Injury" (Protocol ID: 14012346)	January 2014
Recipient: F31 Training Grant (1F31NR014957-01), National Institute of Nursing Research	February 2014 (awarded)
Recipient: Sigma Theta Tau International Honor Society of Nursing, Eta Chapter Research Award	March 2014 (awarded)
Recipient: Neurotrauma Nursing Foundation & American Association of Neuroscience Nursing Research Grant	March 2014 (awarded)
Recipient: International Society of Nurses in Genetics Research Award	November 2014 (awarded)
Recipient: Walter L. Copeland Fund of the Pittsburgh Foundation	June 2014 (awarded);
Comprehensive Examination & Overview	December 2014
Annual renewal of IACUC Approval for Protocol ID: 14012346	January 2015
Annual renewal of IACUC Approval for Protocol ID: 13072038	July 2015
F31 Renewal	August 2015
Annual renewal of IACUC Approval for Protocol ID: 14012346	January 2016
Annual renewal of IACUC Approval for Protocol ID: 13072038	July 2016

1.3.1 Establishing the apoptotic consequences of the model

In this pilot study, western blots were completed with pooled samples of tissue from wildtype (C57BL/6) mice exposed to severe pneumatic CCI (n= 10) or sham surgery (n= 10). Results were semi-quantified using densiometric analysis and normalized to sham. Analysis of the data found that the injury model did lead to apoptosis as evidenced by elevated levels of caspase 3 in the striatum and hippocampus at both 1 day- and 7 days- post-injury (Figure 3). However, as described in Chapter 2, these results should be interpreted cautiously since it was later revealed that many of the mice in this sample did not maintain normothermia during surgery, or did not have their temperature monitored intra-operatively; this is concerning because it is well-established that temperature is an important confounding variable in TBI research with hypothermia being neuroprotective (Bregy et al., 2012; Büki, Koizumi, & Povlishock, 1999; Urbano & Oddo, 2012). Moreover, as discussed in Chapter 3, the change in caspase 3 could not be validated in a Sprague-Dawley rat model.

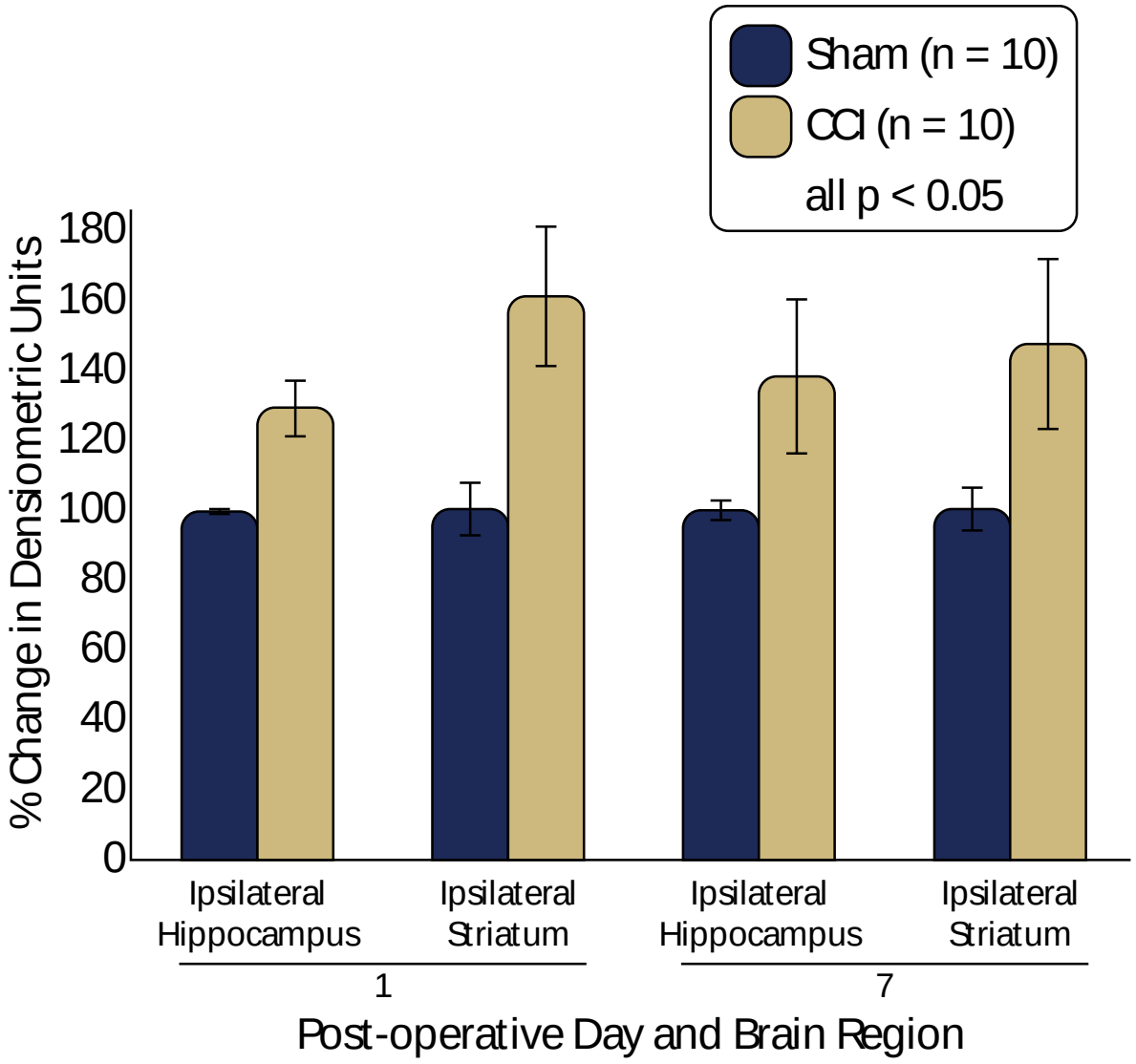

Figure 3: Apoptotic changes (caspase 3) after brain injury in preliminary pilot data

1.3.2 Evaluating endogenous levels of MT1 in the model

In the second stage of pilot work, MT1 protein levels were examined after TBI or sham surgery (modeled as planned in future study activities). Western blots were used to compare pooled samples from 3 mice exposed to severe CCI to pooled hippocampal samples from 3 mice exposed to sham surgery (total n= 6); protein loading was controlled for using beta actin, and normalizing

to sham. Analysis revealed (Figure 4), a statistically significant (p= 0.04) 35% reduction in MT1 levels following CCI (vs. sham); these results are questionable since many of the mice in this sample did not maintain normothermia during surgery, or were not monitored intra-operatively (see Chapter 2).

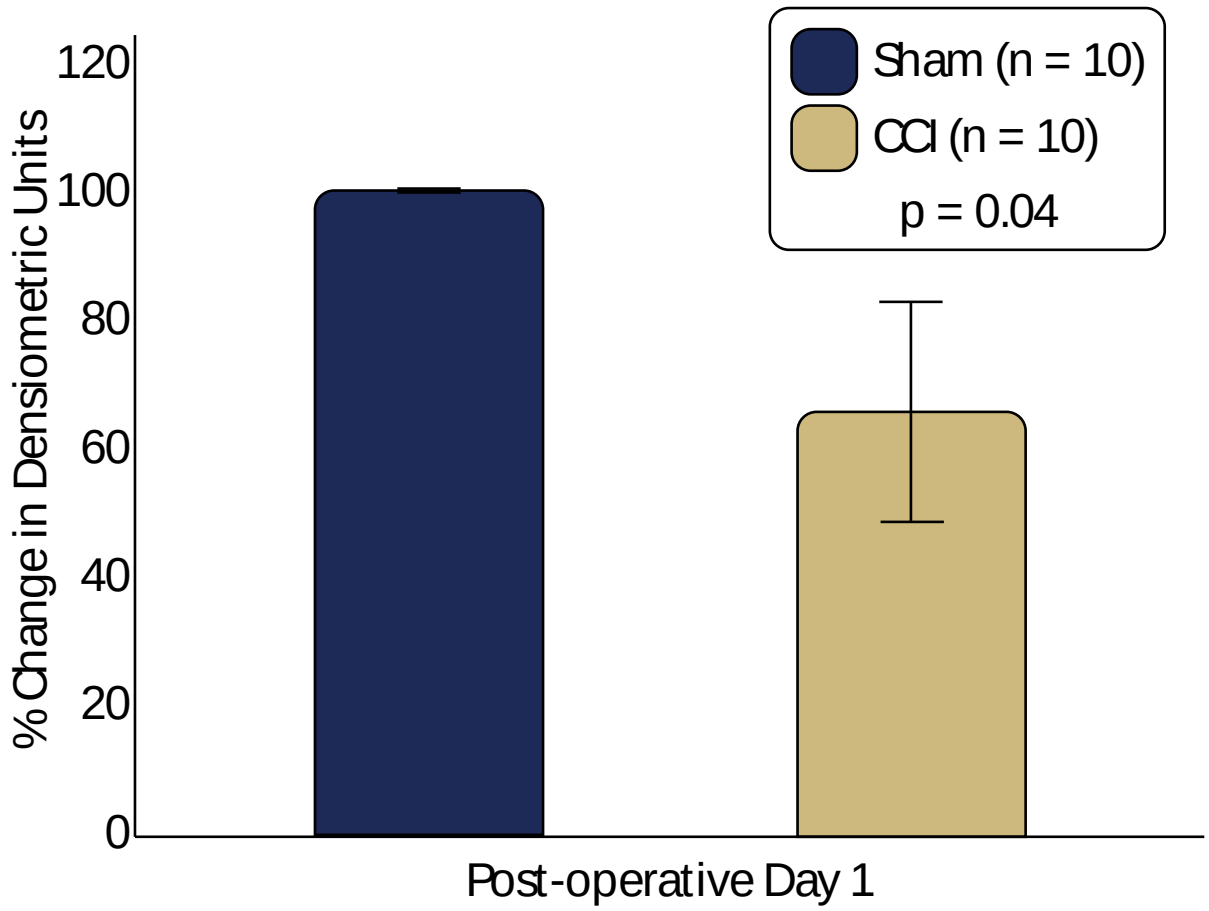

Figure 4: MT1 receptor changes in pooled hippocampal samples after TBI

1.3.3 Establishing behavioral consequences of the model

In the pilot work, the effects of TBI and sham surgery on outcomes of the MWM were tested. The latency period to find the hidden platform was compared in mice exposed to severe CCI (n= 12)

or sham surgery (n= 9). Results presented below (Figure 5), show evidence of a main effect for injury group (p= 0.002) and time (p= 0.005), but no group-by-time interaction effect (p>0.05). These results should be interpreted cautiously since it was determined that mice in this sample did not maintain normothermia during surgery, or did not have their temperature monitored intra-operatively; see Chapter 2 for re-analysis in normothermic mice.

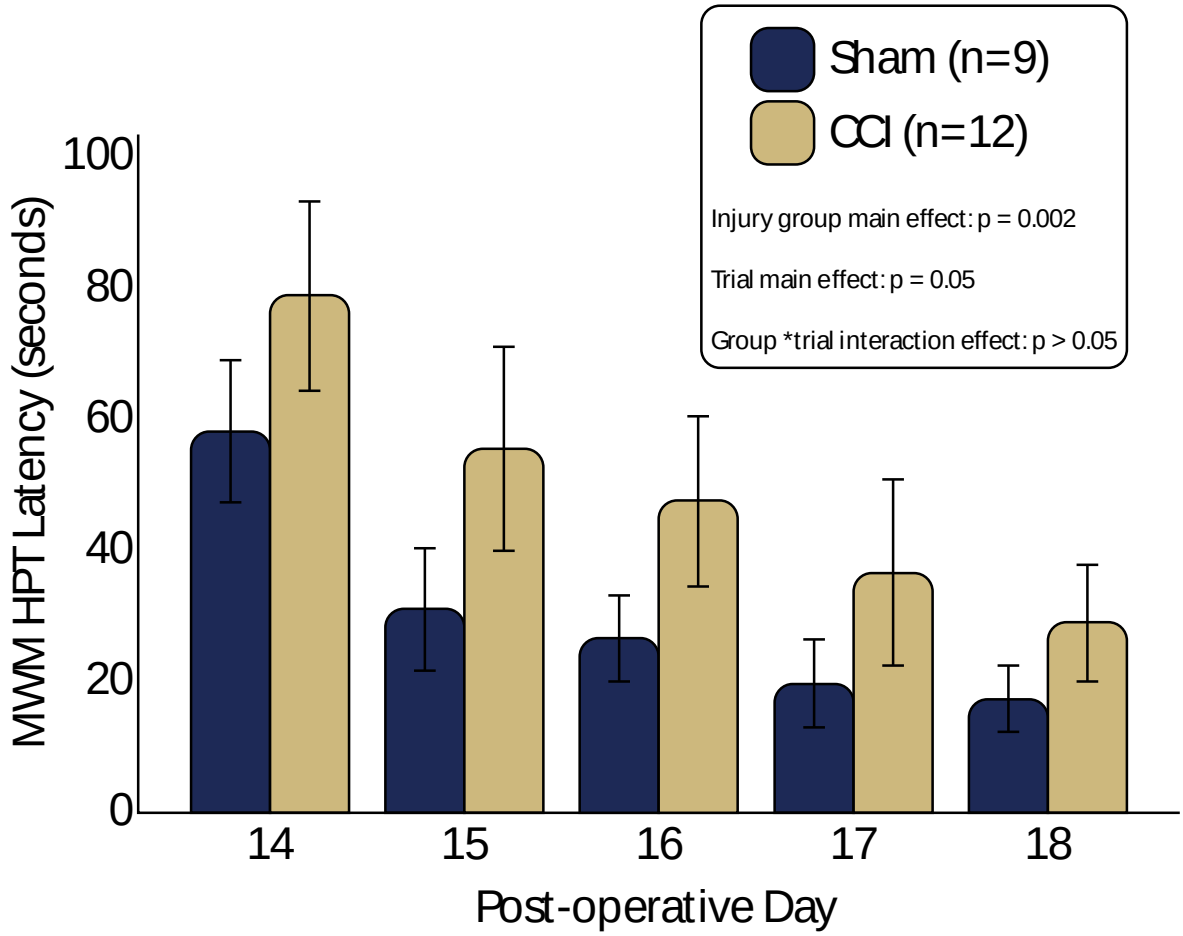

Figure 5: Morris water maze: preliminary data

In addition to the MWM hidden platform task results described above, the probe trial was completed on a sample of mice exposed to severe pneumatic CCI (n= 12) or sham surgery (n= 9). Groups were compared based on percent of probe trial time spent in the southwest quadrant of the

maze (which previously housed the platform). Using the traditional analysis, which compares the two group means using a t-test (Figure 6), there was no significant difference between injured and sham animals, albeit a trend toward significance (p= 0.08). This could mean that the study is underpowered, or it could be that there is just not a significant relationship to detect.

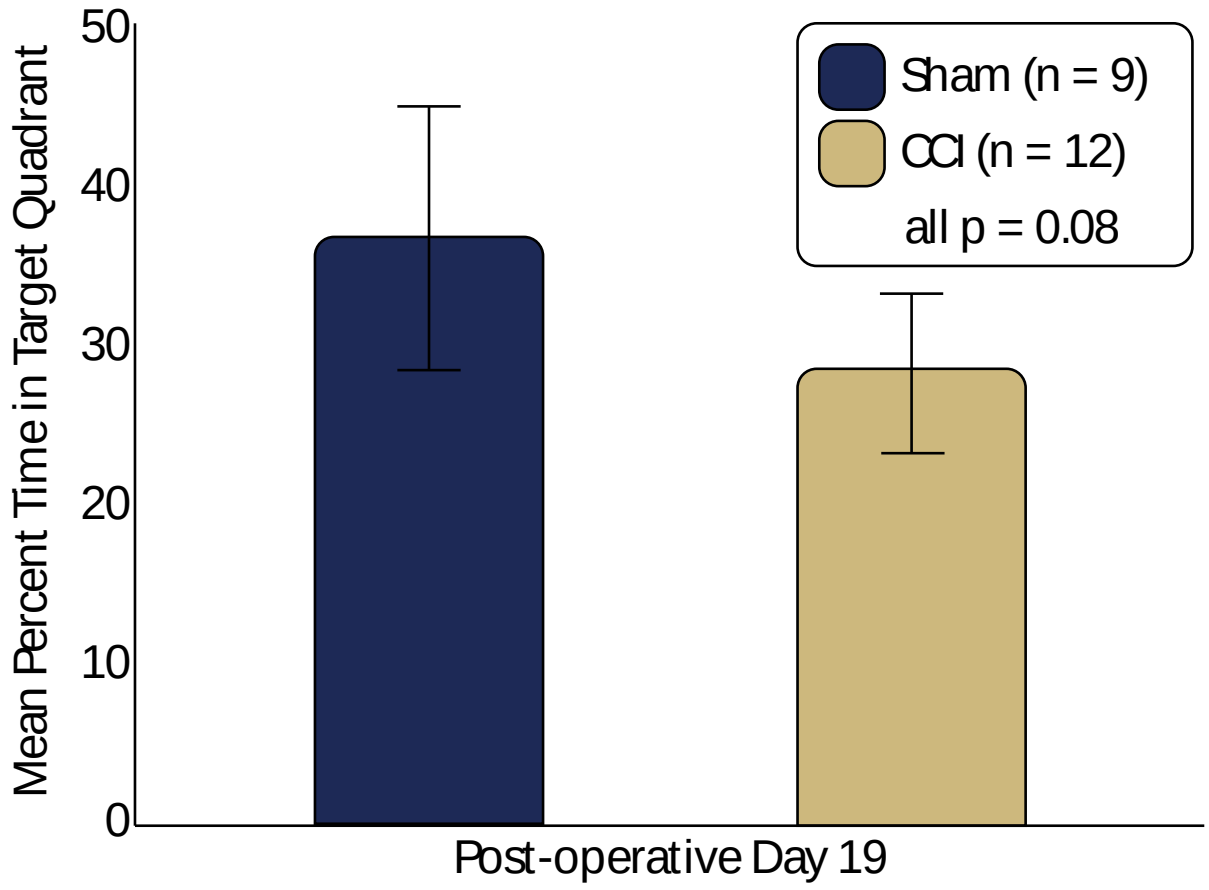

Figure 6: Morris water maze probe trial conventional analysis of preliminary data

When the results were reanalyzed to compare each group to 25% (the amount of time expected to be spent in the target quadrant due to chance alone), there was a significant effect of injury (Figure 7); specifically, CCI-exposed mice did not spend more time in the target quadrant than expected due to chance (p= 0.121) whereas sham mice spent more time than expected in the target quadrant (p= 0.008) suggesting they remembered where the platform had been. It is

important to keep in mind that many of the mice in this sample did not maintain normothermia during surgery, or did not have their temperature monitored intra-operatively; data from mice that met the criteria for normothermia are presented in Chapter 2.

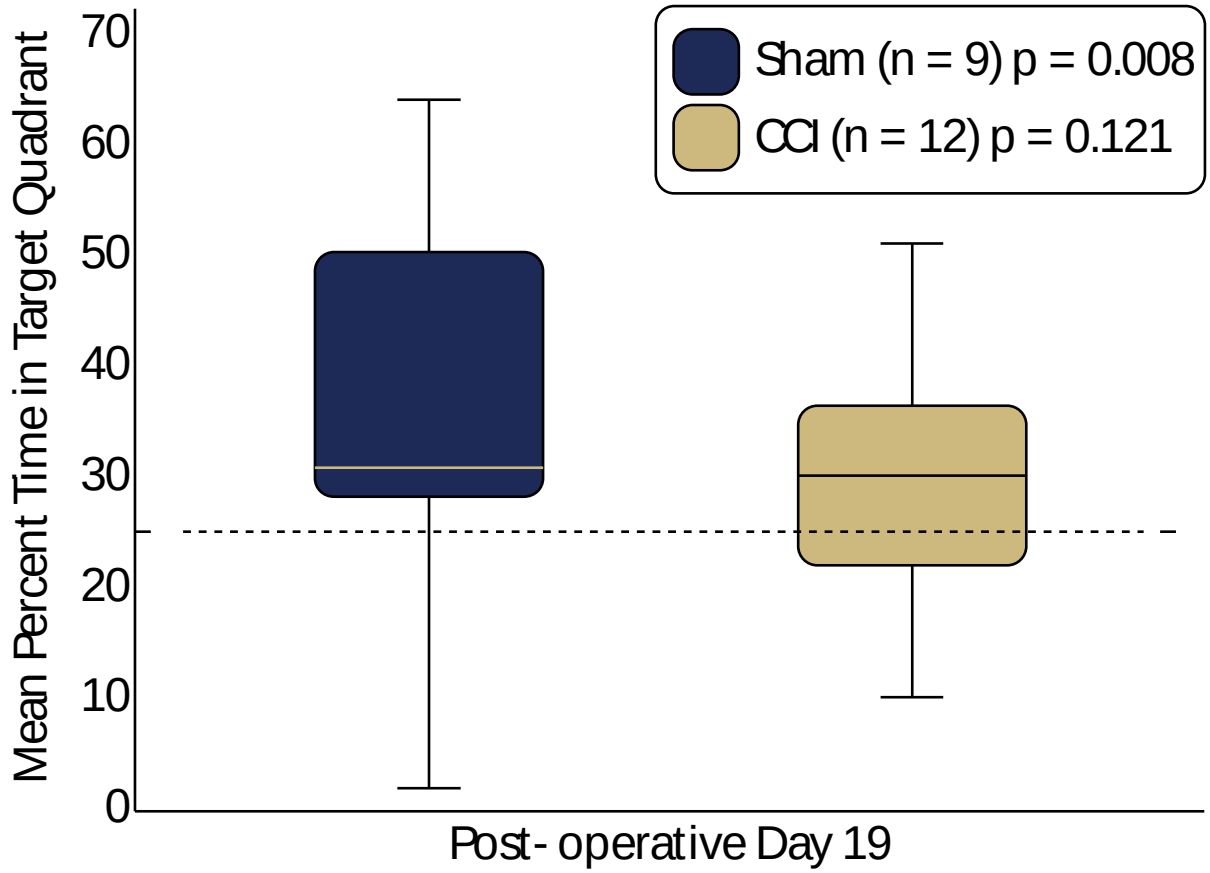

Figure 7: Morris water maze probe trial alternative analysis of preliminary data

1.3.4 Effects of exogenous MEL on behavior after CCI

The NOR task was another assessment in the pilot study. Two groups of mice exposed to CCI were compared: one group received 5 mg/kg MEL (n= 7) and that the other received a vehicle control solution (n= 7), for a total sample size of n=14 mice. The results (Figure 8) showed that melatonin-treated mice had better recognition memory (p= 0.049), as assessed by discrimination

index and discrimination ratio on the NOR task (Antunes & Biala, 2012). Importantly, these results must be interpreted with the confounding variable of temperature in mind. In Chapter 2, the results of analysis using mice that meet the criteria for normothermia are presented.

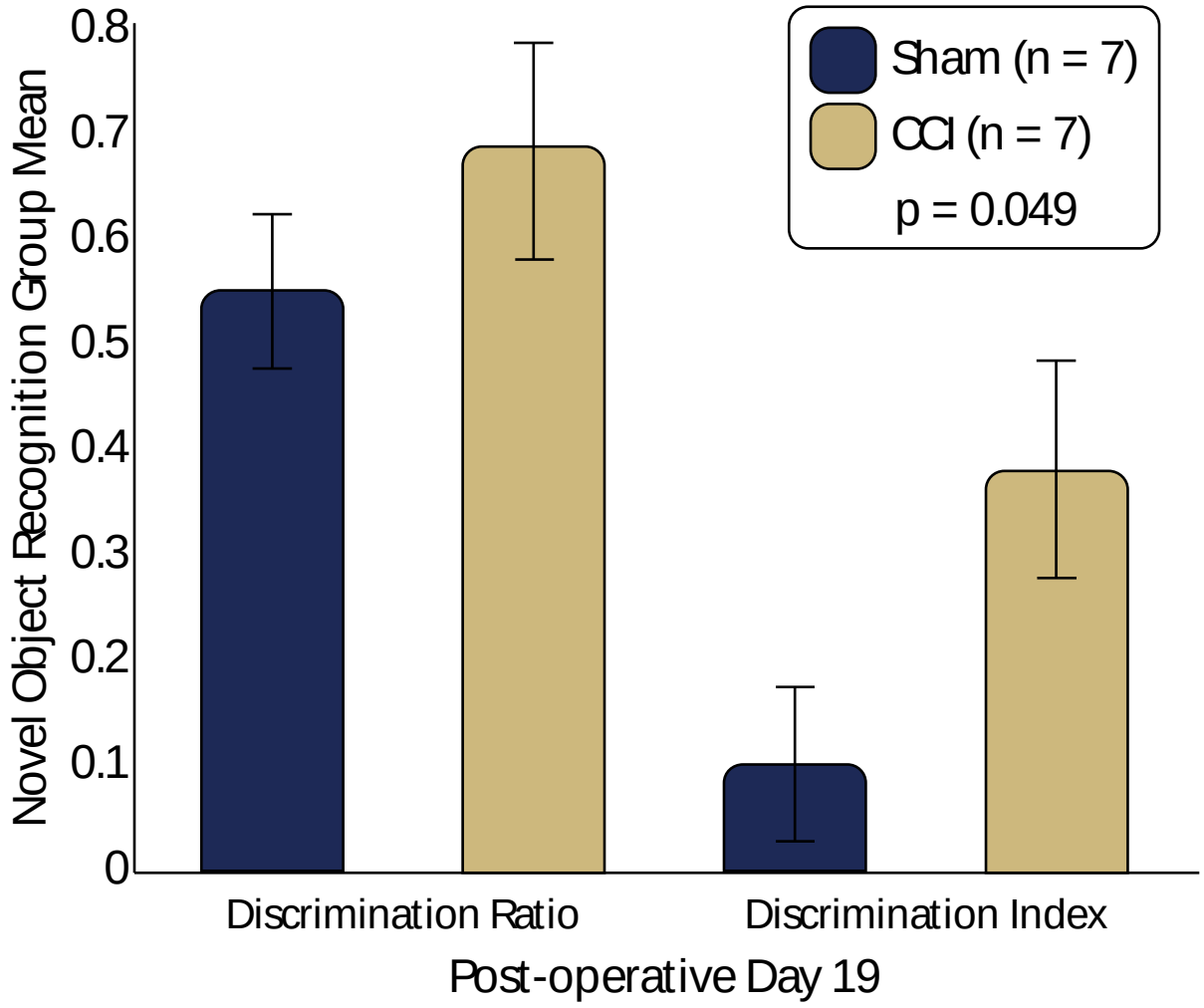

Figure 8: Novel object recognition preliminary evidence in support of melatonin therapy

1.4 RESEARCH DESIGN AND METHODS

Briefly, the proposed dissertation study presented at the time of the Comprehensive Examination and Overview was a prospective 2x2x2 (8-group) experimental study. Mice were randomly assigned into groups and data collectors were blinded to mouse group assignment. Additional study details are provided below.

1.4.1 Animal subjects

Efforts to protect animal subjects were made and are consistent with the guidelines detailed in the Guide for the Care and Use of Laboratory Animals (National Research Council, 2010). All study procedures will be approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC) and were designed to minimize animal suffering. Relevant IACUC approvals were obtained prior to all study activities and maintained throughout the duration of the research (Appendix B). This effort includes keeping the number of test animals to the absolute minimum needed to detect statistically significant differences on the outcome measures of interest. Signs of pain and distress will be monitored daily throughout the course of the study and continuously during the surgery. Animals will be removed from the study and humanely euthanized if they lose 20% of their pre-injury body weight or exhibit signs of infection, distress, or pain (e.g. hunched posture, piloerection, ruffled hair, dilated pupils, chattering on expiration, or shallow respirations). Based on our previous experience and a review of the literature, we anticipate less than 5% of the

sample will meet criteria for premature removal from the study; additional mice will be ordered on an as-needed basis to compensate for attrition.

1.4.2 Overview of study design

This study utilizes a prospective, experimental design that focuses on examining the role of the MT1 receptor after experimental brain injury in a mouse model. IACUC approval was obtained prior to beginning data collection. The timeline for this study is included below (Table 2).

Table 2: Study timeline

Description of Task to be Completed	Year 1 (2011-12)				Year 2 (2012-13)				Year 3 (2013-14)				Year 4 (2014-15)				Year 5 (2015-16)			
	9-11	12-2	3-5	6-8	9-11	12-2	3-5	6-8	9-11	12-2	3-5	6-8	9-11	12-2	3-5	6-8	9-11	12-2	3-5	6-8
Complete Required Coursework																				
Attend NINR's Summer Genetics Institute																				
IACUC Approval & Renewals-Pilot																				
IACUC Approval & Renewals-Dissertation																				
Completion of Pilot Study Activities																				
Analysis of Pilot Study Data To-Date																				
Study Adjustment (Post-CE&O)																				
Execution of Final Study Plan																				
Final Analysis of Dissertation Study Data																				
Preparation of Manuscripts and Presentations																				
Defend Dissertation at Public Defense																				

1.4.3 Sample

To evaluate the role of MT1 in MEL-induced neuroprotection following TBI, WT mice (C57BL/6J) and MT1 KO (B6.129S4-Mtnr1atm1Rep/J) of the same background strain will be used; mice will be purchased from a single commercial supplier (The Jackson Laboratory, Bar Harbor, ME, USA). MT1 KO have been used previously by researchers in other areas (Baba et al., 2009; Comai et al., 2013; Contreras-Alcantara et al., 2010; Dubocovich et al., 2005; Hutchinson et al., 2012; Imbesi et al., 2009, 2008; Kilic et al., 2012; Mühlbauer et al., 2012; Pfeffer et al., 2012; Unfried et al., 2009; Weil et al., 2006; Yasuo et al., 2009), but never in TBI, making this study the first of its kind. The strains selected are widely used in genetic and neuroscience research and do not possess any gross physical anomalies or overt functional deficits likely to confound the results of this study (The Jackson Laboratory, 2013a, 2013b).

Published studies of other neurodegenerative disorders (Wang et al., 2011; Zhang et al., 2013), suggest MT1 alone is involved in neuroprotection, warranting additional research specific to TBI and supporting the use of MT1 KO mice in this study. Beyond the commercial availability of MT1 KO mice and their desirable properties, the University of Pittsburgh research laboratories has specialized equipment for the proposed injury induction and functional outcome assessment (i.e. behavioral testing) in mice. A total sample size of n= 240 mice will be used, with equal numbers of MT1 KO and WT. All of the mice in the proposed study will be young adult males, aged 10-12 weeks at the time of injury exposure; the rationale for these inclusion criteria are that age (Yakovlev et al., 2001; Zhang, Zhang, & Herman, 2003) and sex (Coronado et al., 2011; O'Connor et al., 2007; Ratcliff et al., 2007; Slewa-Younan, Green, Baguley, Gurka, & Marosszeky, 2004) have been found to influence apoptosis and recovery from TBI. Moreover, human males in all age groups are more likely to experience TBI than females, and this disparity

is especially evident among young adults (Centers for Disease Control, 2010). Consistent with recent initiatives by grant funding agencies and the scientific community more broadly, we plan to further explore how sex differences impact response to MEL therapy in future work. To control for the influence of diurnal rhythms on endogenous MEL production (Lynch et al., 1978; Moskała, Krupa, Gościński, & Traczewski, 2004; Nakahara, Nakamura, Iigo, & Okamura, 2003; Reppert, Perlow, Tamarkin, & Klein, 1979; Wright, Lack, & Kennaway, 2004), rooms where the animals are housed will be maintained on a continuous 12 hr light /12 hr dark cycle; the potential confounding effects of diet will be controlled through the use of identical rodent chow. Furthermore, the use of a small mammal (i.e. mouse) model is consistent with the state of the science in this area which is limited to pre-clinical studies. Future work should explore human variation in genes encoding MEL receptors, while considering important confounding factors such as age, sex, diet, comorbidities, and sleep patterns which are difficult or impossible to control in clinical research.

1.4.4 Injury induction

1.4.4.1 Controlled cortical impact model (CCI) TBI will be modeled using CCI, a well-established technique adapted for use in rats by Dr. C. Edward Dixon (Dixon, Clifton, Lighthall, Yaghmai, & Hayes, 1991) and used frequently in the University of Pittsburgh laboratory. Sham animals will receive identical neurosurgical procedures to test animals (i.e. anesthesia, placement in the stereotaxic frame, scalp incision, craniectomy and removal of bone flap, and suturing the scalp), but will not be exposed to the CCI itself. The CCI model was chosen because it results in morphological and cellular-level injury responses that resemble changes characteristic of human TBI, including apoptosis (Dixon et al., 1991; Elliott, Jallo, & Tuma, 2008; Homayoun et al., 2000; Kochanek et al., 1995). Moreover, the CCI model also produces functional deficits (assessed using behavioral testing of motor, learning, and memory) similar to those observed in human patients with TBI, including motor deficits, confusion, amnesia, problems with learning, and memory impairment (Dixon, Kochanek, et al., 1999; Dixon et al., 1991; Fox, Fan, LeVasseur, & Faden, 1998b; Hamm et al., 1992; Onyszchuk et al., 2007; Washington et al., 2012). Thus, CCI is an appropriate model for clinical head injury. This method uses a pneumatic cylinder to induce brain trauma of varying severity, depending on the size of the impactor tip, velocity at which the piston moves, dwell time, and the depth of depression of neural tissue.

Identical neurosurgeries will be performed for the sham procedure and to gain access to brain tissue prior to CCI as follows: adult male mice will be anesthetized with 5% isoflurane in a 2:1 mixture of N_2O:O_2 via nose cone and maintained on a 2% isoflurane mixture throughout the surgery. A stereotaxic frame will be used to secure mice in the prone position with the head held in a horizontal plane with respect to the inter-aural line via ear bars and an incisor bar; topical analgesia (EMLA™ cream, a 2.5% lidocaine/prilocaine mixture) will be applied around the ear

bars. Using aseptic techniques and principles consistent with IACUC surgical policy, the head will be shaved, and swabbed with betadine before a midline incision is made, the soft tissues retracted, a craniotomy will be performed using a dental drill, and marcaine (0.5%) infiltrated into the wound site. Bone flaps will be discarded. To control for the potential confounding effects of therapeutic hypothermia (Bayir et al., 2009; Clark et al., 1996; McIntyre, Fergusson, Hébert, Moher, & Hutchison, 2003; Urbano & Oddo, 2012), body temperature will be monitored continuously via rectal probe and maintained at 37-38°C using a thermostatically controlled pad. Oxygenation levels will be monitored with a non-invasive pulse oximeter (MouseOx, Starr Life Science Corporation, Oakmont, PA, USA). All instruments will be sterilized prior to use. Between surgeries, the impactor tip will be disinfected using cold Gluteraldehyde sterilization solution and other surgical instruments will be disinfected using a glass bead Dry Sterilizer (Germinate 500, Roboz Surgical Instrument Company, Gaithersburg, MD, USA). A single sterilized instrument set will be used on a maximum of five mice. TBI will be induced using CCI with standardized injury parameters described later (see Section 1.4.3). After the CCI, the surgical site will be sutured and anesthesia discontinued. EMLA™ cream will be used immediately after the surgery and for a minimum of 3 days. Animals will be monitored post-operatively for complications as described elsewhere in this document (see Section 1.7).

1.4.4.2 Specific injury parameters As originally planned, the study injury protocol involved using pneumatic CCI at standardized injury parameters. Specifically, the plan was to use a 3 mm round impactor tip. Injury parameters were set as follows: tip deforming the neural tissue a depth of 1.6 mm at a velocity of 4 meters/sec with a dwell time of 0.1 sec; these parameters have previously been successfully used in the University of Pittsburgh laboratory.

1.4.5 Melatonin therapy

A single 0-, 5-, 10-, or 20- mg/kg dose of MEL will be given intraperitoneally (i.p.) 5 minutes after surgery completion, Since MEL readily crosses the blood-brain-barrier regardless of route of administration (Di Bella & Gualano, 2006; Le Bars et al., 1991; R. J. Reiter et al., 2007) the i.p. route was selected based on ease of access in mice. The doses in this study are supported by the literature as being capable of reducing apoptosis and improving functional (i.e. behavioral) outcomes in rodent models of TBI (Jadhav et al., 2009; Kelso et al., 2011; Mésenge et al., 1998; Ozdemir et al., 2005). The rationale for the timing of MEL therapy is that apoptosis begins early following TBI (Barth et al., 2000; Bayir et al., 2007; Conti et al., 1998), but immediate post-injury MEL administration is not clinically relevant. Stock MEL powder from a commercial supplier (Sigma-Aldrich, St. Louis, MO, USA) will be prepared daily by dissolving it in pure ethanol and diluting it with 0.9% saline to a final concentration of 5% ethanoic saline. Control mice will receive an equal volume of 5% ethanoic saline.

1.4.6 Measurement of apoptotic proteins

Apoptosis will be assessed using western blots with antibodies to probe for specific proteins (caspase-3 and cleaved caspase-3) well established in the literature as being good indicators of apoptotic cell death (Chen et al., 2003; Slee, Adrain, & Martin, 2001; Yakovlev et al., 2001) that are detectable within the brain following TBI (Cernak et al., 2004; Tang, Zhao, & Ye, 2003; Tweedie et al., 2007; Yakovlev et al., 1997, 2001). Commercially available antibodies (Cell Signaling Technology, Danvers, MA, USA) will be used following procedures optimized during the pilot work. Cleaved caspase-3, the active form of the protein, is known to be the key executioner of apoptosis by inducing proteolysis and ultimately cell death (Slee et al., 2001); thus, levels of cleaved caspase-3 and its precursor (caspase-3) are excellent indicators of apoptosis.

Tissue preparation for western blot analysis will be completed as follows: after being humanely sacrificed via Fatal Plus injection, the brains will be quickly harvested and tissue from the ipsilateral and contralateral side of the striatum, hippocampus, and frontal cortex will be dissected out via standardized methods used in the University of Pittsburgh laboratory and flash frozen using liquid nitrogen. Tissue samples from the frontal cortex, hippocampus, and thalamus will be stored at -80°C until needed for western blot analysis. These brain regions are involved in cognitive function (Eichenbaum, Yonelinas, & Ranganath, 2007; Moser, Kropff, & Moser, 2008; O'Keefe & Dostrovsky, 1971; Scoville & Milner, 1957; VanElzakker, Fevurly, Breindel, & Spencer, 2008; Voytek & Knight, 2010) and/or known to have an apoptotic response to TBI (Conti et al., 1998; Marciano et al., 2004; Raghupathi, Graham, & McIntosh, 2000; Rink et al., 1995; Yakovlev et al., 1997). Notably, the MT1 receptor has been detected in these regions of mammalian brains (Laudon et al., 1988; Mazzucchelli et al., 1996; Weaver et al., 1989), suggesting that these regions contain the hypothesized target for MEL therapy in WT mice. Samples will be

homogenized and prepared using standard procedures used in the University of Pittsburgh laboratory. Briefly, on the day of analysis, tissue samples will be sonicated in lysis buffer over ice. The lysate will be centrifuged and the supernatant collected. To ensure equal protein loading gel loading volumes for each sample will be based on protein concentrations determined by BCA assay (Bio-Rad, Hercules, CA, USA). Samples will be electrophoresed using a 15% Tris-HCl polyacrylamide gel and transferred to appropriate positively charged membranes. Membranes will be blocked, washed, and incubated with the primary antibody (caspase-3; cleaved caspase-3) overnight, before being re-washed and incubated with anti-rabbit secondary antibody. Chemiluminescent solution (Western Lighting, Perkins Elmer, Boston, MA, USA) will be used to expose autoradiographic X-ray film for protein visualization. Membranes will be stripped with glycine solution and re-stained with β-actin and anti-mouse secondary antibody to ensure equal protein loading. The data will be normalized to β-actin levels to control for any differences in protein loading.

1.4.7 Measurement of functional deficits

Functional deficits common after TBI will be assessed using behavioral testing of mice in the domains of motor, learning, and memory function, as described below. Personnel performing functional assessment will be blinded as to the group to which each mouse is assigned.

1.4.7.1 Morris water maze (MWM)

The MWM is a behavioral test widely used in mice (Ashe, 2009; Creed, DiLeonardi, Fox, Tessler, & Raghupathi, 2011; Galsworthy et al., 2005; Jones et al., 2005; Mannix, Zhang, Park, Lee, & Whalen, 2011) to reliably assess learning and memory function (de Fiebre, Sumien, Forster, & de

Fiebre, 2006). After CCI animals have longer latencies to the hidden platform than their sham counterparts; injured animals also are slower to learn the maze and often spend less time in the target quadrant during the probe trial (Dapul et al., 2013). The MWM uses a pool in a 2.5 meter x 2.5 meter room with extra maze cues that remain constant; the maze itself is 90 cm in diameter and 60 cm high filled to a depth of 28 cm with water maintained at 25-26°C (Figure 9). Hidden in the pool at a depth of 1 cm is a 10 cm diameter platform. A ceiling-mounted video tracking system (AnyMAZE, Stoelting, Wood Dale, IL, USA) records and quantitates only the animals' tracks, not images of the animals.

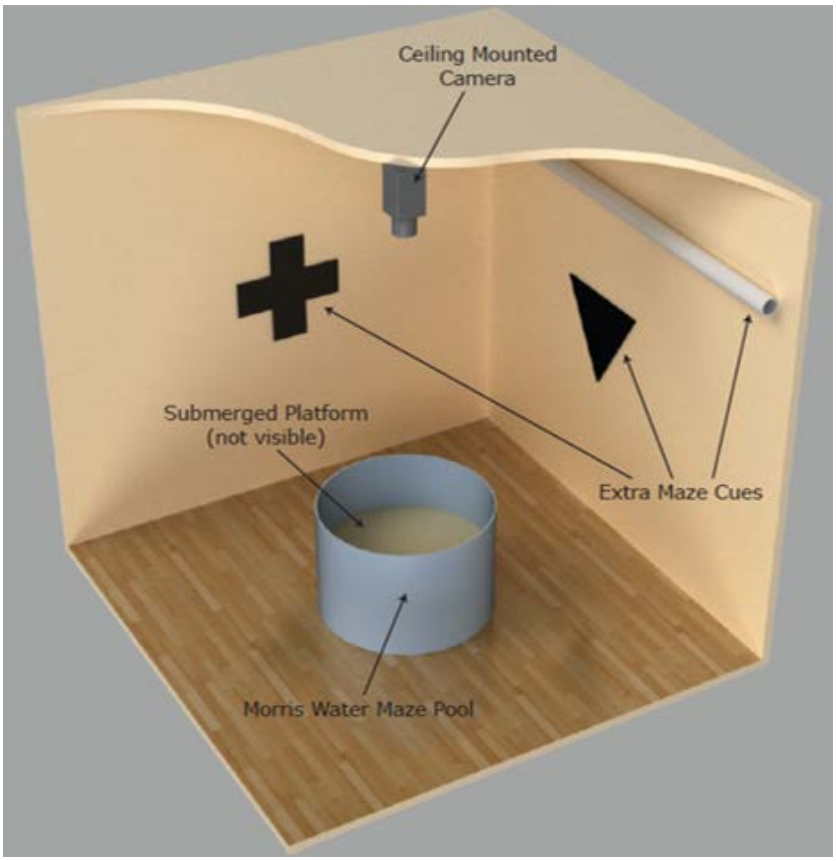

Figure 9: Morris water maze set up

The hidden platform task (Figure 10) will assess each mouse's ability to learn spatial relations between distal cues and the escape platform. Acquisition blocks will consist of four daily trials on each of five consecutive days. At the start of the trial, the mouse will be placed by hand in the pool facing the wall. Latencies are expected to decrease across test days.

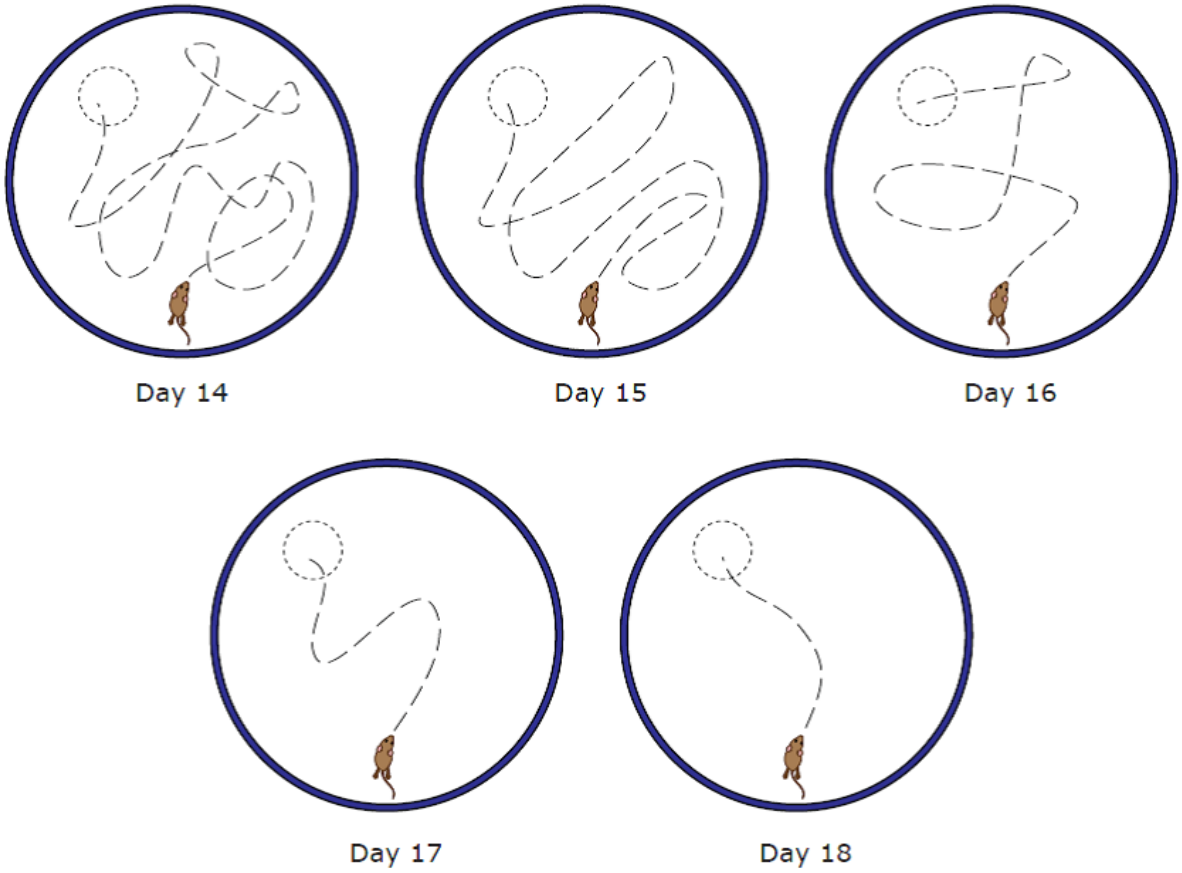

Figure 10: Morris water maze hidden platform task

To control for non-specific deficits in vision and motor function, the MWM visible platform task will be used (Figure 11), in which the platform is raised 2 cm above the surface. Mice will be allowed up to 120 sec to find the platform, after which point they will be placed on the platform for 30 sec before being placed in a heated incubator between trials. There is a 4-minute inter-trial interval. Mice taking the entire trial time will be presumed to have motor and/or vision deficits and excluded from the final analysis.

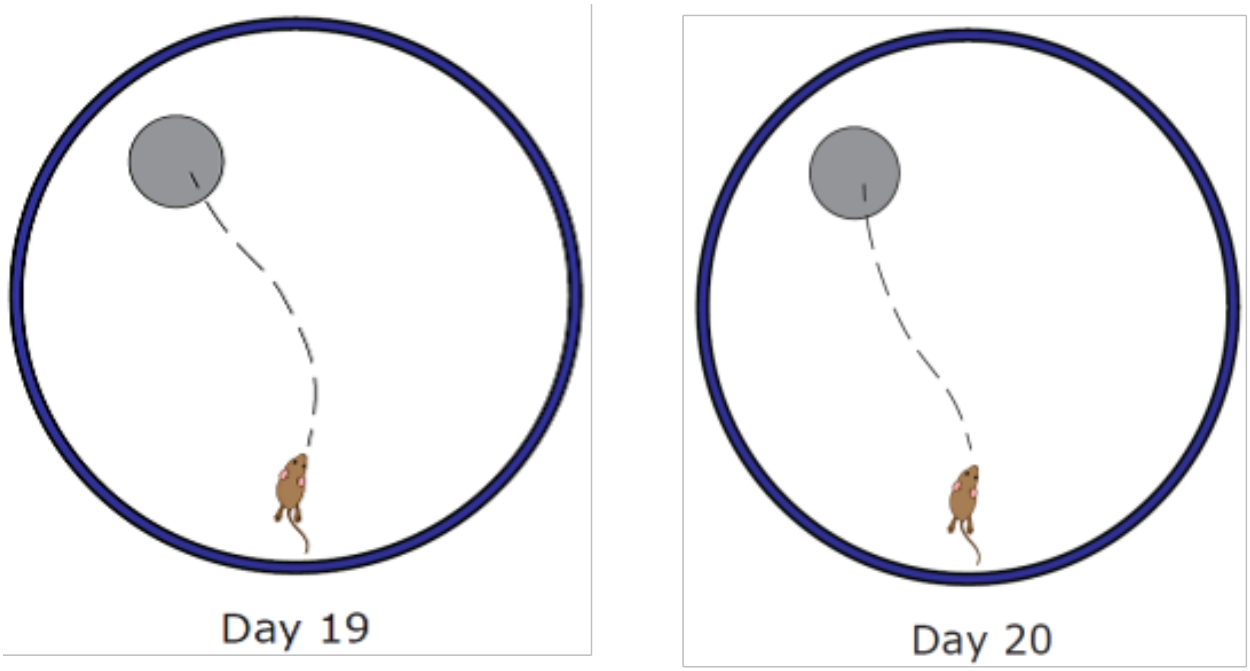

Figure 11: Morris water maze visible platform task

A single probe trial (Figure 12) will be conducted on day 19 to evaluate reference memory by removing the platform and recording the percentage of the trial time swam in the quadrant where the platform had previously been located (i.e. target quadrant). Mice who remember where the platform was during the hidden and visible platform tasks will spend the majority of the testing time in the target quadrant.

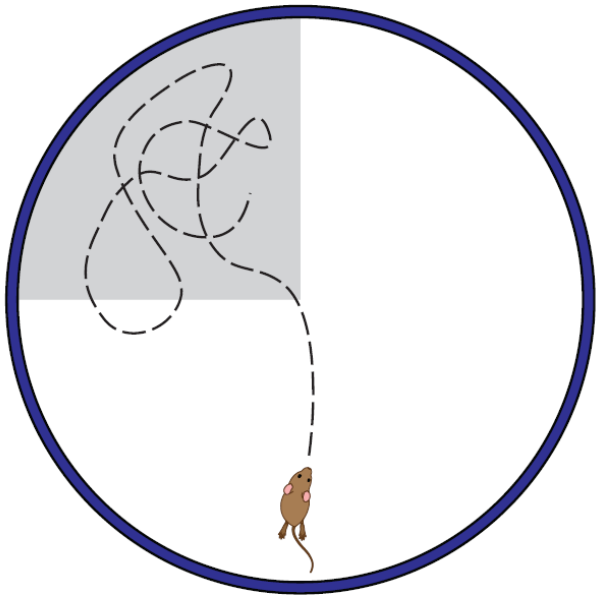

Figure 12: Morris water maze probe trial

1.4.7.2 Beam balance task Gross motor function will be assessed via BBT, a technique used frequently in the University of Pittsburgh laboratory (including the pilot work) and commonly used in research using rodent models of TBI (Chen, Constantini, Trembovler, Weinstock, & Shohami, 1996; Clark et al., 1997; Clifton et al., 1989; Statler et al., 2006; Tehranian et al., 2008; Whalen et al., 1999). Mice will be placed on a suspended, narrow wooden beam (1.5 cm wide) and the latency (time the animal remains on the beam) measured, up to 60 sec (Figure 13); three training trials in the pre-injury period will be used for baseline.

Figure 13. Beam balance task

1.4.7.3 Novel object recognition The NOR task will be performed by placing a mouse in a large open space with 2 objects: one familiar (i.e. object previously exposed to) and one novel (i.e. object never before exposed to). Time spent in proximity to each object in the arena is recorded in seconds (Figure 14). This data will be used to calculate the discrimination ratio (DR) and discrimination index (DI), as described in the literature (Antunes & Biala, 2012).

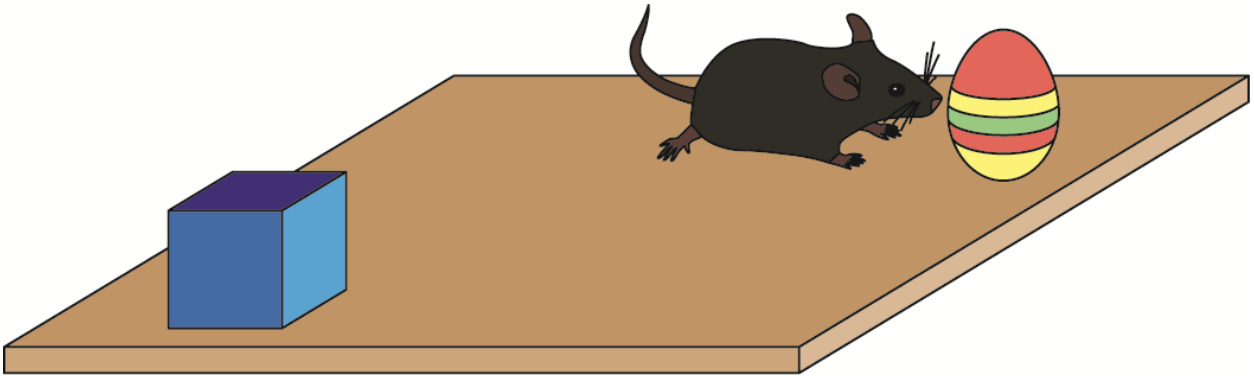

Figure 14. Novel object recognition task

1.4.8 Analysis

1.4.8.1 Sample size justification To date, available data is insufficient to perform effect size calculations and sample size estimations specific to the variables of interest, leading us to base the sample size for this study off the experience of the team, and previous reports of drug trials in animal models of TBI. The results of this study will be used to perform such calculations to ensure that our future work is adequately powered.

1.4.8.2 Preliminary data analysis Univariate and multivariate screening will be used to identify any outliers or invalid values (e.g. latencies exceeding overall trial time). Univariate screening will be done for the injury group and therapy group alone. Multivariate screening will be done for the therapy and injury groups in combination. Results of outlier assessment will be presented in tabular form with the mean, standard error of the mean (SEM), skewness statistic, $SE_{skewness,}$ kurtosis statistic, and $SE_{kurtosis}$. Data accuracy will be assessed based on the possible values for each assessment. For example, MWM hidden platform test has a maximum time of 120 seconds so all values greater than 0 seconds and less than or equal to 120 seconds will be considered accurate.

Missing data will be evaluated for both independent and dependent variables using the frequency command for each variable. Since mice in this study are humanely euthanized at the end of the study, missing data cannot be re-collected. If > 10% of data are identify as missing, the candidate will consider replacing mice with missing data by purchasing new mice for enrollment in the study. If there is loss of animals due to death, the candidate will evaluate whether death occurs completely at random or if one of the experimental groups has significantly higher mortality rates. If attrition is preferentially affecting one group the candidate will discuss with her team and

consider removing that experimental group from the study for ethical reasons (e.g. injury is too severe or treatment results in toxic effects and animals are suffering).

1.4.8.3 Aim 1 analysis Western blot data will be normalized to β-actin levels to control for gel loading differences and semi-quantitated to show relative differences in optical densities between groups via Scion Image PC software (Frederick, MD, USA), as described previously (Shin, Bray, & Dixon, 2012). Group means will be compared using ANOVA; main effects and interaction effects will be tested. Scans of membranes and graphs will be generated using ImageJ software (Abràmoff, Magalhães, & Ram, 2004).

1.4.8.4 Aim 2 analysis For the MWM data, an ANOVA will be used to analyze swim latencies across days post injury with Bonferroni post hoc tests corrected for multiple comparisons; main effects and interaction effects will be explored. Swim speed and latency in the target quadrant will be obtained from the probe trial and analyzed via one-way ANOVA followed by post hoc tests corrected for multiple comparisons. For the BBT, ANOVA will be used to analyze balance latencies across time; Bonferroni tests will correct for multiple comparisons. Should the assumptions of ANOVA not be met (Table 3), mixed modeling will be used as the analysis method.

Table 3: Preliminary analysis test assumptions

Analysis	***Variable(s)***	***Assumption***	***Evaluation of Assumption***
Factorial (2x2x2) ANOVA	Memory (MWM Probe)	Normality	Histograms; formal testing (Kolmogorov-Smirnov; Shapiro-Wilk) *some deviation expected
		Homogeneity of Variance	Box plots; formal testing (Levene's)
		Independence of Observations	By design (individual mice; verify 1 assessment/mouse)
Repeated-Measures ANOVA	Learning (MWM Hidden Platform Task); Motor (BBT)	Normality	As in Factorial ANOVA
		Homogeneity of Variance	As in Factorial ANOVA
		Independence of Observations	By design; independent mice (allow non-independent over-time assessments)
		Sphericity	Mauchly's test

1.4.8.5 Expected findings and interpretation It is hypothesized that outcomes will be moderated by the presence of the MT1 receptor. Specifically MT1 KO mice will have higher levels of known apoptotic proteins than their WT counterparts. Furthermore, MEL therapy will reduce apoptosis and that MEL-treated animals in both MT1 groups (KO and WT) will have lower levels of caspase-3 and cleaved caspase-3 than their counterparts receiving a saline solution as previously reported (Campolo et al., 2013; Jadhav et al., 2009; Kelso et al., 2011; Mésenge et al., 1998; Ozdemir et al., 2005). Lower apoptotic proteins among WT mice (vs. their MT1 KO counterparts) treated with MEL would provide additional support for the hypothesis. Beneficial effects of MEL in MT1 KO are likely due to well-established anti-oxidant effects. With respect to functional outcomes on learning, memory, and motor testing, MEL treatment is expected to have beneficial effects compared to vehicle control with these effects especially evident in WT mice. Favorable outcomes in WT animals support the hypothesis of receptor-dependent effects of MEL on outcomes of TBI.

1.5 LIMITATIONS AND PROPOSED ALTERNATIVES

A primary limitation of the proposed study is the use of an animal model; however, at this time a clinical trial in humans is not yet warranted. The goal is accumulation of enough evidence of a neuroprotective role of MEL following TBI sufficient to justify clinical trials. This study will provide evidence of the potential for apoptosis as a mechanism for neuroprotective effects of MEL after TBI. This study may lead to improved treatment options for TBI patients including the translation of MEL from the bench to the bedside. A second limitation is that data is collected for a limited period (30 days), which is fairly long for an animal study but may obscure effects on even longer-term outcomes. Significant positive results during the 30 day study period will provide

evidence to support additional, more costly and burdensome research, with longer-term outcome measures. A third limitation surrounds the measurement of the cellular- and behavioral- variables of interest. Multiple other ways to assess apoptotic cell death (e.g. TUNEL staining, levels of other apoptotic proteins such as AIF, caspase-9, Bax) are available but not included in this study, as they are less reliable indicators of inevitable cell death than the presence of known executioner proteins. Similarly, other methods of testing behavioral outcomes as a proxy for functional status (e.g. rotarod test; vermicelli test) have been used previously, but were not selected for this study. Additional work exploring alternative endpoints represent a direction for the candidate's future work. Finally, the anesthesia (isoflurane) used may result in neuroprotection and confound study outcomes (Statler et al., 2000, 2006). Sham surgery helps control for the known neurological effects of isoflurane.

Similarly, there are several ways in which the role of the MT1 receptor could be tested empirically. For example, a selective MT1 receptor antagonist could be administered along with the test agent to compete with the ligand of interest (MEL) for the receptor sites (MT1) and outcomes could be compared in mice given MEL with and without the antagonist; however, it is possible that the antagonist would not effectively block all receptors and that some endogenous or exogenous melatonin would still bind. Similarly, a genetic knock in could be used to compare mice with increased expression of the receptor to mice with standard levels of the receptor. However, for this study, a MT1 KO as chosen because this approach results in the complete absence of the MT1 receptor in one group of the test animals, which provides the most compelling evidence for the role of the MT1 receptor (or lack thereof) when compared to the aforementioned alternatives.

1.6 HAZARDOUS MATERIALS AND PROCEDURES

Working with animals, animal specimens, and chemicals involves some risk. To reduce risk to both test animals and investigators, the study will be conducted following extensive training both in-person and online (e.g. bloodborne pathogen; chemical hygiene; etc.). All work will be conducted in approved laboratories and animals will be housed and tested in approved facilities. Appropriate personal protective equipment will be used (e.g. gloves, bonnet, face mask, laboratory coat) as well as safer sharps devices. All team members who contact animals are enrolled in the Animal Exposure Surveillance Program (AESP) at the University of Pittsburgh; all adverse events will be reported to the AESP.

1.7 RESEARCH SUBJECT RISKS, BENEFITS, AND PROTECTIONS

The study presented at the time of the Comprehensive Examination and Overview had a sample comprised of mice, one of the least sentient animals appropriate to meet the experimental goals. Consistent with university and national policy efforts to protect animal subjects were made. Details surrounding general protections for animal subjects, specific risks in this study, benefits, and the data safety and monitoring plan are described below.

1.7.1 Potential risks and protection against risks

Husbandry and veterinary care of the animals will be consistent with institutional and national policies and will be approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh. The mice will be housed, and all experiments conducted within the Safar Center for Resuscitation Research, an approved animal research facility at the University of Pittsburgh. Assessment and monitoring of the animals will be provided by the University of Pittsburgh's Division of Laboratory Animal Resources (DLAR) on a daily basis. The DLAR also provides regular veterinary care of the animals.

Signs of pain and distress will be monitored daily throughout the course of the study and continuously during the surgical intervention. To reduce pain and discomfort, multiple anesthetic and analgesic medications will be administered to the mice at various points in the study. Prior to surgery, anesthesia will be induced using 4% isoflurane (inhaled via nose cone), with a dose of 2% used to maintain the effects. Depth of anesthesia and pain will be assessed using the extended leg-toe pinch withdrawal reflex. Three different analgesics (EMLA cream, bupivacaine, and Buprenorphine) will be used during the study to alleviate pain caused by study procedures. EMLA cream will be used around the ear bars during surgery. Buprenorphine will be administered to alleviate pain associated with TBI or sham operation and will be continued for at least 3 days following completion of the procedure (with the exception of mice sacrificed 24 hours after injury). Prior to discontinuation of the anesthesia, bupivacaine will be administered to the wound site using sterile procedure.

Animals will be removed from the study and humanely euthanized if they lose 20% of their pre-injury bodyweight or exhibit signs of surgical site infection, distress, or pain (e.g. hunched posture, piloerection, dilated pupils, chattering on expiration, or shallow respirations). Based on

experience and a review of the literature, no more than 5% of the sample is expected to meet criteria for premature removal from the study. Animals that complete the study as planned will be humanely euthanized in an IACUC-approved manner; specifically, they will be injected with sodium pentobarbital and decapitated. Immediately following sacrifice, brain tissue will be harvested and frozen. This method of euthanasia is consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

1.7.2 Potential benefits

The results of this dissertation study are not expected to provide direct benefit to study subjects nor be immediately translatable to human subjects. However, findings will increase knowledge of the pathophysiologic mechanisms involved in TBI pathology, specifically as it relates to the melatonergic system. This knowledge could guide future studies aimed at better understanding and eventually therapeutically targeting the melatonergic system after TBI.

1.7.3 Data safety and monitoring plan

This study is not a clinical trial, but rather, a pre-clinical trial using mice; however, the candidate and her team made efforts to monitor and protect data throughout the study. All research data generated during this study will be used solely for research purposes were safeguarded by the candidate and her advisors on password-protected computers. All mice will be assigned to groups using a randomly assigned numerical identifier; all biological specimens will be stored using the unique identifiers as well as information related to the brain region (e.g. hippocampus, frontal cortex, striatum) and side (e.g. contralateral, ipsilateral).

2.0 SUMMARY OF DISSERTATION STUDY

The purpose of this dissertation was to characterize the melatonergic system after experimental TBI. The specific aims as presented at the time of the Comprehensive Examination & Overview were to determine whether the effects of melatonin therapy on apoptotic protein levels and behavioral testing were receptor-dependent. Upon completion of the pilot study, and due to extensive ongoing data monitoring, several important concerns were identified leading to changes in the scope and aims (see Section 2.1). Briefly, behavioral endpoints were de-emphasized and changes in levels of apoptotic proteins and endogenous melatonin receptors in rats became the focus.

The "manuscript dissertation" format was chosen to highlight six of the manuscripts resulting from doctoral training and conduct of the dissertation research: 1 in-press review manuscript, 4 pre-press review manuscripts, and 1 pre-press data-based manuscript. Brief summaries of each of the six manuscripts along with rationale for inclusion are provided. Details regarding where the article was published or submitted for review and consideration for publication are also provided. Full text of the in-press manuscript is provided in Appendix B with the permissions provided in C. Full text of the pre-press manuscripts are provided in Chapters 4-7.

In-Press Manuscript #1 (Appendix C): A review of available experimental TBI models that provides justification for the use of a rodent model for studying TBI and rationale for using

CCI for studying secondary injury cascades, specifically apoptosis. This in-press manuscript titled "Chronic Histopathological and Behavioral Outcomes of Experimental Traumatic Brain Injury in Adult Male Animals," was published during 2015 in the *Journal of Neurotrauma*; permission to reprint this article in the dissertation can be found in Appendix D. *Pre-Press Review Manuscript #2* (Chapter 4): A historical review of the applications of animal models for health science and clinician training titled: "Historical Use of Animal Models in Advancing Health Research and Practice: Paving the Way to 21st Century Healthcare" which was submitted to Nursing History Reviews in July of 2016. *Pre-Press Review Manuscript #3* (Chapter 5): A review of how animal models are useful to nurses and other health science researchers interested in exploring the molecular genomic aspects of various conditions. This in-review manuscript titled: "Animal Models in Genomic Research: Techniques, Applications, and Roles for Nurses" was submitted to *Applied Nursing Research* in July of 2016. *Pre-Press Review Manuscript #4* (Chapter 6): A review of the CCI model, describing the historical development of the CCI model, comparing and contrasting the pneumatic and electromagnetic models, and summarizing key short- and long-term consequences of TBI that have been gleaned using this model. This in-review manuscript titled "The Controlled Cortical Impact Model: Applications, Considerations for Researchers, & Future Directions" was submitted to *Frontiers in Neurology* in June of 2016. *Pre-Press Review Manuscript #5* (Chapter 7): A review of all past publications that have examined melatonin as a therapeutic agent for experimental traumatic brain injury, with a summary of existing clinical evidence. This in-review manuscript, titled "The Effects of Melatonin on Outcomes of Traumatic Brain Injury: A Review of the Literature" was submitted to *Journal of Neurotrauma* in July of 2016. *Pre-Press Data-Based Manuscript #6* (Post-Defense revisions of Chapter 3): A data-based manuscript of the main dissertation findings with discussion focusing on the potential implications

of receptor downregulation, including how it may have contributed to the conflicting evidence to-date surrounding the efficacy of therapeutic MEL after TBI. This drafted manuscript will be revised after the dissertation defense and will be submitted to *Neuroscience Letters* in August of 2016.

Beyond the manuscripts included as part of this dissertation, the doctoral candidate has published two book chapters on the topic of brain trauma that are outside of the scope of the dissertation or already well-covered by another manuscript included in this dissertation. One chapter on the topic of the CCI model (Osier, Korpon, & Dixon, 2015) and one exploring the effects of TBI on the dopaminergic system (Yan et al., 2015). Two additional book chapters on the topic of CCI are in review. Additionally published, but not included in this dissertation are: a first authored peer-review manuscript on the topic of the catecholaminergic system in the context of TBI (Osier & Dixon, 2016); a first authored data-based manuscript that used clinical TBI samples to explore the relationship between PPP3CC polymorphisms and outcomes (Osier et al., 2016) and is in-press in the *Journal of Neurotrauma*; and a review paper written as a collaborative effort on the use of TBI models to understand the biology and behavior of TBI and published in *Neuroscience and Biobehavioral Reviews* (Bondi, Semple, et al., 2014). The doctoral candidate also has a past program of research surrounding weight and weight loss which resulted in collaboration on two published data-based manuscripts, one in the *International Journal of Obesity* (Stommel & Osier, 2012) and another in *Eating Behaviors* (Goode et al., 2016).

This dissertation afforded valuable trouble shooting and problem solving experiences. Several problems arose that the doctoral candidate had an opportunity to identify, address, and evaluate the success of interventions. These problems are outlined in Section 2.1 along with details regarding how the study was modified to address these issues. All changes to the study protocol

were first discussed with the Committee Chair, F31 Sponsor and Co-Sponsors and later approved by the entire committee before the revised plan was executed.

2.1 PROPOSAL CHANGES

Several impediments prohibited the original research for this dissertation from being conducted as initially proposed. First, there were a series unforeseen circumstances and equipment failures. Most notably in 2014 the laboratory where this work was conducted became infected with pinworm (*Syphacia sp.)* curtailing all animal research activities for several months. There were also, issues with intra-operative temperature monitoring and maintenance that compromised the quality of the test outcomes resulting in an insufficiency of pilot data to warrant carrying on with the study as originally planned. Though the measures chosen were well-established in the literature and previously vetted, no injury effect was detected in the pilot study, there for there was no deficit to target with MEL therapy (as described in Section 2.1.2). Finally, recently published evidence has raised addition concerns regarding the state-of-the-science with respect to therapeutically administering MEL after experimental TBI; notably while most of the studies found at least modest effects of MEL therapy, one study found no effect (Kelso et al., 2011) and two studies found adverse effects including increased peroxidation, exacerbation of edema, and deficits on behavioral tests (Cirak et al., 1999; Jadhav et al., 2009). In response to the limitations detected, the sample and methods were adjusted accordingly (see Section 2.1.7).

Therefore, bfefore attempting to target the melatonergic system therapeutically, additional characterization and understanding of the system is necessary. Consequently, the study has been redesigned to focus on changes that occur within the endogenous melatonergic system, particularly

as it relates to levels of melatonergic receptors and apoptotic markers at different time points after injury. Accompanying this increased emphasis on protein markers is a de-emphasis on behavioral outcomes as approved by the dissertation committee.

2.1.1 Animals that were lost or excluded from the pilot sample

At the end of the pilot study a total of 224 mice were enrolled, which included over-sampling for vehicle-treated CCI and sham animals after midpoint analysis revealed high variability and a lack of an injury effect (data not shown). Of the 224 mice enrolled in the study, n= 12 mice were excluded from the final analysis because they either died naturally or were euthanized due to one of 4 circumstances: 1) pre-operative death due to poor health upon delivery (n=1, 0.4% of the sample); 2) intra-operative complication (n= 2, 0.9%); 3) post-operative complication (n= 5, 2.2%); or 4) the mouse was part of a group (n= 4, 1.8%) that was suspected of having had a parasitic pinworm infection known as *Syphacia sp.* These 4 mice fell ill and lost 20% or more of their body weight overnight and either died naturally or were humanely euthanized and were initially presumed to be suffering post-operative complications. Mice that fell ill were given a brief, routine post-mortem examination; however, they were not extensively necropsied or specifically tested for pinworm before disposed as the laboratory had not yet been notified of the pinworm infestation. However, shortly after the 4 mice fell ill, the center where this work was conducted was informed that the building was one of many across the eastern seaboard that had experienced a pinworm infestation. This resulted in the building being closed for fogging and decontamination (September 15, 2016 thru November 1, 2016), after which for a period of several months little-to-no research was conducted and it was recommended that researchers only be in the building on an as needed basis and wear additional personal protective gear including an N95 respirator.

After accounting for the 12 animals that died or were euthanized for health-related reasons, there remained a total sample of 212 surviving mice included in the final pilot dataset. As mentioned previously, the preliminary pilot results presented at the time of the Comprehensive Examination & Overview and published in this document under Section 1.3 (Figures 3-8) was called into question when the data was re-examined. Specifically, many of the mice included in the analysis had not had intra-operative temperatures recorded or the reported intra-operative temperatures were lower than the target range of 36.5°C-37.5°C. Upon completion of the pilot study, 62 of 212 mice (29.2% of the surviving sample) did not have intra-operative temperatures recorded; this omission is attributed to problems with the temperature maintenance system, followed by a subsequent communication failure that, resulted in the problem going unresolved while mice were added to the sample without temperature monitoring. Of the mice that had their temperature monitored only a small percentage (22.2%) met the original criteria for normothermia (36.5°C-37.5°C); there was concern that 47 normothermic mice across 8 groups was not sufficient to detect significant differences between groups (data not shown). Based on available literature suggesting that mild therapeutic hypothermia in the context of experimental TBI is in the range of 32°C-34°C (J. H. Lee et al., 2014), the decision was made to expand the definition of normothermia to 36.0°C-37.5°C, increasing the sample to 85 mice meeting the revised criteria for normothermia.

2.1.2 Concerns raised during analysis of full 8-group pilot sample

The pilot study was initiated with 8-groups made up of 2 injury exposures (CCI or sham) and 4 treatment options (ethanoic saline vehicle, 5-, 10-, or 20- mg/kg of melatonin). After eliminating mice from the sample that failed to have their temperature monitored intra-operatively or those that did not meet the new criteria for normothermia (see Section 2.1.1), a sample of 85 mice were

analyzed. When all 8 groups were compared using repeated-measures ANOVA on MWM HPT performance, as defined as latency to find the hidden platform in seconds (see Figure 15, which plots group mean +/- SEM), there was no main effect for injury group, no main effect for therapy, no main effect for trial day, and no interaction effects (all p>0.05). Notably, the groups displayed variable trajectories with relatively large error bars. An additional concern was that some sham animals had worse performance than expected, despite having a highly experienced surgeon who held potential confounders as constant as possible.

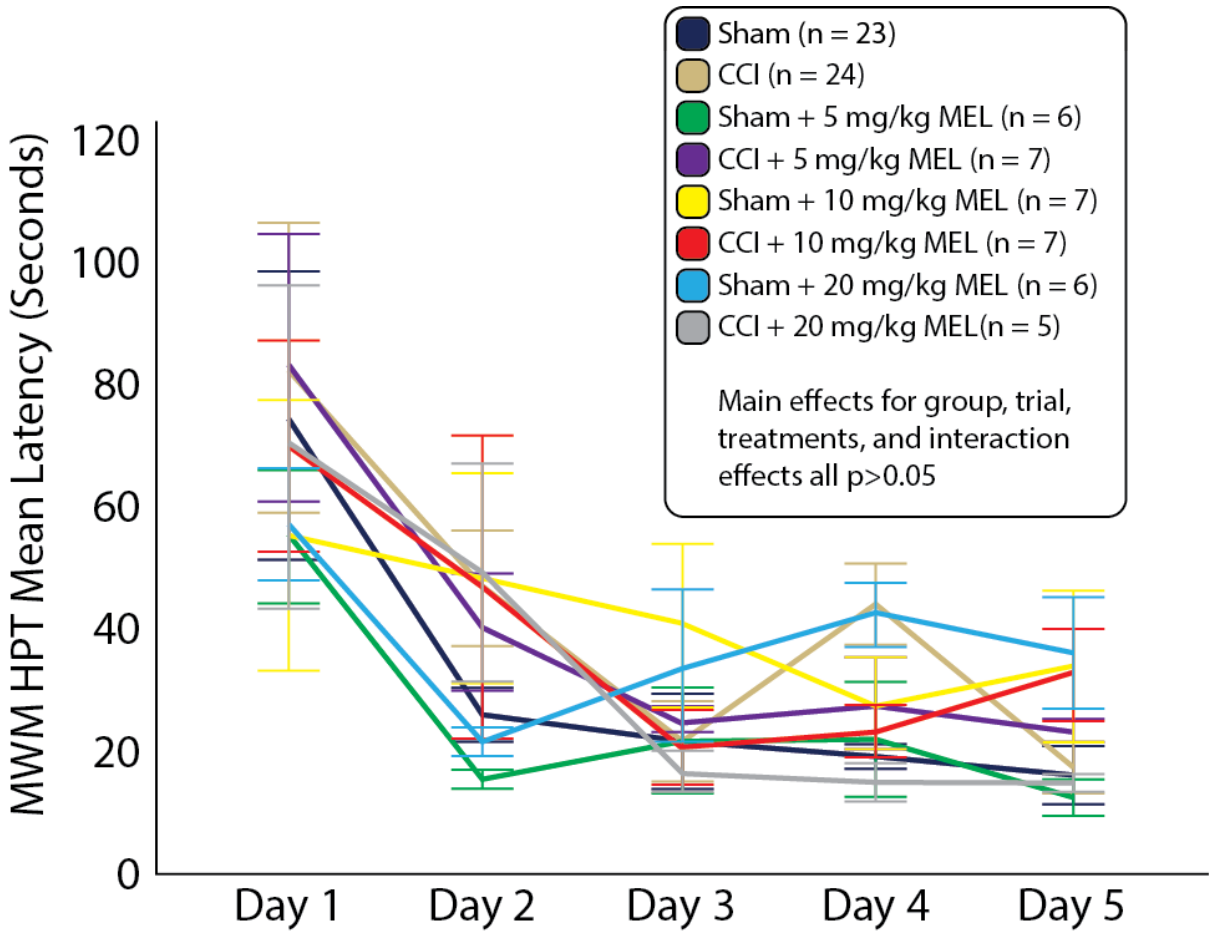

Figure 15: Morris water maze all treatment groups

Similarly, upon comparing all 8 groups on BBT performance (Figure 16) by plotting group mean +/- SEM, as defined as latency balancing on the narrow beam in seconds, there was no main effect for injury group, no main effect for therapy, no main effect for trial day, and no interaction effects (all $p>0.05$). Again, variability was high which complicates the evaluation of the graph. As with the MWM data, some of the sham animals did not perform as well as expected despite having an experienced surgeon who held potential confounders as constant as possible. Concerns about BBT data from the pilot study are described in Section 2.1.3.

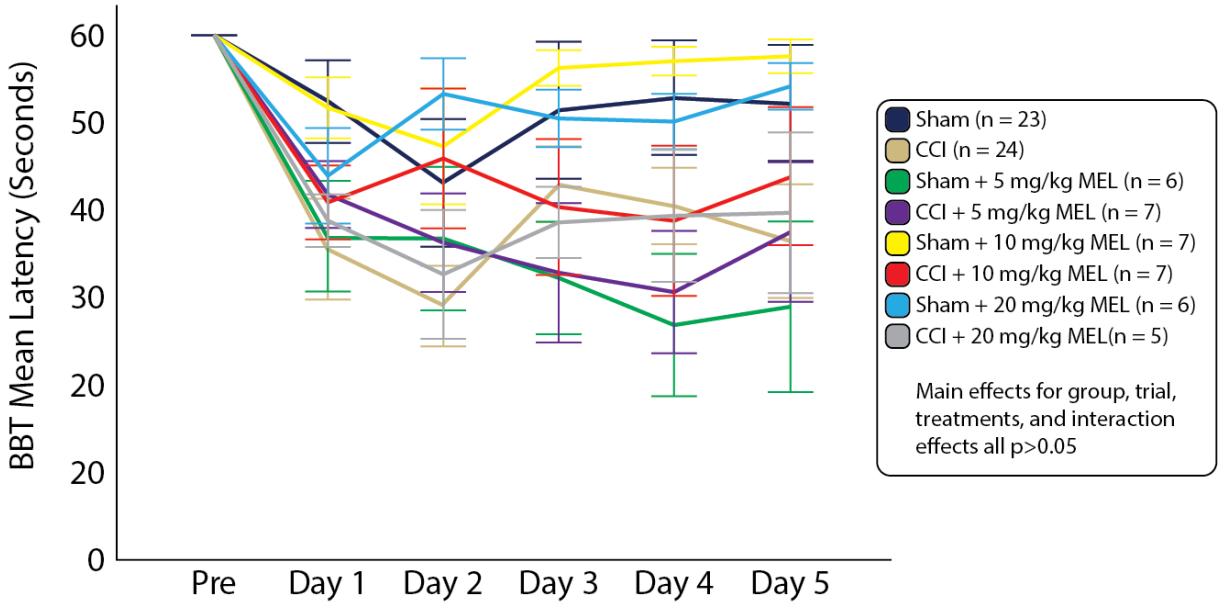

Figure 16: Beam balance task in all treatment groups

2.1.3 Comparison of vehicle-treated animals to establish injury effect

As described in 2.1.2, the graphical displays were muddled for the full pilot sample. Specifically, it was difficult to interpret the graphs when all 8 groups were considered together. Moreover, there

was a lack of significant group differences upon analysis using repeated-measures ANOVA. Some of the data deviated from what was expected as described in section 2.1.2.

Subsequently, the next step was to examine if in vehicle-treated animals there was a significant difference between individuals exposed to CCI and those exposed to sham surgery. It was important to address the question of whether there was an injury effect among the vehicle-treated mice, before moving forward administering therapeutic MEL. Moreover, this was the rationale behind over-representing vehicle-treated animals in the sample, to ensure that a statistically significant injury effect could be detected.

2.1.3.1 Beam balance task There was a main effect for injury group ($p= 0.013$) on motor function as assessed using the average of 3 daily trials on the BBT. Data presented is group means +/- SEM. However, the test did not perform entirely as expected. There was no main effect for trial ($p= 0.302$) indicating no effect of the day of testing (Figure 17) (i.e. no improved performance with practice and repeated-testing). Moreover, there was no group-by-trial interaction effect ($p= 0.957$) which would have indicated the groups had different trajectories with respect to learning to navigate the maze. Thus, in the absence of the expected injury effects, the decision was made to eliminate the BBT from the final analysis.

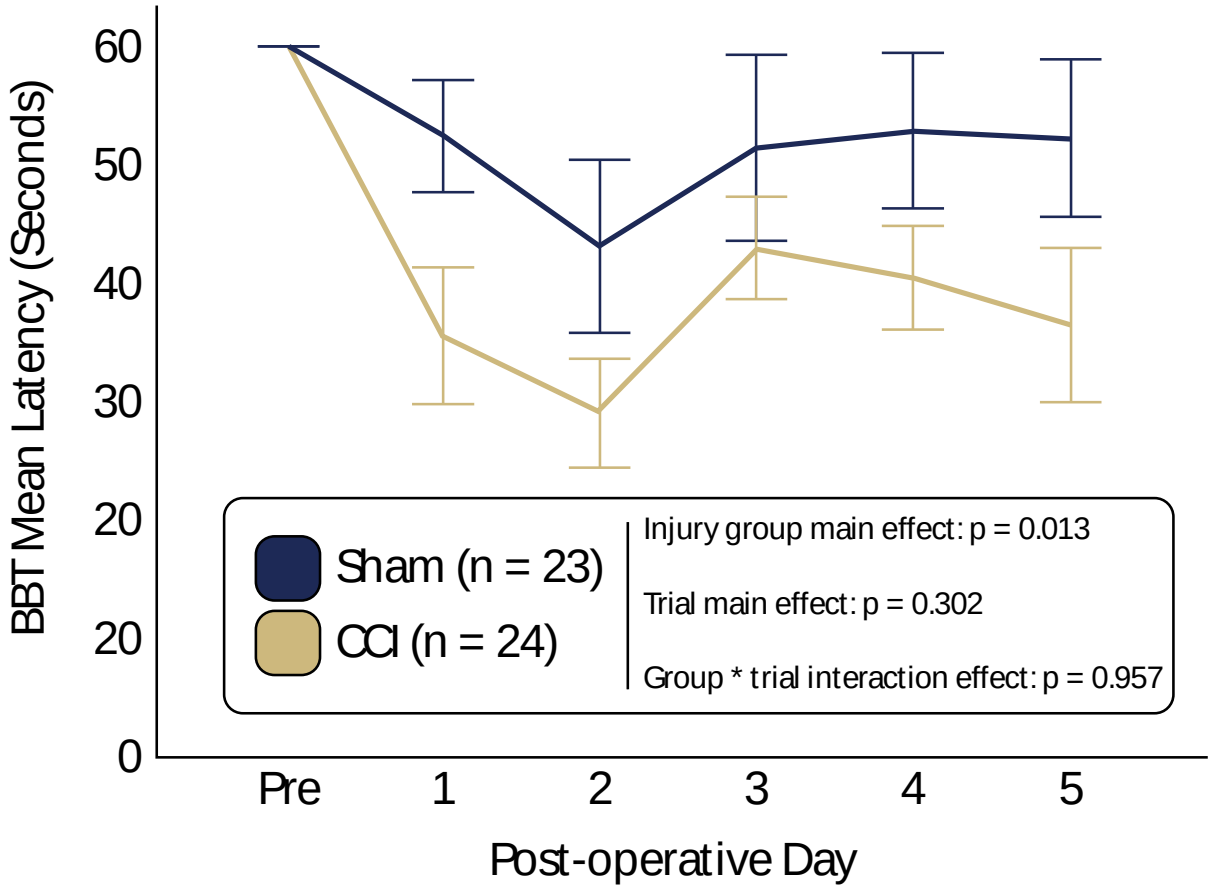

Figure 17: BBT results from the full pilot sample

2.1.3.2 Novel object recognition (NOR) Despite the favorable results in the preliminary analysis of the NOR data prior to the Comprehensive Examination & Overview (Figure 8), the significant effects were lost in the full pilot sample after eliminating hypothermic (or non-monitored) mice. When means in the sham + vehicle and CCI + vehicle groups were compared, there was no significant difference in either the discrimination index (Figure 18), nor the discrimination ratio (Figure 19). Thus, the decision was made to remove the NOR task from the study.

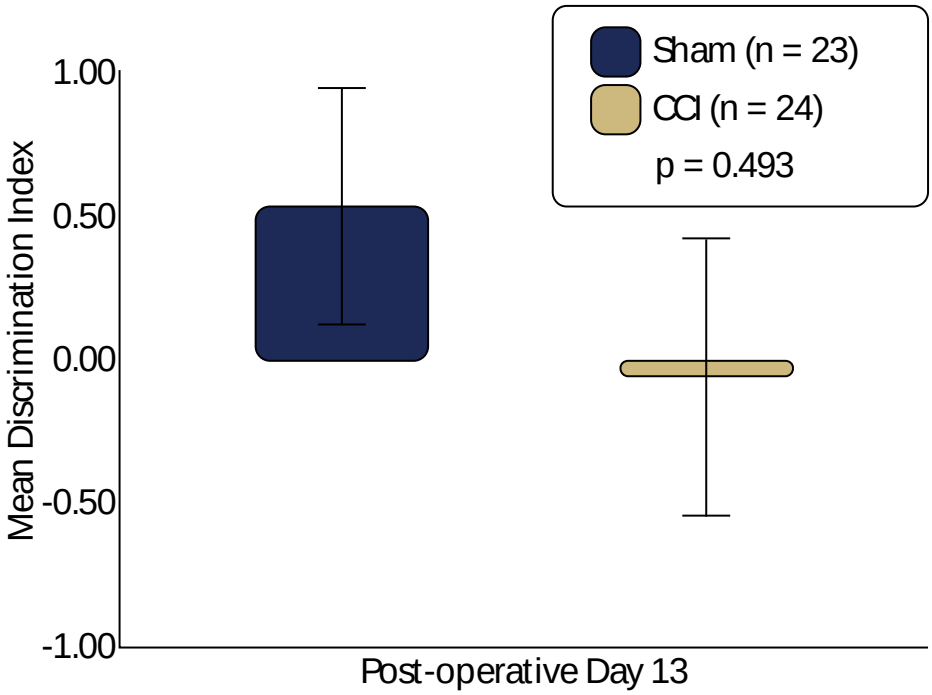

Figure 18: NOR DI results from the full pilot sample

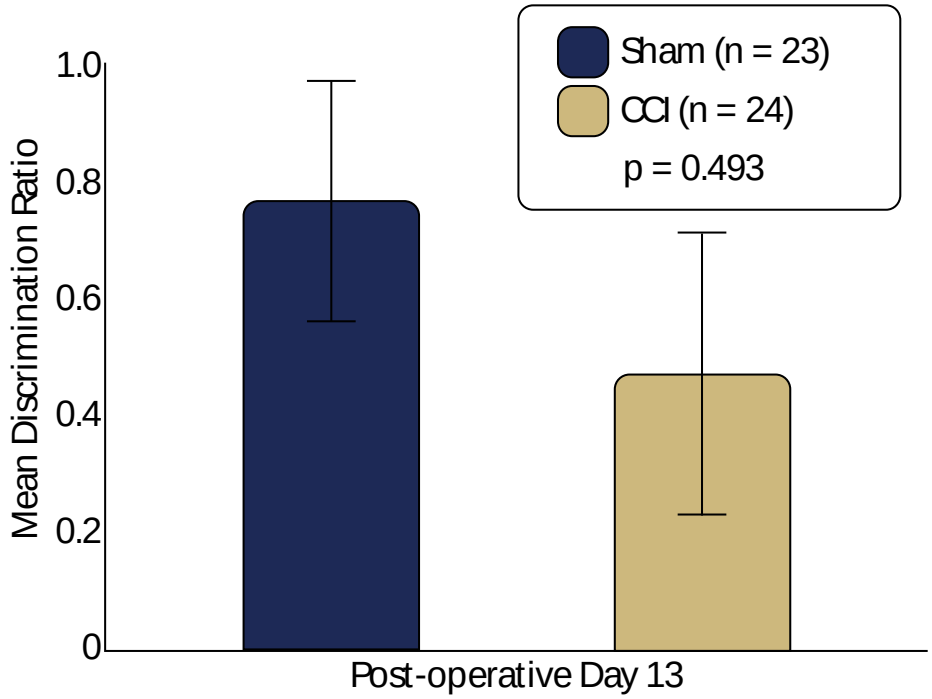

Figure 19: NOR DR results from the full pilot sample

2.1.3.3 Morris water maze (MWM) The MWM is a well-established behavioral test in both mice and rats that has been used extensively in previous TBI studies using mice (Gao et al., 2015; Hu et al., 2015; Xuan, Huang, & Hamblin, 2016; Z. Zhang et al., 2015). In preliminary analysis of the pilot data, there was a significant injury effect (Figure 5); however, it was later determined that many of the mice in this sample were either hypothermic at the time of surgery or did not have their temperature recorded and the decision was made to exclude them from subsequent analysis. When all the valid pilot data was considered together (Figure 20), there was no significant main effect as expected for injury (p= 0.346). The anticipated learning of the maze over time was observed (p= 0.014), though there was no significant interaction effect (p= 0.538).

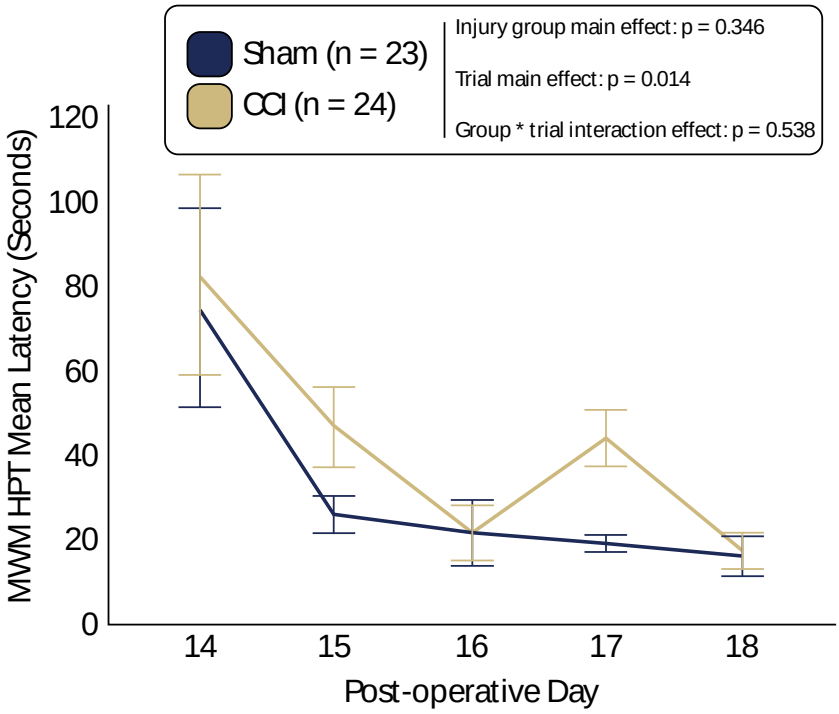

Figure 20: MWM HPT results from the full pilot sample

On the MWM probe trial (Figure 21), there was no injury effect (p= 0.340). This means that CCI exposed animals and their sham injured counterparts did not significantly differ in the amount of time spent in the target quadrant (suggesting poorer memory). This is not consistent with expectations based on past performance of mice where significant injury effects were reported (G B Fox, LeVasseur, & Faden, 1999; Sinz et al., 1999).

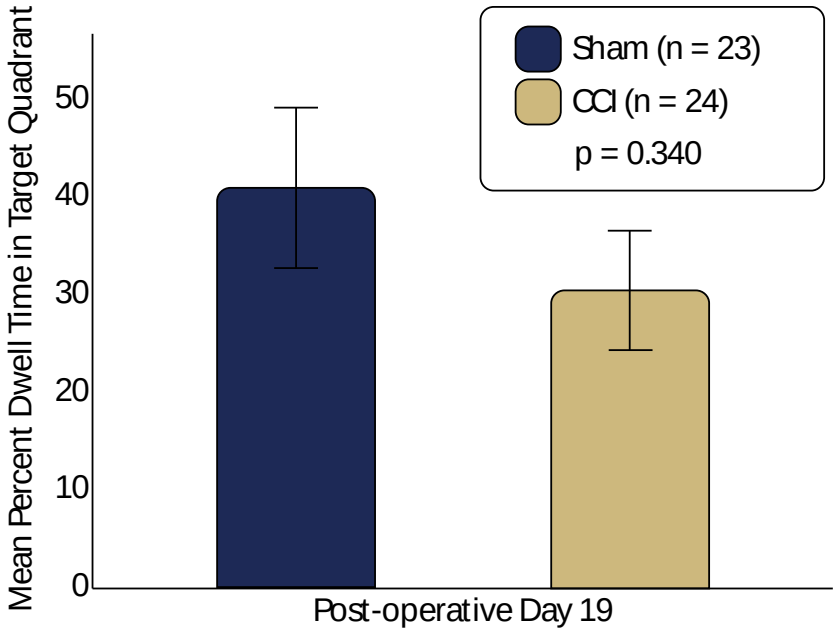

Figure 21: MWM probe trial results from the full pilot sample

Perhaps more concerning was that MWM probe trials variables that were expected to *not* significantly differ across the two injury exposure groups *did* show injury effects. Specifically, the injured animals swam further overall (p= 0.026) but less in the target quadrant (p= 0.002), as plotted in Figure 22. Similarly, CCI-exposed mice swam faster than their sham counterparts both overall (p= 0.025) and in the target quadrant (p= 0.020), as plotted in Figure 23. Based on this evidence the decision was made to exclude the MWM from the dissertation study.

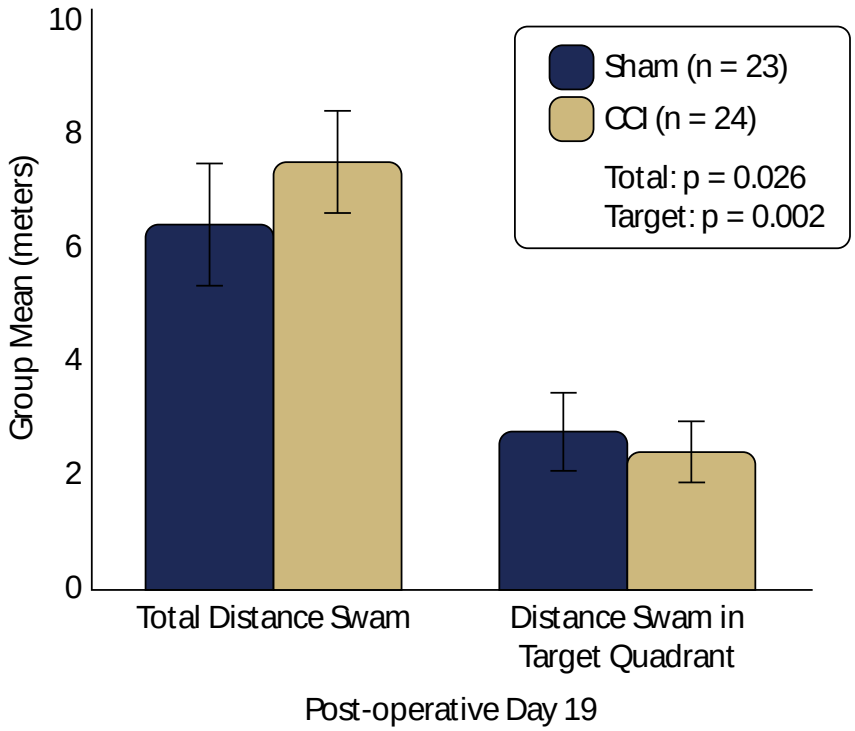

Figure 22: MWM probe distance variables results from the full pilot sample

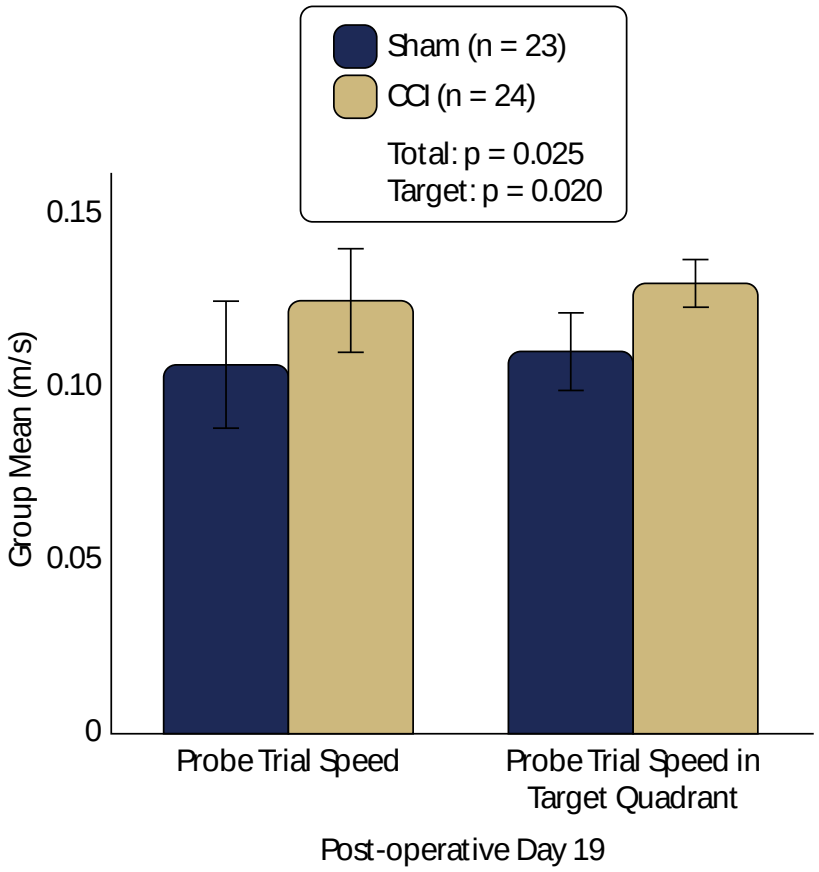

Figure 23: MWM speed variables results from the full pilot sample

2.1.4 Possible sources of error and other issues

There are several sources of errors that are potentially contributing to the lack of injury effects on key variables in this study. One possible confounder is hypothermia, which well documented as being associated with neuroprotection in the context of TBI (Bayir et al., 2009; Clark et al., 1996; McIntyre, Fergusson, Hébert, Moher, & Hutchison, 2003; Urbano & Oddo, 2012). The definition of normothermia was expanded from 36.5°C-37.5°C to 36.0°C-37.5°C based on existing literature regarding hypothermia and the low number of mice meeting the original definition of normothermia. Moreover, there were times when the environmental controls in the mouse holding

room were not within prescribed ranges. On these days, temperatures were as low as 12.78°C and as high as 36.6°C causing conditions which may have impacted the results of behavioral testing. Anesthesia is another possible confounding variable; isoflurane has neuroprotective properties that may alter the results of TBI studies (Statler et al., 2000, 2006). It could not be justified omitting anesthesia or using another type of anesthesia based on the side effect profiles of available alternatives. Additionally, isoflurane is commonly used in pre-clinical TBI research and its use make the research results more easily comparable to the existing literature. A final possible source of error specific to behavioral testing is the large number of technicians the laboratory employs. While all technicians are thoroughly trained and monitored (as well as blinded to group assignment), it is possible that subtle variation in how the tests were conducted may have affected the variability and lack of injury effects observed. For example, one technician may be less proficient when placing the mouse on the BBT apparatus, which could affect performance on the test. Alternatively, there may be minor deviations in stopwatch use.

Beyond the aforementioned issues during the pilot study, additional rationale for modifying the dissertation study comes from recently published evidence, which added to the conflicting evidence base surrounding therapeutic administration of MEL after experimental TBI. Some studies showed beneficial effects (Babaee et al., 2015; Ding et al., 2015; Shochat & Abookasis, 2015), though not necessarily for all variables examined. One study found no beneficial effects of MEL therapy on any of the cellular or behavioral outcomes examined (Kelso et al., 2011). Some of the therapeutic regimens tested resulted in deleterious effects. For example, four i.p. injections of 200 mg/kg MEL was associated with pathology, specifically increased malondialdehyde (MDA) levels indicative of oxidative stress (Cirak et al., 1999). In another study, a single 150 mg/kg injection was associated with edema, as well as worsened outcomes on composite

neuroscore, vibrissae stimulation study, and beam balance task (Jadhav et al., 2009). Moreover, of the studies that found beneficial effects, many pathways were implicated including apoptosis (Babaee et al., 2015; Ozdemir et al., 2005; Yürüker, Naz, & Nilgün, 2014), oxidative stress (Ates et al., 2006; Cirak et al., 1999; Ozdemir et al., 2005), inflammation (Lin et al., 2016), autophagy (Ding et al., 2015), and several intracellular signaling and transport cascades (Kelestemur et al., 2016; Wu et al., 2016). This recent evidence also identified factors that complicate MEL therapy, including a need for early post-TBI administration (Mésenge et al., 1998), differences with daytime vs. nighttime administration (Sarrafzadeh, Thomale, Kroppenstedt, & Unterberg, 2000), and confounding effects of exposure to prolonged darkness (Ucar et al., 2005).

2.1.5 Alternative CCI model trialed

As part of a final effort to salvage the study aims as originally planned, the experimental model was slightly modified. Specifically, instead of the pneumatic CCI model, an electromagnetic model was trialed with an injury depth of 2.5 mm. While the results among normothermic mice were more promising (Figure 24) with the pneumatic model ($p= 0.002$), there remained great difficulty maintaining normothermia intraoperatively, which greatly slowed experimental procedures and still resulted in some mice being excluded due to hypothermia. Based on these difficulties, the decision was made to modify the study substantially, as outlined in section 2.1.7.

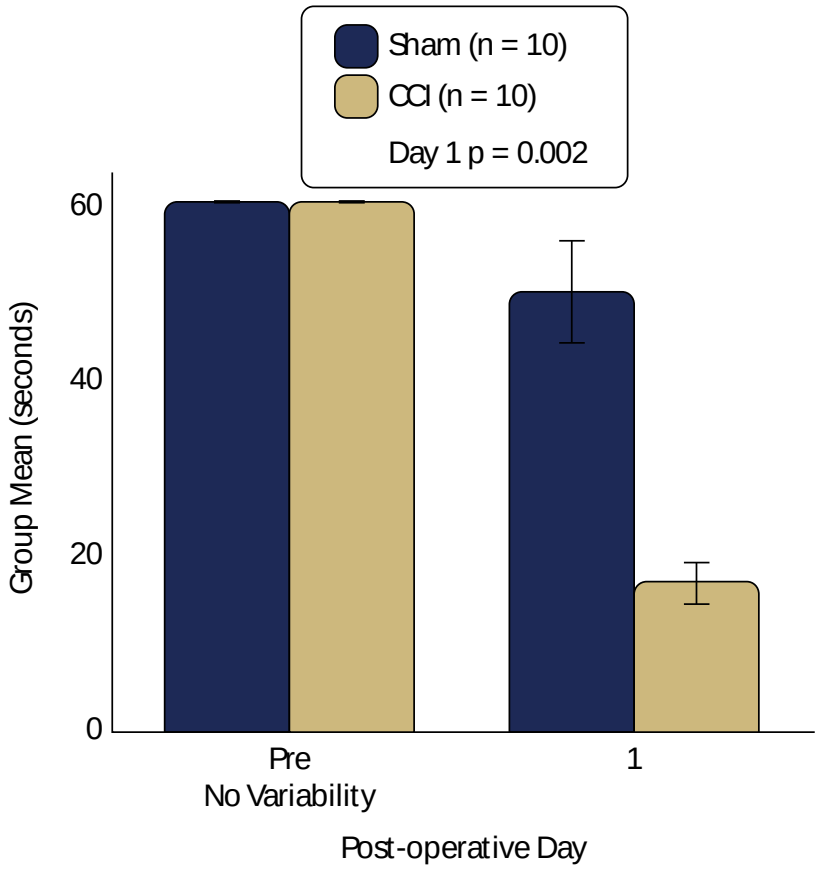

Figure 24: BBT in electromagnetic injury model

2.1.6 Delayed breeding of knockout mice

Another unanticipated and unavoidable barrier to conducting the research as originally planned was that the supplier of the MT1 knockout mice was unable to deliver the necessary test animals on the required schedule to allow for enrollment of these mice in the dissertation study. As these mice have already been paid for, the knockout experiments will be completed as part of a follow-up study.

2.1.7 Changes made to study plan

Based on the aforementioned issues with the pilot sample and lack of significant injury effects in the data, it was not justified to administer melatonin therapeutically. Based on the lack of an injury effect on the behavioral tests of interest, combined with the lack of current evidence supporting these hypotheses, the decision was made to de-emphasize behavior. When the knockout mice become available, behavioral testing will be conducted as originally planned but this data is no longer a part of the planned dissertation study.

2.1.7.1 Refinement of variables The decision to focus on the endogenous changes to melatonergic receptors and post-traumatic changes in the apoptotic cascade was difficult. However, delays in breeding, combined with unexpected results in the pilot study led to modification of the outcome variables of interest in this dissertation project. Behavioral outcomes were de-emphasized for two reasons: 1) evaluation of functional deficits was premature given the current state of the science; 2) in the pilot sample there was no injury effects or interaction effects demonstrated for the functional outcome variables of interest (BBT, MWM, NOR). Thus, the dissertation study was revised to look only at levels of melatonin receptor and apoptotic proteins. To strengthen the study, evaluation of receptors was expanded to include both MT1 and MT2. Notably, the apoptotic marker was also changed, from caspase-3 to Cytochome C (CytC), as continued western blots using several caspase-3 antibodies failed to produce clear and consistent results.

2.1.7.2 Replacement of animal model Mice were initially chosen because there are more knockout strains available than in rats, including a well-characterized MT1 knockout mouse. Due to unanticipated and unavoidable delays in mouse breeding, the knockout mice were not available to be included in this dissertation study. However, once the mice become available, the comparison of MT1 KO and wildtype mice will be ongoing as part of a separate follow-up study. The original pilot study used C57BL/6J mice. The sample was changed to rats for this dissertation project for the following reasons: 1) by replacing the animal model, generalizability is expanded; 2) rats are less prone to the problems effecting mice in the pilot work (e.g. hypothermic temperatures during surgery) and; 3) the laboratory has a history of more consistent results with rat models.

2.1.7.3 Modification of brain injury model With the exchange of the animal model from mice to rats, it was necessary to scale the injury parameters accordingly. The original pneumatic CCI device was used, but the depth of tissue deformation was increased (from 1.6 mm [pneumatic CCI in mice] or 2.0 mm [electromagnetic CCI in mice] to 2.8 mm [pneumatic CCI in rats]). Impact tip size, which was increased from 3 mm to 6 mm (and a larger craniectomy was made to accommodate the larger tip). The dwell time of the CCI device in rats was 150 ms (vs. 100 ms in mice). Another change that applies to both the sham and CCI rats is the use of intubation and mechanical ventilation to provide oxygenation and anesthesia during surgery (vs. a nose cone in mice). These are routine procedures for inducing CCI in rats. Additional minor modifications to the surgical procedure were made as well for practical reasons (e.g. larger hair trimmers were used to prepare the surgical site), though these are not anticipated to affect the data.

2.1.7.4 Revised sample and groups In total, 25 rats were randomly assigned into 4 groups of n= 6-7, divided into 2 injury exposures and 2 sacrifice time points as follows (Table 4). Based on existing literature, a minimum sample size of 6 per group will be sufficient to detect significant differences using western blot (Cutler et al., 2007; J. Y. Ding et al., 2009; Ley, Clond, Singer, Shouhed, & Salim, 2011).

Table 4: Dissertation study sample and groups

	Sacrifice Time Point (Post-Surgery)	
Injury Exposure	*6 hours*	*24 hours*
Sham control	N = 6	N = 6
Severe controlled cortical impact (2.8 mm depth driven)	N = 6	N = 7

2.1.7.5 Revised specific aims Accounting for the changes to the research goals and methodologies that occurred after the PhD candidate passed the Comprehensive Examination & Overview, the aims of this dissertation are:

- *Aim 1:* Evaluate the effects of CCI on levels of melatonin-specific receptor (MT1 and MT2) in the frontal cortex and hippocampus 6 hours and 24 hours after injury induction in a rat model.
- *Aim 2:* Evaluate the effects of CCI on levels of the apoptosis executioner protein cytochrome C (also in the frontal cortex and hippocampus 6 hours and 24 hours after injury) and correlate any changes in apoptosis observed with any changes in receptor levels found in Aim.

2.1.7.6 Revised methods Apart from the aforementioned changes in the sample, de-emphasis on behavior, and scaling of the injury model to produce severe injury in rats, no major revisions to the methods were made. The study still compared pneumatic CCI to sham control. Animals were sacrificed, their brains harvested, tissues of interest dissected and flash frozen for later processing by western blot analysis, probing for MT1, MT2, and known apoptotic protein cytochrome C. In this study only the hippocampus and frontal cortex were analyzed, the rationale being that these regions play important roles in cognitive function, are vulnerable to injury, and are known to express MT1 and MT2.

One minor change was the shifting up of the sacrifice time point to either 6 hr or 24 hr post-injury. The rationale for this change was two-fold. Most importantly, the timeline was truncated because apoptosis is known to occur rapidly after TBI (Barth et al., 2000; Conti et al., 1998; Xiong, Lin, Chen, Peterson, & Lee, 2001). We do not anticipate significant apoptosis to continue beyond the 24 hr time frame. A second reason to shorten the study was to save on the costs associated with

prolonged survival (e.g. housing; husbandry; medication) for behavioral testing, which has been cut from this study due to poor performance of these tests in the large and costly pilot study.

2.1.7.7 Revised analysis In response to the modifications to the sample and methods (i.e. variables included), changes were made to the analysis. First, there are no longer repeated measures assessments so no repeated measures ANOVA were performed. Second, in the western blots, only two groups (CCI; sham) will be compared, leading to the use of independent samples t-tests to compare protein levels in sham vs. injured rats. Correlation analysis will be used to explore if the outcomes of interest are associated.

2.1.8 Summary of study after modifications

Following the modifications of the study based on the above rationale, the dissertation study sample is as follows: N= 25 adult (weighing 275-375 g at time of surgery) male Sprague Dawley rats (Harlan Laboratories, Inc., Indianapolis, IN, USA). All animals were allowed to acclimate to the new housing environment for at least 2 days prior to handling, per institutional protocol. The rats were randomly assigned into two injury groups: CCI (depth of 2.8 mm) or sham control (identical treatment excluding injury induction). The rats were randomly assigned into two post-injury sacrifice time points: 6 hr or 24 hr post-injury.

Animals were sacrificed at one of two randomly assignment post-injury time points (6 hr or 24 hr post-injury). Following sacrifice, brains were harvested and the ipsilateral hippocampus and frontal cortex dissected out and flash frozen in liquid nitrogen. Tissue samples were homogenized and analyzed using western blot; levels of melatonin receptors and apoptotic proteins were semi-quantified using ImageJ software.

2.1.9 Discussion

At the beginning of this study, it was not anticipated that there would be a pinworm outbreak and subsequent building closure; nor was it anticipated that the decontamination efforts would leave the laboratory in a noxious state. These events were beyond the candidate's control and significantly delayed the workflow of the project such that there was not time to continue to optimize the injury parameters in mice and still complete this project in the allotted timeframe (which is dictated by a grant end date and post-doctoral position start date).

Moreover, as discussed previously (see Section 2.1.4), recently published evidence has cast additional doubt on the appropriateness of the original specific aims as presented at the time of the Comprehensive Examination & Overview. The state-of-the-science, combined with the abovementioned problems encountered during the pilot study lead to the decision to alter the study plan (see Section 2.1.7). The decision was made to switch from mice to rats, because the breeding of the transgenic mice is delayed. Moreover, maintaining intra-operative temperature proved to be challenging even when using a heating pad in combination with warming lamps and insulating blankets to keep the heat near the mouse. Rats being larger are less likely to experience significant temperature drops during anesthesia and throughout the surgery, as occurred in many of the pilot studies using mice (see Section 2.1.1).

In originally designing the study, it was acknowledged that there are limitations of behavioral testing in rodents, especially mice. However, based on past performance of the MWM, NOR, and BBT, it was not anticipated that the tests would not produce injury effects and/or injury*trial interaction effects. However, based on the poor performance of these tasks presented in Section 2.1.3, it was not justified to include behavioral testing moving forward. Though rats tend to be more amenable to behavioral testing, the lack of anticipated injury effects in the pilot

study reinforced the need for thorough optimization of study parameters before completing the final study. The inclusion of these variables did not enhance the study in such a way that warranted this effort. The aims and methods were adjusted accordingly as presented in Section 2.1.7.

2.2 STRENGTHS AND LIMITATIONS OF THE REVISED STUDY

A major strength of this study is the responsiveness of the team to the issues that arose and subsequent adjustment to the plan. Moreover, the removal of behavioral variables followed extensive efforts to optimize these variables, including adding mice to the sample, switching to having a single technician perform our behavioral testing, and trialing the electromagnetic CCI model; the pilot data simply did not support the inclusion of these measures and there were concerns that it would be impossible to optimize the variables in time to complete the dissertation study. Similarly, this study is strengthened by adjustment in response to recently published evidence, which suggests that the effects of MEL therapy are conflicting and the mechanism unknown (Cirak et al., 1999; Jadhav et al., 2009; Kelestemur et al., 2016a; Kelso et al., 2011; Sarrafzadeh et al., 2000; Yürüker et al., 2014). Another major strength of this study is the extensive work went into studying the pathway of interest, developing a conceptual framework, and supporting this framework with empirical evidence. Finally, this study is strengthened by its novelty; while molecular-genetic and preclinical research is on the rise in nursing, it is still a relatively new part of the nursing research portfolio.

There were several limitations associated with this dissertation study. The sample size is small though likely to be sufficient for detecting significant group differences using western blot analysis (see section 2.1.7.1). It is also limited in that all study activities use a rodent model

including the pilot study (mice) and dissertation study (rats). The ultimate goal is clinical translation, but at this point the state-of-the-science including this project is limited to pre-clinical efforts. A further limitation is that only cellular endpoints are included; validation of pathology with behavioral deficits would strengthen both the overall quality of the evidence as well as the clinical relevance.

2.3 FUTURE STUDIES AND IMPLICATIONS FOR NURSING

Traumatic brain injury is a global problem that affects individuals across the lifespan, currently there are no FDA-approved treatment known to consistently improve outcomes of brain trauma. Thus, there remains a tremendous impetus to continue to conduct research aimed at better understand the pathophysiology of TBI and target it therapeutically. The melatonergic system has been studied after TBI with inconsistent findings to date. Genetic polymorphisms in genes encoding MEL-specific receptors have been reported (Barrett et al., 1997; Choudhury et al., 2014; Natarajan et al., 2012), attempts to explore the effects of this genetic variation after TBI will enhance the knowledge base. Additional efforts to explore changes in the endogenous melatonergic system after TBI are necessary as well as further attempts to target this system therapeutically. Future studies should expand this work, by diversifying samples (e.g. species/strain; age; sex; genotype) as well as add additional cellular (and behavioral) measures; this will enhance scientific understanding regarding changes to the melatonergic system after TBI and the effects and mechanism of therapeutic MEL. Additionally, efforts to translate the research along the phylogenetic tree and extend the work to more sentient mammals and ultimately clinical trials will be necessary. Since the melatonergic system is affected by lifestyle factors (e.g. exposure

to darkness), it will be challenging to study within the realities of the clinical setting; efforts to control for potential confounders will be necessary.

This pre-clinical study has limited direct relevance to nursing practice at this time. It does increase knowledge of the physiologic effects of the melatonergic pathway, particularly after TBI. This dissertation represents the first step in the principal investigators research career as a nurse scientist so in that way the project indirectly contributes to the nursing profession. Taken together the body of work on this topic which includes basic science, nursing science, and biomedical research has a potential clinical application. For example, melatonin may be translated as a therapy after TBI and genotype may be used to guide to determine who is likely to benefit from such a therapy.

3.0 DATA BASED MANUSCRIPT: MELATONIN RECEPTOR CHANGES AFTER TBI IN RATS

3.1 ABSTRACT

Background: Traumatic brain injury (TBI) is a costly and devastating acquired condition that affects individuals of all ages, races, and geographies via a number of injury mechanisms.

Purpose: The purpose of this study is to garner preliminary evidence which characterizes endogenous changes in the melatonergic system after experimental TBI.

Population: To produce a clinically relevant yet highly controlled model of TBI, adult male Sprague Dawley rats were used with a total of 6-7 rats per group for a total of 25 rats.

Methods: Rats were randomly assigned to receive either controlled cortical impact TBI or sham surgery. This study examined outcomes at 2 post-surgery time points, sacrificing animals at 6 hours (hr) or 24 hr post-injury. Brains were immediately harvested, and the ipsilateral hippocampus and frontal cortex dissected and flash frozen. Whole cell lysates were prepared and the supernatant fluid collected, vortexed, aliquoted, and used for western blot analysis. Primary antibodies were used to probe for cytochrome C, melatonin receptors (MT1 and MT2), and beta actin for normalization. ImageJ and Image Lab software were used to analyze the data with t-tests to compare group means on a single variable; correlation testing was also used to explore the relationship between outcomes of interest.

Results: Melatonin receptors were down-regulated in a brain region- and time point- dependent manner. MT1 was downregulated in the frontal cortex at 24 hr and in the hippocampus at both 6 hr and 24 hr post-injury. Similarly, MT2 was downregulated in the frontal cortex at 24 hr and in the hippocampus at both 6 and 24 hr post-injury. Cytochrome C levels suggested TBI led to initiation of apoptosis, which for some brain regions and time points correlated with receptor changes.

Discussion: This is the first study to report downregulation of MT1 and MT2 after injury, which may affect the efficacy of MEL therapy. Additional research characterizing these changes after TBI is necessary, including efforts to establish the time course and regional patterns, replication in more diverse samples, as well as inclusion of additional cellular, histological, and behavioral endpoints.

Conclusion: TBI in rats modeled using CCI results in acute downregulation of MEL-specific receptors (subtypes MT1 and MT2); replication of these findings is necessary as is evaluations of the implications of lower receptor levels.

Keywords: Traumatic brain injury (TBI); brain trauma; controlled cortical impact (CCI); rat; melatonin

3.2 BACKGROUND AND INTRODUCTION

TBI is a devastating condition that globally affects individuals at all stages of life (Colantonio et al., 2010; Faul et al., 2010; Feigin et al., 2013; Puvanachandra & Hyder, 2008; Reilly, 2007; Roozenbeek, Maas, & Menon, 2013; Scudellari, 2010). TBI comes at high personal and monetary cost. In the United States of America (USA) alone, the direct and indirect costs, in one recent

estimate totaled a staggering $76.5 billion (Coronado et al., 2012). Unfortunately, despite the extensive care received by many TBI survivors including emergent care, intensive care, step down care, physical therapy, and/or occupational therapy, acute and chronic disability remains common (Leo & McCrea, 2016; Wilson, Jones, Weedon, & Bilder, 2015). A recent estimate found 3.2 million Americans were living with one or more TBI-related disabilities (Corrigan et al., 2010). Existing evidence suggests that TBI survivors experience cognitive, behavioral, emotional, and psychological comorbidities (Wilson et al., 2015) that negatively impact quality of life (Anderson et al., 2002; Corrigan et al., 2010; Ergh et al., 2002; McKinlay et al., 1981; Schalen et al., 1994; Tate & Broe, 1999) and cause distress to the survivors and their families (Anderson et al., 2002; Ergh et al., 2002; McKinlay et al., 1981; Schalen et al., 1994).

No novel therapies have demonstrated sufficient efficacy and safety to warrant translation to TBI clinical care. Indeed, as of 2013, countless therapies had been tested in pre-clinical trials, and the most promising 45 were tested in phase II and III clinical trials, yet none culminated in FDA-approved therapies (Gold et al., 2013). Thus, the quest to identify effective therapies for TBI remains a worldwide initiative. Many major barriers to identification of new effective therapies exist. For example, TBI is characterized by a wide variety of cellular and histopathological changes (Osier et al., 2014). Also, in order for a TBI therapeutic to be effective, it must be able to reach and exert its effects in the brain; this is surprisingly difficult, as many pharmaceutical compounds lack the necessary properties (e.g. small; lipophilic) to cross the blood-brain-barrier (Bonate, 1995; Nau, Sorgel, & Eiffert, 2010; van de Waterbeemd, Camenisch, Folkers, Chretien, & Raevsky, 1998).

One promising potential TBI therapeutic is melatonin (MEL), which readily and rapidly crosses the blood-brain-barrier (Di Bella & Gualano, 2006; Le Bars et al., 1991; Reiter et al.,

2007). In addition to being an endogenous hormone produced in various sites, but primarily the pineal gland, MEL is available as a medication and over-the-counter supplement. Importantly, MEL has a known low toxicity profile in both human and animal studies (Jahnke et al., 1999; Seabra et al., 2000; Wiechmann et al., 2008). Existing evidence suggests MEL may be important in the body's response to TBI and there may be an opportunity for exogenous MEL therapy.

MEL levels are altered during the acute post-TBI period (Paparrigopoulos et al., 2006; Seifman et al., 2008; Shekleton et al., 2010). In one study (Seifman et al., 2008), researchers compared MEL levels in CSF and serum after TBI to those in uninjured controls who had neurosurgery for another reason. Biphasic fluctuation of MEL levels in CSF were reported after TBI, whereby MEL gradually increased out to 2 days post-TBI and then decreased to a minimum on day 5 before raising again to a maximum on day 8. With the exception of day 0, 1, and 4, CSF MEL levels were significantly elevated in the TBI survivors (vs. controls). Serum MEL levels also increased from admission to day 2 post-TBI and then reached a minimum level on day 5; however, no significant differences in serum MEL levels between TBI patients and controls was reported (Seifman et al., 2008). Another study (Paparrigopoulos et al., 2006) examined MEL levels in the blood 8 times per day during the first two days after initial trauma but did not include a control group for reference. Still, MEL concentrations in the blood were lower than standard clinical ranges. Moreover, MEL levels in this study were associated with injury extent assessed using the Glasgow Coma Scale (GCS); in the subgroup with a lower GCS, there was greater disruption of MEL levels (Paparrigopoulos et al., 2006). A third study (Shekleton et al., 2010) examined long-term effects of neurotrauma on salivary MEL levels, comparing TBI survivors 6 months after the initial insult (n= 14) to non-TBI controls (n= 14). There was no significant difference in dim light

melatonin onset between injured individuals and controls; however, total MEL production over the entire sampling period was significantly higher ($p= 0.031$) in controls (Shekleton et al., 2010).

In addition to its aforementioned properties, MEL has beneficial cellular effects in pathways activated as part of secondary injury cascades after TBI. To date, most research has focused on the antioxidant and free radical scavenging properties of MEL (Dikmenoglu et al., 2008; Hashimoto et al., 2012; Onur et al., 2004; Tamura et al., 2013; Tan et al., 2002; Taysi et al., 2008), though in some conditions MEL may contribute to oxidative stress (Cirak et al., 1999). Anti-apoptotic effects of MEL therapy have been demonstrated in several organs (Aktas, Kanter, Erboga, Mete, & Oran, 2012; Bai et al., 2013; Cekmez et al., 2013; Celik & Nazıroğlu, 2012; Espino, Rodríguez, & Pariente, 2013; Gürpınar et al., 2012; Laothong et al., 2013; Li, Zhang, & Tang, 2013; Liang et al., 2012; Mukherjee et al., 2012; Park et al., 2012; Patschan et al., 2012; Simsek et al., 2012; Sinanoglu et al., 2012; Sokolovic et al., 2013; Tresguerres et al., 2012; Tuñón et al., 2013; Wang et al., 2013; Wang et al., 2013), including the brain (Alonso-Alconada et al., 2012; Bavithra et al., 2013; Kireev et al., 2013; Ma et al., 2013; Olcese et al., 2009; Ozyener et al., 2012; Reiter et al., 2005; Samantaray et al., 2008, 2009; Suwanjang et al., 2013; Wang et al., 2011; Wang, 2009; Zhang et al., 2013). Pre-clinical evidence from models of TBI demonstrate neuroprotective/anti-apoptotic effects of MEL (Campolo et al., 2013; Jadhav et al., 2009; Kelso et al., 2011; Mésenge et al., 1998; Ozdemir et al., 2005). Apoptosis a highly regulated process (Barth et al., 2000; Bayir et al., 2007; Conti et al., 1998) of genetically programmed cellular death that destroys highly damaged cells. After TBI, apoptosis results in decreased numbers of viable brain cells, as well as correlates with lesion size and functional status (Jain, 2008; Reilly, 2001; Werner & Engelhard, 2007). The early onset of apoptosis and association with symptoms after TBI make it a promising target for drug therapy.

Despite the beneficial characteristics of MEL, current evidence remains limited to 22 published studies (current as of 2016-06-25), which yielded conflicting evidence. Complicating the evidence and making comparisons difficult are differences between studies that include: the therapeutic regimen(s) tested, the nature of the injury, and animal strain. Moreover, some studies failed to report important characteristics pertaining to experimental design that would be necessary to replicate the study. Subsequently, the National Institute of Neurological Diseases and Strokes (NINDS) developed a set of Common Data Elements (CDEs) for use in pre-clinical TBI research, in an attempt to establish standards for reporting (Smith et al., 2015). More concerning, none of the studies to-date have confirmed that a major target for MEL therapy (MEL-specific receptors, MT1 and MT2) is unaltered by TBI; the fact that no study to-date has measured MEL-receptor levels after TBI represents a major gap in the knowledge base. The hypothesis underlying this study is that downregulation of melatonin-specific receptors (MT1 and MT2) may occur in response to TBI, as has also been reported in an animal model of depression (Wang et al., 2012), and has also been reported following treatment with the melatonin receptor antagonist luzindole (Kokkola, Vaittinen, & Laitinen, 2007). The primary goal of this study is to address the gaps in the knowledge surrounding the endogenous changes that occur in the melatonergic system, specifically changes in melatonin-specific receptor levels (MT1 and MT2) within the hippocampus and frontal cortex during the acute (6 hr and 24 hr) post-injury period. A secondary goal is to examine any associations between receptor changes and levels of the known apoptotic protein (CytC).

3.3 METHODS

3.3.1 Methods overview

All experimental procedures were approved by the Institutional Animal Care & Use Committee prior to beginning study activities. The test animals were housed in a climate-controlled housing facility on a 12 hr light/dark cycle. In this study an experimental TBI model was used, specifically pneumatic controlled cortical impact (CCI) in rats. Test animals were randomly assigned to one of four groups: CCI with sacrifice 6 hr post-injury, CCI with sacrifice 24 hr post-injury, sham surgery with sacrifice 6 hr post-injury or sham surgery with sacrifice 24 hr post-injury.

3.3.2 Sample

The target clinical population in this study is survivors of acute, severe traumatic brain injury. However, consistent with the state of the science, a pre-clinical model of TBI was used as this facilitated standardization and control over injury as well as control over other potential confounders; moreover use of a pre-clinical model facilitated analysis of brain tissue at precisely controlled time points, which would not be possible in human subjects research. In this study, Sprague Dawley rats (Harlan, Indianapolis, IN, USA) were used. To control for the confounding effects of age, brain development, and effects of sex on TBI outcomes (Mannix, Zhang, Park, Zhang, et al., 2011; Sandhir & Berman, 2010; Taylor, Smith, Harris, Costine, & Duhaime, 2013;

Yakovlev et al., 2001), all rats were young adult (10-14 week old) males, weighing 275-375g, at the time of injury. The choice of all male test animals better mimics the TBI population which is disproportionally male in every age group (Coronado et al., 2012). In total, 25 rats were included in this exploratory study. This facilitated a sample size of 6-7 rats per group across the 4 groups: severe CCI with 6 hr sacrifice (n= 6), sham with 6 hr sacrifice (n= 6), CCI with 24 hr sacrifice (n= 6), and sham with 24 hr sacrifice (n= 7).

3.3.3 Surgery, injury induction, and post-operative details

Animals in the sham and CCI group were treated identically prior to, during, and after surgery, excluding the impact using the CCI device. Prior to surgery the CCI device was examined and test fired to ensure proper working order (e.g. piston fires freely). Each rat was placed in an anesthesia induction chamber. Anesthesia was induced with 4.0% isoflurane in a 2:1 mixture of N_2O:O_2. The rat was intubated and placed into a stereotaxic frame, secured using bilateral ear bars and a single incisor bar. Isoflurane levels were reduced to a maintenance dose (2.0%) throughout the surgery, unless the test animal showed signs of regaining consciousness, in which case the dose was adjusted accordingly. The animal's head was shaved with electric trimmers and the surgical site prepared using betadine and sterile gauze. A scalpel was used to make a midline incision (~20 mm). The muscles were gently separated and the skin and fascia reflected using a periosteal elevator in combination with microdissecting forceps and cotton-tipped applicators. Once the skull was exposed, a pneumatic dental drill was used to create a craniectomy (i.e. bone window) located between lambda and bregma (anterior-to-posterior) and also between the coronal ridge and sagittal suture (medial-to-lateral) just large enough for unobstructed tip clearance. The detached bone flap was carefully removed using microdissecting forceps so as to not disrupt (i.e. breach) the

underlying dura. The bone flap was discarded, as is commonly done in pre-clinical TBI studies to avoid the associated secondary injury (e.g. increased intracranial pressure).

The piston was gently lowered to ensure that it was centered within the bone window and to confirm unobstructed clearance for the 6 mm diameter tip. The device was zeroed to the cortical surface and gently withdrawn to avoid disruption of the surgical site. The piston assembly was adjusted to reflect the desired impact depth of 2.8 mm, a velocity of 4 m/s, and a dwell time (i.e. duration) of 150 ms. At this point the device was actuated to induce TBI. The CCI device was then removed. The surgical site was sutured, topical anesthetic applied, and anesthesia discontinued. The animal was removed from the stereotaxic frame, extubated, and assessed for righting reflex. Following return of spontaneous locomotion, regular housing and husbandry resumed. Animals were monitored for evidence of pain and distress and analgesic was administered per institutional protocol. Sham animals received identical surgical and post-surgical treatment to injured animals but were not be exposed to the CCI itself.

3.3.4 Sacrifice

Animals were humanely euthanized at one of two post-surgery time points: 6 hr or 24 hr. At the time of sacrifice, animals were injected with Fatal Plus (0.25 mL per rat) and decapitated by guillotine. Brains were rapidly harvested and the ipsilateral fontal cortex and hippocampus dissected, placed in microcentrifuge tubes, and flash frozen in liquid nitrogen. Tubes were stored at -80° C until processed for analysis.

3.3.5 Tissue Processing

Lysis buffer was prepared, composed of: 0.01M Tris-Cl/0.1M NaCl, 0.001M ethylenediaminetetraacetic acid (EDTA), 1 µg/mL aprotinin, 100 µg/mL phenylmethylsulfonyl fluoride (PMSF). An equal volume of lysis buffer was pipetted onto the tissue (200 µL for frontal cortex; 100 µL for hippocampus). A sonicator was used to homogenize the tissue and generate whole cell lysates, which were centrifuged in a cold (4°) room for 30 minutes. Following separation of the layers, the supernatant fluid was collected into a microcentrifuge tube, vortexed to homogenize, and aliquoted out into smaller tubes to minimize the effects of freeze/thaw cycles.

3.3.6 Determine Protein Content

On the day in which the gel was run, protein content of the samples was determined using a Pierce bicinchoninic acid (BCA) assay (Thermo Fisher Waltham, MA, USA). Samples were diluted five-fold and loaded in duplicate into a 96 well plate. For comparison, 8 standards of known protein concentration were loaded in triplicate. A spectrophotometer (Molecular Devices, Sunnyvale, CA, USA), and associated Softmax Pro software (Molecular Devices, Sunnyvale, CA, USA) were used to determine the volume of supernatant fluid needed to load a consistent mass of 20 µg total protein per well.

3.3.7 Prepare Samples

Samples were prepared by combining the volume of sample needed for the desired mass of protein, with Bolt™ Sample Reducing Agent (Thermo Fisher Scientific, Waltham, MA, USA), and Bolt™

LDS Sample Buffer (Thermo Fisher Scientific, Waltham, MA, USA). The purpose of the sample buffer was to create a dye-front. The mixture was centrifuged before boiling for 10 minutes. Boiled samples were allowed to cool and were centrifuged to spin down the fluid.

3.3.8 Gel Electrophoresis

Prepared samples were loaded into a Bolt™ 4-12% Bis Tris Plus 15 well gel (Thermo Fisher Scientific, Waltham, MA, USA). To provide molecular weight comparisons, SeeBlue® Plus2 Pre-stained Protein Standard ladder (Thermo Fisher Scientific, Waltham, MA, USA) was loaded into an empty well adjacent to a sample-filled well. The gel was run at a constant 165 volts for approximately 30 minutes, until the dye front reached the bottom of the gel but did not run off.

3.3.9 Semi-Dry Transfer

A first generation Invitrogen Bolt™ semi-dry transfer system (Thermo Fisher Scientific, Waltham, MA, USA) was used. The transfer stack was prepared following the manufacturer's instructions with the anode stack on the bottom, the polyvinylidene fluoride (PVDF) membrane in the middle, and the cathode stack on top (Thermo Fisher Scientific, Waltham, MA, USA). The transfer program was run for a total of 7 minutes.

3.3.10 Evaluation of Gel

Following semi-dry transfer, the gel was retrieved and placed in a tray. A small volume (~5 mL) of GelCode™ Blue Stain Reagent (Thermo Fisher Scientific, Waltham, MA, USA) was poured

over the gel and was allowed to incubate on a rocker for at least 1 hour. This stain is comprised of a protein-specific colloidal coomassie dye which can be used to ensure that there were no issues surrounding the protein running through the wells during electrophoresis.

3.3.11 Treatment of Membranes

Immediately following transfer, membranes were labeled, rinsed with deionized water, labeled, and rinsed with methanol. Membranes were then washed in Tris-Buffered Saline and Tween 20 (TBS-T) for a total of 15 minutes, divided into 3 washes of 5 minutes each. Next, membranes were blocked for 30 minutes in 5% blotting-grade non-fat dry milk (BioRad, Hercules, California, USA) and incubated overnight in 5% milk with the primary antibodies as described in additional detail below. Following incubation with the primary antibody, membranes were re-washed for 15 minutes (3 washes at 5 minutes each) in TBS-T. Membranes were then incubated in 1% milk with the corresponding secondary antibody described below. A commercially available (Super Signal West Femto Maximum Sensitivity Substrate) two-component chemiluminescent solution (Thermo Fisher Scientific, Waltham, MA, USA) was applied to the membrane (total volume = 1.0 mL per membrane, with the two parts in equal volume). The membrane was imaged using a digital imager (BioRad, Hercules, California, USA). Following imaging, prior to repeating the blocking, staining, and imaging steps for the remaining antibodies, the membranes were washed (15 minutes, as before), stripped with Restore™ PLUS Western Blot Stripping Buffer (Thermo Fisher Scientific, Waltham, MA, USA) for 15 minutes and rewashed (15 minutes, as before).

First, membranes were probed for MT1 (ab184013, 1:1000, Abcam, Cambridge, UK) with goat-anti-rabbit secondary antibody (#31460, 1:5000, Thermo Scientific, Waltham, MA, USA). Next, membranes were probed for MT2 (ab203346, 1:1000, Cambridge, UK) with (#31460,

1:5000, Thermo Scientific, Waltham, MA, USA) goat-anti-rabbit secondary antibody. Third, membranes were probed for Cytochrome C (CytC) (136F3, 1:1000; Cell Signal Technology, Danvers, MA, USA) with (#31460, 1:5000, Thermo Scientific, Waltham, MA, USA) goat-anti-rabbit secondary antibody. Finally, to control for the possibility of differential protein loading, despite attempts to load standard total protein mass based on the results of BCA assay, beta actin (a2066, 1:2500, Sigma Aldrich, St. Louis, MO, USA) was used as a fourth and final primary antibody with (#31460, 1:5000, Thermo Scientific, Waltham, MA, USA) goat-anti-rabbit secondary antibody.

3.3.12 Analysis

Image J software (National Institutes of Health, Bethesda, MD, USA) was used in combination with Image Lab software (Bio-Rad, Hercules, CA, USA) to quantify data for analysis. All analysis was conducted using SPSS version 23 (IBM, Armonk, CA, USA) statistical software. Preliminary analysis was completed as follows: t-tests to compare protein levels of sham vs. injured rats at a single time point of either 6 hr and 24 hr post-operatively. Finally, correlation analysis was used to explore if the outcomes of interest were associated.

3.4 RESULTS

3.4.1 Post-operative outcomes

In this study, there was a 0% mortality rate associated with study procedures. Moreover, neither sham surgery nor CCI caused significant morbidity that would have necessitated a test animal being prematurely euthanized. Similarly, no seizure activity was reported as a result of the experimental procedures.

3.4.2 Protein Levels

Results from western blot analysis are summarized below. Data is also included as part of Appendix A, as a graph in (Figure 25), and as composite gel images for 6 hr cortical tissue (Figure 26), 6 hr hippocampal tissue (Figure 27), 24 hr cortical tissue (Figure 28), and 24 hr hippocampal tissue (Figure 29). The criteria for statistical significance was $p < 0.05$ (Note: criteria for statistical significance in all figures and tables is as follows: *$p < 0.05$; ** $p < 0.01$).

3.4.2.1 Cytochrome C In the frontal cortex, CytC was significantly elevated (p= 0.032) 6 hr after TBI as compared to sham surgery. Notably at the 24 hr time point, cortical CytC was significantly lower (p= 0.006) in the TBI group as compared to sham counterparts. In the hippocampus, CytC was significantly decreased (p= 0.001) after TBI at the 6 hr time point as compared to sham surgery but unchanged from sham levels at 24 hr.

3.4.2.2 MT1 When whole cell lysates from ipsilateral frontal cortex were compared using western blot, MT1 levels were reduced at 24 hr (p= 0.002), though they were unchanged from sham levels at 6 hr. However, in the hippocampus, MT1 levels were reduced at both 6 hr (p= 0.027) and 24 hr (p= 0.011).

3.4.2.3 MT2 As with the cortical MT1, cortical MT2 levels were reduced at 24 hr post-injury (p= 0.010), but unchanged from sham levels at 6 hr. In the hippocampus, MT2 levels were reduced at both 6 hr (p= 0.042) and 24 hr (p= 0.001) post-injury.

3.4.2.4 Beta Actin In all brain regions and time points examined in this study, there was no statistically significant change in beta actin levels after TBI (compared to sham). This is consistent with what has been reported previously (Borán & García, 2007; Budinich et al., 2012; G. Wang et al., 2012). This provided additional support for using actin to normalize the results of this study in an attempt to control for any inconsistencies when loading samples.

3.4.3 Correlations between apoptosis and other proteins of interests

The apoptotic marker in this study (CytC) did not correlate with beta actin levels in any time point and brain regions examined. Notably, CytC was correlated with levels of MT1 in the hippocampus at 6 hr ($p= 0.018$) and 24 hr ($p= 0.016$) after TBI. Likewise, cortical CytC and MT1 levels were correlated at both 6 hr ($p= 0.029$) and 24 hr ($p= 0.002$). For MT2 levels, there was no correlation with CytC in either brain region at the 6 hr time point. However, at 24 hr post-injury MT2 and CytC levels were correlated in both the hippocampus ($p= 0.018$) and frontal cortex ($p= 0.002$). Correlation coefficients and corresponding p-values are presented in Table 5 (see Appendix A).

3.5 DISCUSSION

3.5.1 Study findings and implications

This study is the first to report downregulation of melatonin receptor subtypes 1 and 2 (i.e. MT1 and MT2) after TBI. This may affect the efficacy of MEL therapy after TBI. Notably, past attempts to treat experimental TBI with MEL therapy have yielded inconsistent results, with many studies showing neuroprotective effects after TBI for at least one of the regimens tested (Ates et al., 2006; Babaee et al., 2015; Beni, Kohen, Reiter, Tan, & Shohami, 2004; Campolo et al., 2013; Dehghan, Khaksari Hadad, Asadikram, Najafipour, & Shahrokhi, 2013; Ding et al., 2015; Kelestemur et al., 2016; Mésenge et al., 1998; Ozdemir et al., 2005; Senol & Nazıroğlu, 2014; Shochat & Abookasis, 2015; Yürüker et al., 2014; Sarrafzadeh et al., 2000; Ucar et al. 2005), one showing no effect of therapy (Kelso et al., 2011), and some studies showing adverse effects of one or more of the therapeutic regimens tested (Cirak et al., 1999; Jadhav et al., 2009). Moreover, onset of MEL therapy has been found to be an important factor in therapeutic response, with administration 30 minutes or more after injury, a clinically-relevant time frame, associated with no improvement in outcome in one study (Mésenge et al., 1998). Another study found that MEL needed to be administered during the nighttime to yield therapeutic effects (Sarrafzadeh et al., 2000). Similarly, one study reported an interaction effect of MEL therapy with exposure to prolonged darkness such that MEL therapy was only effective with prolonged darkness (Ucar et al., 2005). Finally, there is known genetic variation in (Barrett et al., 1997; Hernandez et al., 2005;

Langenberg et al., 2009; Li et al., 2013; Natarajan et al., 2012) or near (Chambers et al., 2009) genes encoding MEL receptors, the effect of which was obscured by the use of congenic (i.e. inbred) rats in this study. Replication in different strains of test animal would ameliorate this limitation, as would clinical trials exploring the effects of MEL receptor polymorphisms on TBI outcomes.

CytC was used to validate the extent of injury but was not the focus of this study. CytC is a known initiator of the intrinsic (caspase-dependent) apoptotic pathway (Steel et al., 2004). Specifically, CytC translocates from the mitochondria to the cytosol, where it becomes part of the apoptosome (Li et al., 1997). In human studies of cancer, CytC has been used as an apoptotic biomarker (Renz et al., 2001). In clinical TBI research studies, elevation in CSF CytC has been reported (Darwish & Amiridze, 2010; Satchell et al., 2005; Wagner et al., 2011). Elevated CytC has been associated with poorer outcomes assessed using the Glasgow Outcome Scale and Disability Rating Scale (Wagner et al., 2011); however, one study found elevations in caspase-9, but not CytC were associated with worsened clinical outcomes (Darwish & Amiridze, 2010). Post-TBI changes in CytC have also been explored in pre-clinical studies. One study used quantitative western blot and found CytC was increased in the cytosolic fragment 24 hr post-TBI (Sabirzhanov et al., 2014). Another study reported increased levels of cytosolic CytC after experimental brain trauma (Cao et al., 2016). A third study found that while sham animals had little-to-no detectable cytosolic CytC on days 1 and 3 post-injury, levels were markedly increased on days 1 and 3 after TBI (Chen et al., 2012). Conversely, decreased CytC in the mitochondrial fragment has been reported 4 hr after TBI (Robertson, Saraswati, & Fiskum, 2007). The time course of CytC translocation was explored in one study which found that from 6 hr, to 12 hr, to 24 hr post-injury cytosolic CytC progressively increased and mitochondrial CytC progressively decreased; CytC

levels after injury were significantly different from sham at all time points examined in both the mitochondrial and cytosolic fragments (Sullivan, Keller, Bussen, & Scheff, 2002). Notably, the results in our study differ from what has been reported in the experimental TBI literature, with increased CytC at 6 hr post-TBI in the frontal cortex, decreased CytC at 6 hr post-TBI in the hippocampus as well as at 24 hr post-TBI in the frontal cortex, and no effect on CytC levels 24 hr after TBI in the hippocampus. However, in this study whole cell lysates were used for analysis and fractionization was not performed to isolate the mitochondrial and/or cytosolic fragment(s); one published study found elevation in cytosolic CytC after TBI, but no injury effect when whole cell lysates were analyzed (Sabirzhanov et al., 2014). Unfortunately, at the time of tissue processing, the need for fractionation was not known and the pellet was destroyed, since the original apoptotic outcome of interest was caspase-3. Still, the model and specific injury parameters have been well-validated as reducing the number of viable brain cells and resulting in other pathological changes (Alessandri, Heimann, Filippi, Kopacz, & Kempski, 2003; Bondi, Cheng, Tennant, Monaco, & Kline, 2014; Olsen, Sozda, Cheng, Hoffman, & Kline, 2012).

3.5.2 Limitations of study

All pre-clinical studies have limited clinical applicability and require replication in pre-clinical models before clinical trials can be justified. Importantly, many of the studies that show success in pre-clinical studies, even when replicated, do not demonstrate therapeutic effects in clinical trials. Moreover, the focal nature of TBI induced using the CCI model means that the results may not hold true when polytrauma is present, nor reflect the effects of more diffuse brain injury or milder brain injury. The generalizability of this study is further limited by the homogenous nature of the sample, which was restricted to all young adult male rats. Since sex is

known to be an important factor in brain trauma (O'Connor et al., 2007; Ratcliff et al., 2007; Slewa-Younan et al., 2004), validation of study findings in female animals is necessary. Likewise, replicating the study using pediatric and aging mice would strengthen the evidence base.

Several specific limitations should be acknowledged and considered when interpreting the results of this pre-clinical study. Only one marker of injury severity was used, specifically CytC levels assessed via western blot. Validation using immunohistochemistry, basic histology, or imaging would have strengthened the evidence, though the model is well-established; moreover, the injury parameters used in this study have been well-vetted in past studies out of the laboratory where this work was conducted. Likewise, western blot analysis of MT1 and MT2 receptor levels provides preliminary evidence of downregulation, but additional research is necessary using more sophisticated techniques (e.g. immunohistochemistry; gene expression studies) to further study the precise location and timing of MEL receptor changes. An additional limitation is that in this study, An additional limitation is that in this study, protein measures were conducted using whole cell lysates, which may not completely capture the level of membrane bound receptors (Carrillo-Vico et al., 2003). A final limitation of this study is the exclusion of behavioral endpoints. Though there is evidence of both apoptosis and MEL receptor downregulation after TBI, this may not result in changes in symptom profiles (e.g. cognitive outcomes as assessed using established behavioral tests). Without a meaningful change in functional outcome, or knowledge of how reduced receptors would impact therapeutic benefit of MEL therapy on functional outcomes, the relevance of this data is limited.

3.5.3 Future directions

The limitations of the present study (see 3.5.2) necessitate additional research. Future studies will be strengthened by increased sample diversity (age; sex; genotype) and by evaluation of additional cellular, histological, and functional endpoints. Accumulation of such data would strengthen scientific understanding of the effect TBI has on the endogenous melatonergic system and how to best target it therapeutically.

Eventually, additional clinical trials beyond the ongoing PLAY GAME trial of MEL therapy for pediatric concussion may be warranted (Barlow et al., 2014). As evidence from PLAY GAME and other pre-clinical trials becomes available, testing MEL therapy after more severe TBI in humans may be warranted. Considerations for MT1 and MT2 polymorphisms will be important for future clinical trials, especially for functional polymorphisms. Selection of dose should be informed as much as possible by pre-clinical trials, taking into account the differences in size and metabolism between rodents and humans. Toxicity information from other clinical trials may prove relevant when selecting a dosage to test.

3.6 CONCLUSION

This study is the first to demonstrate that MEL receptors are affected by TBI. Specifically, time point- and region-specific differences in both MT1 and MT2 levels occurred. Replication of these results is necessary using more diverse pre-clinical samples (e.g. other strains/species, females, older/younger animals) and studies with additional cellular and behavioral endpoints. Clinical research exploring the effects of TBI on MEL receptors and trialing the effects of therapeutic MEL

may be warranted. Overall, the results of this study, along with the existing literature, suggest the melatonergic system is implicated in TBI pathology and/or recovery and is worth further studying using test animals, progressing to clinical trials if the evidence warrants.

4.0 REVIEW MANUSCRIPT #1: HISTORICAL USE OF ANIMAL MODELS IN ADVANCING HEALTH RESEARCH AND PRACTICE: PAVING THE WAY TO 21ST CENTURY HEALTHCARE

4.1 ABSTRACT

Animal models have been used in research since antiquity, and remain relevant today. Major advancements in anatomy, physiology, and ontology, as well as the development of many therapies would have been delayed or impossible without pre-clinical research. In the era of molecular genomics and rapid technological advances, animal models remain a mainstay in research; today, animal models shed light on molecular-genomic factors underlying human conditions and ensure safety and efficacy of novel therapeutic strategies before translation to patients. Animal models have been recognized for centuries as a fundamental training tool used to educate and prepare clinicians, including nurses, in areas of anatomy, physiology, assessment, and interventions. The primary purpose of this manuscript is to review the rich history of how animal models contributed to major advancements in health science research and training for nurses and other allied health clinicians. Content is organized chronologically with attention given to relevant legal and ethical considerations during each period. A secondary purpose is to highlight the ways nurses contribute to pre-clinical research and training. Though physicians and basic scientists remain the primary users and developers of animal models, nurses are also key consumers and contributors to the field.

4.2 OVERVIEW AND PURPOSE

It would not be an overstatement to say that modern medical and nursing practice would be significantly delayed, or even impossible, if not for the extensive use of a diverse array of animal models for research and training. Observational and experimental evidence generated from animal models underlie much of the knowledge about factors relevant to human health. For example, animal models have shed light on basic anatomy and physiology, human development from conception through death, the pathophysiology underlying various health states, treatment options for diseases and acquired injuries, as well as the genomic underpinnings of health. Animal models have also played a critical role in testing various interventions and training providers to perform them with proficiency.

The *primary purpose* of this review article is to provide the reader with an overview of key historical and contemporary applications of animal models for health science research and training, using fundamental discoveries as exemplars and highlighting the relevance to nursing. This discussion will be organized chronologically, using the Before Common Era (BCE)/Common Era (CE) dating system preferred by many scholars and historians; this is an equivalent alternative to the Before Christ (BC)/Anno Domini (AD) system. For each time period, relevant ethical and legal considerations will be noted. The *secondary purpose* is to highlight the critical role of animal models in clinician training (e.g. assessment strategies; surgical interventions). The research and clinical training applications are not specific to nurses, but rather shared with other allied health science fields; however, throughout the paper, effort will be made to tailor the discussion to a nursing audience. The *tertiary purpose* is to briefly discuss how clinicians (specifically nurses)

can contribute to pre-clinical studies and describe the expanding presence of the nursing profession in this effort. Notably, this paper is intended to be a brief primer and is not meant to be a comprehensive record of all research conducted using animal models, as doing so would require not just a single manuscript, but rather an anthology due to the widespread use of animal models across the millennia.

4.3 METHOD FOR SELECTING EXEMPLARS

PubMed, Google Scholar, CINAHL, and library resources at a large university were used to identify potentially relevant articles. Animal models have become increasingly common since their initial applications and thus are more frequently used today than they were historically (Figure 25). In this paper, attempts were made to provide a broader, more representative historical overview by providing a similar number of examples for each time period examined. Cited references were chosen based on clinical relevance, inclusion of the nursing perspective (when possible), or fame of the research (e.g. scientific paradigm-shifting; Nobel Prize-winning). All articles cited were written in English or reprinted after translation to English.

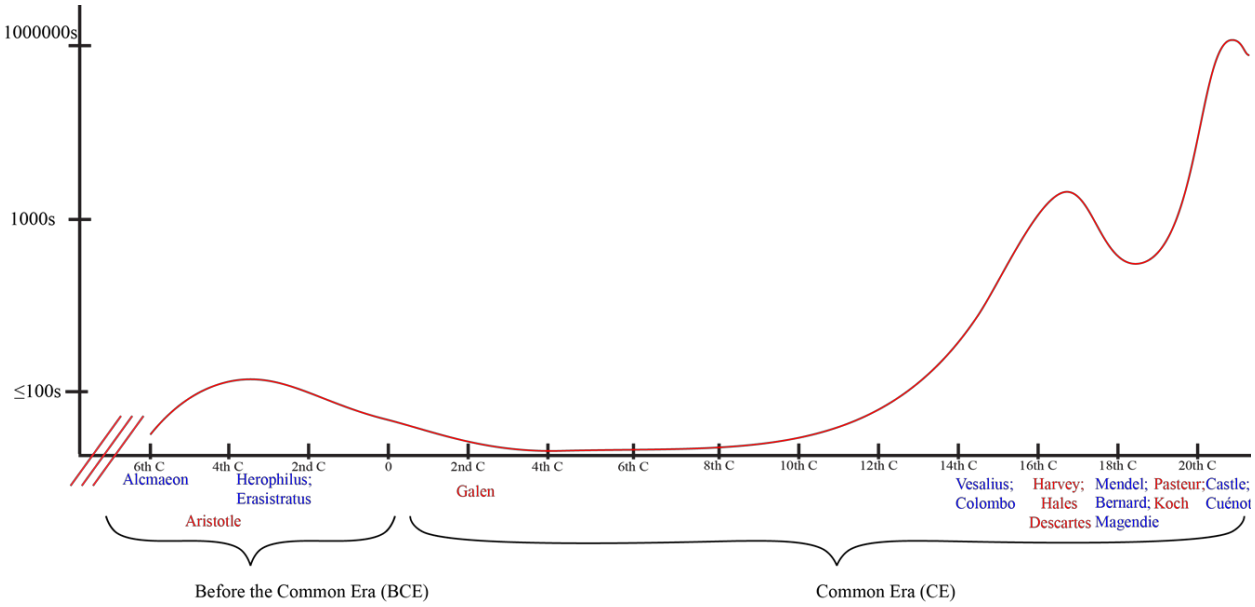

Figure 25: Approximation of the number of research animals used by time period

4.4 HISTORICAL APPLICATIONS OF ANIMAL MODELS

4.4.1 Antiquity through the Middle Ages: Understanding Anatomy & Physiology

Early evidence of animal model utilization dates back to ancient times. By modern research standards, these early efforts would be categorized as observational (i.e. non-experimental) in nature. The fruits of these early efforts led primarily to an enhanced understanding of anatomy, physiology, and ontogenesis (Ericsson, Crim, & Franklin, 2013), the foundation upon which modern nursing and medical practices are based; without a solid understanding of normal physiology and development, the ability to detect and address pathology and developmental issues would be impossible. Examples of such research conducted by ancient scholars are provided in Table 5 and summarized below.

Table 5: Key advancements in animal research by approximate date

Approximate Date	Location	Person	Milestone(s) Relevant to Healthcare
6th to 5th century BCE	Croton (modern day Italy)	Alcmaeon	Identified the brain's role in intelligence and in integrating sensory input using dogs
4th century BCE	Greece	Aristotle	Contributions to ontogenesis and embryogenesis, as well as basic anatomy using several species, commonly chickens
3rd century BCE	Greece	Herophilus; Erasistratus	Identified the heart valves and deduced the role of the heart as a pump using many species
2nd century CE	Pergamon/ Rome	Galen	Pioneered modern vivisection and made substantial contributions to the understanding of many body systems
11th century CE	Germany	Hildegard von Bingen	Pioneered modern medicine through the use of plant therapies and animal models
12th century CE	Moorish Spain	Ibn Zuhr	Evaluated new surgical techniques (e.g. tracheostomy) in animal models
17th century CE	England	William Harvey	Provided the most accurate description of the cardiovascular system to date,

			improving on the Galenic school of thought
1902	United States of America	William Castle	Advent of mouse breeding for genetic research
1905	France	Lucien Cuénot	Determined that mice follow Mendelian inheritance patterns
1940	Australia	John Cade	Tested and translated a treatment for depression based on studies using guinea pigs
1976	United States of America	Rudolf Jaenisch	Development of first transgenic mouse model
1987	United States of America and United Kingdom	Capecchi, Evans & Smithies	Developed the first knockout mouse
2002	Multinational	Many	Sequencing of the first non-human mammalian genome (mouse)
2004	Multinational	Many	Sequencing of rat genome

During the 6th and 5th centuries BCE, Alcmaeon of Croton (540-500 BCE [approximate]) performed exploratory surgery on living animals. Alcmaeon's major contribution to health science pertains to identifying the brain's role in intelligence and integration of sensory information

through his studies using canines (Celesia, 2012; Codellas, 1932; Court, 2005; Debernardi et al., 2010; Ferrarelli, 1952; Zolog, 1994). In Greece, during the 4th century BCE, Aristotle (384-322 BCE) dissected animals for enhanced understanding of anatomy and physiology, particularly as it relates to organismal development across the lifespan. In 350 BCE, Aristotle wrote *History of Animals* (Aristotle, n.d.), which was considered the primary authority on the topic of zoology for two thousand years, and remains relevant and well revered today. Aristotle's work includes extensive characterization of the features possessed by many different species of animals (Smith, 2010; Torras, 2015; Tsuchiya, Kuroki, & Eguchi, 2015). Aristotle also made comparisons between individual animals as well as between groups of animals including humans (Tipton, 2006; von Lieven & Humar, 2008); the multitude of similarities across species further added to the rationale for using animal models to better understand human health.

In the 3rd century BCE, Greek physicians Herophilus (335-280 BCE) and Erasistratus (304-250 BCE) built on earlier findings and expanded upon existing surgical techniques using several species of animals (Imai, 2011; King, 2015; Panegyres & Panegyres, 2016; Reverón, 2014; Wills, 1999). In addition to their pre-clinical work, they pioneered a new technique that had previously been taboo and even outlawed: the dissection of human corpses (Berche & Lefrère, 2011; Moon, Filis, & Cohen, 2010; H. von Staden, 1992). Though the corpses they used were obtained from imprisoned individuals who died in the royal jail, the practice of using humans in any form for research purposes remained highly controversial, and therefore animal models prevailed (S. von Staden & Staden, 1989). Indeed, most ancient Greek physicians and scholars had no moral issue with using animals (living or dead) in research. Rather, the use of animals for anatomy and physiology research only began to appreciably decline after scholars from the empirical school of thought (which predominated between the 3rd century BCE and 4th century CE) raised concerns

regarding the utility of animal models. Specifically, scholars of the day worried that the experimental procedures caused adverse effects (e.g. pain; death) that may impact data, subsequently affecting the conclusions and ultimately the clinical applications.

During the 2nd to 3rd century CE, Ancient Romans used animals exclusively in their studies of anatomy and physiology. Most notably was the work of Galen of Pergamon (129-200 CE), who is credited with making fundamental advancements to techniques used for performing exploratory surgery on living animals, a practice referred to in modern times as vivisection (Goss, 1963; A. Guerrini, 2003; Maehle & Tröhler, 1987; West, 2014). However, the resurgence of animal model usage for health science research was short lived. Following the decline of the Roman Empire (476 CE) and continuing through the Middle Ages (5th-15th centuries CE), there was an increased emphasis on the supernatural and subsequently a decreased interest in scientific exploration (Figure 25). Nevertheless, some research did continue. For example, one Benedictine nun broke the traditional boundaries in Germany during the 12th century CE (Rauch, 2012). Hildegard von Bingen (1098-1179 CE) was renowned for her visionary work with holistic medicine and human etiology (Adamson, 1995; Becher, 2001; T. B. Cole, 2015; Ramos-e-Silva, 1999; Riethe, 2012). *Physica*, a compilation of nine books, was a comprehensive account of her work, which included use of animal models to test scores of therapeutic interventions (Rauch, 2012), such as aloe which remains widely used today (Flanagan, 1998). Hildegard von Bingen is arguably best known for her contributions to dermatology, including early written accounts of several skin conditions (e.g. rosacea, rhinophyma, contact dermatitis, lice, scabies, and other insect bites), many of which are common to both humans and animals (Bork, 1980; Ramos-e-Silva, 1999; Riethe, 2005, 2006). In the Arab world, when Spain was under control of the Moors, Ibn Zuhr (1094-1162 CE), an Arab physician and surgeon used animal modeling to trial novel surgical techniques before translating

them to patients (Hajar, 2011). He pioneered experimental tracheotomy using a goat model and quickly translated the practice to human patients (Missori, Brunetto, & Domenicucci, 2012). He is also credited with publishing an accurate description of several diseases of the gastrointestinal system based on observations using animal models, laying part of the foundation for modern gastroenterology (van den Tweel & Taylor, 2010).

4.4.2 Renaissance through mid-1700s: Resurgence of Animal Models for Research and Training

While animal models had been used in the ancient world, they failed to result in a major paradigm shift in the field of physiology until the Renaissance (14^{th}-17^{th} centuries CE). During this time, animal models rapidly regained popularity (Figure 25); Vesalius (1514-1564 CE), Colombo (1516-1559 CE), and their contemporaries conducted several animal studies in an attempt to better understand anatomy and physiology (Table 5) (Brinkman & Hage, 2016; Ellis, 2014; Hage & Brinkman, 2016; O'Malley, 1964). Vesalius made a considerable contribution to health education when he articulated the skeleton of a notorious felon; this preparation, known as the Basel Skeleton, is the earliest anatomical preparation surviving today (Olry, 1998). Vesalius also wrote prolifically on his work with human bodies, including in the publication entitled *De Corporis Humani Fabrica Libri Septem* (Vesalius, n.d.). Colombo was also a renowned anatomist (Tubbs, Linganna, & Loukas, 2008), who dissected animals and humans and is credited with discovery of the cardiopulmonary circuit (Seaton, 2014). In addition to these research applications, Vesalius and Colombo both recognized the utility of animal models for clinician training and used vivisection in their instruction of medical students in the mid-1500s (Maehle & Tröhler, 1987). However, despite the fact that some concerns regarding the utility of animal models were raised,

vivisection and dissection remained widely used throughout the Renaissance. For example, Vesalius found that many structures present in animals were absent in humans (e.g. harderian gland; baculum). These differences led him to revisit surgical exploration of human cadavers, the same practice that was briefly used in Ancient Greece; despite the resurgence of cadaver research, this practice did not replace, but rather supplemented, animal models (O'Malley, 1964; S. von Staden & Staden, 1989). Moreover, the founder of the modern scientific method, Francis Bacon (1561-1626 CE), deemed vivisection a relevant strategy for both research as well as to promote scientific progress and enhance educational training (Bacon, n.d.; Anita Guerrini, 2013). Ultimately, the use of animal models prevailed throughout the Renaissance (Montague, 1842).

Despite previous concerns that pain response, caused by experimentation methods, could impact study findings and subsequent research quality, there was no consensus within the scientific or philosophic communities regarding animal nociception and distress. Indeed, as late as the 1500s, animals were largely considered incapable of fear and pain; consequently, animal experiments conducted during this timeframe clearly would not have met the standards of committees overseeing animal research in the 21st century (Allen, 2011; Descartes, n.d.; Spinoza, n.d.). René Descartes (1596-1650), in his 1649 text *The Passions of the Soul*, compared animals to machines and justified treated them accordingly. However, by the end of the 16th century, philosopher John Locke (1632-1704 CE) had recognized that animals were capable of feeling pain and cautioned against animal cruelty. Interestingly, his concern was more for the humans involved, rather than the animals themselves. Locke viewed animal cruelty as a wrongdoing against the animal's owner and feared that individuals comfortable with torturing animals may be capable of cruelty toward humans (Locke, 1778). Immanuel Kant (1724-1804 CE), like John Locke, also recognized the sentience of animals but maintained that our loyalty should be to humanity, not to

animals (Kant 1997 [translation]; Broadie and Pybus 1974); thus, to Kant, the use of animals for health science research was justified.

Despite the concerns of some scholars, the overwhelming consensus was that the benefits of animal research outweighed the risks. Subsequently, physicians and scholars increasingly incorporated animal models into their research and educational activities. With the increased utilization of animal models came refinement of research methodologies throughout the 1600s and 1700s CE. Ultimately, this led to substantial improvements in our understanding of human anatomy, physiology, and assessment of health parameters. Without the advancements of the 15th, 16th, and 17th centuries, our current understanding of healthcare and clinical practice would likely have been delayed or even impossible. A few notable examples are discussed below.

William Harvey's (1578-1657 CE) mid-17th century characterization of circulation and heart function made a crucial contribution to the state of Western medicine by addressing significant misconceptions from the Galenic school of thought. Evidence from Harvey's studies using fish and birds contributed in major ways to our understanding of circulation; these studies contradicted the notion that the liver's role was to continually produce blood and that veins transported blood from the liver to other organs, where the blood would be consumed (Aird, 2011; Androutsos, Karamanou, & Stefanadis, 2012; Berche & Lefrère, 2010; McMullen, 1995; Zareba, 2007). Moreover, Harvey identified the role of the arteries in blood transport, which until then had been widely believed to be merely full of air (McMullen, 1995; Shackelford, 2003). Clearly the current state of cardiovascular medicine as well as routine nursing practice (e.g. phlebotomy; auscultation of the heart) is deeply rooted in these important scientific advancements which resulted from animal experimentation. Harvey also made substantial advancements to the field of comparative anatomy through his experiments using live insects, fish, amphibians, and mammals

(Grene & Depew, 2004). In addition to using animal models in his research, Harvey, like Vesalius and Colombo before him, recognized their value for educational purposes (Franco & Henrique Franco, 2013).

Techniques to assess health parameters also improved during this time. For example, Stephen Hales (1677-1761 CE) used animal models in his early work aimed at quantifying pressure within blood vessels (Felts, 1977; Hall, 1987; Lewis, 1994; Smith, 1993); today, blood pressure assessments represent an integral component of routine nursing assessment. Around this time, animal models became increasingly applied to develop and test potential therapies. Richard Lower (1631-1691 CE) had medical applications in mind when he made early blood transfusion and organ transplantation attempts using intra- and inter-species experiments (Hoff & Hoff, 1936). Similarly, Johann Wepfer (1620-1695 CE) recognized the utility of testing potential toxins on animals, to identify poisons that should be avoided by humans, a practice that remains widely used today in medicine and toxicology, as well as testing of products such as cosmetics and toiletries (Maehle and Tröhler 1987; Maehle 1986).

Overall, most physicians and scholars of 14th-17th centuries CE found the use of animal models scientifically and morally justified. However, as was true in ancient times, some scholars had concerns regarding the validity of the models themselves. Jean Riolan Jr. (1580-1657 CE) and Edmund O'Meara (1614-1681 CE) raised concerns that the unnatural conditions under which vivisection is performed may affect the trustworthiness of research findings (Cohen, 1995; Franco & Henrique Franco, 2013; Mani, 1968). Notably, two of the most prominent physiology scholars of the 1700s Hales and von Haller bemoaned the unpleasant aspects of hurting and/or killing animals during the experiments they conducted, though they felt that the overall benefit to science

justified the means. This trend for increasing concern surrounding the potential for animal pain and suffering, along with ways to ameliorate it, increased during the late 1700s and 1800s.

4.4.3 The Late 1700s through the 1800s: Growing Concern and Subsequent Oversight

In the late 1700s, scholars such as Voltaire (1694-1778) criticized the nature of animal experimentation, deeming it cruel and gruesome (Voltaire 2004 [translation]). Indeed, some research questions were best studied at the time using methodologies deemed controversial today. For example, the experiments conducted by Bernard (1813-1878 CE), when studying thermoregulation, and Magendie (1783-1855), when studying the cranial nerves, would not meet today's standards for the care and use of research animals. Notably, most of the abovementioned work was conducted before anesthesia was available; these experiments may have been performed differently if anesthesia were available and routinely used at the time the work was conducted. However, despite the pain endured by individual animals used in these experiments, findings of this work have greatly impacted centuries of research and clinical practice (e.g. cranial nerve assessments). Also worth noting is that even those who conducted gruesome experiments at the time considered themselves to be doing it for the good of humankind. Many even expressed outright condemnation for experiments in humans that were not first vetted using animals (Rey, 1998; Snow, 2008), a practice that remains the gold standard in science today. Overall, the ethics of animal research were being increasingly considered, a trend that continued into the 19th century through the writings of Rousseau (1712-1778), Bentham (1748-1832), Schopenhauer (1788-1860), and Bernard (1813-1878) (Bentham, 1823; Bonnod, 1980; Foëx, 2007; Goffi, 2013; Rousseau, 2007; Schopenhauer, 1903). Despite increasing moral concern, the potential benefits to human health were considered to outweigh the risks, so the animal research continued. Thus, in the late

1700s and 1800s, the question was not whether the use of animals for research and education was justified, but rather, under what circumstances and conditions. Accordingly, discussions focused on how to design and conduct studies to maximize clinical utility while minimizing suffering when possible (Elliot, Walker, & Paddock, 1896; Normandin, 2007; Rudacille, 2001; Ryder, 2000; Tait, 1882). This increased consideration for the ethical treatment of animals paralleled the passage of new legislation.

The Parliament of the United Kingdom enacted the first major piece of animal protection legislation; The Cruel Treatment of Cattle Act of 1822 covered livestock, specifically cows, heifers, steer, oxen, and sheep (Uvarov, 1985). Initially, animal protection laws pertained to livestock, but legislation evolved to protect animals used in research. Later, Parliament passed the Cruelty to Animals Act of 1835 (Tannenbaum, 1995), which added protections for other domesticated or captive animals used for entertainment (e.g. circus) and sport (e.g. fighting), such as: bulls, bears, and dogs. The passage of the 1849 Cruelty to Animals Act reinforced the illegality of overworking, mistreating, and/or abusing animals and imposed a fine for violating the law (Tannenbaum, 1995). The 1876 Cruelty to Animals Act amended the 1849 legislation and for the first time expanded protection laws to research animals. The 1876 amendment served to set limits on animal experimentation and impose a licensing system to facilitate oversight and compliance with the law. One key provision of this law was that research likely to inflict pain upon animals could only be conducted when the work was critically necessary to prolong or save human lives. The act further stipulated that animals exposed to pain must be treated in a way that minimizes discomfort, including administration of anesthesia (which had become available), single use of each test animal, and rapid and humane euthanasia immediately following conclusion of the study

(Kean, 2003). Ultimately, the 1876 amendment laid the foundation for standards regarding the treatment of animals used in research in England, and eventually, worldwide.

Legislative protections for animals were reinforced by the concomitant rise of advocacy groups. For example, in 1824, the Society for the Prevention of Cruelty to Animals (SPCA) lobbied Parliament for additional legislation to protect animals, including the aforementioned 1835 act; Queen Victoria (1819-1901 CE) became involved in the SPCA a few years later, making it a royal society in 1840 (Berkowitz, 2006; Bynum, 1994). Frances Power Cobbe (1822-1904 CE) wrote prolifically on the subject of animal experimentation and founded several groups advocating for animal rights. First was the Society for the Protection of Animals Liable to Vivisection in 1875, the earliest established organization against the use of animals in research experiments in any capacity. Next, she founded the National Anti-Vivisection Society in 1875, which continues to be an active organization today (National Antivivisection Society, 2016). Finally, she founded the British Union for the Abolition of Vivisection in 1898. Through her efforts, Cobbe increased public awareness surrounding animal experimentation, which ultimately contributed to the passage of the 1876 Cruelty to Animals Act discussed above (Finn & Stark, 2015). Despite some individuals' efforts to completely ban animal research, vivisection was allowed to continue under certain oversight, regulations, and restrictions.

Advances in scientific knowledge continued along with these increased ethical standards underlying the use of animal research. Indeed, by the late 1800s, the direct clinical and public health benefits of animal research were becoming widely extolled (Table 5). Notable research from this time period includes Louis Pasteur's (1822-1895) animal studies that led to the germ theory of disease and identification of pathogens, including *Streptococcus spp.* and *Staphylococcus spp.* (Scudder, 1921). Pasteur also applied these findings to eradicate pathogens in milk through a

process that was named after him, pasteurization, saving countless lives. The efforts of Robert Koch (1843-1910 CE) led to the identification of additional notorious pathogens (e.g. *Mycobacterium tuberculosis*; *Bacillus anthracis*) as well as the development of vaccines and earned a Nobel prize for his life-saving work (Gossel, 2000; Gradmann, 2001; Koch & Carter, 1987). Moreover, modern day clinical practice standards regarding hand washing and sterile procedure are deeply rooted in animal research. Ignaz Semmelweis (1818-1865 CE) had previously conjectured that fever and infection were linked to poor physician hygiene (Battistuzzi, 2012; Best & Neuhauser, 2004; Miranda C & Navarrete T, 2008; Rangappa, 2015). However, it was not until Joseph Lister (1827-1912) published the results of his pre-clinical trials in *On the Antiseptic Principle of the Practice of Surgery* (1867) that hygiene practices in the clinical setting were changed for doctors and nurses, which ultimately led to decreased rates of infection. The works of Pasteur, Koch, and Lister together are responsible for saving countless lives by setting the precedent for the hygiene standards now used in healthcare. Indirectly, this served to validate the need for skilled nursing services, since well-trained professional nurses used practices known to reduce adverse events (e.g. infection) and maximize health outcomes.

4.4.4 The 1900s and Beyond: The Need for Animal Models is Clear and Ever-Expanding

By the 1900s, the need for animal research was fully apparent to most scholars and clinicians. Indeed, a number of notable healthcare advancements that relied heavily on animal research were made using many different species. A summary of the species used in research and resulting healthcare advancements are summarized below (see Table 5 and Table 6).

Table 6: Key healthcare advancements made using various animal models

Animal Type	**Major Advancements**
Fruit fly	-Early evidence regarding chromosomal heredity, including genetic linkage
Fish	-Common model for embryology research due to high yield of transparent embryos
Frog	-Understanding of chemical communication between cells
Bird	-Studying the aging process -Contribution to chronic disease research, including cancer and diabetes
Rodent	-Treatment for whooping cough -Treatment for arthritis -Development of chemotherapy -Development of drugs that can cure some forms of childhood leukemia -Cochlear implants
Armadillo	-Treatment of leprosy
Ferret	-Research of the workings of the auditory system -Development of vaccines (e.g. bird flu; swine flu) -Development anti-emetic treatment for cancer patients
Rabbit	-Corneal transplant - Laser treatment to prevention of blindness
Cat	-Treatment of spinal cord injury using methylprednisolone
Dog	-Discovery of insulin -Development of antihypertensive drugs -Advancements in antibiotics (e.g. streptomycin, penicillin, aureomycin)

	-Advancements in critical care research (e.g. cardiac arrest models) -Development of open heart surgery -Development of cardiac pacemaker -Development of coronary bypass surgery -Artificial heart transplant
Pig	-Development of cortisone -Development of techniques to lower cholesterol -Elucidation of the relationship between exercise and heart health -Improvements in skin grafts -Enhanced understanding of traumatic brain injury pathology
Sheep	-Development of heart valve replacement
Goat	Tracheotomy development
Horse	-Vaccination against diphtheria -Vaccination against tetanus
Non-human primate	-Discovery of Rh factor -Development of drugs for anxiety, phobia, and depression -Vaccination against poliomyelitis -Vaccination against rubella -Vaccination against hepatitis B -Control of robotic prosthetic limbs with the brain

Briefly, these include: 1) the discovery of vitamins and hormones (Holst & Frölich, 1907; Larsen et al., 2012; Mellanby, 1918; Edward Mellanby, 1976; Semba, 2012); 2) the extraction and

purification of insulin (Banting, Best, Collip, Campbell, & Fletcher, 1922; Banting, Best, Collip, Campbell, Fletcher, et al., 1922); 3) the development of antibiotics (Aminov, 2010; Ehrlich P., 1910); 4) the establishment and vetting of chemotherapy drugs (DeVita & Chu, 2008; Hirschberg, 1963); as well as 5) improvements in non-pharmaceutical therapies, such as surgical (Dewall et al., 1956) and radiotherapy techniques (Hong et al., 2014; Nikolaou, Cyran, Lauber, Reiser, & Clevert, 2012; Singer, 1979, 2001, 2011). Moreover, modern scientists and clinicians still employ and build on the interventions pioneered by their predecessor. For example, tracheotomy, the life-saving technique developed in the 12th century is still widely used centuries after the initial animal work was conducted; moreover, modern animal studies have tested different airway management techniques (Miller, Guay, Bauer, & Tucker, 1995) and attempted to understand and treat post-tracheostomy stenosis (Borowiecki & Croft, 1977).

Modern management of diabetes relied substantially on animal research. This pre-clinical work helped to transform diabetes from a commonly fatal condition to a manageable one. Working with dogs and rabbits, Charles Best (1899-1978) & J.J.R. Macleod (1876-1935) identified the role of the pancreas in diabetes. They found that surgically excising the pancreas in animals induced diabetes and isolating insulin from the pancreas of healthy animals and injecting it into diabetic animals effectively managed the condition (Banting, Best, Collip, & Macleod, 1922; Banting, Best, Collip, Campbell, Fletcher, et al., 1922; Macleod, 1924; Macleod, 1922). By 1922, insulin had been translated to clinical care by Frederick Banting (1891-1941) and the demand for the hormone was so great that collaborations with slaughterhouses were in place to obtain sufficient quantities. For their efforts, Banting and Macleod were awarded the Nobel Prize in Physiology or Medicine in 1923.

In light of these and other advances, the anti-vivisection groups that had gained momentum in the mid-to-late 1800s had become increasingly uncommon by the 1900s. Moreover, by 1954, the Universities Federation for Animal Welfare had published the first edition of the *Handbook on the Care and Management of Laboratory Animals*, tempering some of the existing concerns. However, anti-vivisection groups regained momentum during the 1970s, though their goal largely shifted to banning the experimental use of dogs and other creatures considered to be companion animals. Moreover, even one of the most prominent writers of the day, Peter Singer (1946-present) felt that animal research was justifiable under certain situations, acknowledging that a cancer patient is capable of more profound suffering than a cancer-stricken mouse (Singer, 1979). This movement, coupled with the lower cost and increased convenience of using small mammals, led to the rising use of rodents for research, which appeased much of the opposition. Still, a handful of scholars preferred an outright ban on animal research (Regan, 1989); with some even going as far as threatening and terrorizing scientists conducting this type of work (Conn & Parker, 2008; Liddick, 2006). Still, the state of modern medical science at the time remained largely rooted in research using animals. Indeed, of the 103 Nobel Prizes awarded between 1901 and 2013 in the fields of physiology or medicine, the vast majority (80.6%) were rooted in pre-clinical research (Franco 2013).

Throughout the 20th century, mice became increasingly utilized in research, especially for studies with a genetic component. Even the earliest genetic research utilized mice. In 1850, Gregor Mendel (1822-1884) used mice to study the heritability of coat color until the local bishop ruled that this effort was not appropriate conduct for a monk, leading Mendel to switch to pea plants as a research subject. In 1905, biologist Lucien Cuénot (1866-1951) determined that mice follow Mendelian inheritance patterns (Cuénot, 1905). Improved knowledge and technology led to Rudolf

Jaenisch's (1942-present) 1976 development of the transgenic mouse (Jaenisch, 1976). In the 1980s Frank Ruddle (1929-2013) and his colleagues performed early genome manipulation in mice (Gordon and Ruddle, 1981). Later, scientists sequenced the genomes of mice (Waterston et al., 2002) and rats (Gibbs et al., 2004). Modern efforts focus on modifying research animals to make them more human-like via the introduction of genes into the genome with the hope that these efforts will facilitate translation from preclinical research to clinical improvements. Major successes in humanizing test animals to date include: 1) producing mice with human-like immune systems to understand severe combined immunodeficiency (Shultz, Brehm, Garcia-Martinez, & Greiner, 2012); 2) creation of mice with humanized livers to better study drug metabolism and liver-specific disorders (Yoshizato, Tateno, & Utoh, 2012); and 3) insertion of the gene encoding the human major histocompatibility locus into rats, leading to an indispensable model for studying autoimmune disorders (Taurog et al., 1999).

Beyond research, animals remain commonly used in clinician training. More recently, nursing-specific clinical training using animals has gained popularity; some schools of nursing have incorporated animal based training into their curriculum. One such program was evaluated empirically and found to improve competency in several domains of nursing practice (e.g. communication; critical thinking; performance under pressure) compared to traditional nursing training without an animal laboratory component (Lin, Wang, & Ye, 2015).

In order to address the shortage of human organs, research in transplantation of animal organs to humans, known as xenotransplants, has been increasingly underway (Cooper, 2012). Many advancements were made during the 20th century, mostly from primates to humans; unfortunately, graft failure most often resulted after transfer, though there were some successes in medical advancements (Bailey, Nehlsen-Cannarella, Concepcion, & Jolley, 1985; Giles, Boehmig,

Amemiya, Halgrimson, & Starzl, 1970; Hardy et al., 1964; Reemtsma et al., 1964; Starzl et al., 1964, 1993; Starzl, Marchioro, Faris, McCardle, & Iwaski, 1966). Examples of this revolutionary research include Keith Reemtsma's (1926-2000) 1963 kidney transplants from chimpanzees to 13 human patients, James Hardy's (1918-2003) 1964 baboon-to-human heart transplants, Leonard Bailey's (1942-present) 1983 baboon heart transplants to neonates, and Thomas Starzl's (1926-present) 1992 and 1993 liver transplants from baboons to humans. Work in transplanting animal organs and tissues is ongoing, most notably using pig-to-nonhuman primate (Butler et al., 2016; Schuurman, 2016) or pig-to-human transplant (Kim et al., 2016). Gene editing is being explored as a means to eliminate human immune system rejection (Hering et al., 2006; Kuwaki et al., 2005; Reardon, 2015).

4.5 THE FUTURE AND ROLE FOR CLINICIANS

The future of biomedical research holds immense promise with the widespread availability of high-quality animal models. The increasing emphasis on translation to clinical practice has led to substantial refinements in available models and efforts to promote thoughtful model selection in the research community. Part of the effort involves diversifying pre-clinical research samples to better represent the complexity of the populations being modeled (e.g. inclusion of male and female animals of different ages). When animal studies have been conducted in a very homogeneous sample of test animals, replication of findings in samples with other characteristics can strengthen the evidence base.

The molecular genomic revolution has made it possible to choose models based on their genetic and genomic characteristics. Moreover, ongoing attempts to humanize animal models

using available genomic technologies are improving models so that they better mimic the human condition. Researchers are increasingly developing appropriate models for clinical applications that have traditionally been difficult to study in animals. For example, recent research in swine led to a cystic fibrosis (CF) model that possessed the hallmark pulmonary symptoms, while previous attempts mainly modeled the gastrointestinal consequences of CF (Stoltz et al., 2010).

There is also scientific movement called "One Medicine," which encourages sharing knowledge, resources, and effort, as well as trans-disciplinary collaboration in an attempt to promote health for all species, especially humans (Ericsson et al., 2013). Through One Medicine, the knowledge generated using animal models can be translated to both clinical and veterinary practice. There is also an increasing trend toward trans-disciplinary communication and collaboration. Subsequently, nurses have become key contributors to pre-clinical research including the planning, evaluation, conduct, and translation efforts.

4.5.1 Nurse Scientists' Role in Animal Research

Overall, there is an increased impetus to conduct multi- and trans-disciplinary research and promote translation to clinical care. Consequently, there is increasing recognition of the role for clinicians across the research spectrum starting with the planning, approval, and conduction of pre-clinical studies, continuing through to translation efforts. While physicians have been involved for centuries in all phases of pre-clinical research, the nursing perspective has only recently been included. Today, nurses contribute to pre-clinical research in various contexts including as scientific reviewers for their local Institutional Animal Care and Use Committees (IACUC), consultants, co-investigators, and principal investigators. In these roles, nurses contribute to pre-clinical research by applying their understanding of patient needs and perspectives and knowledge

surrounding the benefits and adverse effects of available treatments. In this way, nurses promote selection of clinically relevant variables so as to maximize the chance for the research to effectively be scaled up the phylogenetic ladder and eventually translated to humans if the evidence warrants. Some nurses also serve as clinician managers at facilities that do pre-clinical research; in this capacity, the nurse would oversee and participate in the care and monitoring of test animals. Below are several notable examples of contemporary nurse researchers who have contributed to the pre-clinical biomedical and nursing knowledge bases with pre-clinical studies. Note: this is not intended to be a comprehensive list, but rather a primer on the topic.

Dr. Susan Dorsey, PhD, RN, and her colleagues have used animal models to evaluate the genomic underpinnings of neuromuscular function and dysfunction in the context of muscular dystrophy (Dorsey et al., 2012; Khairallah et al., 2012) and neuropathy (Carozzi et al., 2013; Dorsey et al., 2009; Renn, Carozzi, et al., 2011; Renn, Leitch, et al., 2011). Dr. Teresita Briones, PhD, RN, and her colleagues have used pre-clinical models of brain injury and cerebral ischemia to explore factors affecting gene expression, synaptogenesis, neurogenesis, and dendritic growth (Briones, Suh, Hattar, & Wadowska, 2005; Briones, Suh, Jozsa, & Woods, 2006; Briones, Woods, & Rogozinska, 2013). Dr. Nancy Tkacs, PhD, RN, and her colleagues have used rodent models to study severe hypoglycemia (Tkacs, Dunn-Meynell, & Levin, 2000) and infection (Tkacs, Li, & Strack, 1997). Dr. Tina Hines, PhD, RN, and her colleagues, used a rat model to study cardiovascular receptors and reflexes; this work contributed to the cardiac knowledge base, including changes that occur during pregnancy (Hines & Herzer, 2000; Hines & Hodgson, 2000; Hines & Mifflin, 1997). A final example is Dr. Pamela Rowsey, PhD, RN, who has used animal models to explore connections between thermoregulation and the immune system (Rowsey & Gordon, 1999; Rowsey, Metzger, Carlson, & Gordon, 2009; Rowsey, Metzger, Carlson, &

Gordon, 2006); she has also used rat models to identify biomarkers of aging (Gordon, Rowsey, Bishop, Ward, & Macphail, 2011).

Nurses indirectly contribute to the pre-clinical research, by serving on IACUCs, which ensure proposed animal studies meet the institution's ethical standards and comply with federal regulations. Finally, when animal models yield promising evidence, nurses and other clinicians are responsible for much of the effort surrounding clinical trials and translation. Across the research continuum spanning from pre-clinical studies, to clinical trials, and ultimately translation to care, nurses collaborate with basic scientists, clinician researchers, physicians, and other clinicians; the overarching goal of these efforts are to improve clinical outcomes through evidence-based practice.

4.6 CONCLUSION

Animal experimentation has played a critical role in health science research since ancient times. Today animal models have applications in nearly all areas of biomedical research, including basic biology and physiology, infectious disease, gastrointestinal disease, cardiovascular disease, pulmonary disease, neurologic disease and stroke, oncology, and the study of acquired injury (Ericsson et al., 2013). Refinement of existing models, development of new models, and the increasing availability of transgenic animals further enhance clinical relevance and promote translation to patient care. Part of the ongoing pursuit to improve animal research surrounds finding ways to further reduce animal suffering while not sacrificing data quality or study feasibility. One aspect of the effort to reduce animal suffering is to use the least sentient animal appropriate to meet the experimental goals. Another aspect is to design clinically relevant studies so that animal

applications have the best chance of being utilized to promote human health; sometimes this means starting with invertebrates or small mammals and moving up the phylogenetic tree until the work is ultimately translated to humans. A key way to promote clinical relevance is to design research projects based on input from multi- and trans-disciplinary teams. Clinician input beginning with pre-clinical studies and continuing through translation better enables improvements in human health through research. Therefore, nurses and other clinicians should not only be at the table, but also serve as principal investigators on pre-clinical research projects.

5.0 REVIEW MANUSCRIPT #2: ANIMAL MODELS THE ERA OF MOLECULAR GENOMICS: OPPORTUNITIES FOR NURSES IN RESEARCH AND CLINICAL PRACTICE

5.1 ABSTRACT

Animal research has been conducted by scientists for over two millennia resulting in a better understanding of human anatomy, physiology, and pathology, as well as testing of novel therapies; in the molecular genomic era pre-clinical models represent a key tool for understanding the genomic underpinnings of health. The relevance of this line of inquiry has become even more apparent in the era of molecular-genomics. Nurses contribute to improved health by garnering and translating evidence from clinically relevant pre-clinical models. Using animal models, nurses can ask questions that would not be feasible, ethical, or otherwise possible to address in humans, and establish the safety and feasibility of interventions before translating them to clinical trials. Two advantages of using pre-clinical models are: reduced variability between test subjects and the opportunity for precisely controlled experimental exposures. Standardized care controls the effects of diet and environment, while the availability of animals that are genetically identical through inbreeding significantly reduces the confounding effects of genetic differences. Outside of the laboratory, nurses can contribute to the approval and oversight of animal studies, as well as the translation efforts to clinical trials and ultimately patient care. This paper is a primer on nursing

applications of animal models for studying the pathophysiologic and genomic underpinnings of health and disease, testing interventions, and evaluating effects of environment. Nurses also help with translating pre-clinical knowledge to clinical trials and ultimately into patient care. In this review, considerations for researchers are introduced, including discussion of how to choose an appropriate model and controls, potential confounders, as well as legal and ethical concerns.

5.2 DISCLAIMER ABOUT VOCABULARY

This article employs some fundamental *genetics* and *genomics* vocabulary, not all of which is thoroughly defined in this text; a glossary of key terms used in this review *(italicized in the text)* is provided (Table 7). For additional clarification of terms, the reader is encouraged to utilize the freely available Glossary of Genetic Terms published by the National Human Genome Research Institute (https://www.genome.gov/glossary/). It is also important to note that "*animal model*" and "pre-clinical model" will be used interchangeably, though pre-clinical models also include *in vitro* techniques such as cell culture, which are not covered in this review.

Table 7: Glossary of genomic terminology geared toward nursing audience

Term	***Definition***
Allele	A specific version of a given gene. For some genes, there may be one or more allele(s) associated with a change in phenotype (e.g. disease risk) compared to the normal version of the gene (i.e. wildtype allele). For other genes there may be variation as evidenced by two or more

	alleles in the population but there are no known resulting differences in phenotype. Note: Each individual has two alleles for each gene (because humans have 23 pairs of chromosomes, with one member of each pair inherited from their mother and the other from their father). In some cases, one copy of a particular allele results in the associated phenotype and in other cases two copies of the allele are needed to produce the phenotype
Animal model	A non-human animal used to study a clinical problem in biomedical research that shares similar physiological and/or functional characteristics with humans of a particular clinical population. In some instances, the species used is already afflicted by a disease common to humans, and other times, a clinical condition is mimicked in animals as part of the experimental procedures. Animal models are used when a question could not be practically, ethically, or safety studied in humans and they may lay the groundwork for future clinical inquiry and changes in practice.
Autosomal dominant	A pattern of trait inheritance where the given phenotype results when the individual possesses at least one copy of the associated allele, which exists on one of the autosomes (i.e. a numbered chromosome, not the X or Y chromosome). This is in contrast to autosomal recessive conditions, which require 2 copies of the associated allele for the individual to display the phenotype.
Chromosome	A condensed package of DNA found within the nucleus of a cell. Humans have 46 chromosomes in 23 pairs (with one member of each pair coming from each parent). Other types of animals have different numbers of chromosomes. There are two major types of chromosomes: autosomes (numbered chromosomes; 1-22 in humans) and sex chromosomes (X and Y).

Complementary deoxyribonucleic acid (cDNA)	A laboratory-produced doubled stranded DNA molecule. In the context of animal models, cDNA is often used to modify the genomes of test animals as is the case of generating a knockin animal.
Deoxyribonucleic Acid (DNA)	The scientific name for the molecule that serves as the genetic information that encodes all of the proteins comprising a given organism. DNA molecules are double stranded molecules wound together in the form of a double helix.
Conditional (i.e. inducible) mutation	When a given genotype for a particular gene only results in the phenotype of interest under certain environmental conditions. For example, individuals with sickle cell disease only exhibit symptoms of the condition under restrictive environments (e.g. cold; low oxygen; emotional stress).
Gene	A stretch of DNA encoding some trait or protein of interest. Genes are passed on from parents to offspring in the sperm and egg, which contain chromosomes; each chromosome has many genes along it.
Gene Expression	The process of producing a protein using the code contained in the DNA. Each set of 3 DNA bases corresponds to a particular amino acid; consecutive amino acids are strung together as a polypeptide also known as a protein.
Genome	The full set of genetic information for a given organism. Each cell in the organism possesses the full genome within it, mostly in the nucleus (which contains chromosomes) but to a lesser extent in extra-chromosomal mitochondrial DNA.
Genetics	Using scientific techniques to study a particular gene or set of genes.
Genomics	Using scientific techniques to study the entire genome of an organism as opposed to a single gene or small set of related genes (i.e. genetics).
Genotype	The collection of genes possessed by an individual organism that directs protein production and ultimately affects the individual's observable traits (i.e. phenotype)

	Note: depending on the context, sometimes the term is used to describe the composition of alleles that an individual possesses for a particular gene in the genome.
Heterozygous	An individual who has two different alleles for a particular gene, having received different versions from their mother and father.
Homozygous	An individual who has two of the same alleles for a particular gene, having received identical versions from their mother and father.
In Vitro	A process that happens outside of a living organism. The term literally translates to "in glass" because in vitro experiments often happen in in a Petrie dish or test tube.
In Vivo	A process that happens inside of a living organism. The term literally translates to "in life" because in vitro processes occur within a human organism or other animal.
Knockin	Use of molecular genomic techniques to add genetic information to an organism. This is often done as part of the effort to humanize a test animal to better mimic a clinical population.
Knockout	Use of molecular genomic techniques to remove genetic information from an organism or make the copy non-functional.
Locus/loci	The physical location of a particular gene or stretch of genetic material along a chromosome. When more than one locus is being referred to they are called loci.
Mouse model	Use of a mouse to study a condition that affects humans; a specific type of animal model.
Nucleotide	The most fundamental building block of genetic material (e.g. DNA). Nucleotides have 3 components, a sugar (ribose or deoxyribose), a phosphate group, and a nitrogen-containing base (adenine, guanine, thymine, cytosine, and uracil).
Pharmacogenomics	The intersection of genomic information and pharmacology; applications include identifying the correct therapeutic regimen based on a patient's genotype and correlating drug response with genotype.

Phenotype	The traits that can be observed or measured in an individual (e.g. hair color; disease presence/absence; height). The phenotype is produced by the genotype when the gene(s) involved are expressed into protein(s); there may also be an impact of environmental factors (e.g. diet; exercise; sun-exposure) on phenotype.
Strain	A population of test animals that are genetically uniform as a result of inbreeding or genetic engineering.
Substrain	A subpopulation of a strain of test animals because they have some distinguishing feature from the parent strain, usually as the result of genetic changes that accumulate over several generations of breeding.
Transgenic	A transgenic animal is one who has had DNA from another source inserted into its genome using laboratory techniques.

5.3 INTRODUCTION AND PURPOSE

Animal models have been an important research and teaching tool for thousands of years and are becoming even more important in the advent of the molecular-genomic revolution. While the role of molecular genomics in nursing research and clinical practice is increasingly recognized (G. Anderson, Alt-White, Schaa, Boyd, & Kasper, 2015; Blix, 2014; Leach, Tonkin, Lancastle, & Kirk, 2016; Schutte, 2006; Seibert, 2014; Umberger, Holston, Hutson, & Pierce, 2013; Williams, Cashion, Shekar, & Ginsburg, 2016), the relevance of pre-clinical inquiry to nursing practice is not always readily apparent. Still, several published articles address how these areas of inquiry are germane to nurses in both research and practice (Page, 2004; Stanley & Paice, 1997; Tkacs & Thompson, 2006a; Witek-Janusek, 2004). Similarly, within the broader health science research community the role for nurses in various aspects of pre-clinical research and subsequent translation

efforts is often not recognized or fully appreciated. Despite this significant gap in awareness, nurses have a rich history of contributing to pre-clinical research including serving as: 1) members of the research team such as principal investigator (PI), co-investigator (Co-I), or consultant; 2) overseers of pre-clinical research to ensure it meets ethical and legal standards; 3) and translators of findings to clinical trials, and ultimately, the bedside. Increasing understanding of the genomic underpinnings of health and disease, which is typically rooted in pre-clinical research, enables clinicians to better treat patients through precision medicine initiatives.

The primary purpose of this paper is to address the aforementioned gap by highlighting the varied ways that nurses contribute to pre-clinical research efforts, such as study planning, approval, and conduct, as well as efforts to translate findings to clinical trials and care. A secondary purpose is to provide nurse scientists interested in conducting animal research with key considerations for planning and executing animal research studies; in doing so, relevant resources will be highlighted. Though the secondary aim is primarily tailored to a nursing research audience, the information may also be relevant to clinicians in helping them to evaluate the quality of pre-clinical studies. To the authors' knowledge, this is the first publication to focus on a review of *animal models* specifically for *genetic* nursing research and the role of nurses in conducting this type of work.

5.4 THE ROLE OF NURSES

5.4.1 Overview of the rose of nurse in animal research historically and contemporarily

Since antiquity, the vast majority of animal research has been conducted by physicians or bench scientists with training in a scientific discipline outside of nursing (e.g. Biochemistry; Molecular-Biology; Neuroscience; Anatomy & Physiology). Thus, in the 2,500 years of animal research, nurses represent only a small fraction of the scientists conducting this type of work. This can be explained partly by the relatively new nature of the nursing profession broadly and the even newer role of nurses as scientific investigators. Given the ultimate concern of nurses is for their patients, many nurse scientists prefer to use samples drawn directly from the target population; thus human subjects research remains most common in the nursing research portfolio. Moreover, as previously noted, though *animal models* are used in nursing, the number of nurse scientists utilizing them remains small; however, the co-existence of pre-clinical and clinical studies within the nursing research portfolio serves to strengthen the nursing knowledge base (Rodgers et al., 2004). Fortunately, nurses have made – and continue to make – important contributions using pre-clinical research as PIs, Co-Is, consultants, and/or collaborators on animal experiments. Each of these will be discussed in detail below, followed by a selection of exemplar research applications where nurses have contributed to the pre-clinical knowledge base.

5.4.1.1 Nurse clinicians Nurses in clinical practice have several important roles and responsibilities in biomedical research and ultimate translation to the bedside (Sadler, Lantz, Fullerton, & Dault, 1999). Nurses often assist with early-phase clinical trials, which are informed by pre-clinical studies. They may also assist with study design, institutional review/approval, or interpretation of study findings. In clinical research nurses often assist with recruitment, delivery of the study drug, and/or data collection, either on their assigned unit or through additional voluntary or paid experiences. Nurses who specifically seek out the aforementioned opportunities are often motivated by the unique opportunity to contribute to cutting-edge research and an over-arching desire to contribute to improvement in patient outcomes. Some become involved when considering transferring to a nursing position in a research-intensive setting or when pursuing a research-focused PhD degree from a School of Nursing. Others do this simply because it is part of the roles and responsibilities outlined by their employer (e.g. a hospital affiliated with a research-intensive university).

Beyond direct roles in conducting research, nurses also contribute in indirect ways to pre-clinical research, by promoting high-quality studies that meet existing ethical and legal standards. For example, nurses participate on research ethics boards, study sections of grant-awarding agencies, and editorial boards for scientific journals. Nurses particularly interested in promoting laboratory animal welfare may elect to volunteer their time on a research review board such as a university Institutional Animal Care and Use Committee (IACUC). Overall, nurses have a long-standing reputation as trustworthy professionals who, through their holistic perspective, are especially in-tune with patients' perspectives, needs, and realities; thus, nurse scientists are uniquely well positioned to help promote clinical relevance in pre-clinical studies and advance subsequent translation efforts (Tkacs & Thompson, 2006b). Many of the ways nurse clinicians can

contribute to animal research are opportunities for their own learning and professional development, as well as leadership and service experiences that could be listed on a curriculum vita.

5.4.1.2 Nurse scientists The role of the nurse scientist in pre-clinical research is more apparent. Nurse scientists serve as PI, Co-I, collaborators, and consultants on grant applications and associated research projects. Three of the primary reasons why nurse scientists would want to participate in pre-clinical research will be briefly described. First, the unique strengths of *animal models* that allows them to answer a number of health-science research questions in a way that would not be possible in clinical research (see "Strengths" heading for additional details). Second, participation in animal research diversifies the researcher's skills, which enables him/her to be more thoughtful consumer of the pre-clinical knowledge base, and may lead to additional opportunities. Third, inclusion of the nursing perspective into pre-clinical research as part of a collaborative, trans-disciplinary research environment strengthens pre-clinical studies through the incorporation of nurses' unique insights into patient needs, preferences, and experiences.

5.4.2 Nursing research applications

In addition to the nursing perspective strengthening the quality of pre-clinical research, it is also true that pre-clinical research strengthens the nursing science knowledge base (P. J. Rowsey, 2015). *Animal models* allow nurses to address research questions that would be difficult or impossible to answer through clinical research, as described in additional detail under "Strengths and Limitations." For these reasons, many nurse scientists have chosen to employ *animal models* in at least part of their program of research. While the breadth of the literature is beyond the scope

of this review, four key areas of inquiry that nurses have contributed to using *animal models* will be discussed as exemplars, namely: 1) evaluating physiology and pathophysiology; 2) studying the genomic underpinnings of health and disease; 3) testing interventions; and 4) evaluating the effects of environment on health outcomes. In each category, several exemplar studies with known nursing input will be briefly described, and the relevance of the knowledge generated for nursing science and practice highlighted.

5.4.2.1 Application 1: Evaluating physiology and pathophysiology One of the earliest, and thus most well-established applications of pre-clinical research both historically and contemporarily is to explore the physiology of health and disease/injury. While much of this work has been conducted by physician-researchers and other bench scientists, nurses have also made contributions to this effort. While not intended to be a comprehensive review, a few key examples of work in this area that were conducted with one or more nurses on the team (as evidenced by the author byline on resulting publications) are summarized below.

In one study evidence of paravascular fluid circulation within the central nervous system (CNS) was identified (Rennels, Gregory, Blaumanis, Fujimoto, & Grady, 1985). In this study, researchers used microscopic examination of brain tissue harvested from cats and dogs after injection of a tracer; control over sample collection time (which ranged between 2 minutes and 4 hours after initial tracer injection) allowed for examination of the temporal sequence of events, which would not be ethical or feasible in humans. Although, paravascular fluid circulation could not have been as definitively studied in humans, this effort has clear clinical relevance. First, it demonstrated that that solutes within the cerebrospinal fluid (CSF) rapidly have access to the extracellular space (ECS) via microvascular routes; prior to this study, this fluid exchange was erroneously thought to be solely due to diffusion. The authors also acknowledge that this work has

provided avenues to limit paravascular influx of solutes (e.g. if this would result in adverse effects in the ECS) by diminishing or totally inhibiting pulsations (e.g. partial ligation of the brachiocephalic artery). A second study conducted by three of the researchers on the original study (Blaumanis, Rennels, & Grady, 1990) added to our understanding of paravascular circulation. This follow up study examined the effect of edema on previously reported paravascular transfer (Rennels et al., 1985). Cats were subjected to a cold lesion model that resulted in vasogenic edema and a tracer was injected to monitor paravascular circulation. In areas of edema, paravascular transport was greatly reduced as evidenced by sparse tracer levels in edematous regions. Since edema is characteristic of many CNS disorders including traumatic brain injury (Unterberg, Stover, Kress, & Kiening, 2004), stroke (Zheng, Chen, Zhang, & Hu, 2016), meningitis (Citton, Toldo, Calderone, Sartori, & Manara, 2009), and encephalitis (Lan et al., 2016), this line of inquiry has potential far-reaching clinical applications.

A second exemplar of nursing literature characterizing physiology/pathophysiology are the efforts to study the effects of endotoxin exposure. In this line of inquiry, rats injected with endotoxin, a type of toxic compound derived from bacterial cell walls, which could not be ethnically done in a randomized controlled trial in patients; this preclinical research has clear clinical relevance since endotoxin adversely affects humans. Findings from these pre-clinical studies suggest that endotoxin exposure leads to responses in many systems including: insulin, somatostatin, lactate, and the pituitary (Witek-Janusek, 1988; Yelich & Witek-Janusek, 1994). Since these studies, the relationship between endotoxin and hormones has been further validated with human subjects research conducted by non-nurses (Lira et al., 2012), including evaluating inhibition of endotoxin as a therapeutic strategy for diabetes (Wagner, Zierden, Baumeister, Wüst, & Hauss, 1975).

A final line of nursing inquiry is the examination of pathophysiological changes associated with stroke (Ritter et al., 2008, 2011) and strategies to target these changes therapeutically (Funk et al., 2003, 2013; Ruehl et al., 2002). In this context, pre-clinical models are a fundamental tool which facilitates analysis of brain tissue at specified post-stroke time points, which would only be possible in humans using non-invasive methods (e.g. imaging). If researchers were to examine brain tissue after stroke in humans, the timing of tissue collection post-stroke would vary tremendously as patient death could occur at any point after injury. This temporal understanding of pathophysiological changes associated with stroke has relevance to selecting the drug type and regimen. Interestingly, a nurse researcher also published a scholarly dialogue paper in *Nursing Science Quarterly,* detailing the interconnected nature of her pre-clinical mechanistic work and clinical experiences, followed by a discussion on the topic of nursing as a type of basic science (Ritter, 2008).

5.4.2.2 Application 2: Studying genomic underpinnings of health and disease Unsurprisingly, in the genomic era, *animal models* are commonly used in conjunction with molecular and genomic research methods. Other studies indirectly explore the effects of *genes* by using test animals that have had their *genomes* modified in one or more ways. There are many examples of nurses who have contributed to molecular *genetic* research efforts using *animal models*. Several noteworthy examples are discussed below.

Identification of *genes* implicated in various disorders represents an important line of inquiry to which nurses have contributed. One study used a genome-wide screen in mice and identified a novel *gene* (Gan1) implicated in peripheral neuropathy associated with anti-retroviral therapy. One implication is that targeting Gan1 or its protein may obviate neuropathy for individuals taking anti-retrovirals (Dorsey et al., 2009). Moreover, *genetic* variation, including

frameshift and nonsense mutations, in Gan1 has been reported (Bomont et al., 2000; Kuhlenbäumer et al., 2002), which may be relevant to precision medicine initiatives for neuropathy. Another study harnessed the ability of researchers to non-controversially harness molecular techniques to generate mice that have had a particular *gene* removed from the *genome*, also referred to as being knocked out (KO) (Dorsey et al., 2006). In this *mouse model*, KO of TrkB.T1 receptor was associated with improved outcomes including preservation of hippocampal neurons (Dorsey et al., 2006). A follow up study using the same *strain* of mice found that KO of TrkB.T1 receptor is associated with altered sleep, including increased time spent in REM sleep and reduced sleep continuity (Watson, Henson, Dorsey, & Frank, 2015). This information may prove relevant to treating individuals with certain *genotypes* and/or sleep disorders.

Gene expression alterations in the context of various conditions have also been explored by several nurse researchers. For example, expression of the FOS *gene* is induced in the context of infectious disease and endotoxemia (Tkacs, Li, & Strack, 1997; Tkacs & Li, 1999). In another study, non-coma hypoglycemia in a rat model was found to be associated with decreased levels of neuropeptide Y and pro-opiomelanocortin mRNA (N. Tkacs et al., 2000). The aforementioned studies may also lead to precision medicine initiatives. For example, some patients may benefit from antisense therapy which would bind to over-expressed mRNA so that it is not translated to protein (Turner, 1997).

Another study evaluated the effects of 60 minutes of inspiratory resistance loading (vs. sham) in rats and treatment with either dopamine or saline control on *gene expression* of 27 known apoptotic proteins (Goodyear-Bruch, Jegathesan, Clancy, & Pierce, 2008). In the diaphragm, 12 of the proteins were expressed, 2 of which showed higher expression after inspiratory resistance loading when receiving dopamine treatment (vs. saline control). Specifically superoxide dismutase

copper zinc (SOD [CuZn]) and proprioceptive event related potential (PERP) were elevated, suggesting dopamine reduces the apoptotic consequences of this condition (Goodyear-Bruch et al., 2008).

5.4.2.3 Application 3: Testing interventions Of clear relevance to nursing research and practice is identifying the safest and most effective treatments for the many disease conditions and symptoms afflicting patients. The early work in this process requires *animal models* to establish basic safety and feasibility before translation to clinical trials is justified. Thus, it is no surprise that nurse scientists have contributed to *animal model* research testing interventions.

One study used a rat model exposed to various fever inducers to test the possible effects of methyl scopolamine as an anti-pyretic (P. J. Rowsey & Gordon, 2000), a best practice before exploring off-label effects experimentally in humans. In this study, methyl scopolamine (1 mg/kg) was administered intraperitoneally (i.p.) as a single dose. The therapeutic regimen used in this study was associated with marked reversal in the temperature elevation associated with stress (handling of the animal and switching the cage), chlorpyrifos (an organophosphate pesticide), and nocturnal cycles (P. J. Rowsey & Gordon, 2000). The same fever mechanisms were targeted by the known antipyretic sodium salicylate. This study identified a potential avenue for clinical trials and may also be relevant to identification of adverse effects regarding thermoregulation on patients taking methyl scopolamine.

Notably, it is not just novel pharmaceuticals (e.g. pills, injections) that can be tested in pre-clinical models, but rather a diverse array of therapies. In one study (Bennetts et al., 2014) two fluid resuscitation formulations [Lactated Ringer (LR) vs. LR + ubiquinol] were tested as a means of improving outcomes of hemorrhagic shock in rats. Hemorrhagic shock was induced by removing 40% of the total blood volume, treatment administered, and animal monitored for 2

hours prior to sacrifice. The heart, lungs, kidney, and diaphragm were harvested and analyzed. The main finding was that hydrogen peroxide levels and apoptotic proteins were reduced following LR + ubiquinol treatment suggesting protective effects on oxidation and cell death (Bennetts et al., 2014).

5.4.2.4 Application 4: Evaluating the effects of the environment on health outcomes The way environmental and lifestyle factors interact with health can also be easily studied in *animal models*. One study examined the effect of exercise on core temperature in a sample of female rats (P. J. Rowsey, Borer, & Kluger, 1993). In this study, exercise was associated with a higher body temperature both during the period of exercise as well as during rest. A second goal of this study was to identify the role, if any, of prostaglandin E in the elevated body temperature reported. To elucidate the role of prostaglandin E, sodium salicylate was injected in both exercised and sedentary rats; regardless of group this injection was associated with a decrease in body temperature suggesting the increase in body temperature after exercise is not prostaglandin-mediated.

A second line of inquiry evaluated another aspect of exercise, specifically how it relates to susceptibility to environmental toxicants (P. J. Rowsey, Metzger, Carlson, & Gordon, 2003) and alterations in serum cytokine levels (P. J. J. Rowsey et al., 2009). This study built off the nurse-led team's past work which characterized the hypothermic and hyperthermic changes associated with organophosphate pesticide exposure, and factors associated with tolerance to such effects (P. J. Rowsey & Gordon, 1997). Specifically, this study examined the effects of exercise training (vs. sedentary behavior) on the known thermoregulatory consequences of chlorpyrifos, which causes an immediate hypothermic response followed by a temperature spike. Following 8 weeks of exercise or sedentary behavior, rats were treated with either chlorpyrifos or a control solution

administered via gavage over the course of 4 days. In the exercise group, the first dose of chlorpyrifos did not cause hypothermia (as with the sedentary animals); a period of hyperthermia followed the hypothermic events, though this temperature spike was less severe in the exercise group. Overall, this study harnesses the precise environmental control afforded by *animal models* and found that exercise reduced both the hypothermic and hyperthermic consequences of repeated chlorpyrifos exposure (P. J. Rowsey & Gordon, 1997). The follow up study (P. J. Rowsey et al., 2006) further examined the effect of chronic exercise conditioning on thermoregulation following exposure to agents known to be pro-inflammatory (e.g. turpentine and lipopolysaccharide). In this study, female rats were randomized to two activity groups (exercise vs. sedentary) and engaged in the target activity level for 8 weeks before being injected with turpentine, lipopolysaccharide, or a control solution. Turpentine led to a prolonged low-grade temperature that was slightly suppressed by exercise training when core temperatures were assessed during the day (not the night). In the lipopolysaccharide group, exercise training actually compounded the fever. Taken together this evidence suggests that the effects of exercise are different depending on the exposure that leads to fever (P. J. Rowsey et al., 2006).

A final example is a study of the effects of maternal alcohol consumption on neonatal outcomes related to glucose balance (Witek-Janusek, 1986). Clearly, a true experimental study characterized by group randomization would not be ethically possible in clinical research, necessitating an *animal model*. In this study female rats were placed on a liquid ethanol diet, a isocaloric liquid diet, or a standard chow diet beginning 3 weeks prior to mating and continuing throughout the pregnancy. Once the litters were born the ethanol-exposed pups were found to have decreased levels of stored glycogen in the liver. This change in glycogen levels was associated with higher rates of hypoglycemia in the early post-natal period, especially when the pups were

not fed. Notably, maternal liver glycogen stores were also decreased when a liquid ethanol diet was consumed (Witek-Janusek, 1986). This pre-clinical evidence has clinical relevance and may inform future research and interventions.

5.5 CONSIDERATIONS FOR RESEARCHERS

For nurse scientists considering conducting pre-clinical research, there are numerous considerations prior to and during study planning that can greatly affect the quality of the study. For many nurse scientists formal educational training surrounding pre-clinical research design and methodologies is limited, which can complicate the planning process especially when more experienced collaborators are not available. Important considerations, including overall strengths and limitations of pre-clinical research, a discussion of the model selection process, a summary of common genomic modifications and their uses, as well as a discussion of environmental factors that may confound study findings are described below; this discussion will be tailored to a nurse scientist audience. To further assist readers, a summary of available resources relevant to the planning and conducting of pre-clinical research will be provided.

5.5.1 Strengths and limitations of pre-clinical research

5.5.1.1 Strengths *Animal models* have numerous unique advantages allowing questions to be answered that would not be feasible, ethical, or even possible in human research. Arguably the most critical use of *animal models* is to garner fundamental information related to the safety of interventions and products (e.g. cosmetics) before application to humans. Another strength is the ability to collect long-term data without significant risk of sample attrition since test subjects are housed by researchers and monitored by veterinarians. Also, the shorter lifespan of animals enables a researcher to track the natural history of a disease or injury from the time of onset until death (if desired), which would be exceedingly more complicated in humans. Similarly, the ability to euthanize test animals at controlled times allows researchers to study the time course of pathophysiological changes associated with various conditions.

Another advantage of pre-clinical studies is that biological changes that underlie diseases and injury can be studied readily and directly. Consider a researcher trying to understand the effects of ischemic stroke on the functional tissue (parenchyma) of the brain. Extensively characterizing the effects of stroke on human brain tissue would only be possible upon autopsy; moreover, since the time between stroke onset and death can range from seconds to years, it would be difficult to develop a clear understanding of the typical sequence of events. Without *animal models*, researchers would have to depend on indirect measures as a proxy for the state of the brain (e.g. functional magnetic resonance imaging [fMRI]; screening for serum biomarkers), which may obscure scientific understanding of the pathophysiology of various conditions. Using pre-clinical models, brain tissue is readily available and animals can be sacrificed for tissue processing at precisely controlled time points.

Beyond the types of questions that can be addressed in *animal models*, there are other strengths worth noting. First, animals share many similar features with humans, both biologically and behaviorally. It is very likely that if a researcher is interested in some aspect of the human experience there is at least one *animal model* available that is a good representation of the pathophysiology or behavior of interest and established way to measure it. A second asset of *animal models* is that they are always improving. In recent years, *genetic* manipulations are being made to the *genome* of animals; humanization efforts result in test animals that better model a clinical condition. Recently, a *mouse model* was developed that resulted in human-like albumin which has implications for preclinical drug studies since albumin is a possible drug delivery vehicle (Low & Wiles, 2016). In addition to the *genetic* manipulations possible, there are many other practical advantage since the test animal's age, sex, diet, environment, and other factors, can be more tightly controlled. Also worth noting briefly is that while animal research presents its own set of ethical and legal considerations (described elsewhere in this review), generally there are less ethical and legal concerns for pre-clinical research than there are for clinical trials. Ultimately, as the body of pre-clinical evidence grows, additional avenues of clinical research become available. Notably, not all animal studies warrant extension to human studies, rather critical evaluation of a large body of research is necessary. Overall, it is clear that humans benefit in many ways from animal research; indeed, according to a poster published by the Foundation for Biomedical Research using data from the U.S. Department of Health and Human Services the human life span has increased 20.8 years as a result of animal research and subsequent healthcare advances.

5.5.1.2 Limitations While *animal models* play an important role in advancing nursing research and the health sciences knowledge base more broadly, there are several notable limitations. First and foremost, while common *animal models* share important similarities of humans, no pre-clinical model perfectly represents all aspects of the human experience. Moreover, for any condition of interest the combination of the animal (species; strain; sex; etc.) used and the method used to induce the disease or condition of interest (assuming it does not naturally occur in the test animal) must be carefully decided upon; there may be several techniques available to induce the condition of interest in the test animal and each may mimic certain aspects of the pathophysiological and behavioral consequences, but not others. Finally, there are some human conditions for which no *animal model* is available. For instance, rare, or orphan disorders (e.g. Zechi-Ceide syndrome) may have no available *animal model* (Weizmann Institute of Sciences, 2015). However, rare conditions are also challenging to study clinically due to low population sizes and subsequently small samples, which limits study power. Moreover, advancements in the scientific knowledge base and genomic technologies mean that new models may become available.

A second broad-scale concern actually stems from the high degree of control that simultaneously strengthens animal research. While this helps to reducing confounding effects when garnering early evidence, the control characteristic of most animal studies is very dissimilar to what is seen in clinical research. Indeed, clinical trials and subsequent translation efforts are often complicated by the heterogeneity of clinical populations with respect to factors including but not limited to: age, sex, *genotype*, and diet. Thus, when a drug is effective in all male mice of a narrow age range, it may not be easily translatable to a diverse clinical sample. Thus, generalizability of pre-clinical research is limited necessitating accumulation of evidence across several studies; recently, there has also been a push by leaders in the research community and

funding agents alike to diversify samples. Moreover, while a high degree of control is often planned in studies, there are many potential confounders that can accidentally be introduced to the study (see "Unintended consequences and confounders" heading). Moreover, one must balance the ethical considerations of adequately vetting a therapy in preclinical models with the concern of prolonging preclinical trials to the extent that humans are not benefitting from the research.

Beyond these broad limitations, there are also several, specific, practical limitations of animal research. This type of work requires special facilities (e.g. vivarium) and equipment as well as staff trained in how to work safely and ethically with animals. Even when best practices are strictly adhered to, there is the potential of harm or suffering to the animal. Similarly, working with animals poses an inherent risk to the researcher, including the risk of being bitten or exposed to a zoonotic illness that the test animal carries. Of additional concern is that housing, husbandry, and experimentation on animals can be quite expensive. Typically housing costs are paid per day per cage and some animals need to be housed singly due to size or temperament (e.g. C57BL/6 mice are known to be aggressive). Still, it is notable that many pre-clinical studies are less expensive than clinical research, particularly when the cost of participant reimbursement is high or sophisticated molecular-genetic analysis is performed.

5.5.2 Model selection

The most important decision when using an *animal model* is determining which model is most appropriate to meet the research goals. For example, if complex neurological functions such as memory and learning are of interest, mammals whose brains are more similar to humans are preferred over lower vertebrates and invertebrates with drastically different neurological structures. Typically, the extent of genotypic similarity to that of humans is of interest when

choosing experimental models. Many animal *genomes* have been sequenced and analyzed, allowing quantitative comparison between humans and model organisms with respect to number of *nucleotides*, *chromosomes*, *genes*, and proteins. The percent of the *genome* that is *protein-coding*, extent of homology with human *genes*, or presence/absence of a human *gene* of interest (e.g. KO or *knockin* [KI]) can also be considered as part of model selection. Moreover, the *phenotype* of interest is often used to guide model selection. For instance, when evaluating the effect of an antihypertensive drug, the researchers may choose an *animal model* that is taxonomically close to humans vs. a cold-blooded or invertebrate model due to the similarity of cardiac and circulatory systems in mammals.

When certain types of common laboratory animals (e.g. mice) are used, model selection also includes deliberation between many available *strains*, each with their respective set of *substrains*; each *strain* and *substrain* has unique physiological and behavioral characteristics to consider. For instance, researchers testing anti-hypertensive drugs may select BPH/2 mice which have average blood pressures of approximately 145 mmHg, a full 25 mmHg higher than the most commonly used *strain* C57BL/6 (The Jackson Laboratory, 2013b). If the researchers are interested in the effect of a *gene*, they may search for a *strain* characterized by a hallmark mutation of interest. Mice are commonly used to study alcoholism, because some *strains* (e.g. C57BL/6) show a high preference for alcohol (McClearn & Rodgers, 1961), which has been associated with specific genetic *loci* (Melo, Shendure, Pociask, & Silver, 1996). Recently, C57BL/6 mice were used to study the effects of maternal binge drinking on functional outcomes in their offspring (Wagner, Zhou, & Goodlett, 2014).

Choice of a biologically relevant model includes ensuring that the controls are appropriate. If using a *substrain* of mice derived from a commercially available *strain*, a general rule of thumb

is that the main *strain* can be considered a suitable control for your *substrain* if the *substrain* has been maintained through strict inbreeding for at least five generations. It is also relevant to consider whether controls possess any histopathological or behavioral characteristics likely to confound study findings. C57BL/6 mice are prone to age-related hearing loss starting around 10 months, with subsequent degeneration of the organ of Corti (H. S. Li & Hultcrantz, 1994); this hearing loss would confound studying functional outcomes (e.g. fear; cognition; beam walking) in old age using tests that require an auditory cue (e.g. white noise machine).

It is worth noting that some human health disorders may have no single model that fully represents the disease pathology. For example, for type 2 diabetes, there are several available models, each capturing one or more important aspects of the disease. Similarly, traumatic brain injury (TBI) is characterized by complex physiological and behavioral symptoms which persist into the chronic period; however, outcomes vary widely across models with some injury inductions producing more focal effects and others producing more diffuse effects. Researchers should carefully consider the outcomes of interest when choosing a model. Notably, it is also important that researchers report the details of the model (species; *strain*, *substrain*; vendor; etc.) in their publications; this not only enables replication of the study by independent researchers, but also facilitates comparison of study findings with those previously published in the literature. Resources for appropriate selection of a model, strain, and substrain are described later (Table 8).

5.5.3 Common genomic modifications

Modification (either reversible or permanent) to the *genomes* of animals has been well-established and represents a common avenue for research. Most notable are the efforts to humanize animals to further enhance the clinical relevance of pre-clinical studies. While there are many types of

genetic manipulations in use, and commercially available *transgenic* animals, this introductory article will focus only on four common modifications: *knockout* (KO), *knockin* (KI), conditional (i.e. inducible) mutations and *gene* editing.

Researchers can use KO models to determine the role of a *gene*, gene products (i.e. protein), or the consequences of a loss of function mutation in a *gene* in one or both chromosomal copies (LePage & Conlon, 2006). Thus, researchers can identify the effects of a *homozygous* and/or *heterozygous* mutant *genotype(s)* on one or more histopathological or behavioral outcomes of interest. This is especially useful when the *gene* being knocked out is conserved across species and also found in humans. In some cases, especially *autosomal dominant* conditions, a *homozygous* mutant *genotype* is fatal before birth, necessitating the use of a *heterozygous* KO to accurately study the disease. In other cases, *heterozygous* KO animals are chosen because a *homozygous genotype* causes severely impaired development of the animal to the extent that it would interfere with experimental goals. Conversely, researchers may choose a KI model when the goal is to insert a *gene* at a particular *locus* within the *genome*. KI models insert *complementary deoxyribonucleic acid (cDNA)* at specific chromosomal *loci*, typically to create a novel disease model or further humanize a test animal. Researchers can also use KI animals to evaluate the role of the regulatory machinery (e.g. promoters, enhancers) that control *gene expression*.

Conditional mutations are mutant *alleles* that cause a change in *phenotype* from the normal wild-type only when exposed to certain environmental conditions or stimuli (e.g. a diet rich in some nutrient; high temperatures). For example, as seen in sickle cell anemia, carriers of the *genotype* may experience a change in *phenotype* that is conditional on environmental factors such as: exposure to cold temperatures, low oxygen, or another restrictive conditions. Animals with *conditional mutations* are useful to researchers interested in studying disorders that occur later in

life; such medical conditions are difficult to study using KI/KO models, since addition/deletion of genetic material often causes premature death. Conditional mutants are applied to identify crucial developmental *gene expression* events by exposing animals to restrictive conditions at various time points and observing the consequences.

In vivo gene editing represents a new horizon for animal research and, in some international research communities, clinical research. Largely, the prospects of gene editing in humans remains controversial (Hampton, 2016), though research is gaining momentum (Callaway, 2016). To-date the most popular method remains the CRISPR-Cas system (Ledford, 2016). In animals *in vivo* gene editing is an area of rapid research development. For example, gene editing techniques have been used to improve outcomes of muscular dystrophy in mice (Long et al., 2016; Nelson et al., 2016). These techniques have also been used as a simple way to screen human gene loss-of-function (Bhattacharya, Marfo, Li, Lane, & Khokha, 2015).

5.5.4 Unintended consequences and confounders

There are several mundane aspects of experimental design that could potentially confound study findings including housing (e.g. cage type; presence of nestlets/toys/etc.; light/dark cycle), diet (food type; quantity/access) and husbandry. At a minimum, these choices should be informed by a thorough review of the literature. Whenever possible, pilot testing should further inform the environmental exposure selection.

It is also important to note, that in many instances, total control over environmental factors are not possible. Often, studies allow ad libitum (i.e. free, unlimited) access to chow and water. Thus, it is possible that different test animals have different overall intake of food and/or water.

Depending on the experimental procedures, other potential confounders may be introduced. For example, when animals undergo anesthesia, the effects of the agent used on study outcomes should be considered; as an exemplar, isoflurane is associated with neuroprotection (Statler et al., 2000, 2006). Researchers should pay careful attention to potential consequences of experimental procedures and make efforts to reduce their confounding effects.

5.5.5 Resources available

Researchers should identify and utilize available resources at their institutions. Departments overseeing animal studies may offer training opportunities in research methodologies and the responsible conduct of research. Research institutions may also offer other assistance such as shared animal research space, tissue banks, and pilot funding. Furthermore, researchers should carefully consider the available budget, taking into account the anticipated costs of animal acquisition, housing and husbandry, personnel, equipment and supplies. With respect to obtaining test animals, some institutions maintain colonies of one or more species. For those who need to acquire test animals from outside the institution, there are many commercial sources. Researchers using *mouse models* can utilize the many national and international repositories outlined in the International Mouse Strain Resource (www.findmice.org), many of which are highlighted in Table 8. For other species of test animals, there are also resources available as summarized in previous publications (Mashimo & Serikawa, 2009; Matthews, Kaufman, & Gelbart, 2005; Smith, 2012). Regardless of species, researchers are encouraged to learn as much about the test animal as possible. Notably, commercial suppliers of test animal often offer literature, webinars, and other guidance.

Table 8: Resources for pre-clinical research

Resource Name	*Sponsor/ Source*	*Description*	*URL*
Mouse Genome Informatics	The Jackson Laboratory with data contributed form several well-known research groups	International database providing a wealth of data (e.g. genetic, genomic, biological, phenotypic) about laboratory mice used in biomedical research. Selected resources include a glossary, a wealth of readings, data, and other downloadable resources.	www.informatics.jax.org
International Mouse Strain Resource	IMSR funded by National Institutes of Health (NIH)	A database that assists researchers with acquiring information about mice of various strains including sources of these mice for use in research.	www.findmice.org
UCSC Mouse Genome Browser	Genome Bioinformatics Group of UC Santa Cruz	Complete mouse genome information for the most commonly used strain of mice in biomedical research (C57BL/6). Note: genomes of other species of animal are also available through the genome browser, though the link provided is for the mouse browser specifically.	http://genome.ucsc.edu/cgi-bin/hgGateway?org=mouse

Mammalian Gene Collection	NIH	Validated sequence information in a convenient, searchable form. Note: also includes genomic sequence information for human, rat, and cow.	http://genecollections.nci.nih.gov/MGC/
Mouse Genome Assembly Data	Genome Reference Consortium	Searchable website that provide global mouse assembly statistics and data for mice with periodic reviews and patch releases of data. Note: also contains genomic reference information for human, zebrafish, and chicken.	https://www.ncbi.nlm.nih.gov/projects/genome/assembly/grc/mouse/data/
Mouse Genomes Project	Wellcome Trust (WT); Sanger Institute	Provides data and complete sequence information for many inbred strains of mice used in biomedical research. There is also a mouse genome variant querying site. Sequencing reads, variants, and assembled genome sequences are published on this site. With permission, researchers can download data and use it for analysis and publication.	http://www.sanger.ac.uk/resources/mouse/genomes/

Knockout Mouse Phenotyping Project	NIH in collaboration between the Jackson Laboratory, Baylor College of Medicine, the University of California Davis, and others	Provides a functional catalog of a mammalian genome through systematic generation and phenotyping of 20,000 knockout strains. The website provides searchable genotype and phenotype information.	http://www.mousephenotype.org/
Current Lab Codes	Institute for Laboratory Animal Research (ILAR)	International registry of laboratory codes indicating the institute, laboratory, or investigator where a particular strain of test animal was produced and/or is maintained. In addition to strains, substrains, congenic strains, other distinguishable groups of strains, DNA loci, specific mutations, and other chromosomal aberrations may have a laboratory code.	http://dels.nas.edu/global/ilar/Lab-Codes

Knockout Mouse Project (KOMP) Repository	UC Davis	The ultimate goal is a comprehensive resource that is publically available and comprises mouse embryonic stem cells that have knocked out genes of each and every gene in the mouse genome. The repository is updated regularly, following acquisition of new vectors, cell lines, and live mice.	https://www.komp.org/
European Nucleotide Archive	European Molecular Biology Laboratory, Seventh Framework Programme of the European Commission, the British Biotechnology & Biological Sciences Research Council, and the WT	Provides a comprehensive guide to nucleotide sequencing information which covers sequence assembly information, raw data generated during sequencing, and also functional annotation.	http://www.ebi.ac.uk/ena

Mutant Mouse Resource Research Centers (MMRRC)	NIH	A national network of mouse breeding and distribution facilities dedicated to being a high-quality repository of mouse strains, including many that are not commercially available. Researchers can also donate to this resource as part of their obligation to share grant-funded resources (if applicable).	https://www.mmrrc.org/

5.5.6 Summary of ethical and legal considerations for pre-clinical research

As mentioned previously, some studies that would not be ethically acceptable or otherwise feasible in human subjects research may be possible using *animal models*. However, similar to human research, maintaining the highest ethical standards is of utmost importance when conducting animal research. Researchers working with animals should follow institutional and governmental guidelines, all relevant laws (e.g. Animal Welfare Act, 1966), as well as employ the best practices outlined in the G*uide for the Care and Use of Laboratory Animals* (National Research Council, 2010). Moreover, all experimental procedures must be approved by the Institutional Animal Care and Use Committee (IACUC) and strictly adhered to.

Part of the effort to conduct ethical pre-clinical research includes selecting the least sentient animal possible to adequately address the research questions and keeping the sample size to an

absolute minimum as informed by available evidence and, when possible, power analysis. Studies should be designed to minimize pain and suffering. When experimental procedures are likely to induce appreciable pain, anesthesia or analgesia should be used, unless otherwise approved by the IACUC or other pertinent regulatory agency. At the end of the research study, animals should be humanely euthanized if warranted.

5.6 CLOSING REMARKS

The ultimate goal of animal research in the genomic era is to enhance human health and quality of life via improved understanding of disease, development of therapies, and application of novel genetic technologies. This goal is consistent with the overarching goals of nursing science to enhance understanding of unmet patient needs as well as design, test, and translate interventions to address the problems faced by patients. Depending on the clinical population and body of research evidence available, the state of the science may be anywhere from preliminary basic science studies to translation of findings to clinical care; thus, nursing science is strengthened by a diverse research portfolio including both pre-clinical and clinical inquiry (Page, 2004; Rodgers et al., 2004). In addition to nurses' many contribution to the clinical research knowledge base, nurses have also contributed to pre-clinical research targeting a number of health conditions and symptom *phenotypes* (Bond, Heitkemper, & Bailey, 1998; Briones, Therrien, & Metzger, 2000; Dorr et al., 2001; Frazier, Moser, & Stone, 2001; Holden & Naleway, 2001; Holden & Therrien, 2000; Kasper, McNulty, Otto, & Thomas, 1993; Landis & Whitney, 1997; McCarthy, 2000; Page, Blakely, & Ben-Eliyahu, 2001; Stanley & Paice, 1997; Witek-Janusek & Ratmeyer, 1991). Many

of these studies have evaluated molecular-genomic factors underlying health, disease, and injury recovery.

Nursing science, and health science research more broadly, is strengthened by nurses' unique ability to gain patient trust and subsequent insight into their lives, perceptions, and care needs. Thus, the inclusion of the nursing perspective enhances the design of pre-clinical studies by promoting selection of more clinically relevant variables likely to be key contributors to patient well-being and quality of life. As multi- and trans-disciplinary teams are becoming increasingly common, nurses should embrace opportunities to conduct and participate in animal research. Moreover, the climate is right for nurse scientists to further establish themselves as leaders and key contributors to pre-clinical research. Funding agencies within the National Institutes of Health, including the National Institute of Nursing Research (NINR), in addition to other professional nursing organizations encourage and support pre-clinical studies. Among the studies funded are many collaborative research projects with dual-discipline co-investigators (co-I) one of which is a nurse scientist. Moreover, many schools of nursing are becoming home to nurse scientists and other researchers using pre-clinical models in their work, and several schools have in-house pre-clinical research space for housing test animals and completing study activities. Overall, nurses continue to make important contributions to animal research by applying pre-clinical methodologies, reviewing journal articles, being members of ethics review boards, and participating in study sections for granting agencies. Nurses in clinical practice assist with the efforts to translate findings from animal research to the bedside. As the prospects of using *pharmacogenomics* knowledge to inform precision medicine becomes increasingly possible, the applications of pre-clinical research will become even more apparent. Recently the research community has become increasingly interested in how *animal models* can be applied to advance

nursing science, and ultimately translated to nursing practice (Holtzclaw & Hanneman, 2002; Kasper, 2013; Schumacher, 2010; Tkacs & Thompson, 2006b). The research community at large touts a seasoned history of using *animal models* to make substantial contributions to the understanding and treatment of a plethora of human health conditions. While the intersection of nursing science with preclinical *animal models* is relatively new, it represents a promising, robust and growing area of research with a bright future.

6.0 REVIEW MANUSCRIPT #3: THE CONTROLLED CORTICAL IMPACT MODEL: APPLICATIONS, CONSIDERATIONS FOR RESEARCHERS, & FUTURE DIRECTIONS

6.1 ABSTRACT

The controlled cortical impact model (CCI) of traumatic brain injury (TBI) was developed nearly 30 years ago with the goal of creating a testing platform to determine the biomechanical properties of brain tissue exposed to direct mechanical deformation. Initially used to model TBIs produced by automotive crashes, the CCI model rapidly transformed into a standardized technique to study TBI mechanisms and evaluate therapies. CCI is most commonly produced using a device that rapidly accelerates a rod to impact the surgically exposed cortical dural surface. The tip of the rod can be varied in size and geometry to accommodate scalability to difference species. Typically, the rod is actuated by a pneumatic piston or electro-mechanic actuator. With some limits, CCI devices can control the velocity, depth, duration, and site of impact. The CCI model produces morphologic and cerebrovascular injury responses that resemble certain aspects of human TBI. Commonly observed are graded histologic and axonal derangements, disruption of the blood-brain barrier, subdural and intra-parenchymal hematoma, edema, inflammation, and alterations in cerebral blood flow. The CCI model also produces neurobehavioral and cognitive impairments similar to those observed clinically. In contrast to other TBI models, the CCI device induces a

significantly pronounced cortical contusion, but is limited in the extent to which it models the diffuse effects of TBI; a related limitation is that not all clinical TBI cases are characterized by a contusion. Another perceived limitation is that a non-clinically relevant craniotomy is performed. Biomechanically, this is irrelevant at the tissue level. However, craniotomies are not atraumatic and the effects of surgery should be controlled by including surgical sham control groups. CCI devices have also been successfully used to impact closed skulls to study mild and repetitive TBI. Future directions for CCI research surround continued refinements to the model through technical improvements in the devices (e.g. minimizing mechanical sources of variation). Like all TBI models, publications should report key injury parameters as outlined in the NIH common data elements (CDEs) for preclinical TBI.

6.2 INTRODUCTION AND PURPOSE

Traumatic Brain Injury (TBI) is a significant worldwide public health problem (Colantonio et al., 2010; Faul et al., 2010; Feigin et al., 2013; Puvanachandra & Hyder, 2008; Reilly, 2007; Roozenbeek et al., 2013; Scudellari, 2010). Individuals who survive TBI often require extensive care including immediate and emergent care often followed by extensive rehabilitation; taken together, this care is associated with high direct and indirect costs (V G Coronado et al., 2012). Unfortunately, to date, efforts to develop therapies effective at improving outcomes of clinical TBI have fallen short and not led to novel FDA-approved therapies for TBI patients. Continued research is needed, including the utilization of experimental (i.e. preclinical) TBI models before attempts to translate interventions to the clinical setting can be justified.

One of the most widely used models of experimental TBI is controlled cortical impact (CCI). Developed in the 1980s, the CCI model has been adapted and refined extensively in the years since. The purpose of this manuscript is to provide a primer on the past and current applications of CCI and discuss considerations for the future of CCI research. In doing so, this review will: 1) provide an overview of the CCI model, 2) synopsize the history of the model's development, 3) highlight the models strengths and weaknesses, 4) discuss experimental design considerations for researchers using CCI, and 5) identify future directions for CCI research along with ways to improve the model. When relevant, details regarding information that should be reported for CCI studies based on the National Institute of Neurological Diseases and Stroke (NINDS) pre-clinical common data elements (CDEs) will be noted.

6.3 PAST & PRESENT APPLICATIONS: MODEL OVERVIEW, DEVELOPMENT, FEATURES, SUBTYPES, & AREAS OF INQUIRY

6.3.1 Overview of the CCI Model

The CCI model was developed in the late 1980s and rapidly became one of the most commonly used models of pre-clinical TBI. The early devices were pneumatically driven, and more recently electromagnetic devices have become available. In the following sections, the development of CCI will be summarized along with notable applications to date, the key features of CCI will be described, and the different devices (pneumatic; electromagnetic) will be discussed.

6.3.1.1 Model development and applications For over a century, efforts to understand human traumatic brain injury (TBI) have relied on utilization of animal models (Cannon, 1901; Denny-Brown & Russell, 1941; Kramer, 1896) to supplement clinical evidence. Early studies were characterized by small sample sizes, a lack of well-established (i.e. vetted) devices for injury induction, and low levels of control over potential confounders. Starting in the 1970s, efforts to refine, standardize, and quantify experimental brain injury models became increasingly common (Govons, Govons, VanHuss, & Heusner, 1972; Nilsson, Pontén, & Voigt, 1977; Ommaya, Geller, & Parsons, 1971; Ommaya & Gennarelli, 1974; Parkinson, West, & Pathiraja, 1978; Rinder & Olsson, 1968; Sullivan et al., 1976). Contemporary CCI models owe their lineage to the work of Thomas Anderson (Anderson, 1982, 1985), who was the first to develop a neurotrauma model utilizing a constrained-stroke pneumatic cylinder mounted on an adjustable crosshead frame to produced injury with a high degree of mechanical reproducibility. The CCI model was first developed in the late 1980s and early 1990s by J.W. Lighthall and colleagues to induce TBI in ferrets (Lighthall, Goshgarian, & Pinderski, 1990; Lighthall, 1988). By 1991, the device was adapted so that the model could be applied to rats (Dixon et al., 1991). After translation to rats, CCI has since been applied to mice (Fox et al., 1998b; Fox, LeVasseur, & Faden, 1999; Hannay et al., 1999), swine (Costine et al., 2012; Friess et al., 2007, 2009; Manley et al., 2006), and nonhuman primates (King et al., 2010), as described in detail later in this review.

Following the initial development and characterization of the model, several new applications of CCI device have emerged, including options for studying closed head injury (CHI) (Klemenhagen, O'Brien, & Brody, 2013; Petraglia et al., 2014; Shitaka et al., 2011). The first 3 decades of CCI research shows some progression in the types of research questions addressed. Early applications primarily focused on characterizing the model and exploring the biomechanical

and physiological changes associated with injury. Later efforts expanded the histopathological and cellular characterization using brain tissue after CCI to identify putative secondary injury processes that could not easily or ethically be studied in human TBI survivors. CCI has also been used extensively to test novel therapies in the hope of ultimately translating promising drugs to clinical care (Dixon, Kraus, et al., 1999; Giacino et al., 2012; Kochanek et al., 2011; Miyamoto et al., 2013).

More recently, the expansion of transgenic animals has led to applications of CCI to identify important genes and gene products that impact injury severity and recovery profiles (Amenta, Jallo, Tuma, Hooper, & Elliott, 2014; Bachstetter et al., 2013; Raghupathi et al., 1998; Yu, Zhang, Liebl, & Kernie, 2008). Notably, the trend is not linear and ongoing research is being conducted in all of the aforementioned areas, with efforts to increasingly promote rigor and replication through the use of CDEs. Today, CCI remains a mainstay in preclinical TBI research.

6.3.1.2 Key features of the model Traditionally, CCI is a mechanical model of TBI that follows anesthetized craniectomy. The CCI device mechanically transfers energy onto the intact dura mater damaging the cortex, and sometimes the subcortical structures in cases of more severe injury. Applications of CCI are discussed in more detail elsewhere in this review. A key feature of CCI is that the injury parameters (e.g. depth, velocity, and dwell time) can all be controlled for to produce a broad range of TBI severities and well as closed head impact (CHI) by impacting the intact skull.

6.3.1.3 Device types and suppliers Two main types of CCI devices are commercially available: pneumatic and electromagnetic. The original applications of the CCI model used a pneumatic device and pneumatic CCI is still commonly used today. The electromagnetic alternative was developed later but is gaining popularity due to its lower cost and greater portability. Both types of CCI are discussed in detail below including a list of commercial suppliers (Table 9).

Pneumatic

When J.W. Lighthall and his colleagues first developed the CCI model, the device was powered by pressurized gas (i.e. pneumatically-driven). Pneumatic CCI remains widely used to study TBI pathophysiology and test novel therapies (Shin et al., 2015; Songarj et al., 2015; Talley Watts et al., 2013). A typical pneumatic CCI device (Figure 26) includes a cylinder, which is rigidly mounted to a crossbar. There typically are multiple mounting positions on the crossbar so the impactor can be vertical or angled, respective to the skull and underlying brain tissue. Pneumatic CCI devices have a small-bore reciprocating double-acting pneumatic piston with a maximum adjustable stroke length of approximately 50 mm. This piston functions to propel a tip into the exposed neural tissue or, in the case of CHI models, the intact skull (discussed elsewhere in this

review). Depending on the research goals, tips of varied sizes and shapes are available, as described later.

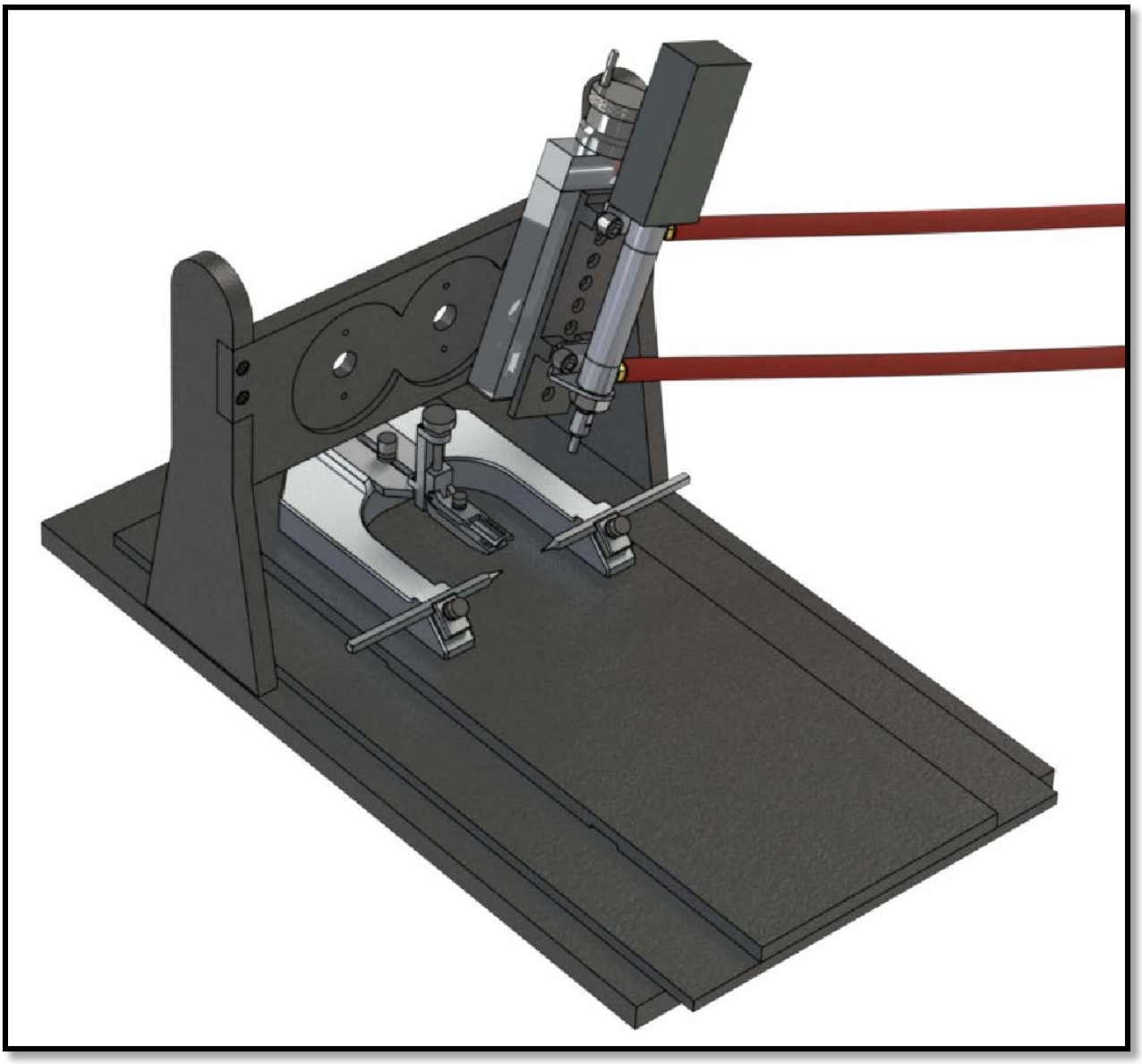

Figure 26: Pneumatic controlled cortical impact device

Electromagnetic

More recently, electromagnetic CCI devices have become available and they share many features with pneumatic devices (Figure 27). As with the pneumatic device, the electromagnetic alternative is traditionally used in combination with a commercial stereotaxic frame, facilitating adjustment of the impactor angle. Some devices are also compatible with an articulated support arm which can elevate the injury device to facilitate modeling CCI in swine and other large animals. The electromagnetic device is potentially more portable than pneumatic devices due to its smaller size and ability to function without a pressurized gas source. A number of options for tip size and shape are also available. Like the pneumatic CCI devices, the electromagnetic devices are also considered to create a reproducible model of brain trauma. Notably, there is little empirical evidence comparing the pneumatic and electromagnetic models; however, one study suggested greater reproducibility with electromagnetic CCI compared to pneumatic CCI (Brody et al., 2007).

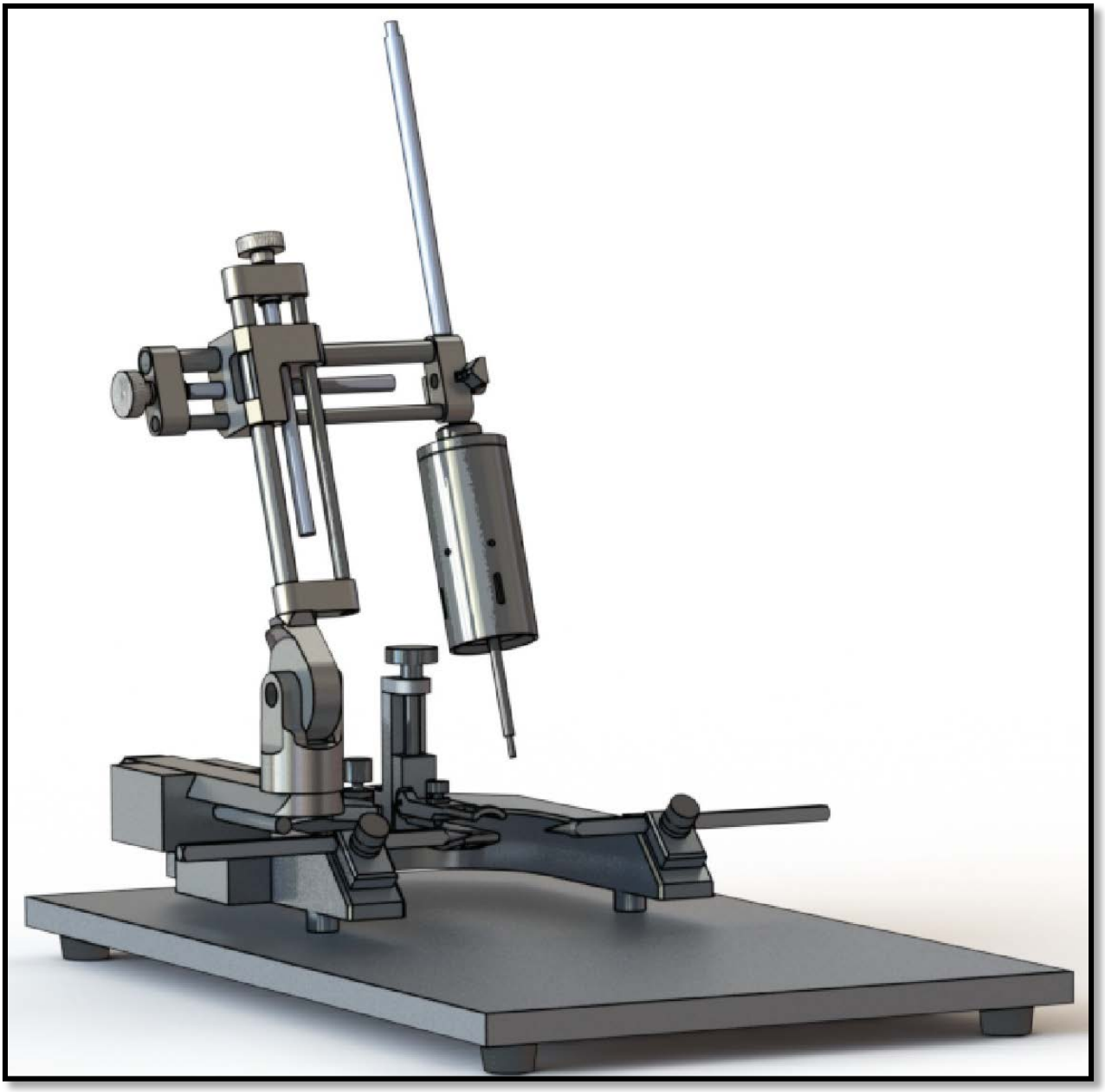

Figure 27: Electromagnetic controlled cortical impact

Commercial suppliers

Over the last decade several CCI devices have become available from commercial suppliers (Table 9). An advantage is that CCI standardization is increased. For each category of CCI, the company

names, locations, and devices are provided. Additional details are also provided including information about the available tips and accessory units.

Table 9: Commercial suppliers of CCI devices

	Company (Alphabetical)	***Location***	***Device***	***Notes/Comments***
Electromagnetic	Hatteras Instruments	Cary, North Carolina	Pinpoint PCI3000 Precision Cortical Impactor	- Removable tips (7 sizes available) -3 system configurations -Suitable for large animal models when used with articulated support arm (accessory unit)
	Leica Biosystems	Buffalo Grove, IL	Impact One Stereotaxic Impactor for CCI	-Removable tips (comes with 1mm, 1.5mm, 2mm, 3mm, and 5mm tips)
Pneumatic	Amscien Instruments	Richmond, VA	Pneumatic (Cortical)	-Accessory unit to measure rod speed is also available

			Impact Device (Model: AMS 201)	
	Precision Instruments & Instrumentation, LLC	Lexington, KY	TBI-0310 Impactor	-Removable tip (3mm and 5mm standard) -Custom tips for sale

6.3.2 Diverse applications & other advantages of the CCI model

Controlled cortical impact has many strengths and consequently the model has had many applications. Below is a brief overview of some of the key strengths of the model. Among the topics included are the use of CCI in many species of test subject, the clinical relevance of the model, scalability, and control over important injury parameters.

6.3.2.1 Control A key strength of CCI is the high degree of control over mechanical factors that may affect study findings including impact depth, velocity, dwell time, and volumetric characteristics associated with varying impact tip sizes. Additional detail regarding how to make decisions about these injury parameters are provided later in this review (see Section 5.1.2.2). Beyond the CCI parameters, there are general experimental conditions that can be controlled, including: housing, husbandry, diet, light/dark cycles, anesthesia (e.g. type; dose; duration), and other factors. While physiological and other confounding factors are not as tightly-controllable, they can be monitored and data recorded, including: temperature, respiration rate, and light/dark cycle. Provided the laboratory is equipped with the necessary and properly maintained and calibrated equipment (e.g. heating pad; temperature probe; ventilation set up), control over these parameters adds little additional burden to the researcher. Details surrounding variables controlled in CCI studies should be reported in accordance with recommended preclinical common data elements (Smith, et al., 2015).

6.3.2.2 Appropriate for use in multiple species Another key strength of the CCI model is its scalability, resulting in the use of the CCI model in multiple species of test animal. For example, CCI has been used in ferrets (Lighthall et al., 1990; Lighthall, 1988), rats (Dixon et al., 1991; Robertson et al., 2013; Shear et al., 2015), mice (Byrnes, Loane, Stoica, Zhang, & Faden, 2012; Fox et al., 1999; Smith et al., 1995; Strauss, Gruzdev, & Zeldin, 2012), swine (Costine et al., 2012; Duhaime et al., 2000; Manley et al., 2006), and non-human primates (King et al., 2010). A summary of species used in CCI research is provided below (Table 10), along with an example of how injury parameters have been set in published studies, though variability across studies exists within a single test species. When scaling injury parameters across species, a recommended starting point is to normalize the percent of brain volume deformed relative to the total brain volume. Beyond scaling the injury parameters, additional modifications to the device may be necessary; for example, the impactor may need to be mounted to an appropriately sized frame or used in combination with an articulated support arm in order to position the tip above the head of a larger animal.

Table 10: Commonly used parameters used in controlled cortical research by species

Animal (Alphabetical)	Injury Site	Depth	Dwell Time	Velocity	Craniectomy Size	Tip Diameter
Mouse	Parietal Cortex	0.5-2 mm	50-250 ms	4-6 m/s	4-5 mm	3 mm
Rat	Parietal Cortex; Midline	1-3 mm	50-250 ms	4 m/s	6-8 mm	5-6 mm
Pig	Frontal Lobe	12 mm	50-400 ms	2-4 m/s	16-18 mm	15 mm
Primate	Frontal Lobe	7 mm	150 ms	3.5 m/s	11-12 m	10 mm

6.3.2.3 Clinical Relevance Overall, CCI is considered a clinically relevant pre-clinical TBI model by virtue of reproducing many aspects of clinical TBI. Topics covered in this review include the pathophysiological and functional consequences produced that are similar to what is seen clinically, the ability to model TBI across the lifespan, as well as applications for evaluating repeated head injury and testing novel therapies.

6.3.2.4 Pathophysiological consequences that mimic TBI pathophysiology One key component of the clinical relevance of the CCI model is its ability to produce many of the histopathological changes seen in TBI patients. Gross histological changes known to follow CCI include: cortical contusion (Atkins, Cepero, Kang, Liebl, & Dietrich, 2013; Dixon et al., 1991; Singleton et al., 2010; Sword, Masuda, Croom, & Kirov, 2013), disruption of the blood-brain-barrier (Dhillon, Donaldson, Dempsey, & Prasad, 1994; Kochanek et al., 1995), hippocampal cell loss (Adembri et al., 2014; Chen, Gao, Zhao, Hu, & Chen, 2015; Pabón et al., 2016; Tsuda et al., 2016; Washington et al., 2012), and overall brain volume loss (Acosta et al., 2013; Dixon, Kochanek, et al., 1999; Vonder Haar, Friend, Mudd, & Smith, 2013). Furthermore, clinical injury is characterized by many secondary injury cascades that have also been reported as activated after CCI, as evidenced by histological markers of several processes, including: apoptosis (Campolo et al., 2013; Chen et al., 2012; Colicos et al., 1996; Kaneko et al., 2012; Schaible et al., 2013), inflammation (Acosta et al., 2013; Gatson et al., 2013; Haber et al., 2013; Khan et al., 2011; Schaible et al., 2013), and oxidative stress (Cheng et al., 2016; Khan et al., 2011; Lazarus, Buonora, Jacobowitz, & Mueller, 2015; Lewén et al., 2001; Miller, Singh, Wang, & Hall, 2015; Zhang et al., 2012). Notably, many of the pathophysiological consequences of TBI have been found to persist into the chronic period, defined in a recent review as persisting at least two weeks following experimental TBI (Osier et al. 2015). For example, CCI is known to result in chronic ventricular enlargement and shrinkage of gray and white matter (Dixon, Kochanek, et al., 1999; Donovan et al., 2014), apoptosis (Colicos & Dash, 1996; Colicos et al., 1996; Patel et al., 2010), necrosis (Fox et al., 1998b; Zhou, Chen, Gao, Luo, & Chen, 2012), axonal injury (Glushakova, Johnson, & Hayes, 2014), and inflammation (Teresita L Briones et al., 2013).

6.3.2.5 Functional deficits that model symptoms experienced by survivors Adding to the clinical relevance of CCI is the functional deficits in many domains, including: overall neurological function (Dixon et al., 1991; Longhi et al., 2011; Xiong, Zhang, et al., 2011), memory (Chauhan & Gatto, 2011; Dixon, Kochanek, et al., 1999; Fox et al., 1998b), learning (Hamm et al., 1992; Z. Zhao, Loane, Murray, Stoica, & Faden, 2012), motor function (Cheng et al., 2012; Fox & Faden, 1998; Hånell et al., 2012), and frontal lobe function (Bondi, Cheng, et al., 2014; Hoffman, Fülöp, & Stein, 1994; Kobori, Clifton, & Dash, 2006). In rodent models, these outcomes can be readily assessed using reliable and valid tests including the Morris water maze (Dixon, Kochanek, et al., 1999; Hamm et al., 1992), Barnes maze (Fox, Fan, LeVasseur, & Faden, 1998a; Fox et al., 1999), elevated plus maze (Washington et al., 2012), rotarod (Murai et al., 1998; Deborah A Shear, Tate, et al., 2011), beam balance task (Cheng et al., 2012; Dixon et al., 1991), beam walking task (C. Dixon et al., 1991; G B Fox et al., 1998a, 1998b; Gerard B. Fox & Faden, 1998; Longhi et al., 2011), and attentional set-shifting test (AST) (Bondi et al. 2014), to name a few. While standardized assessments of functional outcomes are better established in small mammals, there are also options for functional outcome testing in large mammals, though there remains a relative lack of normative data compared to rodents (Friess et al., 2007). Similar to the pathophysiological consequences of CCI, functional deficits often persist into the chronic period (Osier et al., 2014). For example, chronic deficits have been reported in frontal lobe dysfunction (Bondi, Cheng, et al., 2014), overall neurological function (Longhi et al., 2004, 2011; Meng et al., 2011; Xiong, Mahmood, et al., 2011), motor function (d'Avila et al., 2012; G B Fox et al., 1998a; Gerard B. Fox & Faden, 1998; Hånell et al., 2010; Lam et al., 2013; Lindner et al., 1998; Longhi et al., 2011; Deborah A Shear, Lu, et al., 2011), and cognitive function (Byrnes et al., 2012; Chauhan & Gatto, 2011; Cheng et al., 2012; Dixon, Hamm, Taft, & Hayes, 1994; Flygt, Djupsjö,

Lenne, & Marklund, 2013; Fox & Faden, 1998; Han, Hu, Weng, Li, & Huang, 2009; Longhi et al., 2004, 2011; Meng et al., 2011; Tomasevic et al., 2012; Xiong et al., 2012; Xiong, Zhang, et al., 2011; Zhang et al., 2012).

6.3.2.6 Appropriate for use in long-term survival studies and in test animals of all ages The controlled cortical impact model is commonly used and has been used in over one thousand PubMed-indexed manuscripts. In addition to the aforementioned assets of the model, CCI has a low mortality rate, relative to the fluid percussion model. The high survivability of CCI is beneficial in practical ways: it reduces sample attrition, keeps study costs down, and assists in the effort to minimize animal suffering. CCI's low mortality rate also facilitates studying the chronic effects of TBI. For example, CCI is commonly used model for studying the effects of injury on the immature brain as it can be easily scaled down by using a smaller tip and smaller depth (Adelson, Fellows-Mayle, Kochanek, & Dixon, 2013; Costine et al., 2012; Friess et al., 2007, 2009; Kamper et al., 2013; Pop et al., 2013; Robertson et al., 2013). Similarly, CCI has been used to test outcomes of injury later in life, including using mice prone to senescence (Lee et al., 2012; Sandhir & Berman, 2010; Timaru-Kast et al., 2012; Tran, LaFerla, Holtzman, & Brody, 2011). Still, most CCI studies use adult male rodents as the subjects. Rationale for most studies using male animals is that the majority of TBI patients in every age group are male (Faul et al., 2010), and female hormones may influence TBI outcomes (Wei & Xiao, 2013). However, several CCI studies have had samples comprised entirely or partly of female test animals (Geddes, Peterson, Stein, & Sayeed, 2016; Qu, Liu, Xie, Li, & Xu, 2016; Thelin et al., 2016; Wagner et al., 2007).

6.3.2.7 Ability to test the effects of closed head injury & repetitive injury CHI is an additional research application of the CCI device (C. E. Dixon et al., 1994). Beyond studying the effects of single CHI, CCI is also used to test the effects of repetitive impacts (Friess et al., 2009; Petraglia et al., 2014; Shitaka et al., 2011; Vonder Haar et al., 2013). The effects of CHI and repetitive injury are relevant to many clinical populations including: athletes, military personnel, and victims of domestic violence.

6.3.2.8 Testing therapies Despite the wide variety of therapies that have shown benefit in pre-clinical trials including CCI, none have resulted in an FDA-approved therapy for TBI in the United States. Thus, there remains an impetus to identify promising therapies in pre-clinical models and translate them to clinical trials and ultimately clinical care (Kochanek et al., 2011; Kochanek et al., 2015). In this effort, CCI represents an important and commonly used model; evidence from CCI studies in combination with other models has led to identification of therapies for clinical trials, though the success of these trials has been limited. For example, edavarone's effects and therapeutic window were tested in a TBI study (Miyamoto et al., 2013) and later applied to clinical care of TBI in Japan. In the United States, both pre-clinical studies and clinical trials of amantadine have shown promise in promoting neurobehavioral recovery after TBI ((CE Edward Dixon et al., 1999; Giacino et al., 2012). It is also worth noting that several novel therapies (e.g. hypothermia; progesterone; cyclosporine) had successful phase II trials but were unable to withstand phase III trials (Adelson, Wisniewski, et al., 2013; Lulic, Burns, Bae, van Loveren, & Borlongan, 2011; Stein, 2015). Some drugs that had been used clinically for symptom management were tested for their effects on TBI outcomes in studies using CCI and other pre-clinical models, including methylphenidate (Kaelin, Cifu, & Matthies, 1996; Kline, Yan, Bao, Marion, & Dixon, 2000; Plenger et al., 1996; Wagner et al., 2009), amantadine (Dixon, Kraus, et al., 1999), and levetiracetam (Browning et al., 2016; Gabriel & Rowe, 2014; Zou et al., 2013). Thus, translation between pre-clinical and clinical trials of TBI therapeutics is bidirectional.

6.4 LIMITATIONS OF THE CCI MODEL & POTENTIAL ALTERNATIVES

6.4.1 Concerns common to many pre-clinical models

The CCI model shares many of the same limitations as other common pre-clinical TBI models, because some experimental conditions differ substantially from the experiences of TBI patients. Being anesthetized at the time of injury may confound study findings, though these effects are mitigated by the use of sham controls who also receive anesthesia and other identical treatment excluding the injury itself. Concerns regarding the potential neuroprotective or neural suppressive effects of anesthesia as well as ways to mitigate these concerns are discussed later in this review (see "Considerations for Researchers" section). Similarly, many (but not all) preclinical TBI models require a surgical procedure prior to the injury itself. In CCI, this is a fairly large craniectomy, whereas, in fluid percussion injury a smaller craniectomy and placement of the leur-loc device occur. As with anesthesia, use of sham controls mitigates these concerns. Experimental design choices surrounding craniectomy are discussed in additional detail below. A final issue with all pre-clinical models is that each produces one or more aspect of clinical TBI consequences (Osier et al., 2014), but none perfectly mimic the entire human condition; researchers must carefully consider the outcomes of interest in their study and which TBI model(s) best produce deficits in the chosen domains.

It is also worth noting that some of the key assets of pre-clinical models have associated limitations as well. For example, experimental TBI studies are often conducted with unprecedented

control over sample characteristics that would be impossible in clinical trials. This control reduces the possible confounding effects of test subject characteristics (e.g. genotype), environmental conditions (e.g. light cycle; diet), and other factors (e.g. injury location) on study outcomes. However, since human TBI populations are characterized by variability (Saatman et al., 2008), the high-degree of control in pre-clinical models may limit generalizability and slow translation. Commonly, study subjects are comprised of only one sex of animals, most often male, and a narrow age range. However, NIH has recently required that sex be formally considered as a biological variable in new grant applications. Diversification of pre-clinical samples or replication of findings in different sexes, ages, and species of animals strengthens available evidence. Recently, there has been a trend toward diversifying TBI samples when possible, although doing so is associated with increased costs.

6.4.2 Limitations specific to CCI

Limitations specific to the CCI model include: mechanical variation, wear on the device, and limited diffuse effects. Discussion of how experimental design and regular maintenance can help mitigate these concerns is provided in the following section. A primary limitation surrounding the CCI device itself, which tends to be more complex than other models with respect to its mechanical parts. Moreover, the nature of the devices makes some vibration and variation inevitable, which can contribute to change over time of the relationship between the injury parameters and outcome variables. This variability is due in part to the breakdown of wearable materials that comprise the pneumatic model (e.g. seals) and wear due to friction on electromagnetic models; maintenance concerns are discussed later in this review. Another limitation of CCI is that, while it models some aspects of human TBI well, it cannot capture the full breadth of consequences seen in patients.

Moreover, while CCI is an excellent model of TBI with contusion, not all human TBIs are characterized by contusion. Notably, while some diffuse effects of CCI have been reported, they are more limited than with other models (e.g. CHI; blast; fluid percussion injury).

6.5 ONGOING CCI RESEARCH: CONSIDERATIONS FOR RESEARCHERS AND FUTURE DIRECTIONS

6.5.1 Considerations for researchers

To promote the highest quality CCI research possible, and address some of the aforementioned limitations, considerations pertaining to experimental design choices for CCI researchers is provided below. The discussion covers selection of the test animal, decisions surrounding the actual induction of injury (e.g. depth; dwell time, and other experimental choices). Following this discussion, the role of pilot work will be addressed more broadly.

6.5.1.1 Selection of test animal Test animal selection should be based on the study goals, keeping in mind animal welfare goals to reduce, replace, and refine animal models and using the least sentient animal possible to adequately address the research question(s). For example, assessing the relationship between injury biomechanics and outcomes may require use of a primate or porcine brain, which are closer in size to human brains than small mammal models. It is also important to consider practical aspects surrounding the test animal, such as the housing, enrichment, and husbandry requirements, equipment required for behavioral testing, and cost. Finally, ethical considerations for working with various test animals should also be considered; researchers should review the *Guide for Care & Use of Laboratory Animals* and consult their Institutional Animal care and Use Committee (IACUC) for additional guidance.

6.5.1.2 Decisions surrounding injury parameters Beyond the decision to use CCI as the TBI induction technique, there are many additional choices regarding the injury (e.g. depth; tip characteristics; velocity). CCI has been used in a large number of studies, each with different experimental goals; consequently, the CCI literature is characterized by diversity in the specific injury parameters with little standardization. Overall, injury parameters typically scale up with the size of the test animal's brain and desired injury severity. When making decisions surrounding injury parameters, researchers are encouraged to carefully consider the goals of their study, published evidence, and, whenever possible, pilot data. Considerations for the number and location of injury as well as the characteristics of the impactor tip (e.g. size, shape, surface material) will be described below.

Impact location and number

Commonly, a single injury CCI model is used, though repeated injury models have been published (Friess et al., 2009; Petraglia et al., 2014; Vonder Haar et al., 2013). In the authors' laboratory, the preferred injury location for rats and mice is on the cortical tissue of the right hemisphere. Specifically, the craniectomy is centered between lambda and bregma so that the center of the impact tip was AP-4mm, R+4mm (rats) and AP-3mm, R+3mm (mice). The rationale to use a parasagittal injury site is that crossing the sagittal suture (i.e. midline CCI) is associated with increased bleeding (Dixon et al., 1991). Researchers can adjust the location and number of impact(s) to best meet the needs of their study. Some researchers have elected to use bilateral craniectomies in an effort to promote lateral movement of tissue while studying the effects of two contusions (He, Evans, Hoffman, Oyesiku, & Stein, 2004; Meaney et al., 1994). For example, one study found that when animals received bilateral mild-injuries, spaced a week apart, there was damage to myelin within the corpus callosum at 60 days post-injury that was not present after a single contusion (Donovan et al., 2014).

Impactor characteristics: size, shape, depth, velocity, dwell time, angle, & composition

Some of the most important considerations for CCI research surround the impactor tip, including: tip composition, surface material, tip geometry, angle, and dwell time. Depending on the device's commercial supplier, a selection of tips may come standard or researchers may be able to purchase additional tips separately; some vendors offer custom tips of specific size, shape, and/or composition. Researchers can also make after-market modifications to their tips to meet the goals of the study. For example, in one study, vulcanized rubber material was obtained from a lacrosse ball and applied to the tip surface to model sports related concussion (Petraglia et al., 2014; Shitaka

et al., 2011). Notably, the are 8 preclinical CDEs related to the impactor tip including: impactor angle, impactor angle measurement, impactor tip shape, impactor tip rigidity, impactor depth setting, impactor dwell time, impactor velocity, and surface material (Smith et al., 2015).

Tip size is largely dependent upon the test animal, with the tip diameter generally scaling up with the size of the brain (Table 10). For instance, 3 mm tips are commonly used for mice and 15 mm tips for pigs, with intermediate tip sizes used for rats (5-6 mm), ferrets (10 mm), and nonhuman primates (10 mm). Moreover, within an animal model, slightly larger tips may be used to produce more severe injury, though more commonly injury severity is adjusted with increasing impact depth.

The injury depth depends on the zero-point used when setting the desired depth. Notably, there is a lack of standardization across labs with some labs zeroing the tip to the skull, other labs zeroing the tip to the brain tissue (which occasionally herniates slightly after craniectomy), and many groups not specifying in publications which zero point was used. The frame of reference chosen when zeroing the tip should be noted in publications. Importantly, the zero point should be determined while the CCI device is statically pressurized (pneumatic) or energized (electromechanical) to minimize overshoot from the set level of impact depth.

Tip shape is commonly spherical or beveled flat. In the early ferret models (Lighthall et al., 1990; Lighthall, 1988) the tips were spherical. Published CCI studies verify that round tips are still used (Eslami et al., 2015; Mirzayan et al., 2008); however, beveled flat tips have become much more common in recent years (Dennis et al., 2009; Fox et al., 1998b; Hemerka et al., 2012; Monaco et al., 2013; Sandhir & Berman, 2010; Smith et al., 1995). While the emphasis on beveled tips occurs in many animal models of CCI, it is especially true when the test animals are mice. However, this seems to be largely due to convention, as little published empirical evidence

surrounding the effects of tip shape. One notable study in C57BL6 mice found that, compared to round tips, beveled flat tips resulted in a greater extent of both neuronal loss and cortical hemorrhaging (Pleasant et al., 2011).

As with tip size, depth of injury tends to be scaled up with the size of the test animal (Table 10) as well as the desired injury severity; however, it is important to note that within a single test animal, and injury severity, variation in impact depth occurs. Still, scaling impact depth to adjust injury level is common in the literature. One study tested four different injury depths (1.5 mm, 1.75 mm, 2.0 mm, and 2.5 mm) on rat pups (7 and 17 days old, with the older animals receiving greater impact depth); in this study, worsening MWM performance and histological changes were associated with increasing depth of tissue deformation (Adelson, Fellows-Mayle, et al., 2013). An electromagnetic CCI study found that the size of the lesion progressively increased with the impact depth (1.5 mm, 2.0 mm, and 2.5 mm), while holding the tip size, dwell time, and velocity constant (3.5 mm, 0.1 sec, 5.25 m/sec, respectively); progressive increase in cognitive deficits, but not emotional deficits, was also reported with increasing depth (Washington et al., 2012).

Little empirical evidence surrounding how adjusting the velocity affects outcomes. Generally, the consensus is that increasing velocity has some effect on TBI outcomes and the relative contribution of contact velocity to injury outcomes is similar in both midline and lateral CCI (C.E. Dixon, 1994). Notably, beyond 3 m/sec, depth seems to be a greater determinant of injury severity (C.E. Dixon, 1994). While across-study variation in velocity exists in published literature, other methodological differences confound interpretation of how speed affects outcomes of CCI. Additional evidence that depth is more important than velocity in affecting injury severity comes from a finite element simulation of CCI (Mao et al., 2010). In this study, increasing the velocity by 100% was associated with increased maximum principal strains (MPS) of

approximately 9-26.7% depending on brain region (9% in thalamus; 19.5% in deep cortex; 20.2% in hippocampus; and 26.7% in superior cortex). Notably, a 50% increase in velocity was associated with more modest increases in MPS ranging from 1.2% to 13.7% (1.2% in thalamus; 8.5% in hippocampus; 12.2% in deep cortex; and 13.7% in superior cortex). Conversely, a 35% increase in impact depth (from 2.0 mm to 2.7 mm) was associated with increased MPS ranging from 16.6% to 35.7% (16.6% in deep cortex; 25.8% in hippocampus; 26.1% in superior cortex; and 35.7% in thalamus). Thus, larger alterations in intracranial responses occur when depth rather than velocity is scaled up (Mao et al., 2010). Still, researchers should use a velocity sensor to ensure the velocity is consistent with the desired setting; moreover, researchers should report the velocity of impact as part of the TBI preclinical CDEs (Smith et al., 2015).

Other less-well studied considerations regarding injury parameters include the orientation of the tip relative to the brain tissue or exposed skull, the composition of the tip surface material, and the dwell time. With respect to tip surface material, most studies use metal tips. However this need not be the case, and some researchers have modified the impactor tip material to better meet the study goals (Petraglia et al., 2014), as described above. All injury parameter decisions should be informed by a thorough review of the literature and, whenever possible, pilot data. Also, it is important to remember that in order to ensure transparency in research and promote replication of findings, reporting of injury parameters should be done in accordance with the TBI preclinical CDE proposed by the NINDS (Smith et al., 2015).

6.5.1.3 Other important choices

Anesthesia

Choices surrounding anesthesia have the potential to impact experimental outcomes. Indeed, the NINDS recognizes the importance of reporting details about anesthesia as core CDEs for all experimental TBI models, including the type, route, and duration of anesthesia (D. H. Smith et al., 2015). Careful selection and reporting of anesthesia details is important because some agents may confer neuroprotection while others may cause neural suppression. Furthermore, some post-surgical analgesics promote neural suppression and may impair performance on behavioral testing and potentially mask benefits of treatment.

Isoflurane has neuroprotective properties (Statler et al., 2006) and has been associated with less hippocampal damage and fewer behavioral deficits when compared to fentanyl-anesthetized animals (Statler et al., 2000). Still, isoflurane remains a popular and widely used anesthetic for CCI research as well as other models of experimental brain injury. Ketamine is another agent which has been found to have neuroprotective properties attributed to antagonism of N-methyl-D-aspartate (NMDA) receptors (J. W. McDonald, Roeser, Silverstein, & Johnston, 1989). Similarly, halothane has been found to be neuroprotective after CCI (McPherson, Kirsch, Salzman, & Traystman, 1994). Conversely, fentanyl contributes to neural suppression (A.E. Kline & Dixon, 2001).

Notably, the confounding effects of anesthesia are mitigated when sham animals are used as controls instead of naïve animals. There are strategies to reduce the effects of anesthesia. Some researchers perform the craniectomy while the animal is anesthetized but then discontinue anesthesia and perform impact at the time of emergence of the toe-pinch response (Adelson, Fellows-Mayle, et al., 2013). Less commonly, a closed head CCI study was approved that forgoes

anesthesia altogether. One notably study used a cone-shaped bag to comfortably restrain the animals and position the head for the impact (Petraglia et al., 2014). For researchers who prefer to use anesthesia throughout the surgery, the effects of anesthesia can be reduced using proper dosing to avoid under- or over-sedation; standard assessments like the toe-pinch and other tests of reflexes can be used to assess the level of sedation in unparalyzed animals.

Craniectomy

There are several aspects of the craniectomy preceding CCI that can influence the study findings (Osier et al. 2015), including the method used to produce the craniectomy (J. T. Cole et al., 2011), location of the craniectomy (C. Dixon et al., 1991), number of craniectomies (He et al., 2004; Meaney et al., 1994), and whether the bone flap is replaced (Friess, Lapidus, & Brody, 2015; Whalen et al., 1999) or the craniectomy artificially sealed. One study found that regardless of whether the craniectomy was produced using either a manual trephine or an electric drill there were pathophysiological changes including inflammation and evidence of a lesion, when compared to naïve test animals; notably, in this study the extent of pathophysiological changes were greatest when a drill was used (J. T. Cole et al., 2011). Despite this evidence, most researchers prefer using a drill to a trephine because it is a convenient and efficient method. Obviously, researchers using CCI to study CHI do not face these concerns. Those using the invasive CCI model can reduce the deleterious effects of craniectomy through proficiency in the procedure and careful removal of the bone flap. During the procedure, researchers should monitor for bleeding, herniation, and dura breech; these complications may warrant exclusion of some animals from the final sample. Another way to minimize the deleterious effects of craniectomy is to limit heat production during drilling; one strategy is to use a syringe to apply sterile 0.9% saline solution

during the procedure, especially if a drill is used. Overall, consistent and clean craniectomy is fundamental in ensuring high-quality CCI research.

Experimental endpoints

One of the most fundamental choices surrounding all pre-clinical models of TBI is the choice of histopathological and functional outcome and the details surrounding how variables are measured. Choice of outcomes should be based off study goals, and informed by a thorough review of the literature. Specifics regarding the measurement of each outcome should, whenever possible, be based off pilot testing. Beyond choice of a particular behavioral test (e.g. MWM), which subtasks to include (e.g. hidden platform task, visible platform task, probe trial), and additional details (e.g. number of trials; inter-trial interval) must be considered. A review of the techniques for studying the physiologic and behavioral consequences after experimental brain injury, including research using the CCI model, have been described in detail elsewhere (Bondi, Semple, et al., 2014; Osier et al., 2014).

6.5.2 The importance of regular maintenance of CCI devices

As is true for all laboratory equipment, high-quality CCI research depends on a well-maintained CCI device and related equipment. Prior to experiments, researchers should test the CCI device and ensure it is in proper working order and that the piston fires freely. In addition to testing the device immediately prior to use, it is important to perform preventative maintenance, monitor the device's performance over time, and make repairs accordingly, as described below. Failure to do so can negatively affect data quality and result in workflow interruptions.

How and by whom the equipment is maintained varies across laboratory groups. Some groups prefer to have a designated individual who is responsible for all upkeep of the CCI device and other equipment, while in other groups maintenance is a shared responsibility. Regardless of who is responsible for maintenance, it is important that a regular maintenance schedule is adhered to. Researchers should consult with the device manufacturer surrounding the recommended maintenance plan.

Beyond manufacturer recommendations, considerations for the devices parts, their function, and their structure can inform the maintenance routine. For example, pneumatic devices use seals to ensure the cylinder pressurizes and ultimately drives the piston; these seals will wear over time and may result in leaks and subsequent failure of the chamber to fully pressurize, which could affect performance including: reducing maximum speed, or even prevent the piston from firing altogether. Similarly, in devices where the piston's motion is stopped by a soft surface (e.g. rubber), there will be wear over time. Changes in the pressure to velocity calibration curve and new mechanical noises are symptoms of a malfunctioning device. Depending on the device, these parts will need to be replaced periodically; seals may also require periodic lubrication. Leaks in the hoses that deliver air to the cylinder can also occur as the polymer(s) that comprise this component degrade. It is also important to maintain the air compressor (e.g. oil level and tank moisture level), and speed-sensor calibration (e.g. periodically; after long periods of disuse; when other maintenance is performed that results in repositioning of the sensors). Electromagnetic CCI device maintenance includes replacing parts that wear due to friction as the parts move past one another and ensuring electrical components remain in proper working order.

Since much of the wear is use-related, laboratories should tailor their maintenance schedules of the CCI device based on their specific use and needs. Periodic injuring of test animals

to evaluate for a marker of injury severity (e.g. hematoxylin & eosin; cresyl staining), combined with accurate records surrounding how and when the device is used can assist in this effort. For example, by determining how many hits the device can deliver before significant variation occurs, researchers can anticipate changing the seals or chamber itself to avoid negative effects on study data. This information can also be used to ensure replacement parts are available so that there is never an interruption on workflow and study quality. Similarly, high speed videography is a valuable tool that researchers can use to evaluate the approximate extent of mechanical variation and monitor for changes over time. Lastly, changes in brain edema (wet/dry weights) can be used to monitor injury intensity over time.

6.6 REMAINING GAPS AND FUTURE DIRECTIONS

As contemporary researchers build on the rich history of CCI, there remain gaps in the TBI knowledge base and limitations with the model. Future directions for CCI research include making incremental improvements in the device, enhancing control over potential confounders. In the future, findings will be disseminated in a way that promotes replication, by participating in the NINDS's pre-clinical TBI CDEs. Efforts to improve the model should include reducing the mechanical variability of the device and achieving closer tolerances. Ongoing areas of inquiry include application of CCI to more genetically modified test subjects and further evaluation of the genomic, epigenomic, proteomic, and microbiomic factors underlying injury recovery.

6.7 CONCLUSIONS

Over 100 years after the first pre-clinical TBI research, experimental models remain a mainstay. Since its development in the 1980s the CCI model has become one of the most widely used pre-clinical TBI models. Early investigations using this model sought to determine the biomechanical properties of brain tissue exposed to direct mechanical deformation, such as those associated with automotive crashes. CCI has since been applied to evaluate the consequences of open and closed head injury, test novel therapies, as well as explore the molecular-genomic factors relevant to TBI symptom- and recovery- profiles.

CCI has several notable strengths including a high-degree of control over injury parameters (e.g. velocity; depth; duration; and site of impact. The CCI model is clinically relevant in that it produces morphologic and cerebrovascular injury responses similar to aspects of human TBI and can be used to model injury across the lifespan. Commonly observed consequences of CCI include graded histologic and axonal derangements, disruption of the blood-brain barrier, subdural and intraparenchymal hematoma, edema, inflammation, and alterations in cerebral blood flow. Many functional deficits have been observed after CCI as measured using standardized behavioral tests. While CCI is characterized by focal loading with contusion, diffuse effects have also been reported.

Limitations of CCI include the non-clinically relevant craniotomy, use of anesthesia, and potential mechanical variation. Inclusion of surgical sham control groups and thoughtful study design can temper these concerns to some extent. Future directions for CCI research include continuing to make technical improvements in CCI devices and further expanding the CCI

knowledge base. In publishing the results of CCI studies (and other preclinical TBI research), CDEs should be reported in accordance with the guidelines set by NINDS for pre-clinical TBI research.

7.0 PRE-PRESS REVIEW MANUSCRIPT #4: THE EFFECTS OF MELATONIN ON OUTCOMES OF TRAUMATIC BRAIN INJURY: A REVIEW OF THE LITERATURE

7.1 ABSTRACT

Melatonin (MEL) is a hormone that is produced in the brain and known to bind to MEL-specific receptors on neuronal membranes in several brain regions. MEL's documented neuroprotective properties, low toxicity, and ability to cross the blood-brain-barrier make it a promising therapeutic for traumatic brain injury (TBI), a condition with limited therapeutic options. Thus, it is not surprising that studies testing MEL after TBI are increasingly common. The purpose of this manuscript is to summarize the evidence surrounding the use of melatonin as TBI therapeutic, as well as identify existing gaps and future directions. To address this, a search of the literature was conducted using Pubmed, Google Scholar, and the Cochrane Database. In total, 239 unique articles were screened and the 22 that met our a priori inclusion/exclusion criteria were summarized, including the study aims, sample (size, groups, species, strain, sex, age/weight), TBI model, therapeutic details (preparation, dose, route, duration), key findings, and conclusions. The evidence from these 22 studies published was synthesized to draw comparisons across studies, identify remaining gaps, and suggest future directions. Taken together, the published evidence suggests that MEL has neuroprotective properties via a number of mechanisms and few toxic effects. Notably, available evidence is largely based on data from adult male rats and, to a lesser

extent, from mice. Few studies collected data beyond a few days of the initial injury necessitating additional longer-term studies. Other future directions include diversification of samples to include female animals, pediatric and geriatric animals, and transgenic strains.

7.2 INTRODUCTION

7.2.1 Traumatic brain injury

Traumatic Brain Injury (TBI) affects countless individuals worldwide (Colantonio et al., 2010; Faul et al., 2010; Feigin et al., 2013; Puvanachandra & Hyder, 2008; Reilly, 2007; Roozenbeek et al., 2013; Scudellari, 2010). TBI is associated with substantial morbidity and mortality in addition to high direct and indirect clinical care costs (Coronado et al., 2012). Available treatments are not appreciably relieving symptoms and the quest for new therapies has been fruitless; indeed, after thousands of pre-clinical drug trials and dozens of clinical trials, not one FDA-approved therapy has resulted. Thus, identifying novel TBI therapeutics that are safe and effective remains crucial.

7.2.2 Melatonin

Melatonin (MEL) is an endogenous substance synthesized in several sites (Huether, 1993), including the: 1) gastrointestinal tract (Bertaccini, 1960; Söderquist, Hellström, & Cunningham,

2015), 2) retina (Nowak, Urawska, & Zawilska, 1989; Olcese & Møller, 1989), and 3) pineal gland (Bubenik, 2002; Antonio Carrillo-Vico et al., 2005), where the hormone was first isolated (Lerner, Case, Takahashi, Lee, & Mori, 1958). MEL has several known roles in the body, most notably circadian rhythm maintenance (Nakahara, Nakamura, Iigo, & Okamura, 2003; Reiter, 1993). Other well-characterized effects of MEL include reducing oxidative stress (Dikmenoglu et al., 2008; Hashimoto et al., 2012; Tamura et al., 2013; Tan et al., 2002; Taysi et al., 2008) and preventing cell death via anti-apoptotic properties (Bai et al., 2013; Cekmez et al., 2013; Espino et al., 2013; Li et al., 2013; Liang et al., 2012; Sokolovic et al., 2013; Tuñón et al., 2013). Evidence of MEL's therapeutic effects have been demonstrated in the context of cancer (Gonzalez, del Castillo-Vaquero, Miro-Moran, Tapia, & Salido, 2011; Lissoni, Chilelli, Villa, Cerizza, & Tancini, 2003; Wang et al., 2012), bipolar disorder (Fornaro et al., 2013), multiple sclerosis (Natarajan et al., 2012), and various gastrointestinal conditions (Cekmez et al., 2013; Konturek et al., 2008; Lu, Song, Gwee, & Ho, 2009; Song, Leng, Gwee, Moochhala, & Ho, 2005).

While the therapeutic effects of MEL in human TBI remain understudied, some characterization of endogenous MEL after TBI has been published. One study (Seifman et al., 2008) collected cerebrospinal fluid (CSF) and serum out to 13 days post-TBI and compared melatonin levels to that in controls who had a CSF sample collected during surgery for a condition distinct from TBI. In this study, results differed in CSF and blood samples. When the CSF of TBI patients was analyzed, melatonin fluctuated over time in a biphasic fashion; MEL in CSF gradually increased until day 2 post-TBI and then progressively decreased to a minimum on day 5 with levels reaching a maximum on day 8. Overall, and for all days except of 0, 1, and 4, CSF MEL levels were significantly higher in TBI patients than controls. Serum MEL levels also increased from admission to day 2 post-TBI and then reached a minimum level on day 5; no significant differences

in serum MEL levels between TBI patients and controls was reported (Seifman et al., 2008). A second study (Paparrigopoulos et al., 2006) examined MEL levels in the blood 8 times per day for the first two days after TBI but lacked a control group for comparison. The study's main findings were: MEL concentrations in the blood were lower than established clinical ranges and diurnal variation in MEL levels were associated with neurological outcomes assessed using the Glasgow Coma Scale (GCS); specifically patients with lower GCS showed disrupted diurnal variation in MEL levels compared to those with higher GCS (Paparrigopoulos et al., 2006). A third study (Shekleton et al., 2010) recruited TBI survivors 6 months after the initial insult and compared their salivary MEL levels to non-TBI controls. Samples were collected under tight experimental controls including dim light (<10 lux), posture, and activity. Every half hour between 1800 hrs and 0030 hrs participants provided saliva samples which were frozen at -20°C until further analysis. When 14 TBI survivors and 14 controls were compared there was no significant difference in dim light melatonin onset; however, when the total melatonin production over the entire sampling period was compared, significantly higher melatonin production ($p=0.031$) occurred in controls (Shekleton et al., 2010). Overall, the evidence suggests MEL levels go up then down in the acute period and may remain depressed chronically; taken together, it is plausible that MEL is involved in the body's response to TBI but may be inadequate for post-TBI neuroprotection (Paparrigopoulos et al., 2006; Seifman et al., 2008; Shekleton et al., 2010).

Though less evidence is available and largely limited to pre-clinical trials, MEL is a promising therapeutic for neurocritical/neurodegenerative disorders (Naseem & Parvez, 2014; Srinivasan, 2002; Wang, 2009). MEL's possession of many of the fundamental characteristics of a central nervous system (CNS) therapeutic is a primary reason MEL piqued the interest of researchers. MEL has known low toxicity (Jahnke et al., 1999; Seabra et al., 2000; Wiechmann et

al., 2008), there are high numbers of MEL-specific receptors in the CNS (Laudon et al., 1988; Mazzucchelli et al., 1996; Naji et al., 2004; Weaver et al., 1989), and MEL is readily able to cross the blood-brain-barrier (Di Bella & Gualano, 2006; Le Bars et al., 1991; Reiter et al., 2007). Additionally, MEL has demonstrated beneficial effects in pre-clinical models of several CNS disorders, including TBI (Babaee et al., 2015; Campolo et al., 2013; Jadhav et al., 2009; Kelso, Scheff, Scheff, & Pauly, 2011; Mésenge et al., 1998; Ozdemir et al., 2005) and other conditions with similar pathology and symptoms profiles, such as: Huntington's disease (Wang et al., 2011), Alzheimer's disease (Asayama et al., 2003), amyotrophic lateral sclerosis (Weishaupt et al., 2006; Yi Zhang et al., 2013), stroke (Pei, Pang, & Cheung, 2002; Reiter, Tan, Leon, Kilic, & Kilic, 2005), sepsis-induced brain dysfunction (Zhao et al., 2016), and spinal cord injury (Fujimoto, Nakamura, Ikeda, & Takagi, 2000; Liu, Tang, Yang, & Xiao, 2004). In the aforementioned contexts, MEL reduces apoptosis, as well as improves functional (e.g. memory, learning, and motor) outcomes (Olcese et al., 2009; Reiter et al., 2005; Samantaray et al., 2008, 2009; Wang, 2009; Wang et al., 2011; Zhang et al., 2013).

7.2.3 Gap and purpose

There remains a significant gap in the literature pertaining to the role of therapeutic MEL after TBI due to direct injury to the brain or skull. There is one ongoing clinical trial of MEL therapy for post-concussive syndrome, and the rest of the research exploring MEL as a brain injury therapeutic are pre-clinical. Moreover, no recent review has comprehensively evaluated the TBI knowledge base as it pertains to MEL. The purpose of this review is to highlight the state-of-the-science as it pertains to the published evidence of pharmaceutical applications of MEL as a TBI therapy. Published evidence will be summarized in tabular form, including: sample (sample size,

species, and characteristics), groups (number of groups, number of subjects per group; treatment of groups), results (outcomes and details regarding how and when they were assessed), conclusions, and limitations. This review will also identify gaps in the available evidence and suggest future directions for ongoing research.

7.3 METHODS

Initial literature searches were conducted in October 2014 and January 2015; at that time, alerts were set to notify the authors of additional publications on the topic of interest. To ensure no new articles were missed, a second search of the literature was conducted in May 2016. During the primary search of the literature, the following web resources were searched: PubMed, PubMed Central, Google Scholar, and the Cochrane Database. The following search terms were used, alone or in combination: traumatic brain injury, TBI, brain injury, head injury, experimental brain injury, Melatonin, MEL, and pineal. Secondary searches were executed using bibliographies from articles identified in the primary search.

7.4 RESULTS

7.4.1 Summary of articles retrieved

In total, more than 200 unique articles were identified using the key word searches, alone or in combination (N=239). Of these, articles were further screened using the following exclusion criteria: 1) written in a language other than English; 2) tested a condition beyond traumatic injury directly to the brain or skull (e.g. stroke; sepsis-induced neurological deficits; chemically-induced injury); 3) did not test the effect of melatonin; or 4) did not use an *in vivo* model (e.g. neuronal stretch model or another *in vitro* model). After applying the aforementioned exclusion criteria, a total of 22 articles remained.

The global interest in evaluating the therapeutic effects of MEL after TBI is evident by the worldwide research conducted on this topic, including 8 studies from Turkey (Ates et al., 2006; Cirak et al., 1999; Kelestemur et al., 2016; Ozdemir et al., 2005; Ozdemir et al., 2005; Senol & Nazıroğlu, 2014; Ucar et al., 2005; Yürüker, Naz, & Nilgün, 2014), 4 from the United States (Alluri et al., 2016; Jadhav et al., 2009; Kabadi & Maher, 2010; Kelso et al., 2011), 3 from China (Ding et al., 2015a; Lin et al., 2016; Wu et al., 2016), 2 from Iran (Babaee et al., 2015; Dehghan et al., 2013), 2 from Israel (Beni et al., 2004; Shochat & Abookasis, 2015), 1 from France (Mésenge et al., 1998), 1 from Germany (Sarrafzadeh et al., 2000), and 1 from Italy (Campolo et al., 2013).

The majority of the studies used rats, most commonly Sprague-Dawley rats (Jadhav et al., 2009; Kabadi & Maher, 2010; Kelso et al., 2011; Lin et al., 2016; Sarrafzadeh et al., 2000; Senol

& Nazıroğlu, 2014; Ucar et al., 2005; Wu et al., 2016; Yürüker et al., 2014) or Wistar rats (Ates et al., 2006; Cirak et al., 1999; Ozdemir et al., 2005; Ozdemir et al., 2005); N-Mary rats were used in 1 study (Dehghan et al., 2013) and NMRI rats used in another (Babaee et al., 2015). Less commonly, mice were used as the test animals, including the following strains: CD1 mice (Campolo et al., 2013; Ding et al., 2015a), Sabra mice (Beni et al., 2004), BALB/c (Kelestemur et al., 2016a), C57BL/6 mice (Alluri et al., 2016; Zhao et al., 2015), and Swiss mice (Mésenge et al., 1998). Most of the studies conducted experiments on adult test animals with two exceptions testing the effects of childhood TBI (Ozdemir et al., 2005; Ozdemir et al., 2005); one study did not specify the age of the test animals (Cirak et al., 1999). None of the studies explicitly included female mice in their sample, though some studies omitted the sex of the test animals (Alluri et al., 2016; Cirak et al., 1999; Ozdemir et al., 2005; Ozdemir et al., 2005).

Several different injury models were used to garner evidence regarding the therapeutic effects of MEL after TBI. Most commonly used was weight drop injury, which was employed in 13 of the studies (Ates et al., 2006; Babaee et al., 2015; Beni et al., 2004; Cirak et al., 1999; Dehghan et al., 2013; Ding et al., 2015; Mésenge et al., 1998; Ozdemir et al., 2005; Ozdemir et al., 2005; Senol & Nazıroğlu, 2014; Shochat & Abookasis, 2015; Ucar et al., 2005; Yürüker et al., 2014). Of the studies using weight drop, several methods and modifications were used including: Marmarou's model, Feeney's model, and Marklund's model. The second most common method of inducing TBI in the MEL literature was controlled cortical impact (CCI), which was used in 6 studies (Alluri et al., 2016; Campolo et al., 2013; Kelso et al., 2011; Lin et al., 2016; Sarrafzadeh et al., 2000; Wu et al., 2016). Less commonly used models include surgical brain injury (Jadhav et al., 2009), fluid percussion injury (Kabadi & Maher, 2010), and cold-probe injury (Kelestemur et al., 2016a).

Most of the studies tested the effect of melatonin given alone as a single dose (Alluri et al., 2016; Ates et al., 2006; D Ozdemir et al., 2005; Senol & Nazıroğlu, 2014; Ucar et al., 2005; Wu et al., 2016; Yürüker et al., 2014). Several of the studies tested multiple MEL regimens (e.g. 5 mg/kg, 10 mg/kg, and 20 mg/kg) or several dosing schedules (Babaee et al., 2015; Cirak et al., 1999; Dehghan et al., 2013; Jadhav et al., 2009; Lin et al., 2016; Ozdemir et al., 2005; Sarrafzadeh et al., 2000). A small number of studies compared the effects of MEL to another drug with known neuroprotective (e.g. anti-oxidant) properties (Beni et al., 2004; Mésenge et al., 1998; Shochat & Abookasis, 2015). Five studies tested the effects of MEL in combination with another drug (Campolo et al., 2013; Ding et al., 2015b; Kabadi & Maher, 2010; Kelestemur et al., 2016b; Kelso et al., 2011). This evaluation of combination therapy has been suggested as a direction for future TBI researchers, since TBI pathophysiology is complex and may not be realistically targeted with a single drug (Kline, Leary, Radabaugh, Cheng, & Bondi, 2016). All of the studies tested the effects of MEL on cellular and/or histopathological outcomes, including but are not limited to: edema, markers of oxidative stress, inflammatory cytokines, lesion size, autophagy, apoptosis, and mitophagy. Half of the studies evaluated behavioral or functional outcomes after TBI (Beni et al., 2004; Campolo et al., 2013; Dehghan et al., 2013; Ding et al., 2015b; Jadhav et al., 2009; Kelso et al., 2011; Lin et al., 2016; Mésenge et al., 1998; Ozdemir et al., 2005; Ucar et al., 2005; Wu et al., 2016). The behavioral outcomes assessed in the studies included cognitive function assessed using the Morris water maze and motor function assessed using the Beam balance test, wire grip test, or a composite neuroscore.

7.4.2 Literature review table

Below are the results of the literature search in tabular form (Table 11).

Table 11: Summary of published pre-clinical research testing melatonin as a therapeutic for traumatic brain injury

Reference	Sample; Groups	Aim/ Hypothesis	Condition Modeled	Treatment	Results	Conclusion
Mésenge, C. et al. Protective effect of melatonin in a model of traumatic brain injury in mice. J. Pineal Res. 25, 41–6 (1998). PMID: 9694403	Swiss mice (adult males, weighing 25-30 grams). Sample size/groups differed by phase. Groups included: 1) TBI + 1.25 mg/kg MEL given at 20 min pre-TBI, 5 min, 1 hr, and 2 hr post-TBI; 2) TBI + 1.25 mg/kg MEL at 5 min, 1 hr, 2 hr, and 3 hr post-TBI; 3) TBI + vehicle;	Aim: To compare the effects of melatonin and another drug (alpha-phenyl-tert-butyl-nitrone [PBN]), a known free radical scavenger on outcomes of TBI (wire grip & colonic temperature). Hypothesis: Not stated.	TBI (severity not specified) modeled using weight drop. Non-anesthetized mice were assigned to either the un-injured or injured group. Injured animals were subjected to weight drop using a 50 gram weight dropped from a height of 22 cm along a string and onto a	Melatonin was dissolved in HCl and diluted in phosphate buffer to achieve a pH of 7.34. The preparation of PBN is not discussed as MEL is the focus of this review. Both MEL and PBN were administered via 4 i.p. injections. Timing of administration	Colonic temperature was not affected by MEL therapy. Wire grip test scores were improved with MEL (1.25 mg/kg given starting 5 min or 20 min pre-injury) vs. TBI + vehicle. The therapeutic window of MEL was limited to an early post-TBI period. Starting MEL at 30 minutes or later post-TBI was not effective;	To the best of Mésenge et al.'s knowledge, this is the first report to show neuro-protective effects of MEL similar to another free-radical scavenger (PBN). Melatonin reduced

	4) TBI + 1.25 mg/kg MEL at 30 min, 1.5 hr, 2.5 hr, and 3.5 hr post-TBI; 5) TBI + 1.25 mg/kg MEL at 1 hr, 2 hr, 3 hr, and 4 hr post-TBI; 6) TBI + 0.625 mg/kg MEL at times specified in group 5; 7) TBI + 2.5 mg/kg at times specified in group 5; 8) Uninjured controls (n=15); 9-16) PBN groups with different doses/times (not summarized in table).		metal impounder on the skull. Test animals were sacrificed 24 hr after sham or TBI. Some of the injured mice were given vehicle control solution.	varied and included: 1) 20 minutes pre-injury, 5 min, 1 hr, and 2 hr post-TBI 2) 5 min, 1 hr, 2 hr, and 3 hr after TBI 3) 30 min, 1.5 hr, 2.5 hr, and 3.5 hr post-injury 4) 1 hr, 2 hr, 3 hr, and 4 hr post-TBI. Dosed by weight: 0.625 mg/kg, 1.25 mg/kg, 2.5 mg/kg, or vehicle.	starting MEL 20 min pre-TBI or 5 min post-TBI was helpful. <u>Dose response</u> of MEL showed a bell-shaped curve with 0.625 and 2.5 mg/kg being ineffective at improving wire grip performance but 1.25 mg/kg effective. The effects of PBN are not summarized due to the scope of this review.	neurological deficits after TBI as evidenced by improved wire grip. The therapeutic window of MEL is short and the dose is also an important consideration for future studies.

Reference	Sample; Groups	Aim/ Hypothesis	Condition Modeled	Treatment	Results	Conclusion
Cirak, B., Rousan, N., Kocak, A., Palaoglu, O., Palaoglu, S., and Kilic, K. (1999). Melatonin as a free radical scavenger in experimenta l head trauma. Pediatr. Neurosurg. 31, 298–301.	Wistar rats (age sex, weight not specified). N=90 rats across 2 experiments. *Stage 1:* 5 groups (N=50): 1) T0: TBI group sacrificed (sac'd) 15 min post-TBI (n=10); 2) T2: TBI & sac'd 2.25 hr (n=10); 3) T8: TBI & sac'd 8.25 hr (n=10); 4) T 48: TBI & sac'd 48 hr (n=10);	Aim: To test the effects of MEL on outcomes of TBI. Hypothesis: Not specified.	TBI (severity not specified) modeled using Feeney's weight drop method. Anesthetized rats were subjected to sham or weight drop using a 20 gram weight which was dropped along a 10 cm long glass tube to hit the exposed neural tissue. Test animals were sacrificed at one of	Melatonin preparation was not specified. Administered i.p. at one of four post-injury time points: at the time of injury, 2 hr post-injury, 8 hr post-injury, or 48 hr post-injury.	MDA levels were reduced with immediate post-TBI or 2 hr post-TBI administration but raised with 8 hr post-TBI or 48 hr post-TBI administration.	When given early after TBI MEL reduces oxidative stress as evidenced by lowered MDA levels. When given at 8 or 48 hr post-injury MEL actually increased MDA levels for reasons

PMID: 10702729	5) K: controls (n=10). *Stage 2:* 4 groups: 1) TM0: TBI + 200 mg/kg MEL at time of injury & sac'd 15 min post-TBI (n=10); 2) TM2: TBI + 200 mg/kg MEL at 2 hr & sac'd 2.25 hr post-TBI (n=10); 3) TM8: TBI + 200 mg/kg MEL at 8 hr & & sac'd 8.25 hr post-TBI (n=10); 4) TM48: TBI + 200 mg/kg MEL at 8 hr & sac'd 8.25 hr post-TBI (n=10).		4 post-injury time points: -15 min post-TBI; -2.25 hr post-TBI; -8.25 hr post-TBI; -48 hr post-TBI.			which remain to be elucidated.

Reference	Sample; Groups	Aim/ Hypothesis	Condition Modeled	Treatment	Results	Conclusion
Sarrafzadeh, A. S., Thomale, U. W., Kroppenstedt, S. N. & Unterberg, A.W. Neuro-protective effect of melatonin on cortical impact injury in the rat. Acta Neurochir. (Wien). 142, 1293–9	Sprague-Dawley rats (adult males with an average weight of 300 grams). 4 groups (N=45): 1) TBI + 100mg/kg MEL for investigation of contusion volume (n=16) with half the rats dosed during the day and half at night); 2) TBI + vehicle (n=15) for investigation of contusion volume;	Aim: To test the effects of MEL on TBI outcomes (blood gases, contusion volume, blood pressure, edema). Hypothesis was not stated.	TBI (moderate severity) was induced using the controlled cortical impact model. Anesthetized animals were subjected to sham surgery or controlled cortical impact induced using a pneumatic impactor over the poro-parietal cortex. Injury parameters were as follows: 2 mm depth and 7 m/s velocity.	Melatonin was dissolved in a 1:10 solution of ethanol in normal saline. Administered via i.p. injection at 4 timepionts: 20 minutes prior to trauma, immediately after TBI, 1 hr after TBI and 2 hr after TBI.	Blood gases and pressures were not different across the groups. Contusion volume was reduced when MEL was given at nighttime but not daytime. Hemispheric swelling and edema/water content was not significantly reduced with MEL.	This study shows that MEL significantly reduces contusion volume with major effects during the night, which may be due to reduction of early free radical formation. No other variables

(2000). PMID: 11201646	3) TBI + 100 mg/kg MEL for edema analysis (n=7); 4) TBI + vehicle for edema analysis (n=7). Note: sham controls mentioned but sample size nor details of exposure was not provided.		Test animals were humanely euthanized at 24 hr post injury.	Dosed by weight 100 mg/kg. Control animals were given ethanoic saline vehicle.	Intracranial pressure was not different between the groups.	were affected by treatment at the dose given. Other doses during daytime and nighttime should be tested.

Reference	Sample; Groups	Aim/ Hypothesis	Condition Modeled	Treatment	Results	Conclusion
Beni, S. M., Kohen, R., Reiter, R. J., Tan, D.-X., and Shohami, E. (2004). Melatonin-induced neuro-protection after closed head injury is associated with increased brain antioxidants and attenuated	Sabra mice of the Hebrew University Strain (adult males, weighing 30-42 grams). 9 groups (total sample size unclear; groups and respective sample size varied with phase and often presented as a range) *Phase 1: 24 hr* 1) Sham (n=5-11 depending on treatment: vehicle, 1 mg/kg, 5 mg/kg, or 10 mg/kg);	Aim: To evaluate the effects of MEL on TBI outcomes (lesion volume, antioxidant profiles and redox-dependent signaling). *Note: 2-arachidonoyl glycerol (2-AG) was also tested. Hypothesis: Not stated.	TBI (severity not specified) modeled using closed head injury (CHI). Anesthetized mice were immobilized beneath a cylindrical weight drop device and exposed to sham or closed head injury in which a 94g metal rod was dropped from a height of 11-14 cm (depending on the test animal's weight).	MEL was dissolved in a 5% ethanoic saline solution and protected from light until same day use. Administered via a single i.p. injection 1 hr after trauma or sham (following 1 hr neurological score testing). Dosed by weight: 1 mg/kg; 5 mg/kg; or 10 mg/kg in phase 1	<u>Neurological severity score (NSS)</u> pre-treatment groups were similar; 5 mg/kg MEL improved NSS at 24 hr vs. other groups ($p=0.028$). MEL mice did better on 7 of 10 tasks and had higher ΔNSS. <u>Lesion size</u> increased after TBI; 2-fold smaller ($p<0.01$) with MEL vs. vehicle. <u>Brain antioxidants</u> at 1 day after CHI,	This study showed a dose-response effect with 5 mg/kg MEL effective but not 1 mg/kg or 10 mg/kg. MEL. MEL potentiates post-CHI brain antioxidants and blocks the late-phase robust activation of NF-κB and

late-phase activation of NF-kappaB and AP-1. FASEB J. 18, 149–51. PMID: 14597558	2) TBI + vehicle used with MEL (n=10); 3) TBI + 1 mg/kg MEL (n=10); 4) TBI + 5 mg/kg MEL (n=10); 5) TBI + 10 mg/kg MEL (n=10). *Phase 2: 4 or 8 days* 6) TBI + vehicle used with 2-AG (n=5-8); 7) TBI + 5 mg/kg 2-AG (n=5-8); 8) TBI + vehicle used with MEL (n=5-8); 9) TBI + 5 mg/kg MEL (n=5-8).		Test animals were sacrificed in the acute period (24 hr post-injury) or chronic period (4 or 8 days post-injury) depending on the phase of the study.	or 5 mg/kg in phase 2 Vehicle groups received MEL vehicle (5% ethanoic saline) or 2-AG vehicle (a 1:1:18 mix of ethanol : chromophore : saline). Note: details of 2-AG preparation, administration & dosing omitted from this table due to scope of review.	MEL (5 mg/kg) led to 44% increased antioxidants; no sham effect of MEL. Ascorbic acid- MEL increased ascorbic acid 4 days after CHI; no sham effect. DNA binding activity of NF-κB and AP-1- CHI led to increase in nuclear NF-κB. 5 mg/kg MEL blocked the increase on day 8 but not at other time points examined.	decreases AP-1 to half the basal level. Findings suggest MEL exerts effects via ROS scavenging and other nonreceptor -activities. 2-AG treatment was ineffective.

Reference	Sample; Groups	Aim/ Hypothesis	Condition Modeled	Treatment	Results	Conclusion
Ozdemir, D., Uysal, N., Gonenc, S., Acikgoz, O., Sonmez, A., Topcu, A., et al. (2005). Effect of melatonin on brain oxidative damage induced by traumatic brain injury in immature rats. Physiol.	Wistar rats (pups aged 7 days post-natal, of unspecified sex). 3 groups (N=21): 1) TBI + vehicle (n=7); 2) TBI+MEL (n=7); 3) Sham control (n=7). Note: it was unclear if the sham controls received vehicle solution.	Aim: To evaluate the effect of therapeutic MEL on brain antioxidant enzyme activities (e.g superoxide dismutase [SOD]; glutathione peroxidase [GPx]) and indicators of lipid peroxidation (thiobarbituric acid reactive	TBI (unspecified severity) modeled using weight drop. Anesthetized test animals were subjected to sham surgery or weight-drop TBI over the parietal bone surface with a force of 160 g*cm produced by 10-g weight which was guided down a 40 cm long tube onto a footplate affixed to the skull.	Melatonin dissolved in absolute ethanol and diluted with physiologic saline to a concentration of 5% ethanoic saline. Administered as a single intraperitoneal (i.p) injection immediately after TBI or sham.	TBARS levels expressed as nmol/mg protein were significantly increased ($p<0.001$) after TBI within both the contralateral and ipsilateral hemisphere compared to controls. MEL treatment after TBI significantly ($p<0.001$) prevented increase of TBARS in contra- and ipsi-lateral brain,	To the best of Ozedemir et al.'s knowledge this is the first publication suggesting MEL reduces lipid per-oxidation after TBI in immature rats. A single dose of 5 mg/kg MEL prevented

Res. 54, 631–7. PMID: 15720160		substances [TBARS]). Hypothesis: Not stated.	Test animals were sacrificed 24 hr after TBI or sham.	Dosed by weight (5 mg/kg MEL). Controls given equal volume of 5% ethanoic saline vehicle.	restoring TBARS levels to those seen in sham animals ($p>0.05$). SOD and GPx levels showed no significant differences across the study groups.	the increase in TBARS after TBI, suggesting MEL has anti-oxidant properties after head trauma.

Reference	Sample; Groups	Aim/ Hypothesis	Condition Modeled	Treatment	Results	Conclusion
Ozdemir, D., Tugyan, K., Uysal, N., Sonmez, U., Sonmez, A., Acikgoz, O., et al. (2005). Protective effect of melatonin against head trauma-induced hippocampal damage and spatial memory deficits in	Wistar rats (pups of unspecified sex, aged 7 days post-natal) 5 groups (N=63): 1) Control (n=7); 2) Sham untreated (n=14); 3) TBI + vehicle (n=14); 4) TBI + 5 mg/kg MEL (n=14); 5) TBI + 20 mg/kg MEL (n=14).	Aim: To evaluate if therapeutic MEL after TBI would reduce hippocampal damage and attenuate deficits in spatial memory. Hypothesis: Not stated.	TBI (unspecified severity) modeled using weight drop. Anesthetized test animals were subjected to sham surgery or weight-drop TBI over the parietal bone surface with a force of 160 g*cm produced by 10-g weight which was guided down a 40 cm long tube onto a footplate affixed to the skull.	Melatonin was dissolved in absolute ethanol and diluted with physiologic saline to a concentration of 5% ethanoic saline. Administered as a single i.p. injection immediately after TBI or sham.	Hippocampal neuron density was reduced in ipsilateral CA1, CA3, & dentate gyrus in TBI + vehicle group vs. sham/control ($p<0.01$); MEL preserved neurons vs. TBI + vehicle ($p<0.01$), but not to sham/control levels ($p>0.05$). Contralateral neuron loss in CA1 and dentate gyrus were attenuated by MEL.	MEL reduced apoptosis and attenuated functional deficits but there was no difference between 5 and 20 mg/kg. Improve-ment in functional outcome paralleled reduction of cell death.

immature rats. Neurosci. Lett. 385, 234–9. PMID: 15970378			Test animals were sacrificed at either 24 hours- or 3 weeks- post-TBI or sham (half of each group at each time point).	Dosed by weight (5 mg/kg MEL or 20 mg/kg MEL). Controls given equal volume of 5% ethanoic saline vehicle.	TUNEL- positive neurons were detected after injury but less with MEL treatment ($p<0.01$). Spatial memory: TBI + vehicle rats had longer latencies than sham/control animals ($p<0.001$). MEL shortened mean latency on 3rd and 4th days of training vs. TBI+ vehicle ($p<0.01$). Probe trial improved with MEL vs. TBI + vehicle ($p<0.01$).	To the best of Ozdemir et al.'s knowledge this is the first report that MEL improves cognitive outcomes of juvenile TBI. MEL may be a good pediatric TBI therapy.

Reference	Sample; Groups	Aim/ Hypothesis	Condition Modeled	Treatment	Results	Conclusion
Ucar, T. et al. The effects of environmental light--dark changes on experimental mild traumatic brain injury. Acta Neurol. Scand. 112, 163–72 (2005). PMID: 16097958	Sprague Dawley rats (adult males weighing 250-360g). 5 groups (N=56): 1) TBI with normal light/dark cycle (n=14); 2) TBI + 50 mg/kg MEL on a normal light/dark cycle (n=14); 3) TBI + 48 hrs in constant dark (n=14); 4) TBI + 50 mg/kg MEL + 48 hrs in constant dark (n=14).	Aim: To test the effects of normal day/night cycle vs. constant-darkness with or without melatonin therapy on outcomes of TBI. Hypothesis: melatonin secretion after traumatic brain injury would be enhanced by darkness and contribute to	TBI (mild severity) induced using a modified Marmarou weight drop model. Anesthetized animals were subjected to weight drop injury where a 300 gram weight was dropped from a height of 1 meter. Test animals were sacrificed 48 hr after TBI.	Melatonin was dissolved in absolute ethanol and diluted with normal saline to a concentration of 1% ethanoic saline. Administered via a single i.p. injection immediately after TBI. Dosed by weight 50 mg/kg MEL.	Motor function was improved in both melatonin groups (regardless of darkness). EEG recordings were not significantly different with MEL therapy (regardless of darkness). Microscopic examination found MEL led to beneficial effects vs. TBI alone. MEL reduced edema in the perivascular and	Following mild TBI, darkness with or without MEL lead to neuro-protection. Darkness-induced elevation in endogenous MEL secretion contributes to neuro-protection, though other mechanisms

		neuroprotective effects.	Note: There were no sham animals, rather pre-injury baseline was used.		perineuronal regions. MEL preserved neuronal nuclear membranes and euchromatic DNA. MEL also led to healthier neutrophils. Similar effects were seen in the MEL + darkness group though perivascular edema remained. <u>Serum MEL levels</u> only with darkness did MEL therapy raise serum MEL levels.	may contribute.

Reference	Sample; Groups	Aim/ Hypothesis	Condition Modeled	Treatment	Results	Conclusion
Ates, O., Cayli, S., Gurses, I., Yucel, N., Iraz, M., Altinoz, E., et al. (2006). Effect of pineal-ectomy and melatonin replacement on morpho-logical and biochemical recovery after traumatic brain injury. Int. J. Dev.	Wistar rats (adult males 200-250 grams). 6 groups (N=72): 1) craniectomy (crani) for sham group (n=12); 2) crani + TBI (n=12); 3) crani + TBI + 100 mg/kg MEL (n=12); 4) pinealectomy (PX) + crani (n=12); 5) PX + crani + TBI (n=12);	Aim: To examine the effects of TBI with and without pinealectomy (60 days piror to injury) and test the effects of therapeutic melatonin administration. Hypothesis: Not stated.	TBI (severity not specified) using Marklund's modified weight drop model. Anesthetized rats were subjected to sham or weight drop injury using a 21g weight dropped from a height of 35 cm onto a plate resting on the exposed dura. Rats subjected to pinealectomy were allowed to recover for 60	Melatonin preparation was not specified. Administered via a single i.p. injection immediately after injury. Dosed by weight 100 mg/kg MEL. No vehicle control solution was given.	Malondialdehyde (MDA) levels were elevated with PX vs. control or TBI in non-PX rats. MEL decreased MDA more in TBI than PX + TBI. Glutathione (GSH) levels was lower in PX rats than control, TBI reduced GSH; MEL increased GSH. Nitric Oxide (NO) levels were elevated after PX vs. control rats.	This study was the first to explore the effects of PX and MEL therapy in the context of TBI. PX and TBI alone and in combination caused oxidative stress that was prevented with MEL therapy. High-dose

Neurosci. 24, 357–63. PMID: 16959465	6) PX + TBI + 100 mg/kg MEL (n=12).		days prior to other study procedures. Test animals were sacrificed at either 24 hr or 2 weeks post TBI or sham.		MEL after TBI in both PX and non-PX rats reduced NO levels. <u>Xanthine oxidase (XO) levels</u> increased in PX rats vs. control rats. XO levels were reduced in both MEL groups. <u>Histopatholgical lesion</u> was decreased by MEL, larger decrease in trauma alone group than in group with PX + TBI.	MEL immediately post-TBI is neuro-protective in this TBI model and warrants further study.

Reference	Sample; Groups	Aim/ Hypothesis	Condition Modeled	Treatment	Results	Conclusion
Lee, S., Jadhav, V., Ayer, R. E., Rojas, H., Hyong, A., Lekic, T., et al. (2009). Dual effects of melatonin on oxidative stress after surgical brain injury in rats. J. Pineal Res. 46, 43–8. PMID: 18573160	Sprague-Dawley rats (adult males, weighing 200-350 grams). 5 groups (total sample size unclear and group sizes depend on outcome): 1) Sham; 2) TBI + vehicle; 3) TBI + 5 mg/kg MEL; 4) TBI + 15 mg/kg MEL; 5) TBI + 150 mg/kg MEL.	Aim: To test the effects of surgical brain injury on oxidative stress, edema, and neurological outcomes. Hypothesis: MEL will decrease oxidative stress and attenuate postoperative complications (e.g. edema; neurological deficits).	TBI (severity not specified) modeled using surgical brain injury (SBI). Anesthetized test animals were subjected to sham (craniotomy + bone flap replacement) or SBI (right dorsum incision, blunt dissection of skin/ connective tissue, a 5 mm square craniectomy made using a drill such that the lower left edge was at	Melatonin was dissolved in a mixture of 10% ethanoic saline. Administered as a single i.p. injection 1 hour before surgery. Dosed by weight (5 mg/kg MEL, 15 mg/kg MEL, and 150 mg/kg MEL).	Edema (brain water content) was increased after SBI (vs. sham) and not attenuated by MEL 150 mg/kg dose increased edema. Lipid peroxidation (malondialdehyde [MDA] levels); TBI + vehicle had 6x peroxidation vs. sham; 15 mg/kg MEL reduced oxidative stress, but 150 mg/kg increased it.	Overall, the study suggested duel effects of MEL: low doses reduced oxidative stress and protect the brain; high doses, however, have deleterious effects including increased edema, peroxidation

			bregma, incision into the underlying dura exposed the right frontal lobe, incisions made, a piece of brain tissue resected; hemostasis was achieved using intraoperative packing and saline irrigation and the bone flap replaced and skin sutured). Test animals were sacrificed 24 hr post-TBI or sham.	Control-treated mice received an equal volume of 10% ethanoic saline vehicle. All animals received post-operative fluids in addition to above-mentioned treatment.	<u>Composite neuro-score</u> worsened after SBI with or without MEL (150 mg/kg worsened). <u>Vibrissae stimulation study (VSS)</u>:15mg/kg dose improves VSS but 150 mg/kg dose made worse. <u>Beam balance test:</u> showed mixed deficits of SBI and no treatment effect of MEL with some detrimental effects.	and worsened neurological outcomes. The safety of high-dose MEL must be further tested.

Reference	Sample; Groups	Aim/ Hypothesis	Condition Modeled	Treatment	Results	Conclusion
Kabadi, S. V, and Maher, T. J. (2010). Posttreatment with uridine and melatonin following traumatic brain injury reduces edema in various brain regions in rats. Ann. N. Y. Acad. Sci. 1199, 105–13.	Sprague-Dawley rats (adult males, weighing 300-350 grams). 4 groups (sample size unclear; groups depend study phase): *Phase 1- 1 drug only* 1) Sham + vehicle (V) (n=12); 2) Sham + drug: given either 23 mg/kg uridine [UR] or 200 mg/kg MEL (n=6); 3) TBI + V (n=12);	Aim: To test the effects of treatment with melatonin and/or uridine on edema after TBI. Hypothesis: MEL and uridine therapy, alone and in combination, will attenuate edema after TBI induced using the fluid percussion injury (FPI) model.	TBI (severity not specified) modeled using lateral FPI. Anesthetized test animals were subjected to right side craniectomy and FPI (pressure of 2.5–2.8 atms). Test animals were sacrificed 48 hr post-TBI or sham.	Melatonin was dissolved in polyethylene glycol (PEG) 400 in a 1:1 (v/v) solution with sterile water. Uridine was dissolved in saline. Melatonin and/or uridine was administered as a single i.p. injection 15 minutes post-injury.	Edema (wet/dry weight) was observed in the ipsilateral cortex, hippocampus, and striatum after FPI vs. sham. Both doses of uridine reduced edema ($p<0.05$) in the striatum (vs. TBI + vehicle). High dose MEL (200 mg/kg) reduced edema in the striatum ($p<0.05$) vs. TBI + vehicle. No significant edema on the contralateral side, nor was there any treatment effect.	First study to report treatment with uridine and MEL, alone and togeher, reduce edema after TBI. It is important to note that some of the effects of uridine may be due to its hypothermic effects. Moreover, since MEL

PMID: 20633115	4) TBI + drug: either 16 or 32 mg/kg UR or 100 or 200 mg/kg MEL (n=8-10). *Phase 2= 2 drugs* 1) Sham + V (n=9); 2) Sham + drug: given 32 mg/kg UR + 200 mg/kg MEL (n=6); 3) TBI + V (n=9); 4) TBI + drug: given combination of 16 mg/kg UR + 200 mg/kg MEL <u>or</u> 32 mg/kg UR + 200 mg/kg MEL (n=7-8).			Dosed by weight (1 mL/kg of uridine for a total dose of 16 mg/kg or 32 mg/kg and/or 2 mL/kg of MEL for a total dose of 100 mg/kg or 200 mg/kg). Control-treated rats received an equal volume of vehicle.	Both combination treatments failed to reduce ipsilateral cortical edema but reduced ($p<0.05$) hippocampal and striatal edema. On the contralateral side, both combinations reduced ($p<0.05$) striatal edema but only the high-dose uridine with high-dose MEL had less hippocampal edema. No treatment effect in sham groups was reported.	and uridine have different actions, there may be more than one mechanism underlying the effects observed.

Reference	Sample; Groups	Aim/ Hypothesis	Condition Modeled	Treatment	Results	Conclusion
Kelso, M. L., Scheff, N. N., Scheff, S. W. & Pauly, J. R. Melatonin and minocycline for combin-atorial therapy to improve functional and histo-pathological deficits following traumatic brain injury.	Sprague-Dawley rats (adult males, weighing 225-275 grams). 4 groups (N=50) in each of 2 experiments: animals were either sacrificed 12 days post-injury (experiment 1) or 7 days post-injury (experiment 2). *Experiment 1 (n=31):* 1) TBI + vehicle (n=6-9: exact	Aim: To test the effects of MEL and minocycline therapy on outcomes of TBI (cognitive outcomes assessed using Morris water maze, cortical tissue sparing, and ^{3}H-PK11195 autoradiograph y. Hypothesis: combination therapy with	TBI (severity not specified) modeled using the controlled cortical impact (CCI). Anesthetized animals were subjected to sham or TBI using CCI (5 mm impactor diameter, 3.5 m/s velocity, 400 msec dwell time) with a depth of 1.5 mm in experiment 1 or 2.0 mm in experiment 2.	Melatonin and/or minocycline were dissolved in a solution of 2% ethanol in phosphate buffered saline. Administered via 2 i.p. injections. Dosing schedule depended on drug and experiment. *Experiment 1:* MEL administered at 5 and 60 minutes post injury. Minocycline	Cognitive assessment was not significantly different across the groups. Cortical tissue sparing was not significantly different across any of the groups. ^{3}H-PK11195 binding was not significantly different across the groups.	There was no neuro-protective effect of MEL and/or minocycline after TBI in this study. More focal nature of CCI vs. weight drop may have led to less pineal damage and thus less effect of exogenous

Neurosci. Lett. 488, 60–4 (2011). PMID: 21056621	group sizes not specified); 2) TBI + melatonin (n=6-9); 3) TBI + minocycline (n=6-9); 4) TBI + melatonin + minocycline (n=6-9). *Experiment 2 (n=19):* 1) TBI + vehicle (n=6-9); 2) TBI + melatonin (n=6-9); 3) TBI + minocycline (n=6-9).	MEL and minocycline will be more effective than MEL or minocycline alone.	Test animals were sacrificed after cognitive testing completion on either day 12 or 7 post-TBI.	(MIN) was administered on days 1-4 post-injury. *Experiment 2* MEL and MIN were administered at 5 minutes and 90 minutes post-injury. Dosed by weight depending on experiment. *Experiment 1:* MEL= 5 mg/kg. MIN= 40 mg/kg. *Experiment 2:* MEL= 5 mg/kg. MIN=45 mg/kg.		MEL due to endogenous release. Other studies have showed beneficial effects suggesting additional research is needed.

Reference	Sample; Groups	Aim/ Hypothesis	Condition Modeled	Treatment	Results	Conclusion
Dehghan, F., Khaksari Hadad, M., Asadikram, G., Najafipour, H., and Shahrokhi, N. (2013). Effect of melatonin on intracranial pressure and brain edema following traumatic brain injury: role of	N-Mary rats (adult males, weighing 250-300 grams). 5 groups (N=140): 1) Sham (n=28); 2) TBI (n=28); 3) TBI + vehicle (n=28); 4) TBI + 5 mg/kg MEL (n=28); 5) TBI + 20 mg/kg MEL (n=28). Note: The 5 groups of rats divided into 4 subgroups (n=7) to	Aim: To study the effect of MEL on TBI outcomes (edema; ICP; neurological outcome) at different post-injury time points. Hypothesis: Not stated.	TBI (severity not specified) modeled using Marmaou's weight drop model. Anesthetized test animals were subjected to sham surgery or weight drop injury where TBI was produced by dropping a 250g weight onto a steel disk affixed to the skull from a height of 2 meters.	Melatonin was prepared in an ethanoic saline solution (concentration of ethanol was not specified). Administered via 4 i.p. injections given at 1 hr, 24 hr, 48 hr, and 72 hr post-injury. Dosed by weight: 5	Edema higher after TBI; both MEL doses reduced edema vs. TBI/TBI + vehicle. BBB Permeability- Evans blue content higher after TBI, MEL groups had lower content than TBI/TBI + vehicle. ICP increased 1hr post-TBI and was reduced with both MEL doses at 24, 48, and 72 hr post-injury. GPx and SOD levels	Low- and high- dose MEL decreased brain edema and BBB permeability at 72 h after TBI; MEL also improved neurologic scores and ICP. The neuro-protective effects reported

oxidative stresses. Arch. Med. Res. 44, 251–8. PMID: 23608674	examine different endpoints: Subgroup 1: brain water content + neuro-score; Subgroup 2: Blood-brain-barrier (BBB) permeability via Evans blue dye content; Subgroup 3: intra-cranial pressure (ICP); Subgroup 4: oxidant parameters, including malondialdehyde (MDA), glutathione peroxidase (GPx),		Test animals were sacrificed 72 hr post-TBI or sham. Approximately 25% mortality rate occurred in this study.	mg/kg or 20 mg/kg. Control-treated rats received a constant volume of ethanoic saline vehicle (0.33 mL/rat).	increased in MEL groups vs. TBI/TBI + vehicle. (MDA) Level- at 72 hr TBI increased MDA levels vs. sham which was lowered by both MEL doses. Veterinary Coma Scale scores at 1 hr all TBI groups did worse than sham ($p<0.001$). At 24, 48, and 72 hr both doses had better scores than TBI/TBI + vehicle.	may be due to MEL increasing antioxidant enzymes and decreasing oxidant agents (e.g. free radicals). ICP probe insertion could have contributed to elevated ICP.

	Superoxide Dismutase (SOD).					
Reference	**Sample; Groups**	**Aim/ Hypothesis**	**Condition Modeled**	**Treatment**	**Results**	**Conclusion**
Campolo, M. et al. Combin-ation therapy with melatonin and dexa-methasone in a mouse model of traumatic brain injury. J. Endocrinol. 217, 291–301 (2013). PMID: 23532863	CD1 mice (adult males, weighing 25-30 grams and aged 10-12 weeks). 5 groups (N=50): 1) Sham + vehicle (n=10); 2) TBI + vehicle (n=10); 3) TBI + 0.025 mg/kg dexamethasone (DEX) (n=10); 4) TBI + 10 mg/kg MEL (n=10);	Aim: To test the effects of MEL and/or DEX therapy on outcomes of TBI (rotarod test, elevated body swing task, TTC staining, metalloproteina se expression, apoptosis, iNOS expression, and histology).	TBI (severity not specified) modeled using controlled cortical impact (CCI). Anesthetized mice were subjected to sham or CCI (4mm tip diameter, 1.5 m/s velocity, unspecified dwell time) with a depth of 3 mm. Test animals were sacrificed 24 hr after sham or TBI.	Melatonin was dissolved in a solution of 1% ethanoic saline. Both MEL and DEX were administered via two i.p. injections at 1 hr and 6 hr post-TBI. Dosed by weight depending on drug. MEL= 10	Motor function modestly improved with MEL or DEX; MEL + DEX led to greater improvement. Histology revealed damage (gliosis, inflammation, thick vessels); neither MEL nor DEX improved histology; MEL + DEX therapy did. Infarction volume was examined using TTC staining which was attenuated by	MEL + DEX or other multi-drug therapy may be necessary to effectively manage TBI. Combining MEL and DEX therapy has beneficial effects after TBI.

	5) TBI + 10 mg/kg MEL + 0.025 mg/kg DEX (n=10).	Hypothesis: Not stated.		mg/kg. DEX= 0.025 mg/kg. Sham animals were given 1% ethanoic saline.	MEL + DEX not either drug alone. <u>Metalloproteinase expression after TBI</u> was reduced by both therapies alone and even further reduced by combination. <u>iNOS expression</u> was reduced by both MEL or DEX alone; more so with MEL + DEX. <u>Apoptosis</u> was reduced by both MEL or DEX alone; more so with MEL + DEX.	

Reference	Sample; Groups	Aim/ Hypothesis	Condition Modeled	Treatment	Results	Conclusion
Senol, N., and Nazıroğlu, M. (2014). Melatonin reduces traumatic brain injury-induced oxidative stress in the cerebral cortex and blood of rats. Neural Regen. Res. 9, 1112–6. PMID: 25206769	Sprague-Dawley rats (6 mo males weighing 300-340 grams). 4 groups (N=32): 1) Control + vehicle (n=8); 2) Control + 10 mg/kg MEL (n=8); 3) TBI only (n=8); 4) TBI + 5 mg/kg MEL (n=8).	Aim: To evaluate the effect of MEL on TBI outcomes (oxidative stress; antioxidant levels). Hypothesis: Melatonin will modulate oxidative stress and may increase antioxidant levels.	TBI (severity not specified) modeled using Marmarou's weight drop model. Anesthetized animals were subjected to sham surgery or weight drop injury where TBI was produced by dropping a 300 g weight onto a steel disk affixed to the skull from a height of 2 meters.	Melatonin was dissolved in 0.1 mL ethanol and diluted with 0.9 mL isotonic saline for a 0.9% v/w concentration. Administered via i.p. injection 1 hr after trauma or control. Dosed by weight (10 mg/kg in Control + MEL group and 5	<u>Lipid peroxidation levels</u> in the cerebral cortex, plasma, and erythrocytes were elevated in the TBI group vs. controls**.** TBI + MEL therapy lowered peroxidation levels (vs. TBI). <u>Glutathione peroxidase (GPx) activity & glutathione level</u>**;** GPx activity was lower after TBI vs. control; activity did not differ between	In this study, MEL protected against peroxidation caused by TBI and promoted antioxidant activity. MEL has protective effects and may be useful as a TBI therapeutic once better studied. To the best of the Senol et

			Test animals were sacrificed 24 hr after MEL administration.	mg/kg in TBI + MEL group). Vehicle treated rats were given a constant volume of ethanoic saline vehicle (1mL/rat).	TBI and TBI + MEL groups. <u>Antioxidant vitamin levels-</u> vitamin A and E levels in the cortex and plasma were not affected by TBI or MEL therapy. β-carotene, vitamin C and vitamin E levels in the cortex were lower after TBI vs. control. Cerebral cortex β-carotene and vitamin C levels and plasma vitamin C level were increased in the TBI + MEL group vs. TBI alone.	al's knowledge this is the first publication to test MEL's effect on oxidative stress/ antioxidants after TBI in rats.

Reference	Sample; Groups	Aim/ Hypothesis	Condition Modeled	Treatment	Results	Conclusion
Yürüker, V., Naz, M. & Nilgün, Ş. Reduction in traumatic brain injury-induced oxidative stress, apoptosis, and calcium entry in rat hippocampus by melatonin: Possible involvement of TRPM2 channels.	Sprague-Dawley rats (adult males, weighing 340-360g) 4 groups (N=32): 1) control + vehicle control (n=8); 2) control + 5 mg/kg MEL (n=8); 3) TBI + vehicle (n=10); 4) TBI + 5 mg/kg MEL (n=10).	Aim: To test the effect of MEL on outcomes of TBI (oxidative stress, apoptosis, and calcium entry through TRPM2 channels). Hypothesis was not stated.	TBI (unspecified severity) was induced using the Marmarou weight drop method. Anesthetized rats were subjected to sham or weight drop injury where a 300g weight was dropped onto a steel disk on the head from a height of 2 meters. Test animals were sacrificed 24	Melatonin was dissolved in 0.1 mL of ethanol and diluted with physiologic saline to a volume of 1 mL. Administered via a single i.p. injection 1 hr after brain trauma. Dosed by weight 5 mg/MEL.	Calcium (Ca2+) concentration was higher ($p<0.001$) in the TBI group than in the control and MEL groups. MEL reduced Ca2+ entry into hippocampal neurons, likely through TRPM2 channels. Apoptosis (caspase-3, caspase-3) was elevated after TBI vs. control and MEL groups, but lower with TBI + MEL	A significant protective effect of melatonin on Ca2+ homeostasis in hippo-campal neurons was reported. MEL therapy may prevent activation of TRMP2 channels after TBI and provide benefit for

(2014). PMID: 25339252			hours after melatonin treatment, which was administered 1 hr post injury. Note: there was no sham group.	Vehicle control animals were given ethanoic saline vehicle.	than control and MEL groups. Mitochondrial membrane depolarization and intracellular ROS production was higher after TBI when compared to control + MEL groups; TBI + MEL had lower ROS values than TBI + vehicle. TBI + MEL was associated with higher mitochondrial membrane depolarization than in the control group.	TBI outcomes.

Reference	Sample; Groups	Aim/ Hypothesis	Condition Modeled	Treatment	Results	Conclusion
Ding, K. et al. Melatonin protects the brain from apoptosis by enhancement of autophagy after traumatic brain injury in mice. Neurochem. Int. 91, 46–54 (2015). PMID: 26527380	CD1 mice (adult mals, weighing 28-32 grams). Both Nrf-2 wild-type and knock out mice were used. 6 groups (total sample size not specified): 1) Sham (n=6 per assessment); 2) TBI (n=6 per assessment); 3) TBI + saline (n=6 per assessment); 4) TBI + melatonin (n=6 per assessment);	Aim: To evaluate the effects of melatonin on outcomes of TBI (autophagy, apoptosis, and other markers of secondary brain injury). Hypothesis: Not stated.	TBI (severity not specified) was induced using a Marmarou weight drop model. Anesthetized animals were subjected to sham or weight drop injury where a 200 gram weight was dropped onto a disk on the skull before the scalp was sutured closed. Test animals were sacrificed 24 hr after TBI for	Melatonin was dissolved in 5% ethanoic saline and 3-MA was dissolved in saline. Melatonin was administered via 5 i.p. injections at 0 hr, 1 hr, 2 hr, 3 hr, and 4 hr post-TBI or sham. 3-MA was administered in 1 intra-cerebroventric	Neurological severity score was improved with MEL treatment at 1 and 3 days post-TBI but not 7 days post-TBI. Autophagy appeared to be activated with MEL therapy. Apoptosis was decreased with melatonin treatment, which was reversed with 3-MA treatment.	MEL ameliorated secondary brain injury and enhanced autophagy. 3-MA reversed the beneficial effects of MEL in this study.

	5) TBI + 3-MA (n=6 per assessment); 6) TBI + melatonin + 3-MA (n=6 per assessment).		histological analysis or at 7 days for neurological testing.	ular injection 15 minutes prior to TBI. Dosed by weight: 10 mg/kg MEL Control animals received an equal volume saline.		

Reference	Sample; Groups	Aim/ Hypothesis	Condition Modeled	Treatment	Results	Conclusion
Babaee, A. et al. Melatonin treatment reduces astrogliosis and apoptosis in rats with traumatic brain injury. Iran. J. Basic Med. Sci. 18, 867–72 (2015). PMID: 26523219	NMRI rats (adult males, weighing 250-300 grams). 5 groups (N=40): 1) Sham + vehicle (n=8); 2) TBI + vehicle (n=8); 3) TBI + 5 mg/kg MEL (n=8); 4) TBI + 10 mg/kg MEL (n=8); 5) TBI + 20 mg/kg MEL (n=8).	Aim: To examine the effects of melatonin on outcomes of TBI (apoptosis and astrocyte activation). Hypothesis: Not stated.	TBI (moderate severity) was induced using a Marmarou weight drop model. Anesthetized animals were subjected to sham or weight drop injury where a 250 gram weight was dropped onto a steel disk affixed to the skull from a height of 2 meters.	Melatonin was dissolved in 1% ethanoic saline. Administered via 4 i.p. injections at 1 hr, 24 hr, 48 hr, and 72 hr post-TBI or sham. Dosed by weight: 5 mg/kg, 10 mg/kg or 20 mg/kg. Control animals received ethanoic saline vehicle.	Apoptosis was elevated after TBI and reduced with MEL therapy; all 3 doses were effective in preserving neurons compared to vehicle ($p<0.05$). Activated astrocytes were increased after TBI as evidenced by a higher number of GFAP-positive astrocytes; MEL reduced activated astrocytes but did not show dose-dependent effects.	MEL therapy diminished apoptosis and astrocyte reactivity. However, no dose-dependent effects were found. Mechanisms by which melatonin reduces GFAP-positive astrocytes remain to be

			Test animals were sacrificed 72 hours after TBI or sham.			elucidated as do the chronic effects of therapy.

Reference	Sample; Groups	Aim/ Hypothesis	Condition Modeled	Treatment	Results	Conclusion
Shochat, A. & Abookasis, D. Differential effects of early postinjury treatment with neuro-protective drugs in a mouse model using diffuse reflectance spec-troscopy. Neuro-photonics 2,	ICR mice (adult males, weight approximately 40 grams and approximately 12 weeks old). 6 groups (N=60): 1) TBI alone (n=10); 2) TBI + hypertonic saline (n=10); 3) TBI + morphine (n=10); 4) TBI + mannitol (n=10); 5) TBI + melatonin (n=10);	Aim was two-fold: 1) To test the effects of 5 different drugs (hypertonic saline, morphine, mannitol, melatonin, and minocycline) on TBI outcomes; 2) To identify the most promising drug in this injury model. Hypothesis: Not stated.	TBI (severity not specified) induced using a closed head weight drop model. Anesthetized mice were subjected to sham or weight drop injury where a 50 gram cylindrical rod was dropped from a height of 90 cm onto the mouse's intact skull. Test animals were sacrificed 50 minutes after TBI	Melatonin preparation was not described in detail. Administered via a single i.p. injection at 20 minutes post-TBI. Dosed by weight: 10 mg/kg MEL. Note: Details regarding hypertonic saline,	Melatonin had a significant effect on TBI outcomes vs. injury without treatment; specifically MEL decreased HbO_2 and StO_2, stabilized Hbr and hemodynamics, and decreased THC. The end result was that MEL reduced the extent of further brain damage. The results of the other drugs are not summarized in this table given the	To the bet of Shochat et al.'s knowledge, this study is the first to show macroscopic recovery of hemo-dynamic and morph-ologic parameters testing the effects of 5 drugs. Melatonin was not the most

015001 (2015). PMID: 26157981	6) TBI + minocycline (n=10). Note: The paper also mentions a sham group (n=10) that does not appear to be included in the final sample size of 60 listed in the paper.		or sham (30 minutes after therapy was administered).	mannitol, morphine, and minocycline are not the subject of this review and are not included in this table.	scope of this review focuses on MEL therapy.	effective drug tested.

Reference	Sample; Groups	Aim/ Hypothesis	Condition Modeled	Treatment	Results	Conclusion
Kelestemur, T. et al. Targeting different pathophys-iological events after traumatic brain injury in mice: Role of melatonin and memantine. Neurosci. Lett. 612, 92–97 (2016). PMID: 26639427	BALB/c mice (adult males, 23-25 grams). 4 groups (N=30): 1) TBI + vehicle (n=7); 2) TBI + 4 mg/kg MEL (n=8); 3) TBI + 20 mg/kg memantine (n=8); 4) TBI + 4 mg/kg MEL + 20 mg/kg memantine (n=7).	Aim: To test the effects of melatonin and memantine alone and in combination on outcomes of TBI (DNA fragmentation; intracellular signaling; infarct volume). Hypothesis: Not stated.	TBI (severity not specified) was induced using a cold injury model. Anesthetized animals were placed in a stereotaxic device, a craniectomy made, and a liquid nitrogen cooled copper probe (2.5 mm in diameter) paced onto the dura for 60 second before the scalp was sutured closed.	Melatonin was dissolved in 5% ethanoic saline. Administered via a single i.p. injection immediately after injury induction. Dosed by weight: 4 mg/kg MEL and/or 20 mg/kg memantine.	Brain infarct volume was significantly reduced by combination therapy but not either drug alone. DNA fragmentation was decreased with MEL, memantine, and combination. Intracellular signaling after TBI was affected by MEL and memantine alone increasing phosphorylation of	In this study a combination of melatonin and memantine was especially effective in improving TBI outcomes. Further pre-clinical and clinical studies are warranted.

			Test animals were sacrificed 24 hr after TBI.	Control animals were given 100 microliters of 5% ethanoic saline.	JNK1/ERK-1/2 and reducing iNOS activity. Combination therapy reversed the effect of MEL & memantine alone.	

Reference	Sample; Groups	Aim/ Hypothesis	Condition Modeled	Treatment	Results	Conclusion
Lin, C., Chao, H., Li, Z., Xu, X., Liu, Y., Hou, L., et al. (2016). Melatonin Attenuates Traumatic Brain Injury-induced Inflammation: A Possible Role for Mitophagy. J. Pineal Res. PMID: 27117839	Sprague-Dawley rats (adult males, weighing 220-250 grams, aged 8 weeks). The total sample size was unclear, and made up of 5 groups with 5-12 rats per group as follows: 1) Sham; 2) TBI + vehicle; 3) TBI + mitphagy inhibitor; 4) TBI + MEL; 5) TBI + mitophagy inhibitor + MEL.	Aim: To test the effects of MEL on outcomes of TBI (inflammation, immuno-histochemistry, cell death, edema, lesion volume, motor function, cognitive function). Hypothesis: MEL may ameliorate release of pro-inflammatory	TBI (severity not specified) modeled using the controlled cortical impact (CCI) model. Anesthetized rats were exposed to sham surgery or CCI using a 6 mm metal impactor tip to induce injury using the following parameters: 2.5 mm depth, 50 ms duration, and 6 m/s velocity.	Melatonin was dissolved in 2% ethanoic saline. Administered via i.p. injection at 3 timepionts: 0 hr, 2 hr, and 4 hr post-TBI. Dosed by volume: 5 mL/kg with exact amount of MEL used to prepare the solution not specified.	Post-TBI secretion of inflammatory molecules (IL-1β, IL-6, IL-18, caspase 1, and mature IL-1β) was attenuated by MEL. Apoptotic neuronal death was reduced with MEL therapy. Edema was significantly reduced with MEL therapy. Lesion volume was significantly	Melatonin had many protective effects in this study, some of which were reversed with mitophagy inhibitors. Thus, this study suggests melatonin's beneficial effects are partly related to mitophagy.

		cytokines after TBI.	Test animals were sacrificed at 8 hr post-TBI/sham for cellular analysis or 16 days post-TBI/sham for behavioral analysis.	Control animals were given ethanoic saline vehicle. Details of the preparation, administration, and dosing of the mitophagy-inhibitors are not summarized in this table.	reduced with MEL therapy. <u>Motor function</u> on the Beam Balance Task was improved with MEL therapy. Note: Some of the effects of MEL were reversed with co-treatment with mitophagy inhibitor. <u>Cognitive outcomes</u> on the Morris water maze were improved with MEL.	

Reference	Sample; Groups	Aim/ Hypothesis	Condition Modeled	Treatment	Results	Conclusion
Alluri, H., Wilson, R. L., Anasooya Shaji, C., Wiggins-Dohlvik, K., Patel, S., Liu, Y., et al. (2016). Melatonin Preserves Blood-Brain Barrier Integrity and Permeability via Matrix Metalloproteinase-9 Inhibition.	C57BL/6 mice (adults of unspecified sex, weighing 25-30 grams). 4 groups (N=23): 1) Sham (n=6); 2) Sham + vehicle (n=6); 3) TBI + vehicle (n=5); 4) TBI + 10 mg/kg MEL (n=6). Note: cell culture was also used in this study but is not the focus of this review.	Aim: To test the effects of melatonin on blood-brain-barrier breakdown after TBI. Hypothesis: Not stated.	TBI (mild severity) was induced using the controlled cortical impact (CCI) model. Anesthetized animals were subjected to sham surgery (craniectomy) or TBI induced using a pneumatic CCI device with the 3mm diameter impactor tip contacting the brain between lambda and bregma with the	Melatonin was dissolved in a solution of alcohol (50 μg/μL) and diluted in Evans blue. Administered via a single intravenous (i.v.) injection (into the tail vein) and allowed to circulate in the animal for 30 minutes prior to injury (or	Blood-brain-barrier permeability was increased after TBI as evidenced by Evans blue leakage. MEL attenuated blood-brain-barrier permeability.	MEL represents a potential therapeutic for reducing blood-brain-barrier permeability after TBI. The beneficial effects *in vivo* were also paralleled in the *in vitro* component of the study (not described in

PLoS One 11, e0154427. PMID: 27152411			following injury parameters: 2 mm depth, 100 ms duration, and 0.5 m/s velocity. Test animals were humanely euthanized at 24 hr post injury.	sham) induction. Dosed by weight: 10 mg/kg. Note: Other drugs were tested in the cell culture portion of this study but are not the focus of this review.		detail in this table).

Reference	Sample; Groups	Aim/ Hypothesis	Condition Modeled	Treatment	Results	Conclusion
Wu, H., Shao, A., Zhao, M., Chen, S., Yu, J., Zhou, J., et al. (2016). Melatonin attenuates neuronal apoptosis through up-regulation of K + -Cl - cotransporte r KCC2 expression following traumatic brain injury	Sprague-Dawley rats (adult males, weighing 300-330 grams). A total sample of N=156 was divided across two experiments. *Experiment 1:* 7 groups (N=84) divided into 7 post-injury sacrifice groups to examine the time course of K^+-Cl^- cotransporter-2 (KCC2) expression (details	Aim: To test the effects of MEL on neuron-specific KCC2 expression and apoptosis. Hypothesis: Not stated.	TBI (severity not specified) was induced using the controlled cortical impact (CCI) model. Anesthetized animals were subjected to sham surgery (craniectomy) or TBI induced using an electromagnetic CCI device with a 4mm diameter tip and the following parameters: 2 mm depth, 120 ms	Melatonin was dissolved in 1mL of 1% ethanoic saline (vehicle). Administered via 5 i.p. injections at the following time points post-TBI or sham induction: 5 min, 1 hr, 2 hr, 3 hr, and 4 hr. Dosed by weight: 10 mg/kg.	<u>Cortical neuron degeneration</u> (fluoro-jade B staining) was increased after TBI (vs. sham) and attenuated by MEL therapy ($p<0.05$). <u>Brain water content</u> was increased by TBI and significantly reduced ($p<0.05$) by MEL therapy. <u>Neurological deficits</u> using a modified neurological severity score (mNSS) were	Many signaling changes occur after TBI which contribute to diverse histological and functional deficits. MEL shows promise for targeting TBI outcomes. Additional studies are needed especially

in rats. PMID: 27159133	omitted per focus of review). *Experiment 2:* 3 groups (N=72): 1) Sham + vehicle (n=24); 2) TBI + vehicle (n=24); 3) TBI + 10 mg/kg MEL (n=24).		duration, and 3 m/s velocity. Test animals were humanely euthanized at 24 hr post-injury (Note: in experiment 1 the time-course of KCC2 was studied out to 168 hr post-TBI, but there was no MEL therapy in this portion of the study).		pronounced after TBI and decreased ($p<0.05$) with MEL. <u>KCC2 expression</u> was downregulated after TBI and increased with MEL. <u>Expression of BDNF and p-ERK</u> were down-regulated by TBI and attenuated by MEL ($p<0.05$). Apoptosis increased after TBI and was reduced with MEL ($p<0.05$).	those evaluating the longer-term effects of MEL.

7.5 DISCUSSION

Examination of the existing body of literature has led to identification of a major limitation in TBI research; that being that most studies are limited to the acute effects of TBI assessed only within a few days of initial injury. Only four studies (Ates et al., 2006; Kelso et al., 2011; Lin et al., 2016; Ozdemir et al., 2005) examined chronic outcomes defined in a recent review as at least 2 weeks after TBI (Osier et al., 2014). Notably, the longest data-collection period of the studies included in this review was 3 weeks post-TBI (Ozdemir et al., 2005). Future studies will be strengthened by outcome assessment into the chronic period including studies collecting data beyond 3 weeks of the initial injury.

Another limitation in the existing evidence surrounds the type of test animals used. None of the studies retrieved explicitly included female test animals in the study sample, though four studies did not specify sex (Alluri et al., 2016; Cirak et al., 1999; D Ozdemir et al., 2005; Ozdemir et al., 2005). A lack of inclusion of female test animals represents a limitation of the existing knowledge base and an area for future research. While males are more likely to sustain a TBI in all age groups (Centers for Disease Control, 2010), female TBI remains a significant worldwide problem stemming from domestic violence as well as traditional injury mechanisms (e.g. motor vehicle accident; fall). Many agencies that fund research are now requiring studies include female test animals or provide adequate justification for exclusion of female animals.

Similarly, there was an overrepresentation of adult test animals in the studies reviewed with only two studies (Ozdemir et al., 2005; Ozdemir et al., 2005) focusing specifically on juvenile

animals and none focusing on aged or senescence-prone animals. One study did not note the age of the test animals (Cirak et al., 1999). Additional research using representative pre-clinical samples is necessary to promote high-quality evidence that is translatable to clinical trials and ultimately applicable to the TBI patient population. Regardless of the test animals used, it is critical that details pertaining to sex, age, strain, and weight be reported as consistent with the Common Data Elements (CDEs) published by the National Institute of Neurological Diseases and Stroke (NINDS) (Smith et al., 2015). Not all of the studies reviewed adequately provided these details that would be necessary to replicate the research. Another detail that was occasionally omitted from the studies in this literature review was diet (Cirak et al., 1999; Lin et al., 2016; Sarrafzadeh et al., 2000; Shochat & Abookasis, 2015), though most studies specified that animals had *ad libitum* access to food and water.

An important consideration for studies examining the melatonergic system or testing therapeutic MEL pertains to the light/dark cycles on which animals are maintained. The vast majority of studies summarized in this review housed animals on 12 hr light/12 hr dark cycles. One study kept animals on a 14 hr light/10 hr dark cycle (Mésenge et al., 1998), and two studies did not specify (Cirak et al., 1999; Jadhav et al., 2009). Several studies provided additional details about the timing of experimental procedures (Beni et al., 2004; Ozdemir et al., 2005; Ozdemir et al., 2005); this information may be relevant to replication efforts. One study empirically compared the effects of normal 12 hr light/12 hr dark cycles to constant darkness on TBI outcomes (Ucar et al., 2005). Notably, this study found that results differed depending on whether the animal was kept in constant darkness or not, which suggests that this information should be reported in publications and considered when planning experiments evaluating MEL and the melatonergic system.

Taken together the pre-clinical evidence surrounding MEL remains conflicted. The majority of studies showed some beneficial effect of MEL using one or more dosing regimens; however, one study (Kelso et al., 2011) found no beneficial effect of MEL. Moreover, some studies showed dose-response effects. Commonly a bell-shaped curve was reported with the middle dose conferring benefit and low and high doses ineffective (Beni et al., 2004; Mésenge et al., 1998). Notably, the effective doses differed with one study finding 1.25 mg/kg of MEL (but not 0.626 mg/kg or 2.5 mg/kg) effective (Mésenge et al., 1998), and another study finding 5 mg/kg (not 1 mg/kg or 10 mg/kg) effective (Beni et al., 2004). Moreover, some of the regimens studied actually compounded the deleterious effects of injury. In one study, a dose of 150 mg/kg of MEL worsened edema, lipid peroxidation, vibrissae stimulation study, beam balance performance, and composite neuroscore (Jadhav et al., 2009). In a second study, a dose of 200 mg/kg of MEL increased lipid peroxidation (Cirak et al., 1999). Other studies tested different doses but did not report dose-dependent effects (Babaee et al., 2015; Dehghan et al., 2013). Further research is necessary to understanding these adverse effects, especially since doses similar to those that led to adverse effects in one study were associated with neuroprotection in other studies (Ates et al., 2006; Kabadi & Maher, 2010; Sarrafzadeh et al., 2000). Moreover, five of the studies (Campolo et al., 2013; Ding et al., 2015b; Kabadi & Maher, 2010; Kelestemur et al., 2016b; Kelso et al., 2011) tested the effects of MEL in combination with another drug and found the effects of combined therapy were superior to the effects of either drug alone; this is consistent with a growing body of evidence that two or more drugs in combination may be needed to adequately treat TBI (Kline et al., 2016).

Overall, the pre-clinical knowledge base is limited by the lack of a well-tested conceptual framework surrounding the neuroprotective mechanisms of MEL; this limitation affects the researcher's ability to design studies and contributes to the inconsistent results in published studies.

Additional research testing MEL as a potential therapeutic agent is necessary to support clinical trials and translational efforts. Traditionally, the beneficial effects of MEL within the CNS are attributed to its anti-oxidant capacity (Dikmenoglu et al., 2008; Hashimoto et al., 2012; Onur et al., 2004; Tamura et al., 2013; Tan et al., 2002; Taysi et al., 2008). Anti-apoptotic effects of MEL have also been reported in the CNS (Alonso-Alconada, Alvarez, Lacalle, & Hilario, 2012; Bavithra, Selvakumar, Krishnamoorthy, Venkataraman, & Arunakaran, 2013; Bruce-Keller et al., 2007; Kireev, Vara, & Tresguerres, 2013; Ma et al., 2013; Olcese et al., 2009; Ozyener et al., 2012; Reiter et al., 2005; Samantaray et al., 2008, 2009; Suwanjang, Abramov, Govitrapong, & Chetsawang, 2013; Wang et al., 2011; Zhang et al., 2013) including after TBI (Campolo et al., 2013; Jadhav et al., 2009; Kelso et al., 2011; Mésenge et al., 1998; Ozdemir et al., 2005). One study found MEL therapy promoted mitophagy after TBI (Lin et al., 2016).

It is also worth noting that none of the studies reviewed acknowledged the role of endogenous MEL receptors, including MT1 and MT2, two MEL-specific receptors found in the mammalian brain (Mazzucchelli et al., 1996; Naji et al., 2004). This represents a limitation of the research since evidence from a mouse model of Huntington's disease suggests the anti-apoptotic effects of MEL are MT1 receptor-dependent (Wang et al., 2011). The role of MT1 and MT2 remains unclear in the context of TBI and may be relevant to personalized medicine since functional polymorphisms in MT1 have been identified (Barrett et al., 1997; Natarajan et al., 2012).

While additional pre-clinical research is needed, effort to translate MEL to clinical trials has begun. Specifically, there is an ongoing clinical trial in Canada exploring the effects of therapeutic MEL after pediatric TBI (Barlow et al., 2014). The pilot work underlying this clinical trial used a retrospective chart review of 48 pediatric TBI patients being treated for post-traumatic

headache. The authors found that 18 (37.5%) of the patients received MEL therapy, suggesting this drug is administered after TBI in some units, despite the lack of clinical trials and limited pre-clinical evidence. Of the 18 patients receiving MEL, 15 (83%) demonstrated a therapeutic response. Since this was a retrospective study there was no control over therapeutic regimen: 7 patients received doses between 3-5 mg and 8 patients received a dose between 6-10 mg. The ongoing clinical trial entitled the PLAY GAME TRIAL will build on these pilot findings using a randomized control trial. As of 2016-05-13, this study is still in the recruitment phase, with a recruitment goal of 99 males and females aged 8-19 with mild TBI. The study will test the effects of either 3 mg or 10 mg of MEL administered sublingually for 28 days after trauma on symptoms of post-concussion syndrome assessed using the Post-Concussion Inventory (PSCI) score for parents (PSCI-P) and youth (PSCI-Y), the child health questionnaire for both parents and children, the behavior assessment system, and the behavior rating inventory of executive function. In addition to the effects of each dose on outcomes, the authors will explore a possible dose-response effect, as well as whether or not the effect of treatment is independent of the effect of sleep. The proposed PLAYGAME TRIAL end date is November of 2019.

A second clinical trial (Kemp, Biswas, Neumann, & Coughlan, 2004) recruited 7 male TBI patients age 16-65 (average = 39.6 years) who had previously sustained a TBI and suffered a post-TBI history of sleep disturbance. All of the participants were fully oriented and none had a history of neurological insults or dependence on drugs and/or alcohol. Potential test subjects were excluded if they were taking amitriptyline, a drug administered during the study. In this study a randomized double-blind controlled cross-over trial was used to compare the effects of two drugs: 5 mg of MEL or 25 mg of amitriptyline. In this study the effects of drug therapy were modest; MEL improved daytime alertness but did not improve sleep latency, sleep duration, or sleep

quality, nor did it improve mood-related measures or cognitive performance. Response bias is an issue in this study as self-reports were not entirely consistent with the diaries used in data collection. Additional studies testing the effects of MEL after TBI are needed.

7.6 CONCLUSION

There are few studies testing MEL therapy following TBI and, with the exception of two clinical trials (Barlow et al., 2014; Kemp et al., 2004), are limited to preclinical models. The results of the 22 pre-clinical studies identified varied, though most found a beneficial effect of MEL at one or more of the dosing regimens trialed. The studies summarized in this review were characterized by variations in the therapeutic regimen (e.g. dose, timing) and outcome variables chosen and were largely limited to acute evaluation of cellular and histopathological outcomes. Taken together, the evidence suggests that MEL is a safe and low-toxic drug with neuroprotective properties after TBI. Future studies need to expand the pre-clinical samples to enhance generalizability, as well as examine longer-term histopathological and behavioral outcomes.

8.0 APPENDIX A.

TABLES AND FIGURES FOR DATA-BASED MANUSCRIPT

Table 12: Correlations between apoptotic marker and other proteins of interest

6 Hours Post-Operative						
Region	***Hippocampus***			***Frontal Cortex***		
Protein	**MT1**	**MT2**	**Actin**	**MT1**	**MT2**	**Actin**
CytC	r= 0.664 (p= 0.018)*	r= 0.541 (p= 0.069)	r= -0.236 (p= 0.460)	r= 0.626 (p= 0.029)*	r= 0.159 (p= 0.621)	r= -0.038 (p= 0.906)
24 Hours Post-Operative						
Region	***Hippocampus***			***Frontal Cortex***		
Protein	**MT1**	**MT2**	**Actin**	**MT1**	**MT2**	**Actin**
CytC	r= 0.649 (p=0.016)*	r= 0.641 (p=0.018)*	r= -0.367 (p=0.218)	r= 0.773 (p= 0.002)**	r= 0.784 (p= 0.002)**	r= 0.552 (p= 0.071)

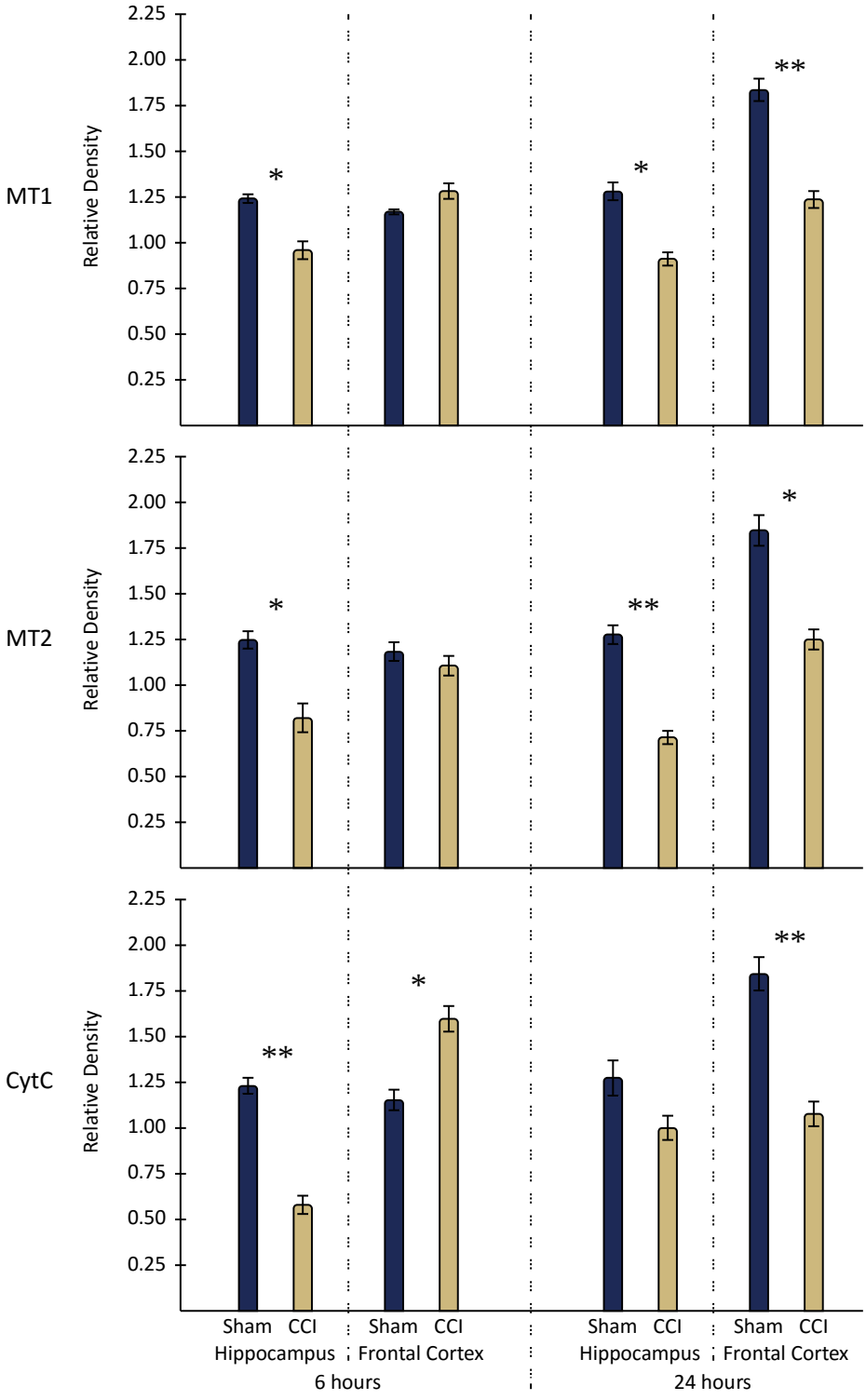

Figure 28: Western blot results for MT1, MT2, and Cytochrome C

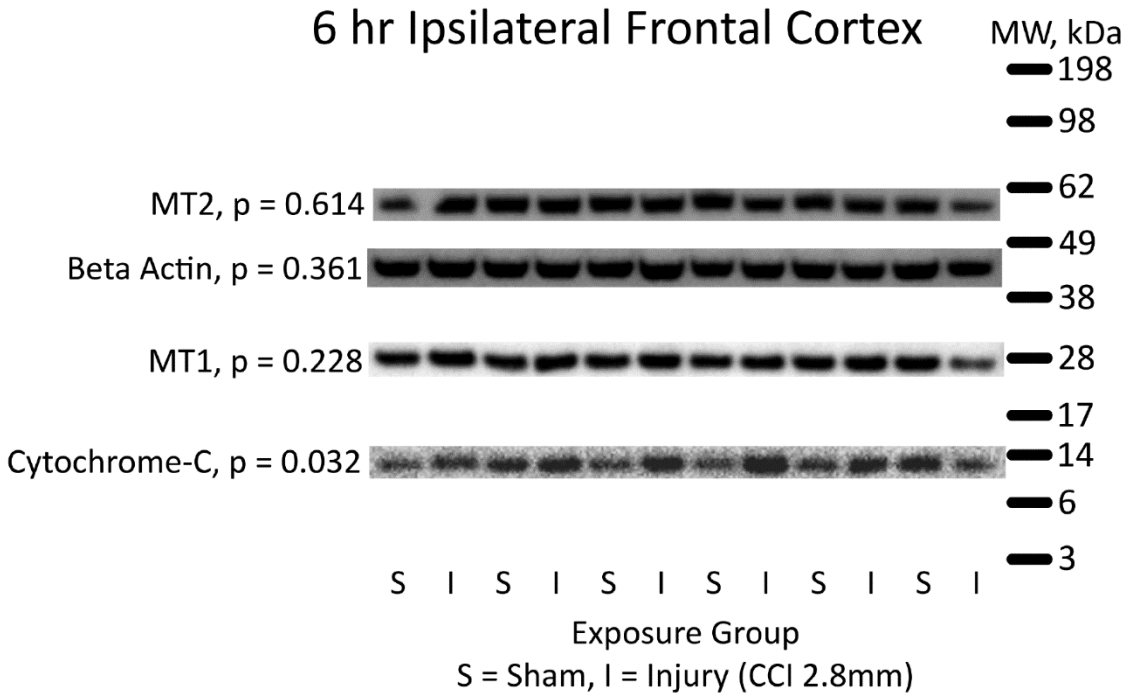

Figure 29: 6 hr post-surgery cortical western blots probing for MT2, Beta Actin, MT1, and Cytochrome C

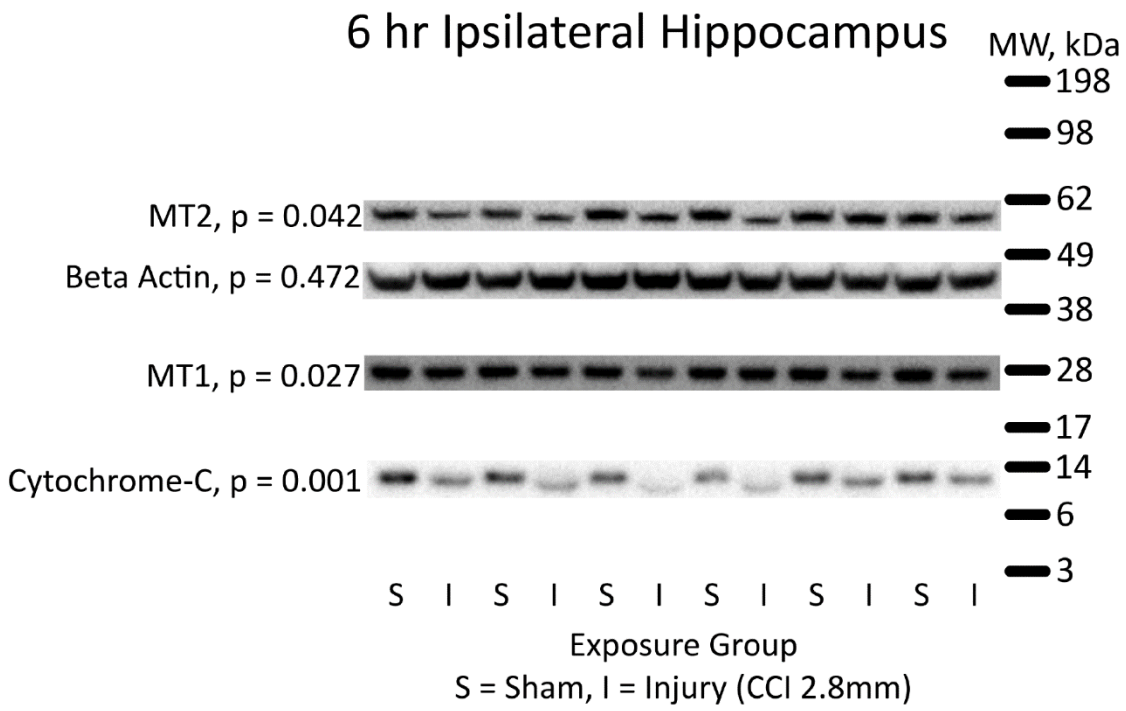

Figure 30: 6 hr post-surgery hippocampal western blots probing for MT2, Beta Actin, MT1, and Cytochrome C

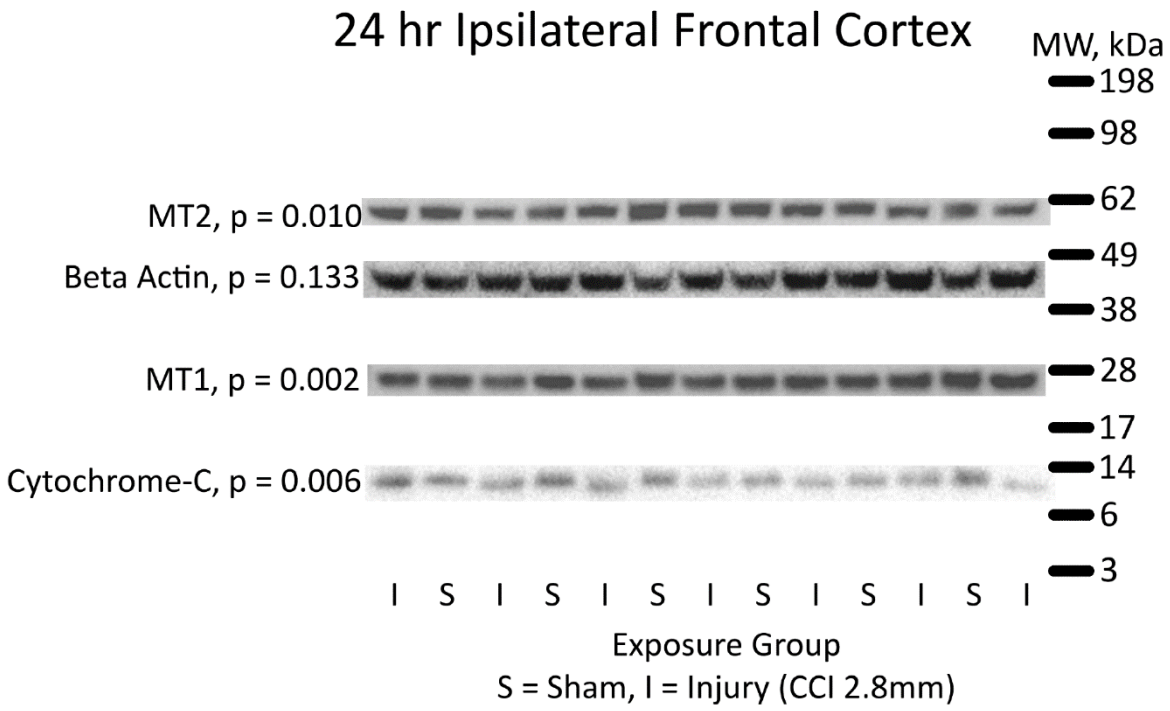

Figure 31: 24 hr post-surgery cortical western blots probing for MT2, Beta Actin, MT1, and Cytochrome C

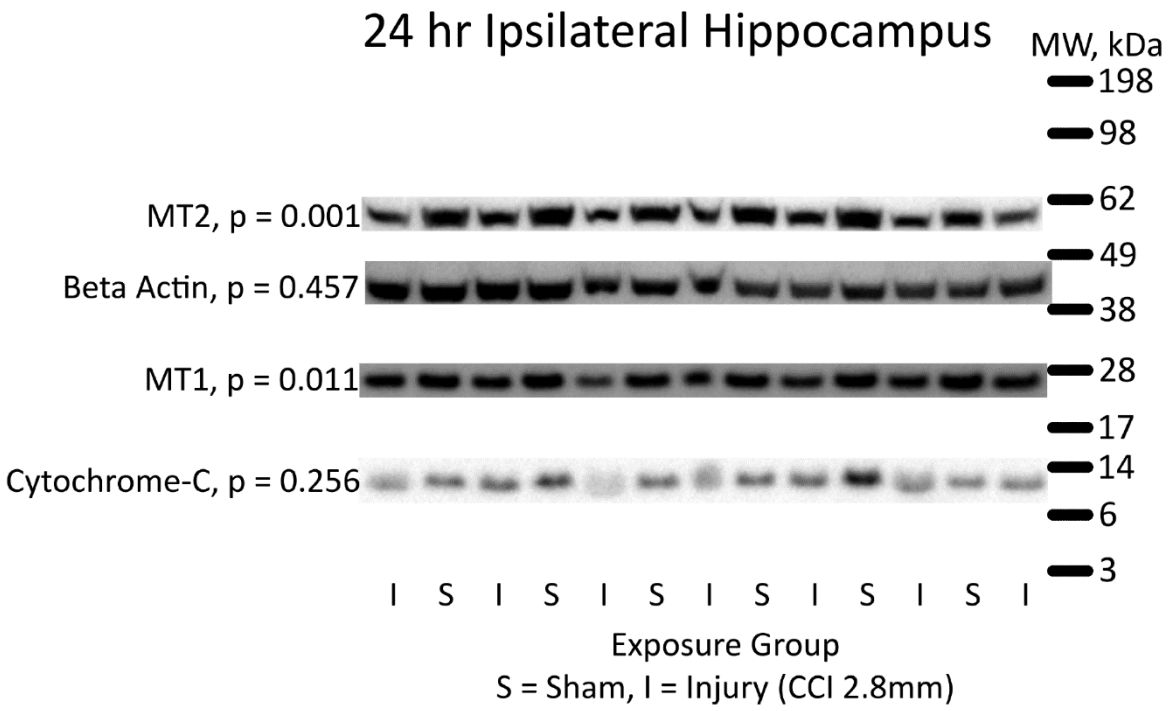

Figure 32: 24 hr post-surgery hippocampal western blots probing for MT2, Beta Actin, MT1, and Cytochrome C

9.0 APPENDIX B.

UNIVERSITY OF PITTSBURGH ANIMAL CARE AND USE COMMITTEE

APPROVAL LETTERS

University of Pittsburgh
Institutional Animal Care and Use Committee
[illegible] 6th Avenue
Suite 206
Pittsburgh, Pennsylvania 15213
Tel: 412-383-4008
Fax: 412-383-2020

IACUC APPROVAL

Protocol #: 13072038
PHS Assurance Number: A3187-01

Principal Investigator:	Clifton Dixon
Protocol Title:	Efficacy and dosing of Melatonin Supplementation after severe TBI in mice
Additional Titles:	
Funding Source(s):	U of Pgh | School of Medicine | Neurological Surgery
Approval Date:	7/10/2013

To Whom It May Concern:

The University of Pittsburgh's Institutional Animal Care and Use Committee has reviewed and approved the research proposal referenced above.

The committee finds that the protocol meets the standards for humane animal care and use as set by the Animal Welfare Act and the NIH Guide for the Care and Use of Laboratory Animals.

Sincerely,

Frank J. Jenkins, Ph.D.
Institutional Animal Care and Use Committee

This letter is valid until 7/31/2014.

IS00002038

University of Pittsburgh
Institutional Animal Care and Use Committee
[illegible] 6th Avenue
Suite 206
Pittsburgh, Pennsylvania 15213
Tel: 412-383-2008
Fax: 412-383-2020

IACUC APPROVAL

Protocol #:13072038

PHS Assurance Number: A3187-01

Principal Investigator:	Clifton Dixon
Protocol Title:	Efficacy and dosing of Melatonin Supplementation after severe TBI in mice
Additional Titles:	
Funding Source(s):	U of Pgh | School of Medicine | Neurological Surgery
Approval Date:	6/5/2014

To Whom It May Concern:

The University of Pittsburgh's Institutional Animal Care and Use Committee has reviewed and approved the IACUC Renewal proposal referenced above.

The committee finds that the protocol meets the standards for humane animal care and use as set by the Animal Welfare Act and the NIH Guide for the Care and Use of Laboratory Animals.

Sincerely,
Frank J. Jenkins, Ph.D.

Institutional Animal Care and Use Committee

This letter is valid until 7/31/2015.

IS00002038

University of Pittsburgh
Institutional Animal Care and Use Committee
[illegible] 6th Avenue
Suite 206
Pittsburgh, Pennsylvania 15213
Tel: 412-383-2008
Fax: 412-383-2020

IACUC APPROVAL

Protocol #:13072038

PHS Assurance Number: A3187-01

Principal Investigator:	Clifton Dixon
Protocol Title:	Efficacy and dosing of Melatonin Supplementation after severe TBI in mice
Additional Titles:	
Funding Source(s):	University of Pittsburgh School of Medicine, Department of Neurological Surgery
Approval Date:	7/31/2015

To Whom It May Concern:

The University of Pittsburgh's Institutional Animal Care and Use Committee has reviewed and approved the IACUC Renewal proposal referenced above.

The committee finds that the protocol meets the standards for humane animal care and use as set by the Animal Welfare Act and the NIH Guide for the Care and Use of Laboratory Animals.

Sincerely,

Denise K. Capozzi, VMD
Institutional Animal Care and Use Committee

This letter is valid until 7/31/2016.

IS00002038

University of Pittsburgh
Institutional Animal Care and Use Committee
[illegible] 6th Avenue
Suite 206
Pittsburgh, Pennsylvania 15213
Tel: 412-383-2008
Fax: 412-383-2020

IACUC APPROVAL

Protocol #: 14012346
PHS Assurance Number: A3187-01

Principal Investigator:	Nicole Osier
Protocol Title:	Mechanism of Melatonin-Induced Neuroprotectioin in Traumatic Brain Injury
Additional Titles:	
Funding Source(s):	NIH National Research Service Award (NRSA)
Approval Date:	1/24/2014

To Whom It May Concern:

The University of Pittsburgh's Institutional Animal Care and Use Committee has reviewed and approved the research proposal referenced above.

The committee finds that the protocol meets the standards for humane animal care and use as set by the Animal Welfare Act and the NIH Guide for the Care and Use of Laboratory Animals.

Sincerely,

Frank J. Jenkins, Ph.D.
Institutional Animal Care and Use Committee

This letter is valid until 1/31/2015.

IS00002346

University of Pittsburgh
Institutional Animal Care and Use Committee
[illegible] 4th Avenue
Suite 206
Pittsburgh, Pennsylvania 15213
Tel: 412-383-2008
Fax: 412-383-2020

IACUC MODIFICATION APPROVAL

Protocol #: 14012346
Modification #: IM-14012346-6
PHS Assurance Number: A3187-01

Principal Investigator:	Clifton Dixon
Protocol Title:	Mechanism of Melatonin-Induced Neuroprotection in Traumatic Brain Injury
Additional Titles:	Effect of Genotype on Response to Melatonin Therapy in Traumatic Brain Injury; Effects of Melatonin on Functional Outcomes in a Mouse Model of Brain Injury
Funding Source(s):	NIH National Research Service Award (NRSA); Copeland Foundation; International Society for Nurses in Genetics; Neuroscience Nursing Foundation; Sigma Theta Tau International Eta Chapter
Modification Approval Date:	9/30/2015

To Whom It May Concern:

The University of Pittsburgh's Institutional Animal Care and Use Committee has reviewed and approved the research modification referenced above.

The committee finds that the modification meets the standards for humane animal care and use as set by the Animal Welfare Act and the NIH Guide for the Care and Use of Laboratory Animals.

Sincerely,

Frank J. Jenkins, PhD
Institutional Animal Care and Use Committee

The protocol will be due for renewal : 1/31/2016.

This modification approval does not change the approval date of the protocol.

IS00002346

University of Pittsburgh
Institutional Animal Care and Use Committee
[illegible] 6th Avenue
Suite 206
Pittsburgh, Pennsylvania 15213
Tel: 412-383-2008
Fax: 412-383-2020

IACUC APPROVAL

Protocol #: 14012346
PHS Assurance Number: A3187-01

Principal Investigator:	Clifton Dixon
Protocol Title:	Mechanism of Melatonin-Induced Neuroprotection in Traumatic Brain Injury
Additional Titles:	Effect of Genotype on Response to Melatonin Therapy in Traumatic Brain Injury; Effects of Melatonin on Functional Outcomes in a Mouse Model of Brain Injury
Funding Source(s):	NIH National Research Service Award, Copeland Foundation, Internation Society for Nurses in Genetics, Neuroscience Nursing Foundation, Sigma Theta Tau International Eta Chapter
Approval Date:	1/11/2016

To Whom It May Concern:

The University of Pittsburgh's Institutional Animal Care and Use Committee has reviewed and approved the IACUC Renewal proposal referenced above.

The committee finds that the protocol meets the standards for humane animal care and use as set by the Animal Welfare Act and the NIH Guide for the Care and Use of Laboratory Animals.

Sincerely,
Frank J. Jenkins, PhD
Institutional Animal Care and Use Committee

This letter is valid until 1/31/2017 .

IS00002346

University of Pittsburgh
Institutional Animal Care and Use Committee
[illegible] 6th Avenue
Suite 206
Pittsburgh, Pennsylvania 15213
Tel: 412-383-2008
Fax: 412-383-2020

IACUC MODIFICATION APPROVAL

Protocol #: 15025455
Modification #: IML-15025455-7
PHS Assurance Number: A3187-01

Principal Investigator:	Clifton Dixon
Protocol Title:	SNARE Proteins and TBI (2)
Additional Titles:	Evaluation of Lithium as a Treatment for synaptic Vesicle Deficits after TBI Lithium as a therapeutic approach to attenuate synaptic deficits after TBI
Funding Source(s):	NIH (NS090748, NS079061)
Modification Approval Date:	5/13/2016

To Whom It May Concern:

The University of Pittsburgh's Institutional Animal Care and Use Committee has reviewed and approved the research modification referenced above.

The committee finds that the modification meets the standards for humane animal care and use as set by the Animal Welfare Act and the NIH Guide for the Care and Use of Laboratory Animals.

Sincerely,

Frank J. Jenkins, PhD
Institutional Animal Care and Use Committee

The protocol will be due for renewal : 2/28/2017.

This modification approval does not change the approval date of the protocol.

IS00005455

10.0 APPENDIX C.

IN-PRESS MANUSCRIPT #1: CHRONIC HISTOPATHOLOGICAL AND BEHAVIORAL OUTCOMES OF EXPERIMENTAL TRAUMATIC BRAIN INJURY IN ADULT MALE ANIMALS

JOURNAL OF NEUROTRAUMA 32:1861–1882 (December 1, 2015)

DOI: 10.1089/neu.2014.3680

Chronic Histopathological and Behavioral Outcomes of Experimental Traumatic Brain Injury in Adult Male Animals

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Abstract

The purpose of this review is to survey the use of experimental animal models for studying the chronic histopathological and behavioral consequences of traumatic brain injury (TBI). The strategies employed to study the long-term consequences of TBI are described, along with a summary of the evidence available to date from common experimental TBI models: fluid percussion injury; controlled cortical impact; blast TBI; and closed-head injury. For each model, evidence is organized according to outcome. Histopathological outcomes included are gross changes in morphology/histology, ventricular enlargement, gray/white matter shrinkage, axonal injury, cerebrovascular histopathology, inflammation, and neurogenesis. Behavioral outcomes included are overall neurological function, motor function, cognitive function, frontal lobe function, and stress-related outcomes. A brief discussion is provided comparing the most common experimental models of TBI and highlighting the utility of each model in understanding specific aspects of TBI pathology. The majority of experimental TBI studies collect data in the acute postinjury period, but few continue into the chronic period. Available evidence from long-term studies suggests that many of the experimental TBI models can lead to progressive changes in histopathology and behavior. The studies described in this review contribute to our understanding of chronic TBI pathology.

Key words: behavior; chronic; function; histopathology; TBI

Introduction

Overview and purpose

IT IS WELL-ESTABLISHED that traumatic brain injury (TBI) leads to diverse histopathological and behavioral consequences that begin in the acute period (hours to days) and persist chronically (weeks, months, and years after injury). Chronic symptoms negatively affect survivors' quality of life and hinder their independence and ability to return to preinjury responsibilities.[1–6] Even with modern medical care, an estimated 3.2–5.3 million Americans are living with one or more residual problem attributed to TBI.[2,7] There is an impetus to better understand the long-term consequences of TBI, given that chronic symptoms are distressing for both TBI survivors and their families and are coupled with significant health service utilization and cost.

Animal models have been a mainstay of TBI research for over a century.[8–11] Several types of experimental TBI models have been developed to model the consequences of TBI, and four of the most commonly used options are discussed in this article: fluid percussion injury (FPI); controlled cortical impact (CCI); blast TBI (bTBI); and closed-head injury (CHI). In this review, separate headings are used for different injury induction methods with the test species, location, or injury severity denoted in the body of the text when relevant. It is important that additional long-term studies of experimental TBI are conducted so that the persistence and progression of TBI pathology may be better understood, given that this information can be applied to guide the development of therapeutic interventions that reduce chronic disability. To date, the chronic consequences of experimental TBI remain less characterized than the acute pathology. This was confirmed by Gold and colleagues, who reviewed 314 data-based publications from experimental TBI studies, which met the following criteria: used rodent models, compared TBI-exposed cases to controls, and included functional outcome assessments.[12] The researchers found that only 32% of all experimental TBI publications meeting the above-mentioned criteria included outcomes beyond 1 month

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11.0 APPENDIX D.

LICENSE AGREEMENT FOR MANUSCRIPT #1

Title: Chronic Histopathological and Behavioral Outcomes of Experimental Traumatic Brain Injury in Adult Male Animals

Author: Nicole D. Osier, Shaun W. Carlson, Anthony DeSana, et al

Publication: Journal of Neurotrauma

Publisher: Mary Ann Liebert, Inc.

Date: Dec 1, 2015

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