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Optimization of a Novel Barnes Maze Protocol for Assessing Antioxidant Treatment Of Traumatic Brain Injury

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OPTIMIZATION OF A NOVEL BARNES MAZE PROTOCOL FOR ASSESSING ANTIOXIDANT TREATMENT OF TRAUMATIC BRAIN INJURY

by

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OPTIMIZATION OF A NOVEL BARNES MAZE PROTOCOL FOR ASSESSING ANTIOXIDANT TREATMENT OF TRAUMATIC BRAIN INJURY

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Current preclinical research into traumatic brain injury focuses heavily upon cellular and molecular testing to determine the effects of injury and potential benefits of neuroprotective treatments. While this may be a useful method, some argue that an increased focus on behavioral testing could lead to better clinical translation as these assays assess the longer term, downstream effects from a brain injury. The most characterized behavioral tests used in traumatic brain injury research are the spatial learning and memory paradigms, Morris Water Maze and Barnes Maze. The Morris Water Maze is the most used of theses paradigms and relies on spatial cues and a platform for the escape from the water to measure spatial learning and memory but has a downside in the endogenous anxiety because of the necessity of swimming. Additionally, previous work with the Morris Water Maze showed issues in finding large differences between injured and uninjured mice. The Barnes Maze offers an alternative to the Morris Water Maze without the added stress caused by forced swimming by instead relying on bright lights to encourage rodents into the dark escape area. Here, a novel shortened Barnes Maze protocol has been developed and optimized to improve upon a traditional Barnes Maze protocol in detecting differences between healthy and injured rodents. Additionally, this protocol is used to assess the efficacy of a novel antioxidant

nanoparticle treatment. Through this testing, additional knowledge regarding the ability and limitations of this experimental procedure are found as well as further knowledge into the benefits shown by a neuroprotective treatment.

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CHAPTER 1: INTRODUCTION

From my previously published literature[1]:

Traumatic brain injury (TBI) is currently the leading cause of injury-related morbidity and mortality worldwide, with an estimated global cost of USD 400 billion annually [2]. Behavioral outcomes associated with TBI begin with primary injury to the brain resulting from an externally applied force [3]. These external forces can originate from direct contact between the brain and an object or through non-impact situations including rotational acceleration and the energy waves produced from blasts [4, 5]. This can result from falls, motor vehicle accidents, assault, domestic violence, military warfare, and even recreational sports including football, hockey, and boxing [3]. These multiple mechanisms of impact generate a broad spectrum of injury severities and behavioral outcomes, leading to difficulties in developing diagnostic and prognostic protocols, let alone effective treatments. Thus, there is still no approved therapy that has shown efficacy in reducing the long-term secondary effects following TBI.

TBI patients have a 2–4-fold increase in the risk of developing dementia later in life due to even a single instance of TBI followed by a loss of consciousness (LOC) [6]. In conjunction with aging, individuals who have experienced mild TBI are at increased risk for developing Alzheimer's disease, at 2.3 and 4.5 times more likely for moderate and severe TBI, respectively [7]. Even repeated mild injuries, such as those among retired professional American football players, have been correlated to long-term cognitive deficits. Retired players who had suffered three or more concussions in their careers had a 5-fold increase in mild cognitive impairments compared to their counterparts with no

history of concussions [6]. Additionally, Parkinson's disease, amyotrophic lateral sclerosis (ALS), Creutzfeldt–Jakob disease, and chronic traumatic encephalopathy (CTE) were also all found to be associated with the progression of chronic TBI [6]. Due to the association of TBI with these progressive neurodegenerative diseases, viable treatment options must be developed with an in-depth knowledge of the injury's pathophysiology, lest the current therapeutic stalemate continues.

Several safety precautions have been implemented to prevent head trauma, including the provision and advancement of helmets, seatbelts, and airbags. However, the major problem facing TBI patients is the spread of secondary corrosive damage to the surrounding brain tissue following this initial impact. This lethal progression of secondary damage is caused by a disruption in the oxidant/antioxidant equilibrium of the brain, which forces a biochemical imbalance, leading to chronic oxidative stress [8]. Oxidative stress leads to the damage of lipids, proteins, and DNA in the brain and creates deterioration similar to the development of some neurodegenerative diseases [8]. Oxidative stress progresses alongside a variety of other biochemical malfunctions, including glutamate toxicity in neurons, mitochondrial dysfunction, and blood–brain barrier (BBB) disruption [9]. Due to this secondary damage, TBI presents with a multitude of physical, cognitive, and behavioral deficits. However, the evolution of these deficits is highly variable and can range from minor concussive symptoms to severe TBI, leading to probable death.

Unfortunately, differences among patients and their injuries provide a variety of complications for medical personnel in determining efficient diagnoses and effective

treatments. From 1993 to 2016, there were 30 failed clinical trials involving various forms of treatment [10]. These treatment options included temperature control, hypertonic saline, progesterone, prostacyclin, surgical intervention, intracranial pressure monitoring, and various pharmacological therapeutics [10]. Although there has been success in Phase II trials, all these treatments have failed during larger, multi-center Phase III trials. These failures have resulted due to a variety of problems during testing for the efficacy of treatments. Progesterone for the Treatment of Traumatic Brain Injury (ProTECT) and Study of Neuroprotective Agent, Progesterone, in Severe Traumatic Brain Injury (SyNAPse) both resulted in negative outcomes during Phase III trials [11]. Researchers postulate that these failures were the result of suboptimal dosing during Phase II trials, suggesting inadequate delivery into the brain and poor target engagement, in addition to heterogeneity between injuries [11]. Other clinical trials have had similar issues, including problems with clinical trial design, lack of accurate injury phenotyping, and inadequate outcome assessment tools [12]. Injury heterogeneity and inadequate outcome assessment tools are capable of being mitigated with effective classification systems. Classification systems have been previously constructed for categorizing the injury severity of TBI in humans immediately following diagnostic exams from medical professionals. Initial methods for classifying TBI in a clinical setting are efficient, but simplistic in approach, leaving room for error between different degrees of human injury. However, recent literature has investigated the most important variables for assessing TBI in the hopes of improving upon the original designs to create a more effective classification system [13, 14].

While methods for classifying degrees of injury in humans have advanced, efforts have also been directed towards developing animal models for TBI to provide an effective comparison to human injuries [15, 16]. These models have been used to understand the pathophysiological mechanism for the progression of different degrees of TBI. Additionally, animal models have aided in the development of potential treatments for the reduction of oxidative stress, BBB dysfunction, and various other biochemical impairments [9, 16]. Recently, Operation Brain Trauma Therapy (OBTT) was developed as a multi-center, preclinical consortium to identify therapies that are beneficial in alleviating damage from head trauma in animal models [12]. The OBTT makes use of several animal models in three distinct injury categories, focal, diffuse, and non-impact injury, creating a broad spectrum of potential pathophysiological outcomes [3, 16]. Each model has unique procedures and outcomes in the hopes of providing a sufficient translation to the variety of head traumas that occur in humans. Through these models, comparisons can be derived between the various degrees of human injury severity, which will ultimately lead to improvements in diagnostics and treatment protocols.

Additionally, these animal models can be used in conjunction with behavioral assessments to identify the cognitive outcomes associated with different mechanisms of injury. These behavioral tasks have been established to address a variety of neurological changes associated with TBI, including deficits in spatial and non-spatial memory. Additionally, impact to specific regions of the brain or spread of secondary injury could result in emotional impairment and deficits in motor coordination, both present in clinical presentations of TBI. In general, we see most of these deficits across all models;

however, behavioral outcomes are highly correlated with levels of injury severity, and repeated injuries result in variable changes in behavior [17].

SECTION 1.1: CLINICAL BEHAVIOR AND ANIMAL MODELS

In addition to the above understanding of TBI, it is important in describing and communicating this research to note the specific behavioral consequences of TBI and the corresponding animal model used throughout the following work. Clinically, TBI leads to a variety of behavioral outcomes including chronic pain, anxiety, depression, aggression, increased incidence of suicide, cognitive deficits, negative impacts to learning and memory, and motor dysfunction [18-23]. While much preclinical TBI research focuses on analysis using histology and biomarkers to determine the molecular mechanisms of injury, behavioral testing has become more broadly utilized to determine how these molecular changes may correlate to behavioral deficits. Some researchers believe that by analyzing behavioral correlates, the transition between preclinical to clinical success may be effectively bridged allowing for the creation of neuroprotective treatments and enhanced rehabilitation [19]. Creating an effective paradigm measuring behavioral deficits is clearly an important endeavor within the realm of preclinical TBI research with clinical success in mind. However, another essential aspect in translating preclinical research lies within the chosen animal model. While various animals, including monkeys, swine, sheep, dogs, and cats are used, the most commonly used animals are rodents, particularly rats and mice [16]. Though there are many methods for producing preclinical TBI models, two of the most widely used are the fluid percussion injury (FPI) and the controlled cortical impact (CCI) models [16]. While the FPI model has many benefits, the CCI model is generally used more due to its reproducibility, highly controllable impact

depth, velocity, and time, lack of rebound injury, and its ability to accurately target a specific area of the brain [16, 19]. Indeed, the CCI model is currently the gold standard in preclinical research and has been well characterized due to decades of use and its similarity to human TBI, including blood-brain barrier (BBB) breakdown [24]. Breakdown of the BBB is an important aspect of TBI, especially in relation to targeted treatment of TBI. With this breakdown, the permeability of the BBB increases dramatically immediately following TBI allowing for intervention into the secondary injury via systemic drug delivery [25]. Nanoparticles (NPs) are a valuable tool for taking advantage of this window of opportunity and providing an effective treatment.

SECTION 1.2: NANOPARTICLE THERAPEUTICS

Research into NP treatments have expanded as many small molecule treatments have failed in Phase III clinical trials, such as progesterone, tirilazad, and superoxide dismutase [26, 27]. Many of these failed clinical trials hold common issues including two areas of improvement: poor delivery and retention in the brain and toxicity away from the targeted treatment area [28]. NPs are particularly suited to overcome these limitations as they have been shown to accumulate and be retained within the area of injury up to 24 hours post-TBI, although with diminishing retention and accumulation the further from the time of injury the NP injection is given [25, 28-30]. Various methods of neuroprotection are used in NP treatment of TBI including antioxidant treatment of oxidative stress, elimination of oxygen radicals, reduction of edema through delivery of encapsulated Cerebrolysin, and delivery of brain-derived neurotrophic factor (BDNF) [28]. Given this information, the potential of NPs as a method of TBI treatment is high with many studies showing that NPs have effectively been able to either be a therapeutic

themselves or deliver therapeutic molecules into the injured area with a higher amount of accumulation and retention when delivered systemically than small molecule treatments.

SECTION 1.3: FORMS OF BEHAVIORAL ANALYSIS

There are a wide variety of behavioral assays one can use to assess behavioral deficits related to TBI. These tasks can be categorized into four distinct groups: spatial learning and memory, nonspatial learning and memory, emotional, and motor coordination. From my previously published research [1]:

Spatial learning and memory are governed by the ability to navigate with two forms, allocentric and egocentric navigation. Allocentric navigation is generally described as using distal spatial cues to guide the direction of movement while egocentric navigation relies more heavily on internal cues such as remembered sequence, speed, the direction of movement, and utilizing closer cues referred to as “signposts”. Important in the discussion of egocentric versus allocentric navigation is distinguishing between “signposts” and “landmarks”. While they provide information for egocentric and allocentric navigation, respectively, signposts do not provide any relational information. Signposts simply convey where to change direction and do not aid in understanding where one is in comparison to other signposts. In contrast, landmarks do not inherently tell you where to change direction but can provide key information regarding one’s placement in relation to other landmarks [31]. To better understand, think of signposts as a particular intersection where you know to turn right to reach your location. Inversely, one could also use the landmark of the street sign and the knowledge of the direction they are approaching from to know to turn right in that situation. While these can sometimes

result in the same or similar choices, such as in this example, that is not always the case. For the sake of consistency, egocentric navigation will be covered as a form of nonspatial navigation; therefore, our focus in this section is the allocentric aspects of each of these paradigms despite the interconnected nature of the two forms of navigation. In order to simplify this section, allocentric navigation will be the only form discussed within this section as it focuses on hippocampal activity even though both allocentric (spatial) and egocentric (nonspatial) navigation systems have an overlap in healthy brains [31].

As opposed to allocentric navigation, as described above, egocentric navigation is a method of determining how to travel similarly to how one might go about a traditional maze, using memory of motions made in conjunction with interior focal points to map out the area mentally. This kind of navigation can be seen in patterns such as the serial and non-spatial navigation shown in the Barnes Maze and Morris Water Maze. While this can occur in many spatial learning tasks such as the Radial Arm Maze, certain variations of spatial learning tasks can be altered to examine nonspatial learning and memory specifically. While the overall administration of these tasks changes for the preclinical models, clinical delayed non-match to sample and VR tasks can also be adjusted to similar specifications to test nonspatial learning and memory.

Emotional changes in human TBI have been well documented. Despite this, many of the tasks used to determine emotional deficits, such as anxiety-like behaviors, lead to directly conflicting results depending entirely upon the paradigm, even within the same procedures. These differences have yielded results determining both high and low levels of anxiety in the same open field test along with equal anxiety when compared to

uninjured counterparts [32]. Many of these tests yield similar conflicts in TBI research. Additionally, human patients have reported near day-to-day variability in their levels of anxiety, depression, and other emotional markers [33]. This may influence attempts to find correlations between preclinical studies of TBI and clinical studies. However, many of these models have been used for drug exploration in other realms such as antidepressants, antianxiety, and other various psychopharmacological drugs. This may redeem some of the criticisms these tasks have been given in the realm of TBI research, though the innate variability of emotional deficits in TBI could also account for that difference.

Motor coordination tasks, otherwise known as vestibulomotor tasks, measure the coordination and physical differences between injured and uninjured rodents. These are the most easily transitional tasks between clinical and preclinical studies as human TBI has been shown to cause adverse effects, at least acutely, to motor coordination and cognition [34].

Even larger than the sheer number of different ways to assess animal behavior are the many data that can be gathered from these assessments. To simplify discussion around data, their meaning, and what paradigms can assess which aspects of behavior, Table 1 in the appendix has a thorough description of each datum, its relationship to TBI, and the meaning behind the results that one could gather.

SECTION 1.3.1: MORRIS WATER MAZE AND BARNES MAZE

Two tests often utilized when determining behavioral deficits in rodent models, which are the most utilized in TBI research, are the Morris water maze (MWM) and the

Barnes maze (BM). Both tasks aim to determine a test subject's spatial learning and memory skills without a restriction to movement. Each test has similar features, such as extra-maze visual cues facing toward the maze in the north, south, east, and west directions. It is worth noting that these are arbitrary distinctions and not related to compass directions. The goal of these tests is to find an escape area, particularly a hidden platform in the MWM and an escape box in the BM, that remains static throughout each week of training, with the start location randomized to ensure allocentric navigation. Additionally, both tests can utilize a reversal trial where the escape area is located opposite of its placement the week prior to test the ability to relearn spatial navigation. Standard protocol usually has these escape areas in the southeast quadrant for the first week and the northwest quadrant in the reversal week [35, 36].

Despite many similarities, there are also various differences between the two maze styles. The MWM differs from the BM as it uses a negative environmental factor, water immersion, to promote learning [35]. Water immersion causes high stress and tends to result in an increase in corticosterone levels in plasma when compared with the BM [37]. While this may be the biggest difference, the MWM also uses a different search strategy analysis due to its vastly different methodology. These search strategies can show if the animal is learning through visual cues, geometric information of the maze, or random behaviors [38]. When quantifying search strategy data for the MWM, three groups of strategies, each with three subgroups, are determined: spatial, non-spatial, and repetitive looping strategies. The subgroups are as follows: for spatial strategies, there are spatial direct, spatial indirect, and focal correct strategies; for non-spatial strategies, there are scanning, random, and focal incorrect; for repetitive looping strategies, there are

chaining, peripheral looping, and circling. These spatial strategies can show differences in learning between the spatial and non-spatial groups versus the repetitive looping groups due to the association between the hippocampus and memory of spatial landmarks in relation to the subject's goal [38]. In comparison, the BM has a much more simplified search strategy analysis which consists of direct, serial, and mixed (or random) strategies [35]. Direct strategies are defined as a direct movement toward the target hole or to the holes adjacent to the target. Serial strategies are defined as strategies where the animal first visits a hole non-adjacent to the target and follows in a clockwise or counterclockwise rotation to each hole until the target is found. Mixed, or random, strategies are defined as a series of hole searches separated by movement across the center of the maze or a generally unorganized search. Figure 16 exemplifies each set of search strategies using previously published examples and new search strategy examples. Other useful data to be gathered from these tasks are the primary escape latency, where the animal first looks inside of the target hole, and the number of primary errors, referring to the number of times the animal attempted to escape through a non-target hole [35].

Both the MWM and BM produce a wide variety of data able to be derived from each experiment. While all data are useful in specific contexts, certain measurements, such as the latency to escape, path length, and cumulative distance from platform for the MWM [36], and the primary latency, primary errors, and total path length for the BM [35], are more useful for TBI testing, while some are just generally more useful and highly utilized in other research contexts.

Due to the widespread use of these mazes in preclinical testing, virtual reality (VR) forms of multiple spatial paradigms have been created to measure cognitive deficits in a clinical setting while remaining both ethical and practical. VR has created a unique opportunity for clinical researchers to draw direct correlations between preclinical and clinical testing by placing patients in a virtual environment similar to that experienced by preclinical rodent models. The MWM VR experience has been highly explored [39]; however, no BM paradigm has yet to be created. Despite this and a lack of endogenous stress in VR, much of the data gathered using the VR MWM may be somewhat translational and help to connect clinical success with preclinical testing. Additionally, VR MWM's have shown a connection between VR testing and rodent testing through the performance relying on hippocampal and medial temporal lobe integrity, among other similarities [39, 40]. These two tests have shown to be incredibly useful and highly characterized through experimentation and thus should play a major role in preclinical research and its translation into clinical success.

SECTION 1.3.2: RADIAL ARM MAZE

The Radial arm maze (RAM) is an eight-armed, walled maze, although variations in the specific number of arms exist. Pre-trial starvation or dehydration is used so food and water can be used as a positive stimulus to encourage exploration (food or water placed throughout the maze) and learning (food or water placed at the end of each arm) [41, 42]. Spatial learning and memory are tested using extra-maze visual cues to allow the animals to create a spatial pattern in their mind or to use nonspatial methods of determining how to most efficiently find all the food in the maze, such as turning only one direction. There are two major RAM paradigms: the delayed spatial win-shift and the

non-delayed random foraging. These paradigms have multiple different characteristics, including the former using arm blocking and two phases, while the latter uses only one phase. Both paradigms bait half of the arms to test learning. While spatial cues are not necessary, they are required to shift this from simply a learning paradigm to specifically a spatial learning paradigm. For a more comprehensive look at a particular protocol, Floresco et al. have provided a comprehensive explanation [43].

Each paradigm produces different specific datasets. The delayed paradigm data are primarily taken from the second part of the test after the delay. At this time, errors are counted as entries into arms that had not been previously blocked during the training phase. Additionally, errors are split into two groups, across-phase and within-phase, which are more thoroughly described in Table 1 [43]. The non-delayed paradigm includes only the single trial of testing and describes errors much more broadly as any re-entry into an arm, whether that arm contains bait or not. However, these are also broken down into two subtypes: re-entries into arms that had been baited at the beginning and re-entry into arms that had not been baited [43]. Both paradigms share total latency and first latency despite their differences. While several types of data can be obtained using this, clinical translation is often very difficult.

Similarly to the MWM, clinical researchers have used VR RAM paradigms to attempt to connect preclinical work with clinical testing. Much like the MWM, the VR paradigm for the RAM shows similarities to results observed in rats. For example, clinical research has been able to demonstrate that the usage of spatial and nonspatial learning corresponded with activation of the brain regions controlling the two forms of

learning, namely the hippocampus and caudate nucleus, respectively, which is also observed in rats [39].

SECTION 1.3.3: T AND Y MAZE

T and Y mazes are similar, based on the same principle of spatial learning and memory. Both mazes function as a two-pronged maze using either positive stimuli (e.g., food, novel objects) [44-46] or negative stimuli (e.g., light, electrical shock and sound, a blocked arm) [47, 48] to promote memorization of the different arms. After training, the stimuli are removed, and animals are tested again to measure memory. Additionally, some variations of the T maze use distal spatial cues to help promote learning and to determine spatial learning in a similar fashion as the MWM and BM tasks [47]. One variation utilizes both positive stimuli during training and spatial cues in a combined system. In this variation, mice are tested for two forms of spatial learning, place learning and response learning [39]. Place learning can be described as the utilization of spatial cues to determine location, while response learning can be described as using internal cues such as the direction of a particular movement. For example, the animal would be using place learning if it turns toward the reward during the probe trial and response learning if it turns away from the reward. Essentially, place learning and response learning can be equated to spatial learning and nonspatial learning, respectively.

The T and Y maze offer very few data, even with the dual-solution T maze which can distinguish between place and response learning in the rodent model [49]. The alternating T maze, which utilizes two phases involving a training phase where one arm is blocked, measures time spent in the unblocked, or novel, arm as a percentage of total time spent in

the maze. While this measurement is a general measurement used in most T and Y maze testing despite the version, the alternating T maze also uses forced alternation as a data point [50].

The T and Y maze have a less significant clinical connection when compared to the VR MWM or RAM. These issues stem from the simplicity of the maze, which is ironically one of the reasons these can be such popular mazes. These mazes have the same issues that plague others, specifically the lack of motivation in humans [39]. Humans do not have the same motivations in VR as animal models do in preclinical testing, such as the potential for drowning, starvation, or even minor annoyances such as the strong lighting in the BM. Therefore, human patients require some outside source to provide a stimulus while the test is taken in VR, such as food or monetary rewards. Regardless of other methods to increase virtual T maze viability, the MWM and RAM VR tasks seem to show much more promise as a viable connection between the preclinical and clinical sides of testing.

SECTION 1.3.4: NOVEL OBJECT LOCATION TEST

In the Novel Object Location test, rodents are allowed to explore an empty open field for 5 min. Animals are then given a 5 min trial one hour later with the objects placed in the open field and then another 5 min trial one hour later with one object in the same place and another object in a new place within the field [39, 51]. The one-hour inter-trial interval forces the animal to rely on the long-term memory rather than short-term memory or luck. Rodents are expected to use their natural curiosity to spend more time examining the object in a novel location as opposed to the object which had not moved.

However, deficits are shown when animals chose to explore both objects similarly to the middle phase prior to object relocation, showing an inability to remember the familiar location when faced with a novel location.

The Novel Object Recognition task is a nonspatial variation of the Novel Object Location task. In this test, rather than one of the same two objects being moved to a new location, the object is instead replaced with a new object the animal is unfamiliar with. Similarly to the Novel Object Location task, it is expected that TBI animals will spend a near equal time exploring both objects while uninjured animals will spend more time exploring the novel object [51].

At this time, human equivalents are only connected to the delayed non-match to sample task, which itself is a behavior test used with animals already [52]. This separate test is administered by giving the subject an initial set of stimuli, generally a set of objects, and providing a separate, novel object after a delay and requiring the subject to select the novel stimulus [52]. The changing of objects can create a thorough connection to the Novel Object Recognition task; however, this is considered to be more similar to the delayed match to sample task as there seems to be some correlation between the slightly different mechanisms of memory used in each task.

Both tasks share data similarities, as time spent with the novel object or location in terms of a fraction of time spent in the maze are the primary data point of measurement. However, a metric called the discrimination index is also used and measured by subtracting the time spent exploring the familiar location or object from the time spent with the novel location or object divided by the total time exploring either object. It is

important to note that this does not mean the total time spent in the open field but rather the summation of time spent exploring either object or location [53].

SECTION 1.3.5: NONSPATIAL VARIATIONS OF SPATIAL LEARNING TASKS

Many paradigms such as the RAM, MWM, and BM can test for nonspatial learning. Indeed, in each task, there are methods with which nonspatial learning can be examined without changing the protocol. Nonspatial search strategies can be present in each task, such as serial exploration in the RAM and BM and MWM strategies that show knowledge of the existence of an escape without a direct understanding of how to get there. Such strategies include serial strategies for the BM, random, focal incorrect, and scanning strategies for the MWM, and chaining or serial strategies in the RAM [31, 35, 54]. However, for researchers interested in limiting these to only nonspatial navigation, several methods have been explored, with the most common being to “drown out” or remove any extra-maze cues. Nonspatial navigation targets a different area of the brain when compared to spatial navigation. Particularly, the area which is most considered to dominate spatial navigation is the hippocampus, while the area most correlated with nonspatial navigation, also thought to be heavily implicated in the same areas as spatial navigation, implicates other brain regions such as the caudate nucleus and entorhinal cortex [55]. While nonspatial learning is a large field within neuroscience, its reasoning is less understood when compared to spatial learning, and therefore, it is less effective when determining differences between injured and uninjured animals or patients

SECTION 1.3.6: FORCED SWIM TEST

The forced swim test was designed originally for testing of antidepressant drugs and is accepted as a preclinical model of depression because of its usage in testing for anti-depressant medication [56]. The protocol for this test requires a 10 cm diameter transparent cylindrical tank filled with water to 15 cm from the bottom. Both diameter and depth can be altered to change behavior, such as the length of time mice were willing to maintain struggle by continuing motor activity which increased with larger tank diameter and deeper water [57]. These conclusions, while important in the field of anti-depressant testing, have less importance within the field of TBI testing, where, for the sake of the effects of TBI on depression, the standard depth and tank width provide sufficient information to researchers. It is worthy to note that the testing performed by Sunal et al. found that larger tanks with a longer duration, namely 15 min, may provide a more accurate measurement without as many issues of false positives [57]. The water should be room temperature and rodents should be placed in the tank gently and remain there for six minutes. Intervention in the test should only be carried out if the rodents cannot maintain swimming or floating, or, in a special case with mice, any diving behavior is observed [56].

The data derived from these experiments have three basic components: time spent inert, time spent climbing, and time spent struggling. While an animal is climbing, it is attempting to come up the side of the vessel of water. While an animal is struggling, it is making active movements to try and stay afloat or get out of the water. While an animal is inert, it is making no movement and can thus be considered as an act of despair, similar

to depressive-like symptoms in humans. The major data point for this test is the time spent inert, which can be interpreted as depressive-like symptoms.

SECTION 1.3.7: OPEN FIELD TEST

The open field test is useful for measuring both locomotion and anxiety-like behaviors in rodents and is one of the most commonly used methods of behavioral testing, especially in rodents. The field consists of a walled area with a light focused directly above the area with a 10 min limit to the test. For anxiety testing, measurements of time spent in the outside area of the maze, known as thigmotaxis, are considered to be a marker of anxiety-like behavior. The more time an animal spends in the center of the arena, the less anxiety-like the animal's behavior. Additionally, movement can be measured with higher amounts of distances travelled being considered as an anxiety-like reaction [58]. When used for motor coordination, the above-described methods are still used, but different measurements are taken. Data for this test include distance moved, time spent walking and running, slower or hyperactive movements, jumping, rearing, and other rodent behaviors described previously. However, the most used and understood data point for motor coordination is the distance travelled [58]. Depending on the timing of this test, one should expect slower movement in TBI mice in the acute phase and more hyperactive movements in the chronic phase, as well as a lower distance moved and higher distance moved for TBI mice in the acute and chronic phases, respectively [59]. Along with the rotarod test, this test is highly characterized and accepted by the behavioral testing community.

SECTION 1.3.8: RESIDENT INTRUDER TEST

The resident intruder test is a common test for aggression. Much of the data gathered from this test are specifically behavioral, relying heavily upon noticing differences, frequency and duration of offensive aggression, defensive aggression, and violence. Each of these categories have well-defined parameters as described by Koolhaas et al. To establish territoriality with rodent models, a male is housed with a sterilized but hormonally intact female companion for at least one week. During the test, the female is replaced with a novel male into the cage and observed to determine a battery of scoring measuring two opposites of behavior, aggression and sociability/anxiety, measured by the Total Offense Score and the Social Exploration Score, respectively [60]. Additionally, latency to first attack is also an often-used measurement to determine aggression with lower latency corresponding to a higher amount of aggression. This protocol can also be adjusted for female mice with almost no change, except to make sure female companions are age-matched to avoid conflict [61].

SECTION 1.3.9: ROTAROD

The rotarod test is a widely used test to determine coordination deficits in rodents. A linearly accelerating cylinder that animals are placed on continues to rotate until all animals have fallen or until the final time point is reached. This is most effective for motor deficits in the acute phase of injury, but may also be used later prior to cognitive testing to ensure there are no motor deficits when using methods such as the MWM, RAM, or other spatial or nonspatial learning tasks. Latency to fall is the most important measurement with this method; however, qualitative analyses can include coordination by way of the method with which the animal stays on the rotarod [19, 59].

SECTION 1.3.10: FOOTPRINT PATTERN ASSAY

The footprint pattern assay is executed by dipping a rodent's paws in different ink colors for the fore and hind paws and leading them down a tunnel lined with paper. Through this method, abnormalities in gait and coordination can be observed. Additionally, many parameters are capable of being measured, such as stride distance, stride length, variability across the center axis of the paper, width between hind paws, step regularity, and step overlap. Many of the most important aspects of the footprint assay include the step length, step duration, and inter-leg coordination, as described in Table 1 [62]. Modernized versions of this assay are automated and also capable of measuring pressure and speed, such as the CatWalk^{TM} system [63-65].

SECTION 1.3.11: DESCRIPTION OF RESEARCH

In this work, I used two different spatial learning and memory paradigms designed to indirectly measure the spread of secondary injury. The rationale for this is that the right hippocampus has been shown to be the dominant side relating to spatial and learning memory in both humans and mice [66, 67]. Using a CCI model with mice, an impact is applied to the left cortex through a craniotomy. After 3 weeks, the spatial learning and memory tasks are given to measure how nanoparticle treatment in the acute phase can affect deficits in learning and memory during the chronic phase of injury. My goal was to identify and develop a paradigm and protocol that generates large differences between uninjured control mice and CCI mice so that various NP-based treatments can be assessed and compared.

CHAPTER 2: MORRIS WATER MAZE

Experiments involving the MWM were done in collaboration with Ali Manske and Dr. Sarah Romereim to test the efficacy of an antioxidant NP treatment on TBI. The MWM was chosen over other paradigms as it is highly characterized in literature and is the most used behavioral test for cognitive deficits [68]. Additionally, mice were tested using Rotarod to determine motor coordination prior to MWM testing.

SECTION 2.1: METHODOLOGY

The antioxidant NPs used in this study were previously reported by Yoo, et al. and are called NP1 [30]. These NPs utilize a thioether bond to scavenge reactive oxygen species (ROS), a cause of oxidative stress and a major contributor to the secondary injury cycle. A volume of 100 µL at a concentration of 1 mg/mL was injected intravenously through tail vein injection immediately following TBI for each NP treated mouse.

From my previously published literature [69]:

SECTION 2.1.1: CONTROLLED CORTICAL IMPACT SURGERIES

All animal procedures were performed in accordance with the approval of the University of Nebraska−Lincoln IACUC. Six-week-old male and female C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME, USA) were acclimated for 2 weeks prior to the procedures. Mice were anesthetized with 3% isoflurane gas via inhalation and were maintained at ∼1.5% with a nose cone on a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). The hair of the scalp was removed with Nair (Church and Dwight Co., Inc., Princeton, NJ, USA), and the scalp was disinfected with a betadine scrub and isopropanol wipes afterward. Lidocaine (0.05 mL of 5 mg/mL) and bupivacaine (0.05 mL

of 0.3 mg/mL) were applied to the scalp, and buprenorphine SR (60 μL of 0.5 mg/mL) was given subcutaneously. An approximately 1 cm midline incision was made on the scalp over bregma. An approximately 2 mm craniectomy was made in the skull over the left frontoparietal cortex (2 mm anterior and 2 mm left of lambda) using a surgical drill. A controlled cortical impactor (Hatteras Instruments, Cary, NC, USA) attached to the stereotaxic frame with a 2 mm convex tip was used to impact the brain normal to the dura surface at a depth of 1.5 mm and a velocity of 4 m/s with a dwell time of 80 ms. Any bleeding was controlled and incisions were closed using tissue adhesive. NP1 (100 μL of 1 mg/mL) was injected through the tail vein immediately after the surgery for the NP1 treated group. With the average weight of 22.24 g for male mice and 16.44 g for female mice, the average dose of NP1 administration was 4.5 mg/kg for male mice and 6.1 mg/kg for female mice. The size of each treatment group is as follows: 15 mice in the control group, 21 mice in the untreated CCI group, and 13 mice in the NP1 treated CCI group. This includes both male (8 CCI, 5 NP1, 5 control) and female (13 CCI, 8 NP1, 10 control) in three separate MWM experiments with two experiments consisting of female mice and one of male mice.

SECTION 2.1.2: ROTAROD

A Rotor-Rod^{TM} motor function system (San Diego Instruments, San Diego, CA, USA) was utilized to assess the motor function and learning of the mice prior to all MWM studies. Rotarod trials were started 3 days post-CCI and were repeated daily for 5 days. Mice were placed onto the cylinders, which then began to rotate. The speed linearly increased from 0 to 50 rpm over 5 min. Latency to fall was averaged over 5 separate runs for each animal each day.

SECTION 2.1.3: MORRIS WATER MAZE

The MWM behavior analysis was executed based on a previously published protocol about assessing spatial learning and memory [36]. The MWM experiment was started 3 weeks post-CCI and consisted of two trials: spatial acquisition and reversal. The mice were trained to find the platform using a visible marker before covering the platform with opaque water (white tempura paint) and removing the platform marker. The platform was placed in the southwest quadrant during acquisition trials with the mice starting randomly in the north, east, southeast, and northwest quadrants. The platform was moved to the northeast quadrant during reversal trials with the mice starting randomly in the south, west, northwest, and southeast quadrants. Spatial cues were placed in the north, south, east, and west directions as extra-maze cues. In both acquisition and reversal trials, the mice underwent four trials per day for four days. Male mice (18 total, 8 CCI, 5 NP1, 5 control) and female mice (31 total, 13 CCI, 8 NP1, 10 control) were employed for the MWM trial. Testing for each sex was done separately from each other. Search strategy analysis was done by two researchers separately then results were combined and analyzed as a group. Results were recorded and data analyzed in GraphPad PRISM 7 (GraphPad Software, CA) using the percentages of each experimental group's usage of each spatial strategy.

SECTION 2.1.4: STATISTICAL ANALYSIS

All the data in this study were expressed as mean ± standard error of the mean (SEM). A $p < 0.05$ was considered statistically significant. For latency to escape, Kaplan-Meier survival analysis with Mantel-Cox log-rank test was employed to account for the non-normal distribution of latencies resulting from the 90 second maximum trial duration.

Other data were evaluated using a two-way analysis of variance (ANOVA) with Tukey's multiple comparisons test and one-way ANOVA for the probe trials. All statistics were analyzed with GraphPad Prism 7 software (GraphPad Software, CA).

SECTION 2.2: RESULTS

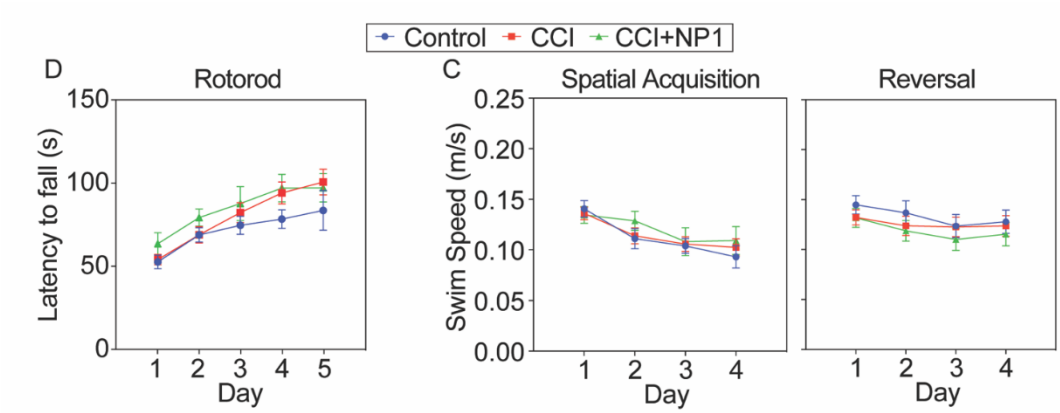

Figure 2.1. Motor coordination results before and during the MWM.

As shown in Fig. 2.1, no significant motor deficits were found during the Rotarod testing in the subacute phase 4 to 8 days post-TBI and were confirmed during both the spatial acquisition and reversal weeks of the MWM using swim speed as a metric for motor coordination. In Fig. 2.2A, only two days were showed statistically significant differences between the control and CCI groups were day 3 of the spatial acquisition week and day 1 of the reversal week. Additionally, significant differences between the CCI and NP1 treated groups on days 3 and 4 of the spatial acquisition week and days 1 and 3 of the reversal week. Other differences during the acquisition and reversal weeks are shown in Fig. 2.2C where significant differences between the CCI and both the control and NP1 treated groups were found when averaging full week of trials for the fraction of time spent in the outer annulus. While statistically significant differences were noticed during each week of training, the probe trial day showed very little significance

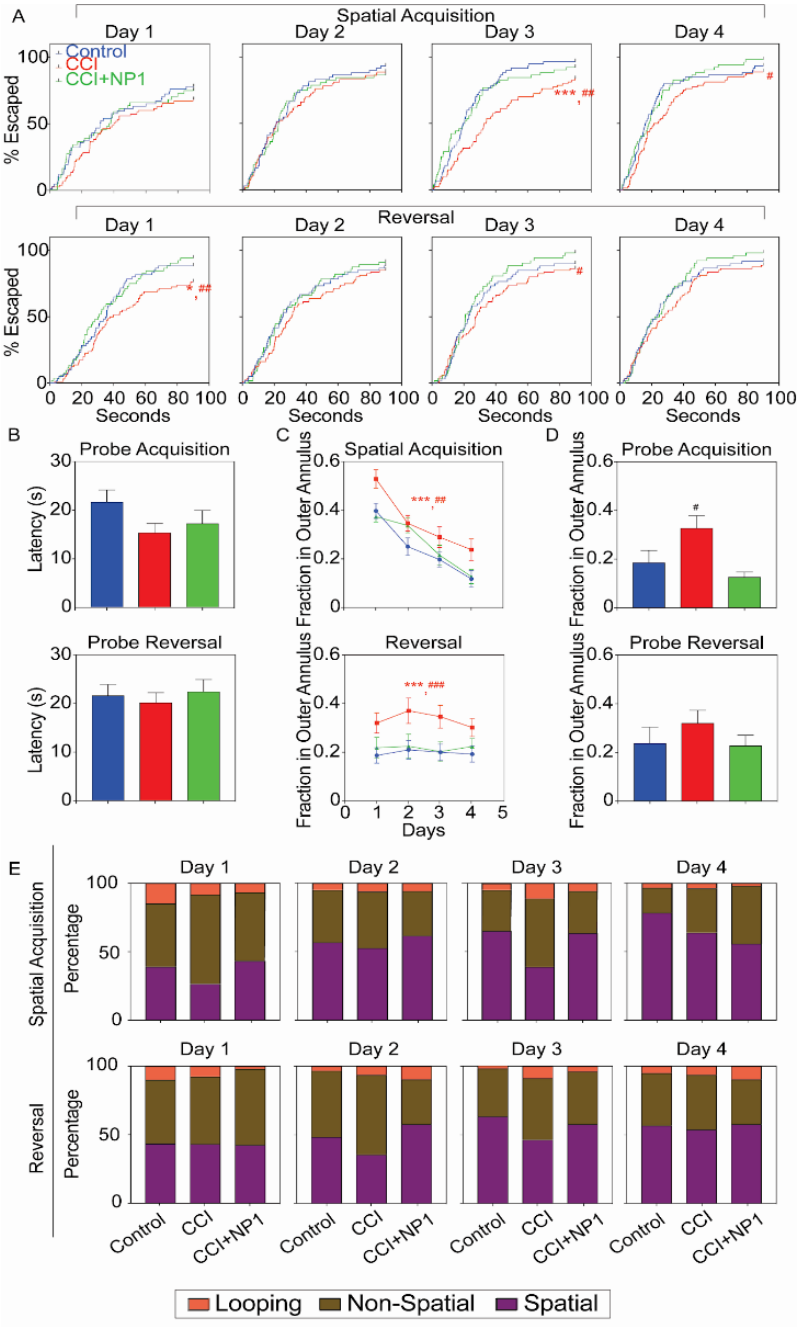

Figure 2.2. A) Percentage of escape based off total latency to escape using Kaplan-Meier regression. B) Probe trial latency to first visit to former target area. C) Fraction of time spent in the outer annulus. D) Probe trial fraction of time spent in the outer annulus. E) Percentages of each search strategy used during the acquisition and reversal weeks. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$ between control and CCI groups, # = $p<0.05$, ## = $p<0.01$, ### = $p<0.001$ between CCI and NP1 treated groups.

probe trial parameters was shown between the CCI and NP1 treated groups on the acquisition probe trial in the fraction of time spent in the outer annulus. There was no significant difference seen between the control and CCI groups in that same probe trial. Additionally, the control and NP1 treated groups began utilizing the spatial search strategy, a sign of increased spatial learning and memory, more than 50% of the time on day 2 of the acquisition week while the CCI group lagged behind and were inconsistent throughout the week, as seen in Fig 2.2E. When the total amount of search strategies are averaged across the spatial acquisition and reversal weeks, control and NP1 treated mice used the spatial search strategy 56.3% and 54.9% of the time, respectively. Meanwhile, CCI mice only used the spatial search strategy 45% of the time, significantly less than both groups ($p<0.05$). When broken down specific to sex, statistically significant differences were even more sparse regarding total latency in both the female and male mice. In Fig. 2.3A, it is shown that the only significant difference between the control and CCI female mice was on day 4 of the spatial acquisition week. Significant differences were seen between NP1 treated female mice and their CCI counterparts on days 1 and 3 of the reversal week. When considering the probe trial statistics (Fig. 2.3B and D) female mice had no statistically significant differences in either probe trial latency or fraction of time spent in the outer annulus. However, it is worth noting that the mean fraction of time spent in the outer annulus by the CCI group is nearly twice that of the control and NP1 groups in both the acquisition and reversal probe trials. Significant differences in the weekly average of time spent in the outer annulus were found between the CCI and controls in both weeks with the female mice (Fig. 2.3C) and only in the reversal week with male mice (Fig. 2.3G). For the male mice, significant differences in

escape percentage were found on between CCI and control mice on days 1 and 3 of the acquisition week and days 1 and 2 of the reversal week (Fig. 2.3E). When it comes to probe trial measurements, male control mice performed significantly worse than CCI mice in the probe trial latency (Fig. 2.3F), however the mean probe trial fraction of time spent in the outer annulus (Fig. 2.3H), while not being statistically significant, shows a nearly 2-fold difference between the CCI and control groups, similarly to female mice. This does not continue for the reversal week. In fact, male CCI mice in the reversal week showed significant differences in fraction of time spent in the outer annulus on average across the week when compared to control mice as shown in Fig. 2.3G. Search strategy analysis revealed some interesting trends when broken down by sex. During the acquisition week, control male mice showed a slightly higher percentage of spatial strategies used at 62.5% compared to their female counterparts who utilized spatial strategies 58.4% of the time. Additionally, the comparisons for the CCI group show male mice utilizing spatial strategies only 38.3% of the time while female mice used spatial strategies 51.1% of the time. While both percentages are lower than the amount used by the control groups at this time, the large difference between male and female mice regarding the effect of injury on spatial learning is worth note. Another interesting aspect of the search strategy results is within the reversal week. When combined, the control group slightly edges out the CCI group in spatial strategies used at 44.8% and 40.3%, respectively. However, when separated by sex, the control males come in at 46.8% and CCI males at 50.3%, showing that male CCI mice seemed to use more spatial search strategies than control. This is countered by the looping strategy comparison, where CCI male mice used those strategies 9.4% of the time compared to 1.3% for controls. Female

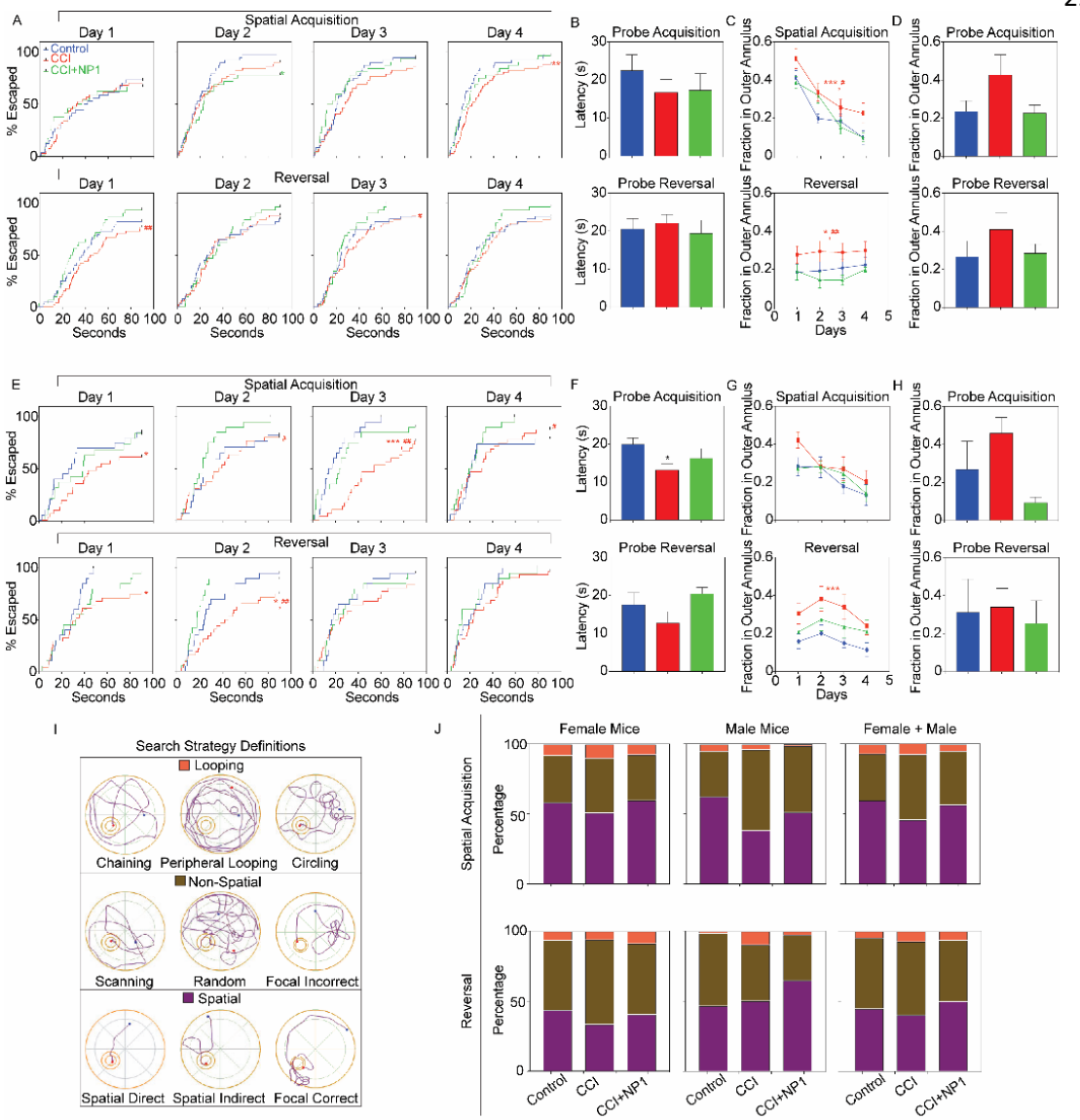

Figure 2.3 A) Percent escaped for spatial acquisition and reversal trials, female mice. B) Probe trial latency to first visit to target, female mice. C) Fraction of time spent in the outer annulus, female mice. D) Probe trial fraction of time spent in the outer annulus, female mice. E) Percent escaped for spatial acquisition and reversal trials, male mice. F) Probe trial latency to first visit to target, male mice. G) Fraction of time spent in the outer annulus, male mice. H) Probe trial fraction of time spent in the outer annulus, male mice. I) Search strategies examples used in the MWM. J) Percentage of each search strategy used separated by week and sex.

mice, however, showed a significant decline across the board for spatial strategies with 43.8% for controls and 33.5% for CCI and the opposite situation for looping strategies with controls and CCI groups using this strategy 6.3% and 5.9% of the time, respectively.

SECTION 2.3: DISCUSSION

While our MWM data showed promising results, the separation between control animals and CCI animals were not large enough to show many significant differences on either week combined or within either sex. Search strategies and fraction of time spent in the outer annulus proved to be the most effective methods in measuring spatial learning and memory deficits in the combined statistics and male mouse groups. This is useful for assessing NP efficacy; however, the fraction of time spent in the outer annulus can also be connected to search strategy analysis as a peripheral looping strategy, one of the three looping strategies most often associated with spatial deficits, often result in animals spending an excess amount of time in the outer annulus. These looping strategies were also shown to be higher in CCI mice in the male, female, and combined groups on both weeks, with some notable exceptions in the spatial acquisition week for males and the reversal week for females. While this may suggest a difference in cognitive flexibility, the lack of probe trial differences and separation between control and CCI mice in total latency seem to instead suggest that there was an issue with the MWM resulting in suboptimal results. When looking at the escape percentages, very little difference was observed in both the sex-separated data and combined data. Despite being a measurement of the training weeks, this lack of separation is especially concerning considering the lack of differences in the acquisition and reversal probes, indicating learning within each group is much less than what is needed for effective testing. When focusing on the

separated sex data, it seems that there is a more noticeable gap in total latency between control and CCI groups within the male mice. This was an expected result as male mice have been noted to be outperformed by female mice when both have undergone CCI [70].

SECTION 2.4: CONCLUSIONS

NP1 had some effect when averaging the whole of the groups; however, the lack of probe trial differences between control and CCI mice weaken our ability to claim NP1 efficacy. The MWM showed minimal separation between injured and uninjured animals except on day 3 of the spatial acquisition week and day 1 of the reversal week for the combined data. However, these findings allowed us to determine a moderate neuroprotective role of NP1 when in tandem with various molecular and histological correlates that showed significant differences between CCI and control groups (not part of this thesis), even despite the mild-to-moderate injury model used. Most importantly, this work allowed us to identify two potential opportunities for improvement in our behavioral testing. One involves increasing CCI injury severity, which could aid in creating greater differences between control and CCI groups. Secondly, a separate paradigm with less endogenous anxiety, more time for exploration, and more data to describe how the mice are learning could help improve performance of control mice and increase observable differences from CCI mice.

CHAPTER 3: BARNES MAZE OPTIMIZATION

We chose the BM to test spatial learning and memory without the anxiety created by placement in water as with the MWM. Male mice were the original focus of these tests as they have been known to perform worse than their female counterparts post-TBI and are

expected to perform better than female mice when healthy, as has been reported in both rats and humans [71, 72]. In mice, repetitive concussive TBI has shown lower impairments chronically than males, however CCI models have shown mixed results regarding cognitive deficits [70, 73]. Additionally, previous work into spatial learning and memory as it pertains to TBI showed males with slightly more pronounced deficits. While MWM data on these differences has been mixed in mice, BM reports suggest that these differences exist, whether that be from sex hormone or a sex-dependent effect on injury or anesthetic [70]. This study focused on determining the most efficient method for expanding deficits between CCI and control groups while being able to ensure learning in both groups.

SECTION 3.1: METHODOLOGY

SECTION 3.1.1: TRADITIONAL BARNES MAZE

One protocol we used was a standard and widely accepted protocol adapted from Gawel, et al. 2019 [35]. This protocol consisted of 2 weeks with 5 days of spatial acquisition trials in week 1 and 5 days of reversal trials in week 2 with one probe trial on day 6 of each week. Training trials were 180 seconds with 2 trials per day and a 30 second period either after escape or at the end of the 180 seconds for the mice to stay in the escape hole with the top covered. At the end of each training trial, if the mouse had not escaped, the mouse was placed in the escape. For each probe trial, the escape box was removed and the mice were allowed to explore the maze for 90 seconds. In both the

training and probe trials phases, four spatial cues were placed and labeled as the north, south, east, and west (Figure 3.1).

Figure 3.1 An image of the Barnes Maze that was used in these experiments with one of the two 50 W bulbs and two of the four spatial cues showing.

One day prior to the spatial acquisition week, the mice were allowed to explore the maze for 60 seconds with the spatial cues hidden and both 50 W lightbulbs on and directed toward the maze. After these 60 seconds, the mouse was led to and placed in the escape box for 120 seconds with the escape hole covered. After each trial, the maze and escape box were cleaned with 70% ethanol and wiped clean before the next trial began. All testing took place on a 93 cm diameter platform with 20 holes placed evenly around the platform, just inside the diameter an equal distance from the edge (Noldus Information

Technologies, Leesburg, VA, USA). Videos of each trial were acquired with a 1080p high-definition camera then converted into mp4 format and analyzed using EthoVision XT (Noldus Information Technologies, Leesburg, VA, USA).

SECTION 3.1.2: UPDATED BARNES MAZE

We designed a new, shortened BM that used the same platform, lights, spatial cues, cleaning, and data acquisition process and software. Based on our finding from the traditional BM protocol, we altered the number of trials per day, length of the acquisition week, and purpose of the probe trials. Additionally, removing the reversal week allowed for a more focused approach on measuring short-term and long-term memory as opposed to the standard cognitive flexibility measurements gained from the reversal week. This updated BM is comprised of one spatial acquisition week lasting 6 days with three 180 second trials per day. Like the former protocol, if the mouse did not escape in 180 seconds, it was then led to and placed in the escape hole. However, in this protocol the mouse was led to the escape using a clear 2-liter beaker and allowed to enter the escape hole on their own. The probe trials took place on days 7 and 10 as a potential method of measuring short-term and long-term memory. Each probe trial lasted 90 seconds with the escape removed in the same manner as the old protocol. Further differences lie in the pre-training trial (day 0) where instead of the spatial cues being hidden, they were visible to the animal from the platform. Additionally, after the first 60 seconds to explore the maze, the mouse was then led to the escape hole using a 2-liter beaker and allowed to enter the escape on their own before the hole was covered and mice are given 120 seconds in the escape hole.

SECTION 3.1.3: CONTROLLED CORTICAL IMPACT

All animal procedures were performed in accordance with the approval of the University of Nebraska−Lincoln IACUC. Seven-week-old male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME, USA) were acclimated for 1 week prior to the procedures. Mice were anesthetized with 3% isoflurane gas via inhalation and were maintained at ∼1.5% with a nose cone on a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). The hair of the scalp was removed with Nair (Church and Dwight Co., Inc., Princeton, NJ, USA), and the scalp was disinfected with a betadine scrub and isopropanol wipes afterward. Lidocaine (0.05 mL of 5 mg/mL) and bupivacaine (0.05 mL of 0.3 mg/mL) were applied to the scalp, and buprenorphine SR (60 μL of 0.5 mg/mL) was given subcutaneously. An approximately 1 cm midline incision was made on the scalp over bregma. An approximately 2 mm craniectomy was made in the skull over the left frontoparietal cortex (2 mm anterior and 2 mm left of lambda) using a surgical drill. A controlled cortical impactor (Hatteras Instruments, Cary, NC, USA) attached to the stereotaxic frame with a 2 mm convex tip was used to impact the brain normal to the dura surface at a depth of 2.5 mm and a velocity of 4 m/s with a dwell time of 80 ms. Any bleeding was controlled and incisions were closed using tissue adhesive. The size of each treatment group is as follows: 10 mice in the old protocol control group, 10 mice in the old protocol CCI group, 10 mice in the new protocol control group, and 7 mice in the new protocol CCI group. The control experiments were done in groups of 5 mice separated into different weeks while the CCI groups were done in tandem with a group of 5 NP treated mice. This latter group will be discussed further in Chapter 4.

SECTION 3.1.4: STATISTICAL ANALYSIS

All the data in this study were expressed as mean ± standard error of the mean (SEM). A $p < 0.05$ was considered statistically significant. Escape frequency using total latency was analyzed using Kaplan-Meier survival analysis with a Mantel-Cox log rank test. Weekly training statistics were analyzed using two-way ANOVA or a mixed-effects model when applicable. Probe trial data was analyzed using an unpaired t test with Welch's correction. All data analyses were performed using GraphPad Prism 9 (GraphPad Software, CA).

SECTION 3.2: RESULTS

Fig 3.2A shows the full week of escape frequency by total latency throughout using the new protocol. An increase in escape frequency across the six days of the updated BM was observed. On day 1, 10% of control mice escaped within 143 seconds. On day 2, there was a decrease in escape frequency and increase in latency for control mice with only 3.3% of mice escaping before 172.9 seconds. On day 3, 10.1% of control mice escaped before 98 seconds while nearly 21% escaped prior to 172.8 seconds. CCI mice performed the same on days 1, 2, and 3 with zero escapes in the 180 seconds given. On day 4, CCI mice were able to escape 4.76% of the time prior to 159.3 seconds, however there were no further escapes. Control mice on the same day, however, surpassed a 50% escape frequency by 179.3 seconds with 51.87% escaping prior to that point. Notably, 33.333% of control mice had escaped prior to 113.3 seconds. On day 5, these differences continued to expand with CCI mice showing a 28.57% escape at 166.6 seconds and control mice escaping at 73.333% by 142 seconds. Additionally, control mice reached a 50% escape frequency at 60.8 seconds. On day 6, control mice reached

50% escape at 15.3 seconds and reached a peak escape frequency of 76.667% at 111.6 seconds. CCI mice reached a peak escape frequency of 38.1% at 141.8 seconds. Statistically significant differences were noticed on days 3, 4, 5 and 6 between the control and CCI groups when using the updated BM protocol.

In Fig 3.2B, the same data as discussed in the last paragraph is reported for the traditional protocol. Day 1 had a peak escape frequency of 36.8% and 25.3% at 169.8 and 180 seconds for the control and CCI groups, respectively. Day 2 showed a decrease in the frequency as well as a decrease in total latency for both control and CCI groups. Control

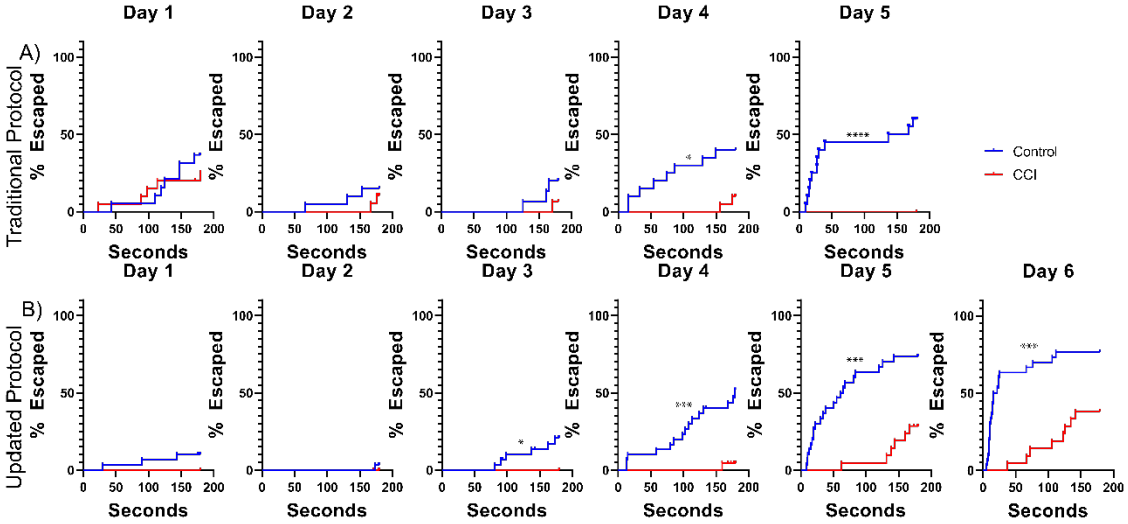

Figure 3.2 Comparison in escape frequencies by day between the traditional and updated Barnes Maze protocols. A) Traditional protocol escape percentages for the spatial acquisition week. B) Updated protocol escape percentages for the spatial acquisition week. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001

mice had an escape frequency of 15% before 153.7 second and CCI mice had a frequency of 10.5% before 176 seconds. Day 3 also showed a decrease in escape frequency and latency but only for the CCI group with an escape frequency of 6.667% at a time of 169.7

seconds. Control mice had 20% of the group escape before a time of 165.4 seconds. On day 4, the escape frequencies were statistically significantly different with control mice escaping 40% of the time before 149.7 seconds and CCI mice escaping only 10% of the time by 174.4 seconds in the maze. Finally, on day 5, significant differences were again seen, and control mice reached a 50% escape frequency at 136.2 seconds. CCI mice, however, highly underperformed and had zero escapes throughout the full 180 seconds.

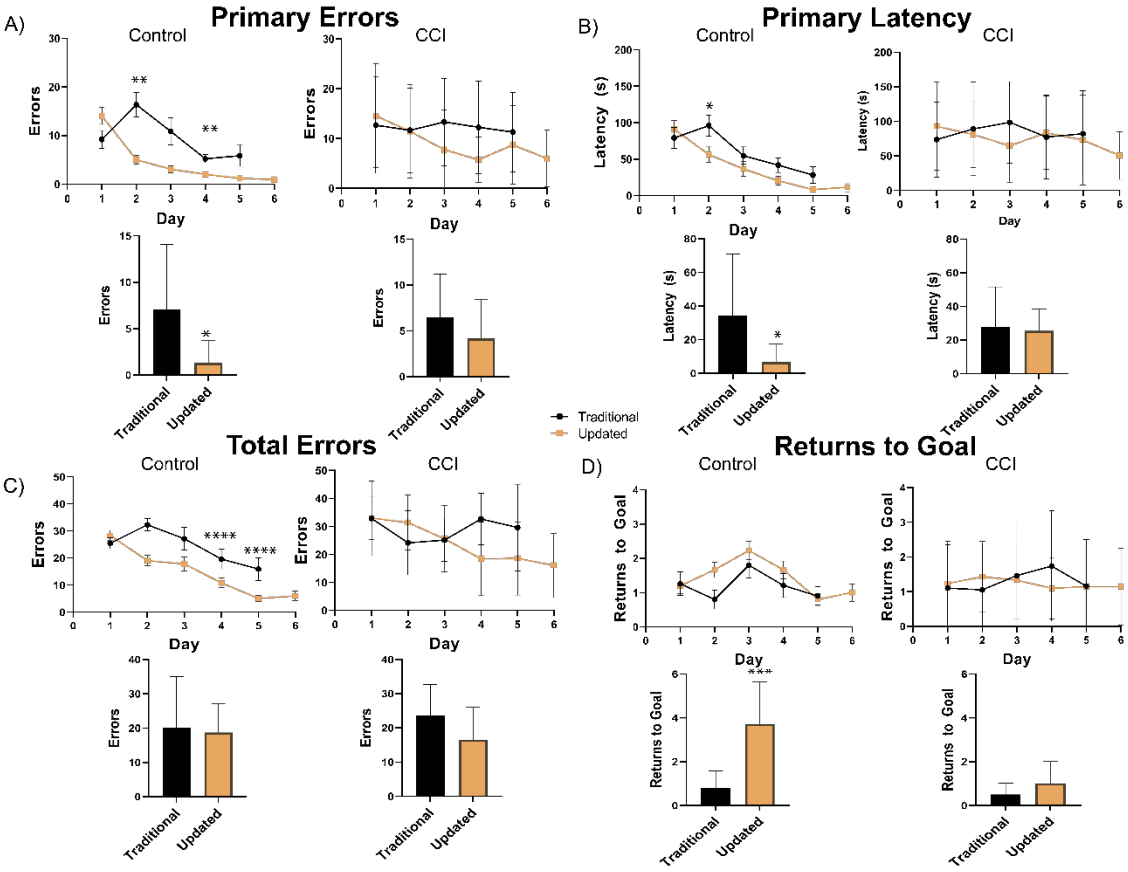

Figure 3.3 A) Primary error comparison of both protocols control (left) and CCI (right) groups. B) Primary latency comparison of both control (left) and CCI (right) groups. C) Total errors comparison of both protocols control (left) and CCI (right) groups. D) Returns to goal comparison of both protocols control (left) and CCI (right) groups.

The primary errors between control and CCI groups were compared to determine the effect the updated protocol had on spatial learning (Fig. 3.3A). For the sake of this comparison, the acquisition probe from the traditional protocol is compared to the short-term memory probe from the updated protocol. For control mice, the updated protocol showed significant differences on days 2 and 4 of the acquisition comparison compared to the traditional protocol as well as a significant difference in the probe trial day. CCI

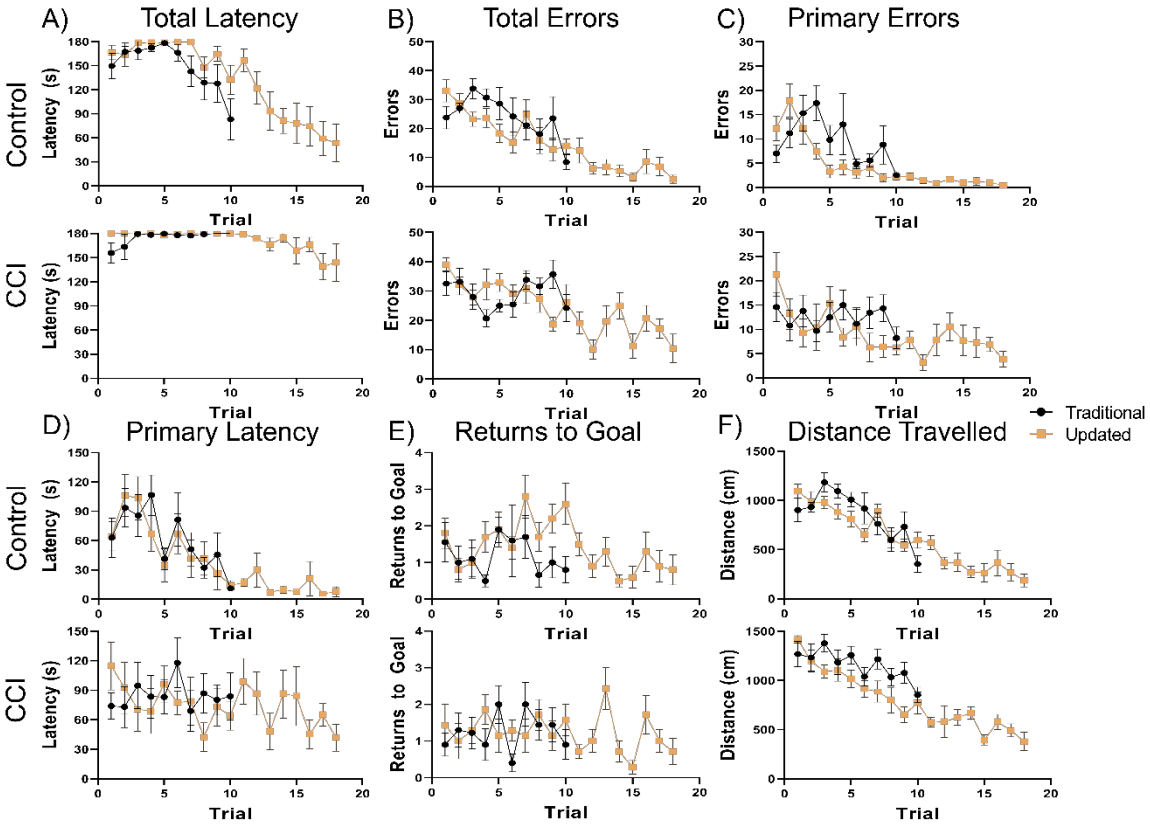

Figure 3.4 Control and CCI group protocol trial by trial comparisons. A) Total latency by trial B) Total errors by trial C) Primary errors by trial D) Primary latency by trial E) Returns to goal by trial F) Distance travelled by trial.

mice, however, showed no differences but showed high variability independent of the protocol used and a lower mean in the probe trial when going through the updated protocol. Similarly, primary latency showed significant differences between the control groups in the probe trial while the CCI mean was also lower using the updated protocol. During the week, CCI animals again showed high variability and no notable differences while the controls groups are significantly different on day 2 as seen in Fig. 3.3B. When looking at the differences between total errors in Fig. 3.3C, no observable changes regarding the probe trials, however the mean value of total errors between the protocols in the CCI group is lower in the updated protocol on days 4 and 5 than in the traditional protocol. This also is shown in the control groups where the total errors are significantly different on days 4 and 5 with the updated protocol seeing decreased errors.

Alternatively, returns to goal showed no significant differences during the week in both the control and CCI group comparisons, however the probe trial control comparison showed significant differences between the traditional and updated protocols (Fig 3.3D). The CCI comparison, while not statistically significant, was trending toward better performance in returns to goal during probe trial for the updated protocol when compared to the traditional protocol.

With significant differences were seen between the traditional BM protocol when compared to the updated protocol, we wanted to identify whether these differences were based off increased learning due to additional trials or an increased learning within the same number of trials. When comparing trial-by-trial rather than day-by-day, both protocols seemed to follow the same trend regardless of treatment. The high variability

within each trial made it difficult to notice any major differences; however, some noteworthy comparisons were seen in the total latency (Fig. 3.4A), primary errors (Fig. 3.4C), and returns to goal (Fig 3.4E). While total latency for the CCI comparison was relatively the same except for an initial better start for the traditional protocol, it seems that the tenth trial between the controls of each protocol showed a lower mean latency for the traditional protocol. However, after further trials, the updated protocol surpasses the best total latency the traditional protocol was able to obtain. When looking at primary errors, the traditional protocol control means had much less of a continuous decrease when compared to the updated protocol control. Returns to goal also had some notable control comparisons with the traditional protocol means being much lower than the updated protocol means in trials 8, 9, and 10.

SECTION 3.3 DISCUSSION

Firstly, with little differences shown and no significant differences in the trial comparisons, there is reasonable evidence that the updated protocol provided no difference to increasing learning and memory in the BM on a trial-by-trial basis. This ultimately leads to the conclusion that the differences seen in Fig 3.1 and Fig. 3.2 are based upon the increased volume of trials per day and the addition of a 6^{th} day. The addition of this day increased learning dramatically and allowed for a wider gap for measuring deficits between the control and CCI groups both during the week and in the probe trials. A major focus of this section has been the protocol differences. While the data showed a growing gap between the control groups of each protocol, the CCI groups stayed relatively similar in much of the data. This indicates an increased separation between the control and CCI groups within the updated protocol. Much of the CCI data

shows similarities between the updated protocol and traditional protocol with most of the major differences occurring in escape frequency. When looking at the escape frequency data of the traditional protocol, the data showed that both the control and CCI groups started with higher percentages of escape and total latencies and ultimately bottomed out on day 3, apart from the CCI group which showed its lowest values on day 5. The lack of escapes on day 5 for the CCI group compared to the nearly 30% escape on day 5 for the updated protocol itself exemplifies the increased ability of the updated protocol on spatial learning. Indeed, the whole of the escape frequency data gives strength to the validity of the updated BM protocol between the steadily increasing escape frequencies for both the control and CCI groups, larger gap in final day escape percentage, and four days of significant differences. While the training days offer a great insight into how the two different protocols compare, the ultimate test is in the probe trials. According to O'Leary and Brown, primary errors and primary latency are critical data [35]. Due to this importance, the significant differences in primary errors and primary latency between the traditional and updated protocol controls is essential in confirming the validity of the updated protocol. An additional benefit is the lower means in both primary latency and primary errors of the CCI group comparison, adding more evidence that even the injured animals have showed increased learning. More support for this new protocol is shown in the significantly different total errors between each protocol controls during the acquisition week and the significant difference between the returns to goal probe trial between the controls in each protocol.

SECTION 3.4: CONCLUSIONS

The increased learning visible in the various provided data for both the control and CCI groups in the updated protocol provides strength to the argument that this novel updated BM protocol is capable of more precisely measuring TBI-based deficits than the traditional BM protocol. While there is no difference on a trial-by-trial basis, the increased number of trials over a similarly long period of time with additional day of training led to stronger spatial learning and memory. While removing the reversal week removes testing for cognitive flexibility, in the realm of TBI work short-term and long-term memory are a more useful metric for preclinical testing as the focus on memory and memory consolidation shows a clearer target than separating results between two different abilities of the brain. Thus, this novel BM protocol will allow us to provide more powerful testing of TBI therapeutics.

CHAPTER 4: ASSESSING NANOPARTICLE TREATMENT USING BARNES MAZE

With a novel BM protocol capable of showing significant deficits in both the training week and probe trials between control and CCI mice, we utilized this paradigm for the testing of TBI therapeutics. For this, an antioxidant NP similar to the one used in the MWM study was applied to a group of animals that had undergone CCI surgery [29].

SECTION 4.1: METHODOLOGY

The BM protocol utilized is described above in Section 3.1.2 with all aspects remaining the same as they did for prior protocol comparison testing.

SECTION 4.1.1: NANOPARTICLE DESCRIPTION

The NP used has been previously described [29]. For use in this study, the NP treatment, labelled as LIPOMA, was injected at a concentration of 2 mg/mL and a volume of 100 μL immediately post CCI. This NP is similar in nature to the previously discussed NP1, however where NP1 was specifically an ROS scavenger, LIPOMA serves a dual purpose in both ROS scavenging and the neutralization of lipid peroxidation products (LPOx).

SECTION 4.1.2: CONTROLLED CORTICAL IMPACT

All animal procedures were performed in accordance with the approval of the University of Nebraska−Lincoln IACUC. Seven-week-old male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME, USA) were acclimated for 1 week prior to the procedures. Mice were anesthetized with 3% isoflurane gas via inhalation and were maintained at ∼1.5% with a nose cone on a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). The hair of the scalp was removed with Nair (Church and Dwight Co., Inc., Princeton, NJ, USA), and the scalp was disinfected with a betadine scrub and isopropanol wipes afterward. Lidocaine (0.05 mL of 5 mg/mL) and bupivacaine (0.05 mL of 0.3 mg/mL) were applied to the scalp, and buprenorphine SR (60 μL of 0.5 mg/mL) was given subcutaneously. An approximately 1 cm midline incision was made on the scalp over bregma. An approximately 2 mm craniectomy was made in the skull over the left frontoparietal cortex (2 mm anterior and 2 mm left of lambda) using a surgical drill. A controlled cortical impactor (Hatteras Instruments, Cary, NC, USA) attached to the stereotaxic frame with a 2 mm convex tip was used to impact the brain normal to the dura surface at a depth of 2.5 mm and a velocity of 4 m/s with a dwell time of 80 ms. Any

bleeding was controlled and incisions were closed using tissue adhesive. The size of each treatment group is as follows: 10 mice in the control group, 7 mice in the CCI group, and 8 in the LIPOMA treated group. The control experiments were done in groups of 5 mice separated into different weeks while the CCI groups were done in tandem with LIPOMA treated mice.

SECTION 4.1.3: STATISITCAL ANALYSIS

All the data in this study were expressed as mean ± standard error of the mean (SEM). A $p < 0.05$ was considered statistically significant. Escape frequency using total latency was analyzed using Kaplan-Meier survival analysis with a Mantel-Cox log rank test. Weekly training and probe trial statistics were analyzed using two-way ANOVA or a mixed-effects model when applicable. All data analyses were done using GraphPad Prism 9 (GraphPad Software, CA).

SECTION 4.2: RESULTS

Figure 4.1A shows the distance travelled in centimeters between all groups of throughout the training week. While significant differences were noticed between the control group and both the LIPOMA and CCI groups across the week, the greater distance travelled is on the part of the CCI and LIPOMA groups. Concern could be taken for motor coordination deficits if the opposite had been true as a decrease distance travelled in tandem with decreased velocity could mean that injured animals experienced long-term motor impairment and are thus compensating by travelling less distance.

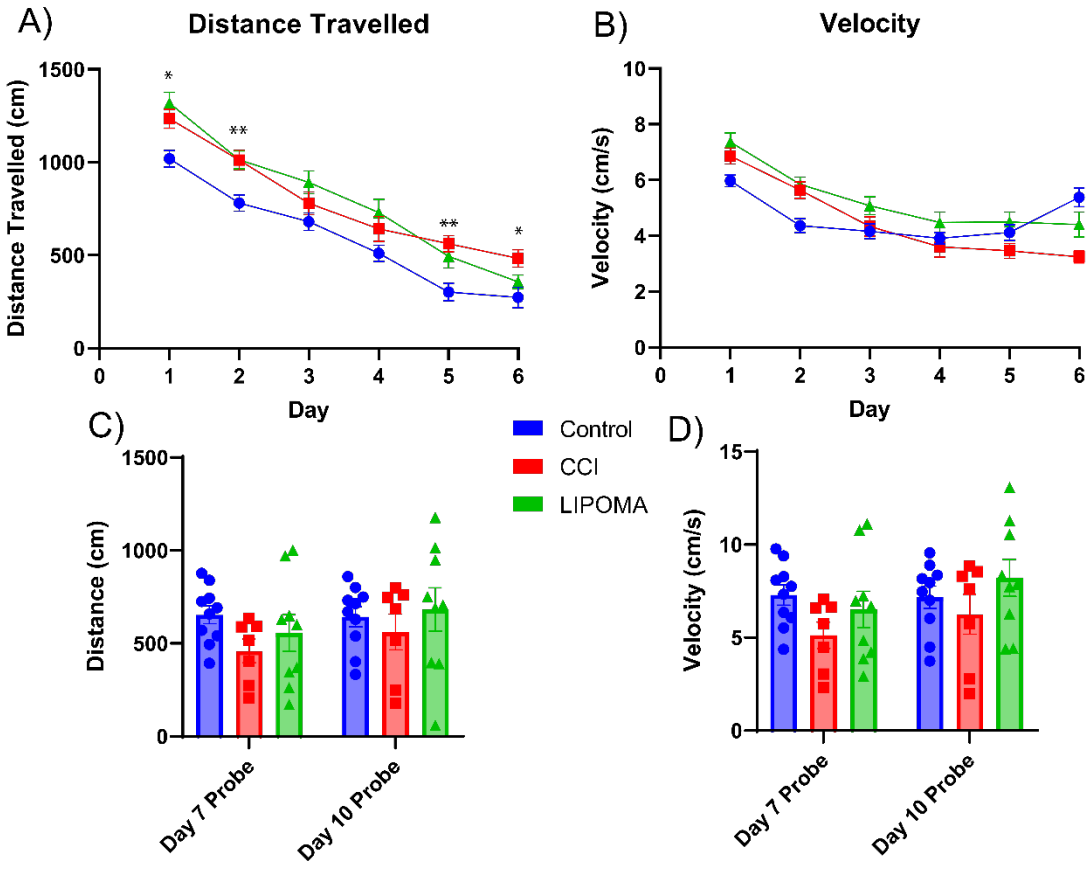

Figure 4.1 A) Training week distance travelled in centimeters B) Training week velocity in centimeters per second C) Probe trial distance travelled in centimeters D) Probe trial velocity in centimeters per second. * = $p < 0.05$, ** = $p < 0.01$ for control vs CCI comparisons

Additionally, to confirm the lack of motor coordination deficits, we analyzed both distance travelled and overall velocity among the groups. Fig. 4.1B confirms that velocity was not significantly different between all groups. Both distance travelled and velocity showed no significant differences during the probe trial days as well; therefore, motor coordination deficits at any point during the behavioral testing can be ruled out for any other deficits we observed.

In Figure 4.2A, days 3 through 6 show statistically significant differences between the control and CCI groups with day 3 also showing significant differences

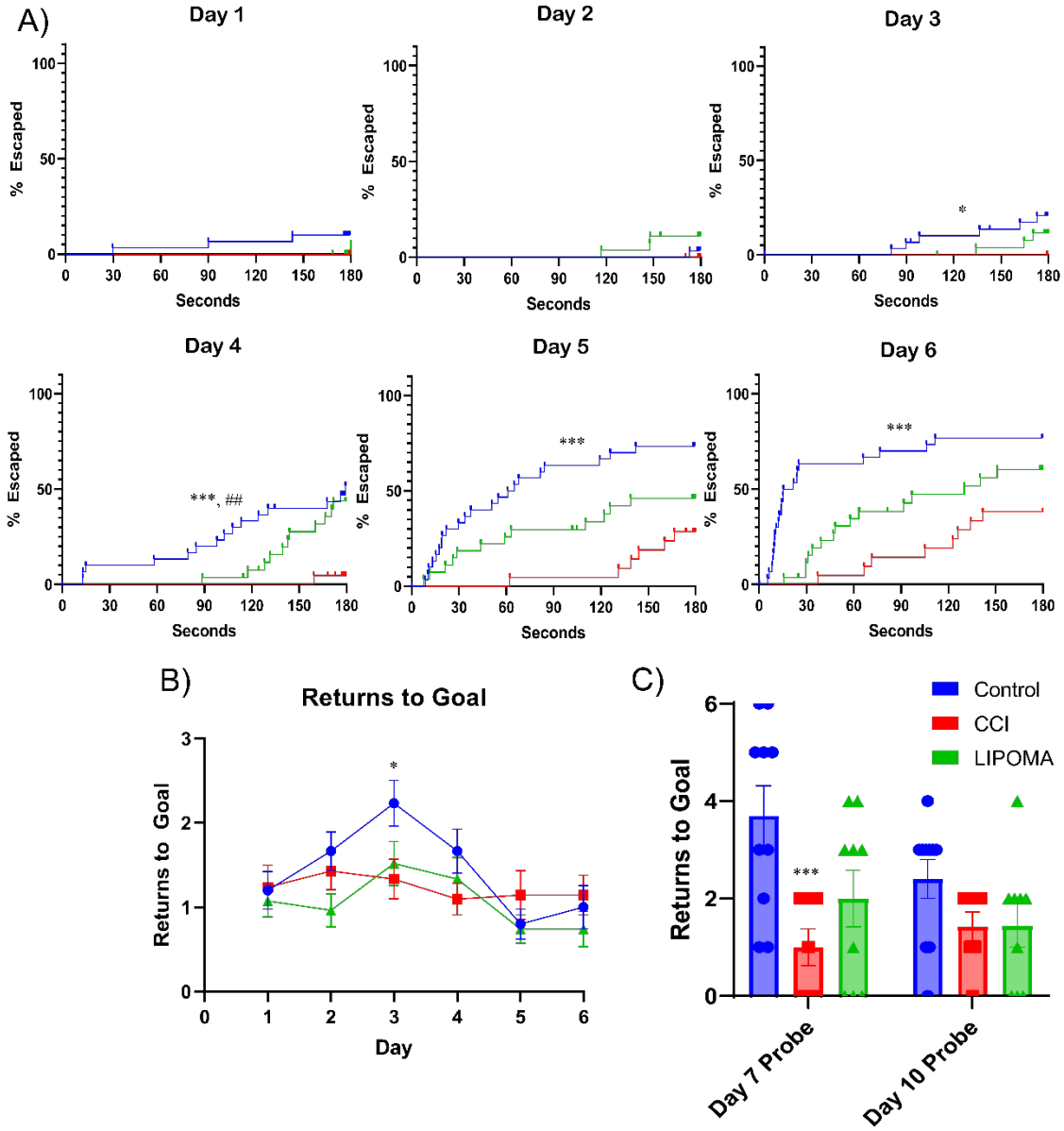

Figure 4.2 A) Escape percentages for the 6 training days. B) Returns to goal across the training week. C) Returns to goal in the probe trials. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ for control vs CCI comparisons, # = $p < 0.05$, ## = $p < 0.01$, ### = $p < 0.001$ for LIPOMA vs CCI comparisons.

between the LIPOMA and CCI groups. Going further into these results, peak escape frequencies on day 1 occur at 10% and 7.7% at 143 seconds and 180 seconds for the control and LIPOMA groups, respectively. On day 2, the control group peaks at 3.333% before 172.9 seconds while the LIPOMA group peaks at 11.111% before 147.7 seconds. Day 3 again showed results for the control and LIPOMA groups with peak escape percentages of 20.8% and 11.5% at 172.8 seconds and 170.3 seconds, respectively. On the 4th day, the CCI group had its first escapes with a 4.76% escape percentage at 159.326 seconds. The control and LIPOMA groups had peak escape percentages of 47.1% at 176.4 seconds and 43.7% at 171.8 seconds, respectively. Day 5 resulted in higher escape percentages across the board with 73.333% of control animals escaping at or before 142 seconds, 28.571% of CCI animals escaping at or before 166.6 seconds, and 46.187% of LIPOMA animals escaping at or before 138.87 seconds. Finally, on day 6, the escape percentages of 76.667%, 38.095%, and 60.381% for the control, CCI, and LIPOMA groups at the latencies of 111.6 seconds, 141.8 seconds, and 150.9 seconds, respectively. While examining the returns to goal, day 3 shows a significant difference between the control group at a mean of 2.233 returns and the CCI group at a mean of 1.333 returns. LIPOMA mice had a mean of 1.519, however this was not statistically significant when compared to either the control group or the CCI group. With the probe trial, while the day 10, or long-term, probe trial did not elicit any statistically significant results, the day 7, or short-term, probe showed significant differences between the control

group with a mean of 3.7 returns and both the CCI and LIPOMA groups with a mean of 1 return and 2 returns, respectively.

Primary latency is shown in Fig. 4.3A. Statistically significant differences were noticed between the control and CCI groups on days 4, 5, and 6. Additionally, as shown in Fig. 4.3B, there are statistically significant differences between the control and CCI groups on days 2, 3, 5, and 6. Looking at the probe trial data, no significant differences

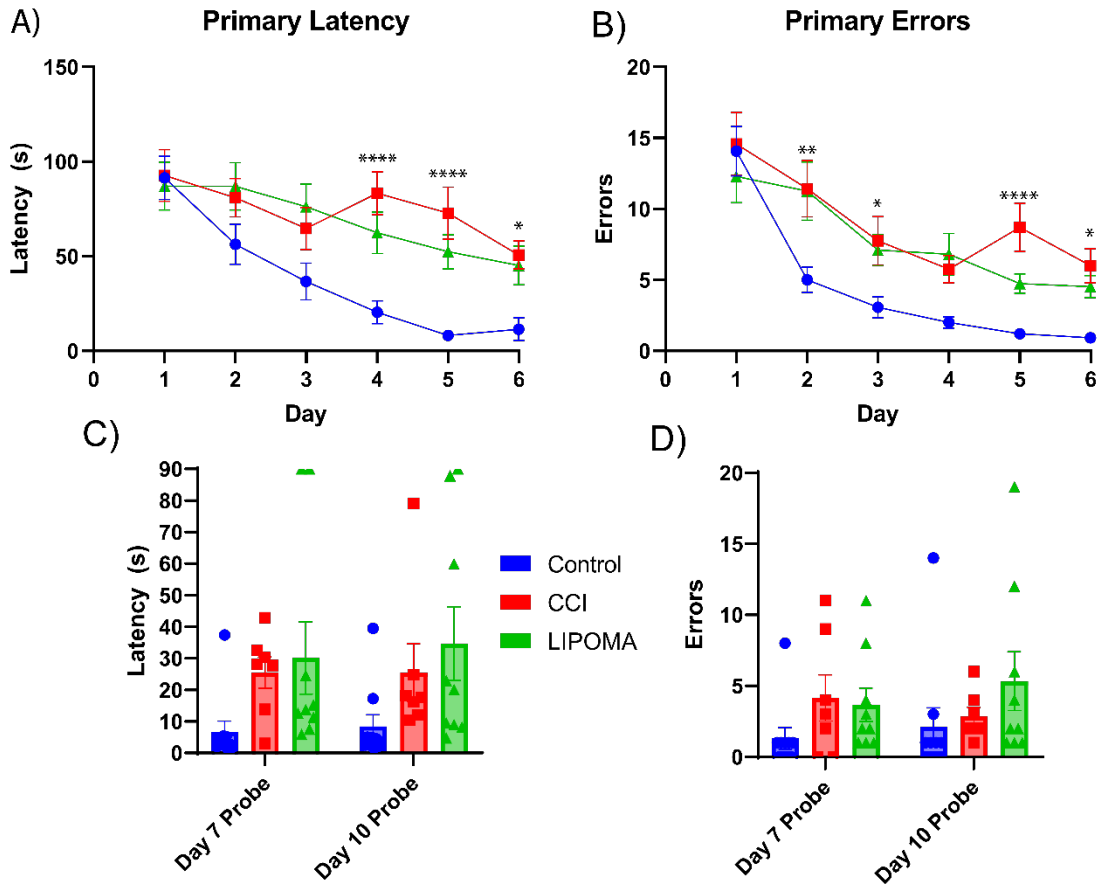

Figure 4.3 A) Training week primary latency in seconds B) Training week primary errors C) Probe trial primary latency in seconds D) Probe trial velocity in centimeters per second. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ for control vs CCI comparisons

are seen in both the primary latency and primary errors between any group. However, the mean control probe trial primary latency for day 7 is 6.633 seconds and for day 10 is 8.405 seconds while the same primary latencies for the same days for the CCI group are 25.48 seconds for each day and for LIPOMA are 30.09 seconds and 34.67 seconds, respectively. Similarly, primary errors also have large differences in the same vein as primary latencies. Day 7 showed 1.3, 4.143, and 3.667 errors for the control, CCI, and

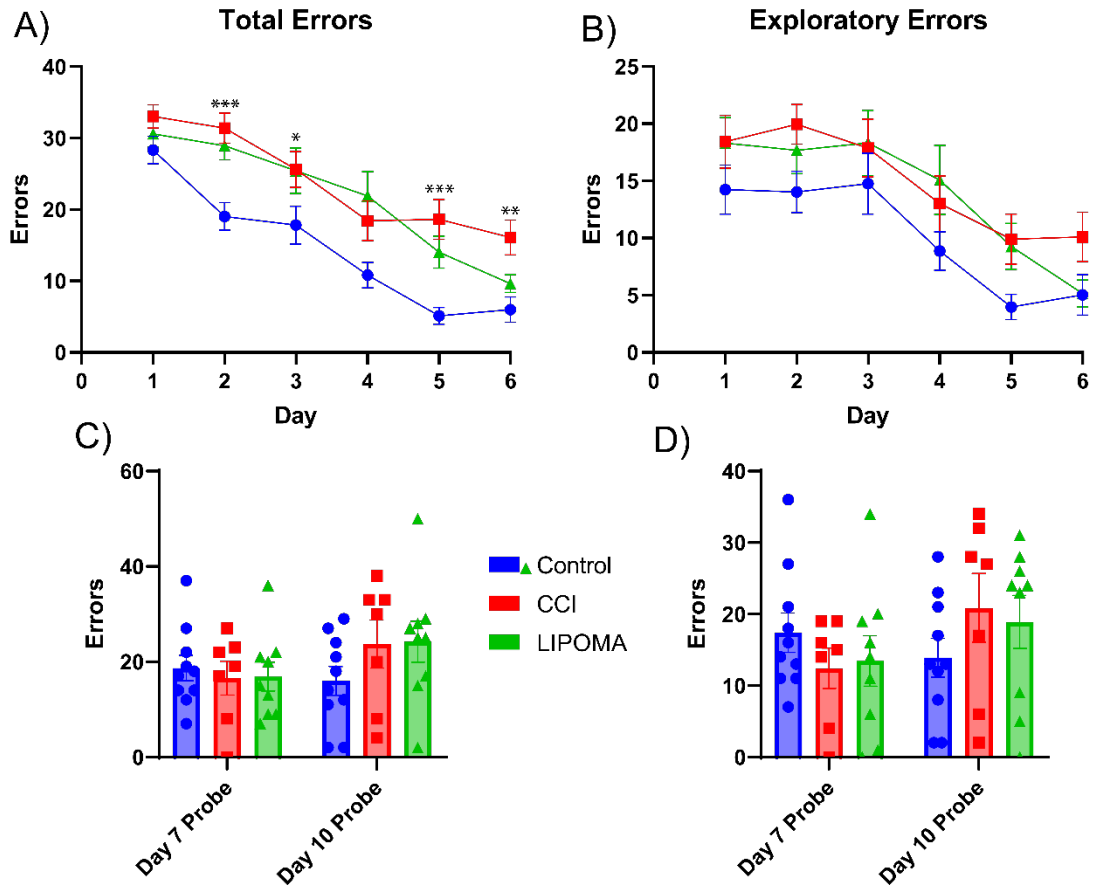

Figure 4.4 A) Training week total errors B) Training week exploratory errors C) Probe trial total errors D) Probe trial exploratory errors. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ for control vs CCI comparisons

LIPOMA groups, respectively, while day 10 showed 2.1, 2.857, and 5.333 errors for the control, CCI, and LIPOMA groups.

Figure 4.4 outlines total errors and exploratory errors to assess the amount of total exploration and exploration after the first visit to the escape. We define exploratory errors as the difference between total errors and primary errors. This metric is mainly used to measure the number of errors made after the first visit to the escape, and thus the number of errors caused purely from exploration and not from active searching. While there were no significant differences in exploratory errors, on day 6, both control and LIPOMA animals had a mean of approximately 5 errors compared to the mean of 10 for the CCI animals. For total errors, significant differences were noticed between the CCI and control groups on days 2, 3, 5, and 6. While this should bode well for the probe trials, no significant differences were noticed in either probe trial for total errors. The result of the exploratory errors probe is much the same as there are no significant differences, however the means of the control and CCI group on the day 10 probe trial (13.9 and 20.86, respectively) show some separation.

SECTION 4.3: DISCUSSION

While the total latency, escape percentages, and returns to goal probe show some benefit to spatial learning and memory due to LIPOMA treatment, the high primary latency and number of primary errors more closely follow those of the untreated CCI group. These data suggest that LIPOMA has a neuroprotective effect; however, not a large one. An accurate description of the level of neuroprotection LIPOMA was able of achieving is highlighted in the probe trial returns to goal. While a significant difference

was found in the short-term between CCI and control, LIPOMA also outperforms the CCI group. In the long-term returns to goal probe, the control group outperformed both the CCI and LIPOMA groups with the latter two groups performing similarly. However, both the short-term CCI and LIPOMA difference and all long-term differences are not significant, likely due to the high variability within both injured groups. One major source of variability and something that is to be addressed in future work is the low number of animals in each group. While the 10 for the controls was enough to see differences in protocol comparisons, it is entirely possible that smaller differences, such as those between CCI and LIPOMA treated animals, would be missed with only 7 and 9 animals, respectively. Additionally, impact depth in the CCI surgeries could be a factor as well as the moderate-to-severe depth of 2.5 mm could lead to the full destruction of the left hippocampus, an injury of which only the most substantial recovery would be noticeable. While additional testing is needed to determine the extent of the benefit, these results seem to suggest increased neuroprotection from intervention immediately after injury. Indeed, the differences shown in escape frequency are enough to warrant further investigation into not only LIPOMA, but other neuroprotective NPs as well.

CHAPTER 5: CONCLUSIONS

In this work, a MWM study was used as a benchmark to establish the base for another spatial learning and memory paradigm capable of testing TBI. Two BM protocols were established as potential replacement tests, allowing to control for the endogenous anxiety which may have been a confounding factor in measuring both sex differences and ensuring a large enough gap between control and injured animals that the effects of a NP

therapeutic could be accurately and measured. We developed and optimized a BM protocol based on a traditional protocol that has been highly characterized and widely used. Our work utilizing the traditional protocol did not provide ample separation and learning expected from what has been seen in various TBI literature and what was being seen in the molecular and histological correlates from colleagues within the lab. Due to these shortcomings, a novel, shortened BM protocol was established pulling from various literature sources discussing the importance of increased trial number and a long-term memory probe was added to determine if differences might occur there as well. While the protocol has shown a mild neuroprotective effect with LIPOMA, the success of the BM in this testing opens the ability for testing of other antioxidant NP therapeutics.

SECTION 5.1: SHORTCOMINGS

Behavior is highly variable and thus must be highly controlled to maintain consistency. As seen in the trial results shown in Chapter 3, the variability between subjects can be very high in injured animals resulting in a low statistical power. The most common method of increasing power, increasing the number of animals tested in each group, is a potential way of dealing with this issue; however, the length and speed at which behavioral testing can be done is intrinsically slow. Another shortcoming of the updated BM protocol is the removal of the reversal week and therefore the potential to test for cognitive flexibility. While this is a shortcoming, it is less important in the current research as our focus is purely on learning and memory for initial testing as it is more important to establish a significant treatment effect before exploring other effects of NP treatment. Another possible shortcoming of this work could be in the high variability in CCI mice. This variability may be inherent due to injury and more testing is required to

determine the extent of this variability and how much it can be reduced. Indeed, while control mice have continuously smaller variability throughout the week, CCI mice seem to have a relatively consistently high variability with, arguably, a slightly lower variability by the end of the week due to the innate variability in individual reactions to TBI and an inability to consolidate memory as efficiently as healthy mice.

SECTION 5.2: FUTURE WORK

Future work following the optimization of the protocol lies in four main areas and several less directly important ones. Firstly, increasing the number of animals in each group, especially the CCI and LIPOMA groups, can help to decrease variability and establish outliers more consistently while inherently bringing down variability within the injured groups. For example, from the returns to goal of this current data, we would need 16 mice in each group to notice differences between the CCI and LIPOMA groups at a power of 80%. Alternatively, if we find a NP with stronger neuroprotective effects, NP mice would need to average a latency of 6 seconds with the current amount of variability and 17 mice in each group to see significant differences in primary latency with 80% power. While this seems low, the current LIPOMA standard deviation is 26 seconds. When compared to the control group at 10.3 seconds and the CCI group at 12.1 seconds, LIPOMA variability is extremely high. If we assumed a similar variability to CCI, approximately 13 seconds, NP treated mice would need to have an average primary latency of 13.2 seconds, twice that of the control mice, with 14 mice in each group to see significant differences against the untreated group at 80% power. All of these power analyses were done using the short-term memory probe-trial. This highlights a further need to understand the sources of increased variability of LIPOMA mice to determine if

this is the result of nonperformers or variability inherent to different reactions to LIPOMA. Second, expanding research into female mice can help to gain a further perspective into a quickly growing realm of preclinical research aimed at increasing clinical translation by introducing biological heterogeneity more similar to a clinical population while still keeping many of the variables under control. This will also allow us to identify any possible biological sex-based differences in antioxidant NP treatment effects that exist. Third, lowering the injury severity may help in better assessing therapeutic outcome as the immense damage caused by the tested injury depth may not be able to translate into behavioral changes in the ways we would hope and expect from other forms of NP efficacy testing. Finally, determining a quick and efficient method of imaging to determine approximate injury depth is another primary focus to measure injury variability and potentially rule out any injuries that fall outside the expected depth. While this variability should be very small given the reproducibility of CCI, it is an especially important to be precise and careful in behavioral testing with an already high amount in innate variability.

Briefly, some additional areas of research important to the field lie in several realms. Testing the effect of the therapeutic window on behavior seems to be an important step forward after the main three areas of future work can be addressed. Additionally, the development of a behavioral battery measuring deficits from the acute to subacute to chronic phases of injury could be a beneficial method of categorizing animals better to increase the ability of all behavioral tests in the battery to determine deficits and assess how neuroprotective therapeutics may cause varying changes to behavior dependent on the injury phase. Finding molecular correlates to spatial memory

consolidation may also be a promising area of study as recent work has pointed to potential sex-dependent differences in fear memory consolidation [74]. Understanding those mechanisms may also open opportunities for therapeutic advancement that we have not yet explored.

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TA BLE 1. Key data generated from behavioral paradigms expected changes following TBI. Adapted from [1].

Behavioral Task	Data Type	Description	Expected Result (Compared to Control Group)	Meaning of Result
Spatial Learning and Memory				
MWM	Latency to Platform (s)	The amount of time it takes an animal to escape the maze.	TBI should take longer	Decreased latency shows a higher amount of spatial learning.
	Percent in Quadrant (% or fraction)	The percentage of time spent in a specific quadrant over the total time in maze.	TBI should spend less time near the escape and more time in quadrants away from the escape	High percentages in the quadrant of the platform show higher learning; however, high percentages in the reversal week in the former escape quadrant show an inability to relearn.
	Percent of Time in the Outer Annulus (% or fraction)	The percentage of time spent in the outer annulus of the	TBI should spend more time in the outer annulus	Higher percentages in the outer annulus show thigmotaxis, which shows no learning or confusion.

Behavioral Task	Data Type	Description	Expected Result (Compared to Control Group)	Meaning of Result
Spatial Learning and Memory				
MWM	Latency Path Length (cm)	The length of the path made while moving through the maze.	TBI should have a large path length	Higher path length shows more movement and a lower understanding of how to escape the maze and thus, less ability to learn and memorize the maze.
	Cumulative Distance from the Platform (cm or m)	The distance, measured every few seconds or milliseconds, from the platform.	TBI should have a larger cumulative distance	Longer distances show a lack of spatial or non-spatial search strategies, which indicate worse learning or memory.
	First Bearing (Degrees or radians)	The angle between the first movement of the animal and a direct line to the platform.	TBI should have a larger degree of first bearing	Higher degree of first bearing shows a deficit in memory of where the platform lies spatially.
	Search Strategy	The strategy (i.e., spatial, nonspatial, or random) the animal uses to find the platform.	TBI should use more random or nonspatial strategies	Higher use of random search strategies indicates lower learning and memory while the inverse of higher spatial strategies shows an increase in learning and memory.

B

Behavioral Task	Data Type	Description	Expected Result (Compared to Control Group)	Meaning of Result
Spatial Learning and Memory				
MWM	Probe Trial Time in Target Quadrant (% or fraction)	The time spent in the quadrant where the platform should be as a percentage of total time.	TBI should spend less time in the target quadrant	Higher percentage of time in the target quadrant shows an increased ability in learning and memory of the maze.
	Probe Trial Platform Crossings (Frequency)	The number of times the area where the platform should be is passed over.	TBI should pass over less	Higher frequency of platform crossing shows better learning and memory.
	Swim Speed (m/s)	The velocity at which animals are travelling in the maze	TBI should be relatively similar to rule out motor deficits; however, this is specific to post-acute phase testing	Lower swim speed shows either a motor coordination deficit, or, potentially but unlikely, a lower ability to learn and remember the maze. These should, in most circumstances, be very similar.
BM	Primary Latency (s)	The amount of time it takes an animal to find the escape and enter (head only).	TBI should take longer	Lower primary latencies show a better understanding of the escape and how to reach it via nonspatial navigation or spatial navigation, depending on search strategy.

C

Behavioral Task	Data Type	Description	Expected Result (Compared to Control Group)	Meaning of Result
Spatial Learning and Memory				
BM	Total Latency (s)	The amount of time it takes an animal to find and fully enter the escape hole	TBI should take longer	Lower total latency shows learning and memory into which method will provide escape the quickest.
	Reference Errors (Frequency)	The number of times an animal enters a non-escape hole with its head.	TBI should have more errors	Higher reference errors show a decreased ability to learn and memorize the maze.
	Working Errors (Frequency)	The number of times an animal makes a reference error after having visited that hole before.	TBI should have more errors	Higher working errors show a decreased understanding of the maze along with potential confusion regarding visited areas, showing a lack of memory.
	Persevera-tive Errors (Frequency)	The number of times an animal repeats searching the same hole before moving on	TBI should have more errors	Higher perseverative errors show a lack of learning and memory of places previously visited and may, in reversal trials, indicate
	Primary Errors (Frequency)	The number of times an animal enters a non-escape hole with its head before finding the escape hole.	TBI should have more errors	Higher primary errors indicate deficits in learning and memory of the maze.

Behavioral Task	Data Type	Description	Expected Result (Compared to Control Group)	Meaning of Result
Spatial Learning and Memory				
BM	Total Errors (Frequency)	The number of times an animal enters a non-escape hole with its head before entering the escape hole with its whole body.	TBI should have more errors	Higher total errors indicate deficits in learning and memory of escape of the maze, or, when combined with low primary latency, more curiosity from the animals, indicating comfort in the maze.
	Hole Deviation Score	The number of non-escape hole visits between the first visited hole and the escape.	TBI should have a higher score	Higher hole deviation scores show a lack of learning and memory when related to finding the correct path in the maze. Spatial learning will show lower scores than nonspatial learning.
	Primary Path Length (cm)	The distance an animal has travelled before reaching the escape hole with only its head.	TBI should have a longer distance	Path length, in either context, shows a decreased ability to understand and memorize the maze

Behavioral Task	Data Type	Description	Expected Result (Compared to Control Group)	Meaning of Result
Spatial Learning and Memory				
BM	Total Path Length (cm)	The distance an animal has travelled before entering the escape hole with its whole body.	TBI should have a longer distance	Path length, in either context, shows a decreased ability to understand and memorize the maze.
	Search Strategy	The strategy (i.e., direct/spatial, serial, or mixed/random) the animal uses to find the escape hole.	TBI should use more mixed/random strategies and fewer direct/spatial strategies	Higher use of mixed/random search strategies show a decreased ability to learn the maze; however, an increase in serial strategies after a large number of spatial strategies show complacency within the maze
	Velocity (cm/s)	The change in distance over time at which animals are travelling in the maze.	TBI should be similar during chronic phase, acute phase measurements may be lower for TBI	Lower velocity can indicate motor coordination issues within the maze. These should stay relatively similar throughout both weeks of trials.

Behavioral Task	Data Type	Description	Expected Result (Compared to Control Group)	Meaning of Result
Spatial Learning and Memory				
RAM	Errors (Frequency	For delayed test, the number of entries into non-baited arms. For the non-delayed, re-entries into the arms entered previously that trial.	TBI should have more errors	Higher frequency of errors shows a lack of memory.
	Across-Phase Error (Frequency)	Entry to an arm previously entered during the training phase (delayed test only).	TBI should have more errors	Higher frequency shows a poor ability to learn from the training phase and thus a worse long-term memory
	Within-Phase Error (Frequency)	Entry into an arm entered within the test phase (delayed test only).	TBI should have more errors	Higher frequency shows a poor ability to remember what has been visiting, showing a worse short-term memory
	Baited Arm Re-entry (Frequency)	A second entry into an arm that had been baited at the beginning of the trial but was already discovered (non-delayed test only).	TBI should have more errors	Higher re-entries of this type show a lack of learning
	Non-baited Arm Re-entry (Frequency)	The time it takes for the animal to first visit a baited or non-baited food cup.	TBI should have more errors	Higher re-entries of this type show a lack of memory.

Behavioral Task	Data Type	Description	Expected Result (Compared to Control Group)	Meaning of Result
Spatial Learning and Memory				
RAM	First Latency (s)	The time it takes for the animal to first visit a baited or non-baited food cup.	TBI should take longer	Higher first latency shows a hesitancy to explore the maze and potential deficits in memory or learning. This may also indicate a nonperformer.
	Total Latency (s)	The time it takes for the animal to retrieve all food pellets.	TBI should take longer	Higher total latency shows a lack of learning and memory.
T and Y Maze	Time Spent in Novel Arm (% or fraction)	The amount of time the animal spends in the opened arm during the second trial (alternating T/Y maze only).	TBI should spend about equal time exploring both arms	A lower percentage of time spent in the novel arm shows memory deficits.
	Forced Alternation (% or fraction)	The percentage of animals that enter the novel arm first during the second trial (alternating T/Y maze only).	TBI should enter the novel arm less	A lower percentage of forced alternation shows a lack of learning.
	Place Versus Response Learning	When the entrance arm direction is switched, the animal will use spatial learning and turn toward goal or nonspatial learning and turn the direction turned during training.	TBI should more often use nonspatial learning and turn in the direction it did during training	This shows the difference between place learning (spatial learning) and response learning (nonspatial learning).

Behavioral Task	Data Type	Description	Expected Result (Compared to Control Group)	Meaning of Result
Spatial Learning and Memory				
Novel Object Location	Percent of Total Investigation Time (% or fraction)	The time spent exploring the novel location divided by the total time spent exploring either object.	TBI should spend about 50% of the time or less exploring the novel location	A lower percentage of novel investigation shows an inability to remember the familiar object.
	Discrimination Index	The time spent exploring the novel location minus time spent exploring the familiar location divided by total time exploring either object.	TBI should be closer to zero; positive values show more time investigating the novel location	A higher discrimination index shows a preference to explore the novel object rather than the familiar object
Nonspatial Learning and Memory				
Novel Object Recogni-tion	Percent of Total Investigation Time (% or fraction)	The time spent exploring the novel object divided by the total time spent in the exploring either object.	TBI should spend about 50% of the time or less exploring the novel object	A lower percentage of novel investigation shows an inability to remember the familiar object.
	Discrimination Index	The time spent exploring the novel object minus time spent exploring the familiar object divided by total time exploring either object.	TBI should be closer to zero; positive values show more time investigating the novel object	A higher discrimination index shows a preference to explore the novel object rather than the familiar object

Behavioral Task	Data Type	Description	Expected Result (Compared to Control Group)	Meaning of Result
Nonspatial Learning and Memory				
Nonspatial Variants of Spatial	Same data as described above	Nonspatial variants simply take away spatial cues for each task.	Refer to above corresponding expectation for spatial tasks.	
Emotional				
Forced Swim Test	Time Spent Immobile (s)	The time spent not attempting to climb, move, or leave the swimming column.	TBI should spend a longer time immobile; however, depression-like activity is still controversial	A longer time spent immobile shows a larger number of depressive-like symptoms.
Dark/Light Avoidance Test	Time Spent in Either Zone	The time spent in either the light or dark zones. These will amount to complimentary measurements.	TBI should spend more time in the dark zone	Longer time spent in the dark zone shows a higher level of anxiety-like behaviors, while a longer time in the light zone shows the inverse.
	Distance Travelled in Each Zone (cm)	The distance travelled while in either the dark or light zone. This will also contain two separate data points for light and dark zones.	TBI should travel a greater distance in the dark zone	Higher distance travelled in the dark zone shows a higher level of anxiety-like behaviors, while a higher distance travelled in the light zone shows the inverse.

Behavioral Task	Data Type	Description	Expected Result (Compared to	Meaning of Result
Emotional				
Dark/Light Avoidance Test	Latency to Light Zone (s)	The amount of time it takes an animal to first explore the light zone.	TBI should take longer to explore the light zone	A greater latency to the light zone shows an increased amount of anxiety-like behavior.
	Number of Entries into the Light Zone (Frequency)	The number of times an animal enters and renters the light zone.	TBI should have fewer entries into the light zone	A lower number of entries into the light zone shows an increased amount of anxiety-like behavior.
Open Field Test	Time Spent in the Outer Zone (s or %/fraction)	The amount of time the animal stays on the outside of the open field, measured either as seconds or as a percentage or fraction of total time spent in the open field.	TBI should spend more time in the outer zone	A longer time spent in the outer zone infers an increased anxiety-like response to the open field.
	Time Spent in the Central Zone (s or %/fraction)	The amount of time the animal spends in the center of the open field, measured either as seconds or as a percentage or fraction of total time spent in the open field.	TBI should spend less time in the center zone	A higher amount of time spent in the central zone shows a decrease in anxiety-like responses.

Behavioral Task	Data Type	Description	Expected Result (Compared to Control Group)	Meaning of Result
Emotional				
Open Field Test	Total Distance Travelled (cm)	The distance the animal travels through the entire trial regardless of zone.	Differences could be from locomotor issues or a greater stress response from a change in general activity. It is important researchers take notice when using this measurement.	Total distance travelled should, normally, be relatively similar. However, a greater total distance travelled along with a significantly larger time spent in the outer zone may show increased anxiety-like behaviors. Additionally, decreased total distance travelled along with a significantly greater percentage of time spent in the center may show a decrease in anxiety-like behaviors.
Resident Intruder Test	Attack Latency (s)	The amount of time between introduction and the first clinch attack for either animal.	TBI should attack earlier and usually first	Lower attack latencies show a higher aggression if the animal attacking is the resident animal.
	Total Offense Score	The sum of lateral threat, upright standing, clinch attacking, keeping down the intruder, and chasing.	TBI animals should have higher total offense scores	A higher total offense score shows a higher level of aggression.
	Social Exploration Score	The sum of social exploration, genital sniffing, and social grooming.	TBI animals should have lower social exploration scores	A higher social exploration score shows a lower level of anxiety.

Behavioral Task	Data Type	Description	Expected Result (Compared to Control Group)	Meaning of Result
Motor Coordination				
Open Field Test	Total Distance Travelled (cm)	The distance the animal travels through the entire trial regardless of zone.	TBI animals should have less distance travelled. This is mainly true for the acute phase of injury.	Lower distance travelled can mean worse motor coordination. See above for the relation between total distance travelled and anxiety-like behaviors. Time after injury is an important parameter when interpreting these results.
Rotarod	Latency to Fall (s)	The amount of time it takes an animal to fall off of the rotating rod.	TBI animals should perform worse during the acute phase	
Footprint Assay	Step Length (mm)	The distance between steps of the same paw.	Dependent on time; TBI animals should show differences during acute and subacute phases	A shorter step length in the acute and subacute phases shows poor motor coordination.
	Step Duration (ms)	The length of time one step takes.	Dependent on time; TBI animals should show differences during acute and subacute phases	A shorter step duration in the acute and subacute phases shows poor motor coordination.

Behavioral Task	Data Type	Description	Expected Result (Compared to Control Group)	Meaning of Result
Motor Coordination				
Footprint Assay	Inter-Leg Coordination	The coordination to keep both legs on each respective side within a straight line. This datum is quantitative.	Dependent on time; TBI animals should show differences during acute and subacute phases	A worse outcome of inter-leg coordination in the acute and subacute phases shows poor motor coordination