George Mason University

Protocol No: 0488

IACUC APPLICATION
FOR ANIMAL RESEARCH AND TRAINING PURPOSE

SECTION I

Please complete this cover page AND provide the Protocol information requested on this form. Please submit this form and all supporting documents through IRBNet. If you have any questions, please feel free to contact the Office of Research Integrity, & Assurance at 703-993-6118.

1. SUBMISSION REQUIREMENTS

One (1) copy of the entire IACUC Application (Sections I-III) and one (1) copy of any related grant application are required in order to process the proposal for committee review. Insufficient information may result in delay of the review process. The IACUC reserves the right to request additional information.

2. APPLICATION DATA

TITLE OF PROTOCOL: The Effects of Young Blood Plasma Treatment following Repetitive Mild Traumatic Brain Injury in Mice

STATUS: ☒ NEW SUBMISSION ☐ 3 YEAR RE-SUBMISSION

PRINCIPAL INVESTIGATOR: Jane Flinn

SECONDARY INVESTIGATOR(S) WHO WILL BE WORKING WITH ANIMALS [redacted]

STUDENTS WHO WILL BE WORKING WITH ANIMALS: [redacted]

STAFF WHO WILL BE WORKING WITH ANIMALS:

TRAINING FOR ALL PERSONNEL WORKING WITH ANIMALS ON THIS PROTOCOL:

Name	Specific procedures or duties in this protocol	Experience working with species and specific procedures as assigned in this protocol	Training Received (CITI, EH&S, specific procedures, etc.)
Jane Flinn	Principal investigator, will oversee all aspects of the project	Experience working with mouse models and all behavioral paradigms. Has numerous publications using behavioral assays in mice	CITI, EH&S, handling, breeding, ear punching, behavioral assays
[redacted]	PhD student, will perform all aspects of this study, breeding, weaning, ear punching, handling, rmTBI, tail vein injections, terminal cardiac bleeds, behavioral assays and euthanasia	~ 4 years of experience working with mice including handling, breeding, weaning, ear punching, performing behavioral assays and euthanasia, ~ 3 years of experience administering rmTBI and ~ 2 years of experience with tail vein injections and terminal cardiac bleeds	CITI, EH&S, handling, breeding, ear punching, various behavioral assays, tail vein injections, terminal cardiac bleeds manual restraint and rmTBI administration
[redacted]	PhD student, will assist with breeding, weaning, ear punching, handling, rmTBI,	Has ~4 years of experience with working with mice: handling and breeding mice, performing husbandry duties, behavioral assays and	CITI, EH&S, handling, breeding, ear punching, manual restraint, various behavioral assays,

	injections, behavioral assays and euthanasia	euthanasia, and has experiencing administering rmTBIs	rmTBI administration, euthanasia additional training through [redacted] husbandry animal care program, will be fully trained on tail vein injection prior to assisting with this study
[redacted]	PhD student, will assist with breeding, weaning, ear punching, handling, rmTBI, injections, terminal cardiac bleeds behavioral assays and euthanasia	~ 5 years of experience with mice: has experience with breeding and handling mice, has performed several behavioral assays and euthanasia.	CITI, EH&S, handling, breeding, ear punching, manual restraint, various behavioral assays, euthanasia, will be fully trained on tail vein injection, terminal cardiac bleeds and rmTBI administration prior to assisting with this study
[redacted]	PhD student, will assist with breeding, weaning, ear punching, handling, rmTBI, terminal cardiac bleeds injections, behavioral assays and euthanasia	~1 year experience with mice, has experience with breeding and handling mice, has performed several behavioral assays	CITI, EH&S, handling, breeding, ear punching, various behavioral assays, will be fully trained on tail vein injection, terminal cardiac bleeds and rmTBI administration prior to assisting with this study
[redacted]	MA student, will assist with breeding, weaning, ear punching, handling, rmTBI, terminal cardiac bleeds injections, behavioral assays and euthanasia	~1 year experience with mice, has experience with breeding and handling mice, has performed several behavioral assays	CITI, EH&S, handling, breeding, ear punching, various behavioral assays, will be fully trained on tail vein injection, terminal cardiac bleeds and rmTBI administration prior to assisting with this study
[redacted]	MA student, will assist with breeding, weaning, ear punching, handling, behavioral assays and euthanasia	~1 year experience with mice, has experience with breeding and handling mice, has performed several behavioral assays	CITI, EH&S, handling, breeding, ear punching, various behavioral assays
[redacted]	Undergraduate RA, will assist with breeding, weaning, ear punching, handling, rmTBI, terminal cardiac bleeds injections, behavioral assays and euthanasia	~1 year experience with mice, has experience with breeding and handling mice, has performed several behavioral assays, multiple terminal cardiac bleeds, tail vein injects and euthanasia	CITI, EH&S, handling, breeding, ear punching, various behavioral assays, tail vein injections, terminal cardiac bleeds and euthanasia, will be fully trained on rmTBI administration prior to assisting with this study

[redacted]	MA student, will assist with breeding, weaning, ear punching, handling, rmTBI, terminal cardiac bleeds tail vein injections, behavioral assays and euthanasia	~1 year experience with mice, has experience with breeding and handling mice, has performed several behavioral assays, multiple terminal cardiac bleeds, tail vein injects and euthanasia	CITI, EH&S, handling, breeding, ear punching, various behavioral assays, tail vein injections, terminal cardiac bleeds and euthanasia will be fully trained on rmTBI administration prior to assisting with this study
[redacted]	MA student will assist with breeding, weaning, ear punching, handling, rmTBI, terminal cardiac bleeds tail vein injections, behavioral assays and euthanasia	Less than 1 year experience Has a basic understanding of mice behavioral, physiological, and anatomical characteristics	CITI and EHS training Will be fully trained on all procedure prior to assisting with this study
[redacted]	MA student will assist with breeding, weaning, ear punching, handling, rmTBI, terminal cardiac bleeds tail vein injections, behavioral assays and euthanasia	~2 years of experience with mice Has a basic understanding of mice behavioral, physiological, and anatomical characteristics Has previously worked in an animal vivarium and was responsible for daily health checks and recognizing pain and distress in animals	CITI and EHS training Will be fully trained on all procedure prior to assisting with this study
[redacted]	PhD student will assist with breeding, weaning, ear punching, handling, rmTBI, terminal cardiac bleeds tail vein injections, behavioral assays and euthanasia	Less than one year experience Has a basic understanding of mice behavioral, physiological, and anatomical characteristics	CITI and EHS training Will be fully trained on all procedure prior to assisting with this study

DEPARTMENT/COLLEGE: Psychology

CAMPUS ADDRESS: 4400 University Dr. MSN 3f5, Fairfax, VA 22030 EMAIL: jflinn@gmu.edu PHONE: 703.993.4107

3. FUNDING SOURCE. Check the appropriate spaces and/or provide the information requested.

☐ None ☒ Departmental/Internal Funds (ORG. # 102201)

☐ Other External Funding Source: OSP/Routing Form #:

Note: For accurate maintenance of institutional records, the title of the project on the grant application must be identical to the title on the IACUC Application. Additional titles can be added by submitting a Request for Change form.

STATUS OF GRANT APPLICATION.

☐ Application submitted – Date:

☐ Application to be submitted – Date:

☒Not applicable

Unusual deadlines or considerations

4. CERTIFICATION OF PRINCIPAL INVESTIGATOR

I certify that the information provided in this project is correct and that no other procedures will be used in this protocol. I agree to conduct this research as described in this project and adhere to the USDA regulations described in the Animal Welfare Act, the PHS Policy on Humane Care and Use of Laboratory Animals, the ILAR Guide for the Care and Use of Laboratory Animals and Mason policies and SOPs. I will request and receive approval from the IACUC for changes prior to implementing these changes. I will comply with all IACUC policies and procedures in the conduct of this research. I agree to promptly report any unexplained adverse event that causes pain or distress beyond what was originally approved to the IACUC for review before continuation of the project. I will assure that animals will not be transferred between investigators without prior written approval of the IACUC. I will be responsible for ensuring that the work of my co-investigator(s)/student researcher(s) complies with this protocol. I will be responsible for ensuring that only properly trained and IACUC approved individuals will participate in this project. I understand that I am ultimately responsible for the entire conduct of this research.

SECTION II

1. CATEGORY OF RESEARCH: The investigator should check the highest appropriate category of experimentation.

☐ B **Animals will be bred, conditioned, or held for use in teaching, testing, experiments, research, or surgery but not yet used for such purposes. (For example:** Some breeding colonies, conditioning periods as well as field studies with only observation and no animal contact would qualify for Category B.)

☐ C **Animals upon which teaching, research, experiments, or tests will be conducted involving no pain, distress, or use of pain-relieving drugs.** (For example: Simple procedures such as observing feed selection preferences; injections of relatively harmless substances such as therapeutic levels of antibiotics, and blood collection from peripheral veins; physical examinations; food and water deprivation for short periods measured in hours; standard methods of euthanasia which induce rapid unconsciousness, such as anesthetic overdose; holding or weighing animals in teaching or research activities; ear punching/tagging of rodents; or tail snips in mice ≤ 21 days old.)

☒ D **Animals upon which experiments, teaching, research, surgery, or tests will be conducted involving accompanying pain or distress to the animals and for which appropriate anesthetic, analgesic, or tranquilizing drugs will be used.** (For example: Exposure of blood vessels or implantation of catheters under full anesthesia; behavioral studies on conscious animals which involve short-term stressful restraint; noxious stimuli from which escape is possible; surgical procedures under deep anesthesia that may result in only some minor post-operative discomfort or pain; and tail snips in mice > 21 days old.)

☐ E **Animals upon which teaching, experiments, research, surgery, or tests will be conducted involving accompanying pain or distress to the animals and for which the use of appropriate anesthetic, analgesic, or tranquilizing drugs are not used because this would have adverse effects on the procedures, results, or interpretation of the teaching, research, experiments, surgery, or tests.** (For example: Severe burn or trauma infliction on anaesthetized animals or without the use of analgesics following awakening from anesthesia; application of noxious stimuli (as in shock for behavioral studies), killing by nonapproved methods which may involve severe distress or pain before unconsciousness is obtained.)

In order for a protocol to **qualify** as category D, **appropriate** anesthetics/analgesics **must** be used **if** the animal will experience **more** than momentary slight pain. Momentary slight pain is defined as pain **no greater** than the **level** and **duration** of pain attending a routine injection. Alternately, the animal **must** be immediately euthanized upon evidence of such pain or the protocol classified as category E.

2. ANIMAL CHARACTERISTICS: The investigator must specify the number of animals by species and source that are required for the research project.

Species	Sex	Age/Weight	Animal Vendor/Source (e.g., breeding colony, transfer)	Total
C57BL/6J	M/F	3 weeks upon arrival, breeding begins at 5 weeks	Jackson laboratory(breeders) /in house breeding in [redacted] (experimental mice and donor mice)	39 breeders, 26 females, 13 males
C57BL/6J	M/F	Testing begins at 8 weeks	In house breeding in [redacted] (experimental mice and donor mice)	520 total, 200 experimental, 320 donors
C57BL/6J (Preliminary work breeders)	M/F	4 Weeks Upon Arrival, breeding begins between 5 and 6 weeks	Jackson laboratory(breeders) /in house breeding in [redacted] (experimental mice and donor mice)	6 Breeders, 2 males and 4 females

C57BL/6J (Preliminary work mice)	M/F	Testing begins at 8 Weeks	In house breeding in [redacted] (experimental mice and donor mice)	~30 mice any additional mice will be used as breeders
C57BL/6J (Additional plasma donor breeders)	M/F	4 Weeks Upon Arrival, breeding beings between 5 and 6 weeks	Jackson laboratory(breeders) /in house breeding in [redacted] (additional donor mice)	16 females, 8 males
C57BL/6J (Additionally plasma donor mice)	M/F	Testing begins at 8 weeks	In house breeding in [redacted] (additional donor mice)	320 additional donor mice
	...			
	...			
	...			

This experiment will use a total of 939 mice including the breeders, experimental mice, preliminary work mice, and the donor mice used to extract the blood plasma.

3. HAZARDOUS MATERIAL: If the experiments involve hazardous materials, the appropriate category should be checked and specific chemicals listed.

☐Carcinogens/Mutagens ☐Acutely Toxic Chemicals ☐DEA Controlled Substances (Schedule 1 through 5)

Please list: _____

4. BIOLOGICAL/RADIOLOGICAL MATERIAL: If the experiments involve biological or radiological material, the appropriate category should be checked. A Project Review Form (PRF) must be completed and submitted to the Laboratory Safety Office at labsafe@gmu.edu. PRFs must be approved prior to ordering or otherwise obtaining animals for this protocol. Letters of approval from safety committees should be submitted to the Office of Research Integrity & Assurance.

☐Infectious Agents ☐Recombinant or Synthetic Nucleic Acid Molecules ☐Transgenic Animals

☐Biologically-derived Toxins (i.e. tetrodotoxin, picrotoxin, conotoxin, etc.) ☐Field Study

☐Potential Infectious Material (i.e. human or nonhuman primate blood, serum, cells, tissue)

SECTION III

INSTRUCTIONS: The IACUC requests the information described in the following section pursuant to its charge by the PHS Policy on Humane Care and Use of Laboratory Animals and USDA Animal Welfare Rules (9CFR2.31). Submit all information **using the headings** listed below in **boldface** type. Address each item independently, according to the **specific** information requirements of each item, without reliance on information covered under other items. **Do not** include information which is not pertinent to the specific information requirements of the item and avoid redundancy whenever possible.

Include sufficient information to allow reviewers to judge whether the research merits the use of animals and whether the animals will be treated humanely. Do **not** copy and paste major sections of your grant proposal into the application or include excessive detail of assays not directly related to the use of animals (e.g., chemical assays, molecular biology, in vitro tests).

The IACUC Application is the official institutional record of your research and serves to document its potential value and humaneness. The IACUC Application may be subject to on-site review by the PHS, USDA and AAALAC.

All abbreviations and terms not part of common usage should be clearly defined. Remember that not all reviewers are familiar with your area of research. Consult the IACUC Guidelines during completion of this form.

1. Abstract. Provide a summary of the study in **lay terminology**. The abstract must be understandable to non-scientists.

Traumatic brain injuries (TBI) are an alteration in brain function or evidence of brain pathology that is caused by an external force or blow to the head (Menon et al., 2010) and symptoms can manifest as confusion, altered state of consciousness, seizure, coma, and behavioral and neuropsychological symptoms (Bruns & Hauser, 2003). Over 80% of the brain injuries that occur are mild, rather than moderate or severe (Bruns & Hauser, 2003). However, once one injury is sustained, the brain is more vulnerable to a second concussive impact for at least 72 hours (Laurer et al., 2001). There is a high prevalence of mild TBIs (mTBI) in athletes and military personnel, many of which may go unreported, so the risk for sustaining a second concussive impact while within the window of vulnerability is high (Langlois et al., 2006). Repetitive mild traumatic brain injuries (rmTBI) increase the risk of developing a neurodegenerative disease later in life in both humans and in animal models of rmTBI. One of the most well studied neurodegenerative diseases that is seen following rmTBI is chronic traumatic encephalopathy (CTE) with is characterized neurologically by an increase in tau tangles, neuronal loss, chronic inflammation and occasionally increases in beta-amyloid deposition (McKee et al., 2015). Those clinical features have overlapping pathologies with other neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and frontotemporal dementia (FTD), which are also at an increased risk following TBI and can be seen comorbidly with CTE. TBI and neurodegenerative diseases have similar behavioral and cognitive symptoms including learning and memory deficits and difficulty performing activities of daily living (Iverson et al., 2015). With these long-term consequences of TBI, it is therefore important to develop effect treatment methods. This study will be administering young blood plasma to mice who sustained rmTBI, as young blood plasma treatment has been shown to target several of the pathologies that are seen following TBI including reduction of tau tangles (Hernandez, 2019) and inflammation (Villeda et al., 2012 Villeda et al., 2014) as well as remediation of cognitive and behavioral deficits including spatial learning and memory (Middledorp et al., 2016). Mice will receive injections at two different time points after injury: one subset of mice will receive injections immediately following the final rmTBI and a second subset of mice will receive injection one month after the final injury. These two time points were chosen as inflammatory processes peak within one week after injury; however, chronic inflammation is still seen at least a month after injury (Taib et al., 2017; Tweedie et al., 2020; Kumar & Loane, 2012). When translating this research to humans, it is more likely that humans who sustain rmTBIs will not seek treatment immediately due to the mild nature of the injury, so it is important that this treatment be tested after a delay following the final injury.

Results from a pilot study conducted in our lab showed that there were no significant differences in behavior between wild-type mice that received blood plasma and wild-type mice that received saline. They showed normal circadian rhythm as well as normal behavior in the open field tests. Both groups showed identically normal health after injections. These results indicate that the blood plasma transfusions were done properly and there were no infections, since there were no signs of health deterioration. A study by Hernandez (2019) in our lab found that plasma from young mice injected into older mice who display tau tangles was able to reduce the presence of tau tangles in the brain and a study being completed currently in our lab by Pedemonte (2021) with tau mice shows promising preliminary data that young plasma injections improves nest building behavior and spatial memory functioning.

2. Purpose of the Study. What are the specific scientific objectives (aims) of the research? If the research has more than one objective, they should be stated separately in **numbered** sequence.

The main objectives of this study are as follows:

1. To determine the effect of young blood plasma treatments on the behavioral deficits seen following rmTBI to determine if young blood plasma can restore behavioral functioning including learning and memory deficits, abnormal circadian activity, anxiety, and impaired activities of daily living
2. To determine the effect of young blood plasma treatments on the morphological and biochemical changes seen after rmTBI after the conclusion of behavioral testing

3. To compare the efficacy of treatment in both behavioral and neurological outcomes with young blood plasma at two time points after injury, with treatment starting one day and one month after the final injury

3. Potential Value of the Study. What is the potential value of the study with respect to human or animal health, the advancement of knowledge or the good of society? Identify the information gaps the project is intended to fill. *Note: The relative value of the study is a particularly important consideration in potentially painful experiments where it is imperative that the potential benefits of the research clearly outweigh any pain, discomfort or distress experienced by the animals.*

The specific objectives of this study, as stated previously, are to determine the effect of young blood plasma treatment on animals that have sustained rmTBI to do preliminary work to determine if young blood plasma injections will be a beneficial treatment to ameliorate the behavioral and neurological deficits that are seen after rmTBI. The cost associated with impairments following TBI is high, with about 5 million people in the US alone and $9-10 billion spent on acute TBI and rehabilitation services with an additional estimated $1 billion cost due to lost wages and increased public assistance (Popescu et al). As TBIs also increase the risk of developing neurodegenerative diseases later in life, this cost is even higher. It is therefore necessary find effective treatments that can reduce pathology and decrease the risk of developing neurological diseases later in life. Current treatments of TBI mainly target one modality of injury. With its effect on several different mechanisms, including reducing inflammation, increasing synaptic plasticity, and restoring learning and memory deficits, young blood plasma injections may prove to be a beneficial multimodal treatment that can target several of the pathologies associated with TBI.

4. Duplication. Does the study or do portions of the study duplicate previous experiments? If yes, explain why the duplication is scientifically necessary. If there is no duplication of previous research, this should be stated. This section must also include a description of all the search strategies used to explore duplication (e.g., databases consulted, key words used). *Note: Research involving animals must not unnecessarily duplicate previous experiments conducted at GMU or elsewhere.*

This study is not duplicated from any previous experiments. Past research from our lab has used similar methodology, however, no experiment has looked at whether young blood treatment is an effective method for treating rmTBI. Hernandez (2019) and Pedemonte (2021) were both using young blood plasma injections to determine the effect on mice that contain human tau, however, these studies were focuses on Alzheimer's disease pathology rather than traumatic brain injury studies. Craven (2018) used this rmTBI method in a dual transgenic mouse that contained both human amyloid and human tau to look at the effect of rmTBI on the progression of Alzheimer's disease. No studies have yet looking at rmTBI in mice followed by treatment with young blood plasma. Literature reviews to determine whether this experiment duplicates previous experiments was completed in April 2021.

Description of search strategies used to explore potential duplication (PubMed)

Search	**Total Hits**	**Potential Duplication Hits**	**Similar Studies**	**Duplication?**
Repetitive mild traumatic brain injury AND ((plasma injections)) AND (mice)	0	0	0	No
Traumatic brain injury AND (plasma injections) AND ((mice))	24	0	0	No
Traumatic brain injury AND (plasma injections)	70	0	0	No
Traumatic brain injury AND (young plasma) AND (mice)	8	0	0	No

5. Previous IACUC Approved Application(s). Does this project contain Category D or E procedures that have been approved in previous applications by the principal or secondary investigators? If yes, indicate the IACUC approval number(s) and the procedures.

Yes, [redacted] have previously approved terminal cardiac extractions and plasma and saline tail vein injections and [redacted] have previously approved the rmTBI method.

6. Alternatives to Animal Use. What alternatives to the use of live animals have been considered? What reasons did you have for rejecting them? If specific alternatives to live animal use do not exist, this should be stated and justified appropriately. This section must also include a description of all the search strategies used to explore alternatives (e.g.,

databases consulted, key words used). *Note: When a research objective(s) can be achieved using reasonably available non-animal models (e.g., mathematical or computer) or in vitro models (e.g., tissue culture), the investigator should choose the alternative thus avoiding the need to use live animals.*

For this study there are no alternatives to animal use. An important feature of this study is to determine whether blood plasma injections following rmTBI can rescue behavioral deficits seen after TBI and there are no alternative to an animal model of rmTBI that will model behavioral changes. Additionally, due to the heterogenous nature of TBI in human patients, studies with animal models are important as the researcher is able to control for factors such as impact force and depth, the severity of the impact and the time between injuries. Cell cultures will not allow for assessment of changes made to the whole brain following TBI and subsequent treatment, nor will they allow for testing of behavioral effects. Computer programs are also unable to capture the changes made to behavior and the specific changes that will occur throughout the brain. Alternatives to animal use was searched using George Mason's Library databases including APA PsychNet and PubMed on April 20th, 2021. Keywords used include TBI and cell cultures, TBI and cells, TBI and computer models, TBI and in vitro models, TBI and technology, young blood plasma and cell cultures, and young blood plasma and computer models. Searches returned no relevant information that would allow for the proposed experiment to be done using cell cultures or a computer program.

7. Species Justification. What anatomical, physiological or other characteristics/factors did you use to select the species in consideration of the scientific objectives of the research? Strong justification from the scientific literature, preliminary data, or other relevant considerations **must** be provided for applications proposing to study only one sex. *Note: The investigator should choose the most appropriate species for the research project.*

Male and female C57BL/6J wildtype mice will be used for both the experimental mice and the donor mice who will give the blood plasma. This strain of wildtype mice is the most frequently used for research according to Jackson Laboratories. This mouse will show the inflammation, cell loss, morphological and behavioral changes that occur following TBI and the effect on these parameters due to blood plasma injections. Donor mice will be used from the same strain to increase compatibility of blood donation between mice.

8. Procedures. Describe, sequentially, with a reasonable degree of detail **all** procedures (surgical and non-surgical) to be carried out **on** live animals under **each** specific objective (aim) as listed in Section III(1). When chemical agents are administered, specify the dose, frequency, and route of administration. If blood samples or other invasive tissue recoveries are employed state the source, method, volume and frequency of sampling. The endpoints of the experiment(s) and the time frame over which the experiment(s) will be conducted must be clearly defined. *Note: The procedures section should clearly indicate how each objective of the research will be achieved. Surgical and other interventions should be fully described, e.g., a brief technical description of the surgical procedure, suture material, suture pattern(s) and time to suture removal. Do not describe the preparative regimen, pain control or postprocedure care in this section. In vitro assays should be identified but not described in detail. Do not refer to procedures approved in previous IACUC Applications in lieu of providing a description.*

Does the project include breeding of animals? If yes, please check the box, complete and submit Appendix A: Breeding Colonies. If no, please check the box.

☒ Yes. Appendix A attached.
☐ No breeding will be done.

A. Animal Housing/Cages/Handling:

All breeder males will be housed separately for the duration of the study. This is to ensure that the female harem is being exposed to only the scent of the male they are being placed into a cage with, rather than a group of males. Breeder females will be housed in harems of 2. Cages contain a running wheel, igloo, and Nyla bone, and as such additional enrichment is not necessary. Placing the males back into group-housed cages, even if they were only singly housed for the period in which they are paired with the females, leads to cage-mate aggression. Experimental male animals will be housed in cages of no more than 4. In the event that separation of animals is necessary (aggressive behaviors causing injury), the cage will be monitored, and the aggressor will be removed. In this case, because we house males in cages of 4, there will still be other mice left in the cage after removing the aggressor, and as such, the non-aggressor mice will not be singly housed. Experimental female mice will be group housed in cages of no more than 6 mice. Water and unautoclaved Envigo 7012 mouse feed will be provided to the mice *ad libitum*. Water will be provided through the automatic water system via the lixit of the cage rack.

In order to identify cages, cards will be placed in holders already on the cages. They will designate the number of mice in the cage, sex, source of the animal, strain, names and contact information for responsible investigators, date of arrival, date of birth of litters, date of weaning, protocol number, and animal IDs. Cage cards will also indicate which experimental condition the animals are in (TBI vs SHAM and saline vs plasma). Males will be housed 4 to a cage, whereas females will be housed 6 to a cage. In the case of cage-aggression, animals will be separated out and housed singly if necessary. All cages will have a regular 'igloo' with another 'igloo' that has a running wheel, and Nyla bones for chewing. Rat cages will be necessary for this experiment, as running wheels and igloos will not fit in a standard mouse cage and this will ensure consistency with previous studies of similar nature conducted in our lab. In order to keep everything consistent, all experimental groups will need to be in rat cages. These forms of enrichment are vital to the well-being of the mice, as mice are very active during the nighttime. Without a running wheel we will run the risk of cage-mate aggression, which we have experienced in our lab in the past. A running wheel and igloo help to decrease the probability of aggression and therefore any injuries. In the event of cage-mate aggression where a male-aggressor becomes singly housed, we will provide that cage additional enrichment. Mice that will be used for blood donation purposed only will be housed in mouse cages to conserve space in the animal colony if needed. Mice housed in mouse cages will also receive food and water *ad libitum* as well as a tube that can be used for nest building and shelter within the cage. These mice will also be monitored for aggression, and aggressive mice will be removed and individually housed for the duration of the study.

B. Breeding paradigm:

1 male mouse will be paired with 2 female mice for a period of two weeks. Two weeks prior to pairing, all breeders will receive 2 pellets each of "Love Mash" which is commercially available through Bio-Serv, a certified company that has developed diets and enrichment products for research animals. This is used as a supplement to the normal diet and was developed to help increase fertility and milk production in mothers and also decrease stress. Two pellets will be added to the food source every other day. This supplementary diet was recommended by The Jackson Laboratory and is used in Jackson facilities. Starting one week prior to pairing, bedding from the female cage will be added to the male cage and bedding from the male cage will be added to the female cage to introduce the scent of each of the breeding pairs. The female mice will all be housed together for 3 weeks during pregnancy and separated just prior to birthing to avoid cannibalization of pup, unintentional fostering and overcrowding with pups. Researcher will check for vaginal plug, the date when the plug is observed will be gestational day 0 to 0.5. However not all females will plug, thus, to ensure females are pregnant researcher will observe their body shape daily. If their abdomen has a bulge giving them a pear-shaped appearance, or enlarged nipples, they will be considered pregnant and separated out. Upon separation, female mice will be housed singly for the duration of their pregnancy until pups are weaned between p21-28. Once pups are weaned, females will be returned to their harem cages, until the next breeding cycle, which will occur NO SOONER than 2 weeks post weaning. Mice are weaned 3-4 weeks after birth. We will assess whether or not mice are ready for weaning based on activity level. The mice will have adult fur, teeth, and their eyes will be fully open before weaning is completed. Mice will be closely monitored for the first few days after weaning in order to be sure they are properly adjusting.

C. Ear punching

In order to properly identify mice from their cage-mates during behavioral testing, ear punching will be utilized. This procedure is widely used and simple. It is completed on weanlings between 21 and 28 days old after weaning. The left or right ear will be punched using a specialized ear punch device. One individual will scruff the mouse while another marks ear with the tool. The ear punch device will be properly sanitized before the procedure on each animal by dipping it into ethanol. This method is used instead of ear tagging because it only momentarily induces discomfort and provides a way to permanently identify an animal. In addition, it is inexpensive, simple, and requires no anesthesia (Wang, 2005). Daily monitoring and health assessment from Lippi et al. (2014) has shown that there is little to no injury (i.e. blood, swelling, infection) and little to no scarring of the animals' ears after the initial punch. In addition to this, ear tags have the potential to get caught on igloos and running wheels that are placed in animal cages, leading to potential injuries. Only experimental mice will be ear punched, donor mice will only be weaned and separated into cages by sex without being ear punched as identifying between donor mice is not necessary.

D. Handling and Restraint Acclimation

Handling will occur 1 week after the animals arrive at [redacted]. Handling will be conducted under a biosafety cabinet in the housing room and will occur on 3 times a week through the duration of the study. Researchers will gently pick up the animal and place the animal in the palm of their hand. The researcher will firmly yet carefully hold on to the tail of the mouse, permitting movement on the palms of their hands for approximately 60 seconds. The researcher will then return the animal to its homecage.

Approximately 7 days before the first transfusion, researchers will acclimatize older mice to the restraint device (Fisher Scientific). Mice will be brought to [redacted] for acclimation. The restraint device has a 2.5 cm-diameter tube and is approximately 9.5 inches long and is constructed out of plastic and is ideal for injections or blood sampling. The tube itself can support 15 to 30 g of weight; therefore, it is possible that the device will be taped down to the bench top after the animal is placed into the device to prevent the animal from moving the device during injections. The animal will be placed into the restraint device once per day and will remain in the restrainer for no more than approximately 1 minute. After 1 minute has elapsed, the researcher will remove the animal from the restrainer and return it to its homecage. Researchers will monitor any aggression that could result from stress due to short-term restraint.

E. Blood Collection

1. Blood collection will occur in [redacted]. Only donor C57BL/6J mice will be used for this procedure. Mice will be purchased from The Jackson Labs and bred for the purpose of blood donation. Collection will occur when mice are between 8 and 10 weeks of age.
2. Preparation: 1ml syringes and 25-gauge x 5/8" needles will be used for collecting blood from the heart. Prior to starting, the researcher will coat the inside of the needle and syringe with ethylenediaminetetraacetic acid (EDTA) and push out excess fluid. EDTA will prevent the blood from clotting and will allow more blood withdrawal.
3. Anesthesia: Delivery of the inhalant will be conducted in a closed chamber with a tight-fitting lid. The animal will then be put under a deep general anesthesia with an induction phase of 4% isoflurane admixed with 1 – 1.5 L/min O2 delivered by a precision vaporizer into an induction chamber. Researchers will assess the level of anesthesia by gently tipping the container to evaluate the loss of the righting reflex. Once the loss of the righting reflex has been confirmed, then the researcher will wait an additional 10 seconds before quickly and carefully removing the animal from the container. The researcher will pinch paws and agitate whiskers. If the rodent is responsive to these stimuli, then it will be returned to the container until the rodent has stopped

responding. The researcher will close the induction chamber after the animal is removed and will quickly transport the animal to a wax board. A nose cone will be placed over the mouse's head to maintain anesthesia and anesthesia will be reduced to 2 - 3% admixed with 1 - 1.5 L/min O_2 as for a maintenance phase. The researcher will pinch paws and tail to confirm proper anesthesia.

4. Procedure: the researcher will wipe down the chest area of the mouse with 70% ethanol. The researcher will pierce the animal's left ventricle at the apex using the EDTA-coated syringe, taking care to not puncture the above atrium. Opening the thorax to visualize the heart for blood collection, will also be done if necessary. The researcher will collect blood until clotting prevents further collection or no more blood can be drawn from the ventricle. Time of collection is expected to last no more than one minute.
5. Confirming death: death will be confirmed using rapid decapitation or cervical dislocation. Alternatively, since more than 50% of the animal's blood volume will be collected, the animal will be euthanized by exsanguination under deep anesthesia, followed by bilateral thoracotomy.
6. Gas scavenging. Blood collection procedures involving isoflurane will occur over a downdraft table. The lid of the induction chamber will remain closed until all procedures are complete. After all procedures, researchers will remove the lid of the chamber and orient the chamber and mask facing the downdraft. Valves on the vaporizer will be open to allow excess gas in the tubing to the induction chamber and mask to flow out and be scavenged by the downdraft table. The researchers will thoroughly clean the induction chamber and gently wipe the mask with 70% ethanol or quatricide.
7. Plasma storage: blood will quickly be placed into 1.5 mL centrifuge tubes coated with EDTA. The tubes will then be centrifuged at 1000g, plasma separated and collected, aliquoted, and stored at -80°C in the dark until further use (Middeldorp et al., 2016).

F. rmTBI

The first rmTBI will occur at 8 weeks of age, followed by 4 subsequent TBIs with 48 hours in between each (see timeline). First, mice will be anesthetized with isoflurane until unresponsive to tail or paw pinch in a sealing container. While under anesthesia the mouse will be quickly positioned so that its head is directly in the path of the CCI device by placing the device on the scalp midline between bregma and lambda. The Leica CCI device will be pre-set to the desired force (3.0 m/s) prior to anesthesia and while mouse is under anesthesia and placed on the apparatus will be set to administer a hit with a depth of 5mm. Once depth is set, the mouse will be removed from anesthesia and 30 seconds post-anesthesia will be struck by the CCI device and rotate on to the foam pad after the stage falls. The mouse head will not be shaved prior to undergoing TBI. Immediately after falling on to the foam pad, we will start a timer to determine time to righting (standing with all four legs on the ground) and time to ambulation (able to walk normally). The mouse will then be placed individually in a mouse cage containing bedding for subsequent monitoring post-injury for overall health (signs of stroke, brain bleed or any permanent damage) using the clinical scoring sheet. Some signs associated with this are abnormal gait and circling behavior, both of which are assessed using the clinical scoring sheet. After righting time has been established and the mouse has shown no neurological damage, the mouse will be placed in their home cage and observed approx. every 30 minutes for two hours' post injury (4 checks total) and then assessed once the following day and once the morning before the subsequent injury. Following the last injury, the mice will be observed for 3 days post-injury. Checks will include grooming and social behavior, as well as signs of neurological damage and discomfort such as porphyrin staining. The clinical scoring sheet provides a detailed description of how the mouse's health and well-being will be assessed. This sheet is identical to those used in previous experiments. No significant skin damage is expected from the blunt force trauma since we are only using a force that will amount to 2-2.5minutes of unconsciousness and in prior studies using this method in our lab, no skin damage has been seen. In the event any significant skin damage is seen we will consult with the vet immediately, however this is considered a mild TBI in the field and therefore we should not see any skin damage. If any signs of brain bleed or stroke are observed we will immediately euthanize the mice as described in item III.17 of this protocol: premature euthanasia. Following TBI, any animals who do not recover from consciousness within 2.5-3 minutes after the cessation of anesthesia or experience a time to righting of longer than three minutes will be euthanized. Following TBI animals will be monitored closely for any signs of stress or discomfort (e.g., lethargy, neurological damage, etc.). If an animal displays lack of grooming, non-social behavior (in the social group) or porphyrin staining, the veterinarian will be consulted. Animals that reach a clinical scoring level of 11 or greater will be euthanized. Sham animals will undergo anesthesia and will be placed on the device platform but not in the path of the CCI device. After 30 seconds, the device will activated but will not strike the mouse. Following this the animal will be placed in a recovery cage until it rights itself and then be placed in its home cage. This is the exact protocol as the injury with the only deviation being the injury itself.

G. Transfusion and saline injections:

1. Administration will occur in [redacted]. Some mice will receive blood plasma, while some will receive saline. A subset of animals will receive injections beginning 24 hours after the final brain injury; a second subset of mice will receive injections beginning 30 days after the final brain injury.
2. Animals will be weighed the morning of the first injection before the start of saline/plasma transfusion for

record keeping. Animal weight will be monitored on a weekly basis and records of weights and dosing will be kept in a laboratory notebook in the housing room.

3. *Preparation of plasma:* Prior to administration, plasma will be dialyzed using 3.5 kDa Slide-A-Lyzer Dialysis Cassettes (ThermoFisher Scientific) to remove EDTA (Middeldorp et al., 2016, Villeda et al., 2014). The plasma will be prepared and placed in 150 microliter aliquots, ready for injection.
4. *Injection schedule.* Animals will receive saline injection or plasma transfusion once every 72 hours for a total of 16 injections. Saline-assigned animals will receive 150 microliters of 0.9% saline, and blood plasma-assigned animals will receive 150 microliters of plasma from a young donor during a single injection (Middeldorp et al., 2016).
5. *Tail vein exposure and restraint*. Animals will be individually placed in a transport cage approximately 30 cm under a heat lamp to expose the tail vein. Researchers will visually inspect the animal to determine when the tail vein is visible. Once the vein is visible, the researcher will remove the animal from the cage and place it into the restraint device. The researcher will then dip the tail for five seconds in warm water to help dilate the tail vein more. One researcher will hold the base of the animal's tail and the animal is introduced into the restrainer, and another researcher will quickly secure the end piece to ensure that the mouse does not escape. The restrainer will then be taped down to the bench top to prevent the animal from moving the restrainer during injection.
6. *Injection*. While firmly holding the mouse's tail, the researcher will then insert the tip of the needle into one of the tail veins. The researcher will then inject saline or transfuse plasma, not to exceed 150 microliters, using a commercially sterilized, 1 cc syringe with a 27 G x ½" gauge needle. A bolus intravenous injection slow rate of 1 minute will be used to transfuse, as recommended by "A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes" (Karl-Heinz et al., 2001). In the event that the researcher does not enter the vein but causes bleeding due to the needle, then the researcher can attempt a second injection using the same side of the tail vein, but in a different location. After two attempts on the same side, the researcher will attempt to inject the other tail vein on the opposite side of the tail. These attempts will be done quickly in order to minimize restraint and distress. In the event of bleeding, even after successful injection, the researcher will firmly hold the tail and apply a styptic powder containing benzocaine on gauze pads for several seconds, then follow with hydrogen peroxide (as not to reinitiate bleeding) swabs to clean the area around the injection site.
7. *Evaluation of acute signs of distress/injury.* Animals are expected to have slight bruising around the injection site. Proper styptic powder containing benzocaine application and hydrogen peroxide swab cleaning will reduce bleeding and will reduce the chance of swelling and/or infection. Researchers will monitor animals for several minutes after the animal is removed from the restrainer and note any abnormal behavior, swelling, or bleeding. Seventy-two (72) hours between injections will allow the site to heal from injections. Researchers will attempt to not inject in the same location multiple times in order to reduce swelling and discomfort. Trained researchers will evaluate animals on a daily basis during the four weeks of injections. In the event that an animal's health declines, the researchers will notify the attending veterinarian. Mice will be treated on a case-by-case basis in the event that additional monitoring is warranted. A clinical scoring sheet is attached as a separate document in IRBNet

Behavioral Measures

H. Rotarod

The rotarod test is a behavioral test that measures the coordination and balance of rodents and provides a measure of locomotor activity (Pritchett et al., 2003) This is a test that is commonly used to assess motor function in mice that have sustained TBI and has been shown to be more sensitive than other tests of motor functioning. (Hamm et al., 2009) The rotarod is comprised of a rotating cylinder upon which an animal is placed. As the cylinder rotates, the animal must move forward to keep from falling off. The cylinder is placed just above a foam pad to reduce the risk of injury when the animal falls off. If the mouse falls off, the researcher will place the mouse back on the rotarod and allow it to run for the rest of the time allotted. Each trial will run for 5 minutes (300 seconds). Animals with balance and coordination problems will fall off the cylinder more quicky than animals with normal motor function. A researcher will time with a stopwatch the latency to fall off the platform and count the number of times the animal falls off the rotarod.

I. Nesting

For small rodents, nests are important in heat conservation as well as reproduction and shelter. Nesting is an easy measure of "activities of daily living" in mice, and most researchers assess this using pressed cotton squares or shredded paper. Nesting has been shown to be sensitive to brain lesions, pharmacological agents and genetic mutations (Deacon, 2006). Our lab has recently discovered that shredded paper is a better measure of nest building behavior (Neely et al., 2019). For nesting, mice will be singly housed in individual cages. Enough corncob (Teklad laboratory) bedding will be put down in the bottom of each of the cages. Corncob bedding is used to ensure that mice do not attempt to make a nest out of

the bedding. 3.5 grams of shredded paper with no ink that has been autoclaved will be scattered evenly around the bottom. Animals will have access to food (un-autoclaved standard 7012 food) and water through a water bottle placed at the top of the cage *ad libitum*. The testing room will be maintained on a 12-hour light/12-hour dark cycle consistent with colony conditions. Researchers will take pictures of the nest 2 hours and 24 hours after the mouse has been added to the cage. After the final check at 24 hours, mice will be returned to their home cage. Nests are scored on a 1-5 scale. 1: the shredded paper appears to be untouched; 2: there was some attempt to build a nest, but the majority of paper is still scattered throughout the cage; 3: a nest was constructed using a majority of the paper but there still remains several pieces of shredded paper around the cage; 4: a nest was constructed with very few papers not incorporated; 5: all of the paper has been used in the building of the nest. Blind raters will score the nests after all testing and euthanasia is complete and inter-rater reliability will be measured. The nests are scored by undergraduate research assistants who are not familiar with the test (i.e., they were not told about it and have not helped with set-up). This is accomplished by showing them pictures of the nests that were built and then having them score each one individually. The blind raters do not come into contact with the mice while they are in the nesting assay. These raters will be added to the protocol once they are identified.

J. Burrowing

Burrowing is a common behavior in rodents that provides shelter but also allows for defense against larger predators and storage of food (Deacon, 2006). The burrowing test is relatively easy to set up and can allow a quick assessment of a behavior which may point toward neurological deficits - particularly deficits in activities of daily living. One paradigm for measuring burrowing in rodents, as characterized by Deacon (2012) has shown that burrowing behavior is a sensitive marker for hippocampal lesioned and scrapie containing mice. The paradigm outlined by Deacon (2012) suggests establishing a baseline in burrowing behavior (although this baseline is ideally needed when using drug treatments). This paradigm would be optimally started three hours before the lights off cycle so that a preliminary assessment of burrowing can be made an hour before lights go out in the facility. A hollow tube with one end closed will be filled with 250 grams of peashingles (small rocks) and weighed after a mouse has interacted with it for 2 hours and will be weighed again the following morning. In order to perform the burrowing assay, each mouse will be individually housed for a period of less than 24 hours (~6PM - 10AM) and have a tube filled with 250 grams of peashingles. An hour before lights out, each tube will be weighed to note how much material has been burrowed and then will be replaced in the cage with no additional peashingles added back in. The next morning, the burrows will be weighed again and the animals will be placed in back in the cages to look at nesting behavior. Besides the tube with peashingles, no additional enrichment items will be placed in the cage. This is to ensure we are obtaining accurate data from burrowing behavior and so that we do not see a ceiling effect, which may occur if the tubes are not weighed before lights out. No additional enrichment will be provided to mice while individually housed during this assay beyond the tube with peashingles (small rocks). Individual housing of animals is necessary in this assay because we are measuring how many peashingles (amount in grams) are removed before lights out and overnight. If multiple animals are in a cage during this procedure, it is impossible to say how much a single animal may have burrowed. Mice will have access to food (un-autoclaved standard 7012 food) and water through a water bottle placed at the top of the cage *ad libitum*. The testing room will be maintained on a 12-hour light/12-hour dark cycle consistent with colony conditions.

K. Morris Water Maze (MWM)

The Morris water maze task is used to assess spatial memory. Animals learn to locate a platform hidden just below the water to allow for escape from the water. Testing takes place over 8 consecutive days with 3 trials per day, except for days 7 and 8 which will only consist of 1 and 2 trials, respectively. Each day, all animals are transported to the dimly lit testing room where they will habituate undisturbed for at least 10 minutes before testing begins. The metal Morris water maze tub is filled with opaque water to keep the animals from seeing the platform; visual cues are images located around the tub which is surrounded by a white curtain. One at a time, each animal is placed in the metal tub facing a wall. The submerged escape platform is in a fixed location for the trials but where the animal starts changes for each trial, forcing them to use the cues around the pool to recall the platform location. The animal will be given 60 seconds to find the hidden platform while being tracked by the camera (using the Videotrack tracking system). Distance traveled, latency to find the platform, and time spent in the target quadrant will be measured. In addition, thigmotaxis will be measured. This is a measurement of how much time the mouse spends in the outer 10% of the pool and can be considered an “alternative search strategy” indicating that the mouse is not in fact using spatial memory to recall the platform location (Brody & Holtzman, 2006). At the end of 60 seconds, if the mouse fails to find the platform, the animal will be gently guided to the platform where it will remain for 10 seconds before being removed. Upon completion of the trial, the animal will be dried with a towel and placed under a heating lamp for 45 seconds before beginning the next trial. Once the animal has completed all 3 trials, it is finished for the day. On days 2, 4, and 6, trial 3 will be a probe trial. The probe trial consists of the platform being lowered so that the animal is unable to locate it. They are given 60 seconds before the platform is raised and they are guided to it. The purpose of the probe trial is to measure the amount of time the animal spends searching in the target quadrant where the platform is located. The more time spent there suggests that the animal remembers where the platform should be. Day 7 consists of only 1 trial which is a probe trial. This trial is used to assess their long-term memory. Day 8 consists of 2 trials using a visual

platform to ensure there are no visual abnormalities. However, these mice have been shown to have no visual deficits. In the event that a mouse is unable to complete all trials, this mouse's data will be removed from the experiment and the mouse's overall health will be assessed and the vet will be consulted. However, we have never had a mouse that was unable to complete all the trials in our laboratory and do not expect this to occur.

L. Circadian Activity

Circadian running wheel activity will be assessed through the use of Actiview Biological Rhythm Analysis software (Minimitter Co.). Circadian rhythm disruption and sleep disturbances are commonly seen after TBI (Lucke-Wold et al., 2015). Animals will be placed into individual cages equipped with running wheels and their activity will be continuously monitored for 6 days (144 hours) to assess activity levels through different periods of the day. Each wheel is hooked up to a computer that monitors rotations/minute. The testing room will be maintained on a 12-hour light/12-hour dark cycle consistent with colony conditions. Due to the nature of this task, animals cannot be housed together during this test. Mice will have access to food (un-autoclaved standard 7012 food) and water through a water bottle placed at the top of the cage *ad libitum*. Mice will continue to undergo health checks throughout the duration of the test.

M. Open Field

The open field test is a measure of exploratory behavior and general activity and is commonly used as a "control" assay for other behavioral tests that involve activity (e.g., Morris Water Maze, Elevated Zero Maze). It is a simple test in which the animal is placed into a box without any type of stimuli and is allowed to explore for 5 minutes. The apparatus will consist of two identical 18 inch by 18 inch by 9.5-inch-tall boxes constructed out of blue plexiglass (Clever Sys., Inc., Reston, VA). Animals will be transported individually to a dimly lit testing room where they will be allowed to habituate for 10 minutes prior to testing. Animals will be gently placed in the apparatus and distance traveled will be recorded over a 5-minute period using the Videotrack tracking system (Clever Sys., Inc., Reston, VA). The software measures total distance traveled as a measure of general locomotor activity. Time spent in the center of the open field, a measure of anxiety-like behavior, will also be recorded. The container will be thoroughly cleaned with quatricide to reduce olfactory cues between animals. This test allows us to infer whether or not mice may have any potential motor deficits. This is important, as we would be unable to assess the Morris Water Maze or elevated zero maze data accurately if the effects, we see in those tests are due to motor deficits. Open field allows us to rule out this possibility.

N. Elevated Zero

The elevated zero maze is a measure of anxiety. The apparatus is an "O" shaped platform that is raised from the ground and is divided into two sections with 31cm-high walls surrounding the edges (100cm x 70cm) and two open sections (100cm x 70cm) with no surrounding walls. All animals will be transported down to a dimly lit testing room where they will habituate for 10 minutes. Each animal will be given a single trial that begins with being placed in an open portion facing a closed portion (portion used instead of 'arm'). Once the animal is placed, data will be collected for 5 minutes using a ceiling-mounted camera positioned over the elevated zero maze (TopScan, Clever Sys., Inc., Reston, VA). Software will measure the total distance traveled, time spent in the closed arms, time spent in the open arms, and the total number of entries into each arm. The maze will be completely wiped down with 70% ethanol to reduce olfactory cues between animals upon completion of each animal's single trial.

O. Gait assessment

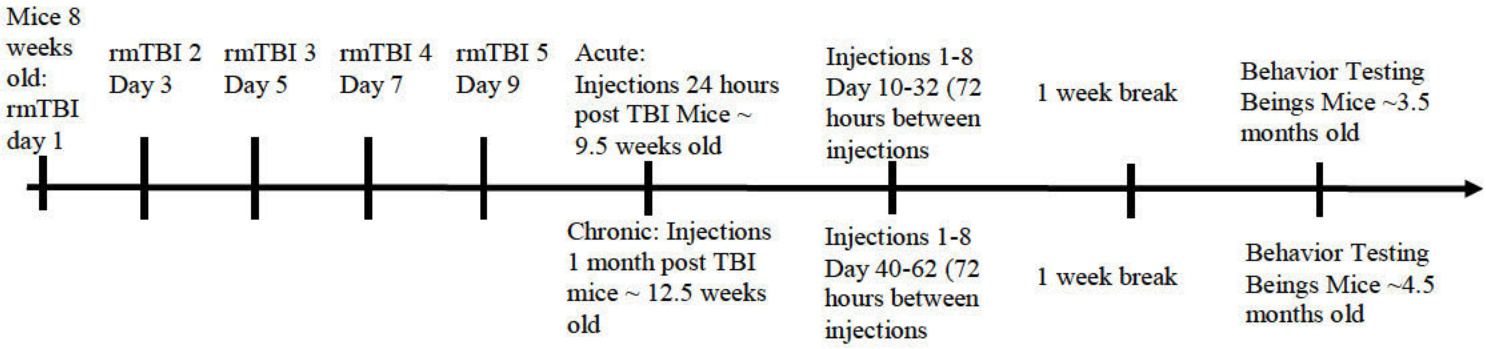
Animals who undergo mild repetitive traumatic brain injury (rmTBI) often have subtle motor deficits (Mouzon et al., 2012). This method of measuring motor deficits is a simple but effective measure of gait that other tests of motor deficits do not show. The gait assessment test measures the way the animal steps, so we are able to assess stride length, stride width and toe spread while being sensitive enough to pick up the subtle motor differences that occur following rmTBI. Procedure: Mice will undergo the gait assessment test two days after the completion of the Morris Water Maze behavioral test in the experimental timeline. Mice will have a day of rest between the Morris Water Maze test and the beginning of the gait assessment test. Mice will also have a day of rest in between the gait assessment test and the beginning of the nesting assessment. Mice will be placed in a goal chamber that is 4 inches wide and 12inches long. Watercolor paper will be cut into individual strips to fit underneath the goal chamber. Two contrasting colors of non-toxic washable water based paint will be painted onto the forelimbs and hindlimbs of the mouse. One color will be placed on the hindlimbs and the contrasting colors will be placed on the forelimb. Mice will be scruffed while paint is being applied to paws. Mice will the be placed a the start of the goal chamber and allowed to walk all the way into the goal chamber and to the end. The mouse is then removed from the goal chamber, paint cleaned from its feet with a water-dampened cloth and returned to its home cage. Only one trial of the test will be completed per mouse. Mice will be excluded from the trial if they have an injury or health condition that prevents them from participating in behavioral testing, as determined by the veterinarian.

P. Experimental Timelines:

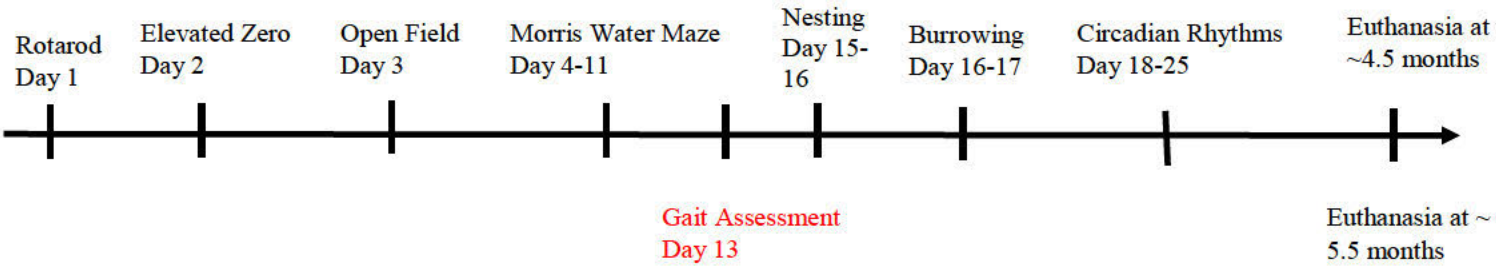
Donor mice will undergo terminal cardiac blood extractions and euthanasia between 8-10 weeks of age.

Starting at 8 weeks of age, rmTBI will be administer to all mice with 1 hit occurring every 48 hours for a total of 5 hits over 10 days. Following completing of rmTBI, mice will be divided into two groups, an acute treatment group and a chronic treatment group. Plasma or saline injections will begin for the acute treatment group 24 hours after the final TBI. For the chronic treatment group, plasma or saline injections will begin 30 days after the final TBI. After the completion of injections, both groups will have a one-week break before behavioral testing beings. For the acute treatment group, behavioral testing will occur when mice are 3 months old; for the chronic treatment group, behavioral testing will occur when the mice are 4 months old. Behavioral testing will run for approximately 2.5 weeks. Mice will be euthanized within 48 hours after the final behavioral test.

rmTBI and Injection Schedule:



Behavior Schedule



Q. Euthanasia: Mice will be euthanized within 48 hours of the completion of the final behavioral test using one of the three methods listed below in Section III Item 14: Method of Euthanasia.

R: Description of Preliminary Work As Taken from Minor Amendment in Package #2

Previous research in our lab has shown inconsistent "time to righting" and "time to ambulation" in mice between researchers following the repetitive mild traumatic brain injury (rmTBI) paradigm with the dropping platform. "Time to righting" is described as the amount of time it takes for a mouse to stand with all four paws on the ground following rmTBI and "time to ambulation" is described as the amount of time it takes for the mouse to walk normally following rmTBI. We've noticed in our lab, that the rmTBI paradigm with the dropping platform produces inconsistent results in time to righting and time to ambulation depending on the researcher who is administering the TBI. For example, in a method study describing the device ([redacted]), when one researcher was administering the rmTBIs, mice had a time to righting of an average of 45 seconds and in another study when another researcher was administering the rmTBIs for a different rmTBI study ([redacted]), mice had an average time to righting of 19 seconds. This difference is statistically significant ($p < 0.05$) even though both groups were wildtype mice undergoing the same rmTBI paradigm. This is concerning, as the same procedure should elicit the same results. Inconsistencies were only

found in the rmTBI with the dropping paradigm platform in time to righting response when different researchers were administering the TBIs

Several histopathology procedures have been run on these mice to attempt to determine where the inconsistencies are occurring. Cresyl-violet staining was used to determine the extent of overt neuronal loss. Western blot immunoblotting analysis was performed with markers of inflammation to determine whether there were differences in neuroinflammation following rmTBI. However, as this injury is mild, there were not significant differences in levels of neuroinflammation or levels of neuronal loss even between animals were received rmTBIs and animals who did not, in the seven brain regions that were assessed. Brain regions that were assessed include cortical areas under the site of impact, and other regions that typically see damage following TBI including the infralimbic regions of the prefrontal cortex and regions within and surrounding the hippocampus. So determining differences within the groups that received rmTBI with different researchers using histopathology staining did not reveal anything of value.

The researchers also had an extensive conversation to make sure they were performing the rmTBI paradigm in the same way. They ensured that the force and impact depth were set to the same force and depth, that the mice were being anesthetized to the same levels (unresponsive to paw and tail pinch), that the stopwatch to measure the time to righting and time to ambulation reflexes was being started at the same time. That conversation revealed no differences in the way that the researchers were administering the rmTBIs.

The proposed amendment seeks to determine that the rmTBI method is consistent within researchers to ensure that the variance seen within experimental groups comes from true individual differences and not experimenter error. This will hopefully reduce the variance in the behavioral tests, increase statistical power, and therefore may reduce the number of mice needed for rmTBI experiments in the future.

To assess the consistency of these two methods, three cohorts of mice will be used. Each cohort will consist of 10 mice, 5 mice receiving rmTBI with a dropping platform and 5 mice receiving rmTBI with a stationary platform.
A full experiment is not necessary to find the desired information that this amendment proposes. There was inconsistency in time to righting following rmTBI with the dropping platform when different researchers administered the TBI, and this preliminary study is solely to determine if only one researcher is administering the TBIs, if the results in the time to righting reflex are consistent across different cohorts. We have no information on this to this point, as all previous TBI studies used different researchers for different cohorts to administer the TBIs. This study is not yet at the point of trying to determine the cause of inconsistencies between researchers. At this point, the goal of this amendment is to determine if rmTBI with a dropping platform will give consistent time to righting reflex with a single researcher administering the TBIs at different time points. This will be compared to rmTBI mice with the stationary platform, as inconsistencies in time to righting were less seen in that method. However, the dropping platform is preferable to use for other reasons, including a reduction of apnea following the hit, reduction of compression on the spinal cord, and more translatable to human injuries. Though, if the injury administration is inconsistent, the stationary platform will be preferable as to reduce the variance seen within groups and get more reliable results.

Breeding: 4 female and 2 male C57BL/6J wildtype breeder mice will be obtained from Jackson Laboratories. Animals will be paired in cages with 2 females to one male for a period of two weeks. Upon separation, female mice will be housed singly for the duration of their pregnancy until pups are weaned between p21-28. Additional breeders are requested to produce the mice for the preliminary study, as by the time a third litter can be bred, the original donor mice will be about 6-7 months old. In the past, our lab has been behavioral differences in mice that were produced from older parents. Therefore, in this case, a third cohort will not produce optimal results.

No animals that will be bred for this preliminary study will go unused. Any animals over the number that are needed for the preliminary study will be used as donor mice for the main project.

Method: To assess the internal consistency of the method, preliminary work will need to be done to determine if time to righting and time to ambulation is consistent when the same researcher is administering the rmTBIs with a dropping platform. These results will be compared to an rmTBI with a stationary platform to determine which method results in greater consistency. Mice will be given the first TBI at 8 weeks of age. Wildtype mice will be given either 5 hits at 48 hour intervals on a stationary platform or 5 hits at 48 hour intervals on a dropping platform. Five mice will be used per group, with three different cohorts to test consistency for a total of 30 additional experimental mice. After completion of traumatic brain injury paradigms, mice will be euthanized 24 hours after the final hit was administered. Anesthesia, TBI administration procedures, and post-TBI monitoring will remain the same as seen in the original procedure.

Three cohorts of mice will be used to compare each cohort to each other. This is necessary, as the objective of this amendment is to determine whether this method is consistent when the same researcher is administering the rmTBIs. This necessitates performing the rmTBIs on different days. Thus, the mice will be split into three different cohorts. The three cohorts will undergo the rmTBI paradigm on different days to determine that the experimenter performing the rmTBIs can be consistent across different days of administration.

9. Justification of the Number of Animals Requested. What is the justification for the number of animals requested? This justification must include a clear description of the experimental design (not methods or procedures) and specification of the number and species/strain of experimental/control animals per group/subgroup in each experiment/procedure. The scientific/statistical rationale which supports the size of the N in each group should be included. **A table or block experimental design must be provided facilitate the review of this section.** *Note: This section should clearly reflect that the number of animals to be used is both justified and minimized to the greatest extent possible consistent with sound scientific and statistical standards. Detailed procedures should not be described in this section.*

A G*power analysis was completed to determine the minimum number of animals needed to reach significance in the behavioral tests. The two separate time points were entered separately. For the treatment immediately after injury, there are 4 groups (SHAM saline, SHAM plasma, rmTBI saline and rmTBI plasma), the entered effect size is .20, with an alpha level of .05 and a power of .8 that yields a total sample size of 76 animals.
A G*power analysis was also conducted for determine the minimum number of animals needed to reach significance in the brain analysis that will be conducted after euthanasia. There will be 5 separate analyses conducted. A G*power analysis was conducted for each of the brain analysis. These are more sensitive than behavioral tests so for each of the 4 groups, the effect size was entered as 0.35, with an alpha level of .05 and a power of .8 which yields a total sample size for one brain analysis of 20 mice, 5 per group. Across 5 brain analysis, this will work out to 100 animals for the immediate treatment after injury.

The treatment at one month subset was also entered using the same parameters and the same groups (SHAM saline, SHAM plasma, rmTBI saline and rmTBI plasma) as the immediate treatment group also yielded a sample size of 76 animals. Again, the brain analysis that will be conducted require 5 per group across 5 different tests which yield a total of 100 animals per group. This project has a total of 8 group, with 25 animals per groups, so a total of 200 experimental animals will be used across both treatment time points.

The mice will be bred in house in two cohorts (See breeding appendix below). 10 females and 5 males will be used. As female mice typically yield litters of 10 mice, 100 mice will be obtained from the first cohort. Pairs will be bred again to reach the total of 200 mice needed.

Across both groups, half of the mice will be receiving plasma and half will be receiving saline, for a total of 100 mice receiving plasma. The plasma will be taken from donor mice. About .6ml of blood are able to be retrieved from each donor mouse, and that is spun down to about .45ml of plasma for about a 3/4 ratio of plasma to total blood. Treatment mice will receive 0.15ml per injection over 16 injections for a total of 2.4 ml of plasma per mouse. 2.4 ml times 100 mice is 240ml of total plasma that will be used for this experiment. With the ¾ ratio of plasma to total blood, we will need about 320ml of total blood to get the 240ml of plasma which will require approximately 640 donor mice.

Donor mice will also be bred in house using 16 females and 8 males which will yield 160 mice for the first cohort and 160 mice in the second cohort for the 320 total mice.

A second set of donor mice will be bred in house using an additional 16 female and 8 male breeder mice which will yield 160 mice for the first cohort and 160 mice in the second cohort for an overall total of 640 donor mice.

Acute treatment group (Injections 24 hours after final TBI):

	No rmTBI SHAM	**rmTBI**
Plasma Treatment	n = 25	n = 25

Saline Treatment	n = 25	n = 25

Total: 100 mice

Chronic treatment group (Injections 1 month after final TBI):

	No rmTBI SHAM	**rmTBI**
Plasma Treatment	n = 25	n = 25
Saline Treatment	n = 25	n = 25

Total: 100 mice
Total Experimental mice = 200

Across both groups, half of the mice will be receiving plasma and half will be receiving saline, for a total of 100 mice receiving plasma. The plasma will be taken from donor mice. About .6ml of blood are able to be retrieved from each donor mouse, and that is spun down to about .45ml of plasma for about a 3/4 ratio of plasma to total blood. Treatment mice will receive 0.15ml per injection over 16 injections for a total of 2.4 ml of plasma per mouse. 1.2ml times 100 mice is 120ml of total plasma that will be used for this experiment. With the ¾ ratio of plasma to total blood, we will need about 320 ml of total blood to get the 120ml of plasma which will require approximately 640 donor mice.

Total Plasma Mice	**Total Saline Mice**
n = 100	n = 100

200 experimental mice + 640 donor mice + 63 breeder mice = <u>903 mice</u>

Sample Size After Breeding for Preliminary Work	Stationary Platform	Dropping Platform
rmTBI (5 hits)	5 x 3 cohorts: 15 total	5 x 3 cohorts: 15 total

A total of 36 additional mice will be used including the 30 experimental mice and the 6 breeders.

903 mice for the main study + 36 for preliminary work = 939 total mice

A G*power analysis was completed to determine the minimum number of animals needed to reach significance in time to righting and time to ambulation measures. The entered effect size is .20, with an alpha level of .05 and a power of .8 that yields a total sample size of 30 animals.

10. Painful Procedures. Does the project include any potentially painful procedures (Category of Research D or E)? If yes, please check box the box, complete and submit Supplement 1. If no, please check the box.

☒ Yes. Supplement 1 attached.
☐ No painful procedures will be performed.

11. Site of Animal Housing. Identify the facility where the animals will be housed.

Animals will be housed in the [redacted] in room [redacted]

12. Site of Study. Where will the procedure(s) (including any preoperative and postoperative care) be carried out on live animals? Identify the building and room number(s) if applicable. *Note: Any site where animals are being held must be approved in advance by the Chair of the IACUC.*

Behavioral experiments will be carried out in approved testing suites in the available behavioral testing rooms in the [redacted]. rmTBI, tail vein injections, terminal cardiac bleeds and euthanasia will all occur in the [redacted] surgery room.

13. Restraints. Does the project include any prolonged restraint? If yes, please check the box, complete and submit Supplement 2. If no, please check the box.

☐ Yes. Supplement 2 attached.
☒ No prolonged restraint will be used.

14. Method of Euthanasia. What method(s) of euthanasia will be used? Include the dose (e.g., mg/kg) and route (e.g., IM, IV), of any injectable agent(s). In addition, provide the rationale for selecting this method of euthanasia. *Note: The method of euthanasia should be based upon the species, size of the animal, the scientific objectives of the experiment and its ability to quickly and painlessly produce a loss of consciousness and death. All methods of euthanasia must comply with AVMA guidelines. If animals are not to be euthanized this should be stated and their final disposition explained.*

Within 48 hours of the final behavioral assay (see the Experimental Timeline **Section III Question 8" Procedures: subsection "O": Experimental timeline**") the animals will undergo one of three euthanasia methods.

1. Exsanguination/perfusion under deep anesthesia: All procedures will be conducted on a downdraft table. While deeply anesthetized with isoflurane (unresponsive to paw and/or tail pinch), animals will be completely exsanguinated and decapitated using a guillotine for tissue collection. All procedures will occur on a downdraft table. Mice will be anesthetized using isoflurane. The vaporizer will be turned off and the lid will remain shut once the animal is removed from the chamber. A researcher will perform a thoracotomy to expose the heart, will sever the heart adjacent to the aorta using a small pair of scissors, and will then pierce the apex of the animal's left ventricle with a 25 – 30-gauge needle. The heart will be perfused using the needle puncture with 0.9% saline in a 30 mL syringe. The researcher will continue to perfuse with saline until fluid exiting the severed aorta runs clear. The mouse circulating blood volume is typically between 2.25 and 3 mL total; therefore, it is not expected for all 30 mL of 0.9% saline to be used. The researcher will then continue to perfuse the animal using 4% paraformaldehyde (PFA) in order to fix the tissue. Lightened color of the liver due to fixation will indicate that perfusion is near completion. Perfusion in a mouse takes approximately 8-10 minutes. Mice will then be decapitated using a guillotine specialized for rodents, and brains will be collected for histology. Carcasses will be placed into a designated body disposal bag and placed into the carcass freezer. Commercially sterilized needles and syringes will be used between mice.

2. Decapitation under deep anesthesia: Mice will be anesthetized using isoflurane as described above. The researcher will deeply anesthetize the animal and perform euthanasia by rapid decapitation using a guillotine after exsanguination.

3. Decapitation using gradual carbon dioxide (CO_2) exposure for deep anesthesia: for the mice that will not be exsanguinated, we will utilize gradual CO_2 asphyxiation as a means of anesthesia. Animals will be anesthetized using gradual CO2 exposure, then euthanized by rapid decapitation using a guillotine after exsanguination. Mice will be kept in their home cages or transferred to another appropriate euthanasia chamber and the procedure will be performed on a downdraft table. A specially fitted lid that is connected to a CO2 cylinder will be placed on top of the chamber. A CO2 flow meter is used to regulate the flow of CO2 into the chamber. CO2 delivery to the chamber is accomplished by turning the CO2 cylinder valve and flow meter on so that animal(s) are slowly exposed to increasing levels of CO2 at the determined flow rate (directions are posted in [redacted]). CO2 should be introduced into the chamber at a rate of 30-70% of the chamber volume per minute to reduce distress to the rodent (s). Once loss of consciousness is observed, paw pinches and tail pinches will be done in order to ensure the proper amount of anesthesia. Personnel will perform rapid decapitation using a guillotine to perform euthanasia. Brains will be collected for histological purposes.

We will ensure that the guillotine is sharpened prior to decapitation procedures. Our lab manager will assess sharpness by cutting pieces of paper without any sticking or tearing. In addition, the lab manager will assess sharpness by cutting through a rubber band without any sticking or tearing. This method is used at other institutions such as the University of California at Los Angeles and the University of Michigan. The lab manager will maintain records in his office.

Record of guillotine use will be in a notebook that will be stored in [redacted]. The record will contain the following: which animal, date, and reason.

15. Criteria for Euthanasia. Describe the criteria for euthanasia of the animals (e.g., endpoint of experiment, specific time period, tumor size, etc.). *Note: Animals should be euthanized at the earliest possible endpoint in consideration of the scientific objective(s).*

Animals will be euthanized after all behavioral testing is completed as described in the section III.8 timeline.

16. Criteria for Death. What are the criteria that will be used for determining that euthanized animals are dead? *Note: The investigator must ensure that animals are dead by using physiological parameters such as cessation of heartbeat and respiration for a suitable length of time.*

Criteria for death include the physical separation of the head from the body or the clearing of visible blood from the perfusate.

17. Criteria for Premature Euthanasia. What are the criteria for premature euthanasia of the animals (e.g., signs of significant pain/sickness/morbidity, inability to feed, etc. that cannot/will not be treated)? *Note: Animals in chronic experiments must be monitored and euthanized as soon as possible upon evidence of pain/sickness/morbidity that cannot/will not be treated.*

Animals will be observed on a daily basis. Animals will be prematurely euthanized if they are found to display signs of overt neurological damage, distress, illness, or inability to feed. If animals show sign of stroke follow rmTBI, mice will be immediately euthanized. Following TBI, any animals who do not recover from consciousness within 2.5-3 minutes after the cessation of anesthesia or experience a time to righting of longer than three minutes will be euthanized. Following TBI animals will be monitored closely for any signs of stress or discomfort (e.g., lethargy, neurological damage, etc.). If an animal displays lack of grooming, non-social behavior (in the social group) or porphyrin staining, the veterinarian will be consulted. If an animal scores between a 7-11 on the clinical scoring sheet will also received increased monitoring and the veterinarian will be consulted. Animals that reach a clinical scoring level of 11 or greater will be euthanized. Animals that are underweight, appear lethargic or are showing signs of increased distress/illness will be closely monitored. This includes any animals that display these signs of necrosis of the tail and/or body, or other signs of distress throughout tail injections. Additional adverse events seen with a blood transfusion may include fever and allergic reaction, fluid overload, lung injury or breakdown of red blood cells due to a mismatch between the donor's and recipient's blood type. Since mice of the same strain are being used, reactions of blood transfusions are expected to be rare. Determination of these symptoms will be confirmed with the veterinarian. Clinical evaluation logs will be maintained in the colony. Refer to Section III Item 8 above for additional details on early removal criteria.

Or, if premature euthanasia will not be necessary because the research involves **only** a terminal procedure, this should be stated.

18. References. Provide a list of *key* references supporting the statements contained in Section III of this form.

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Kumar, A., & Loane, D. J. (2012). Neuroinflammation after traumatic brain injury: Opportunities for therapeutic intervention. *Brain, Behavior, and Immunity*, *26*(8), 1191–1201. doi: 10.1016/j.bbi.2012.06.008

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George Mason University

IACUC APPLICATION
FOR ANIMAL RESEARCH AND TRAINING PURPOSE

SUPPLEMENT 1

PAIN, DISTRESS OR DISCOMFORT INVOLVEMENT

Painful Procedures. Does the project include any potentially painful procedures?

A. Justification for <u>Any</u> Painful Procedures. The use of procedures potentially causing more than momentary or slight pain or distress to an animal even though that pain will be relieved by administration of proper drugs, requires investigators to consider alternative procedures and provide a written description of the methods and sources used to determine that alternatives were not available and/or were scientifically unacceptable. *Note: In designing the experiment, the investigator should choose procedures that have the least amount of potential pain, discomfort, distress or morbidity in consideration of any limitations imposed by the objectives of the research. Examples include reduction of the number of required surgeries, less invasive surgery, use of a less toxic adjuvant, selection of the earliest possible endpoint, use of a modified LD50 test. This section should clearly justify why less potentially painful procedures cannot be used to achieve the specific objectives of the research. This section must also include a description of the search strategy used to explore alternatives (e.g., databases consulted, key words used). Do not discuss pain control in this section.*

Our procedure for repetitive mild traumatic brain injury (rmTBI) has been found to be the most translational in relation to humans, as 80% of human TBIs considered to be mild (Bruns & Hauser 2003). It is also the least stressful to the mice when compared with a single moderate or severe injury. For instance, a mortality rate of 64% has been cited in one particular method (Marmarou et al., 1994). While some methods of TBI involve anesthetic followed by a craniotomy and direct impact to the skull (Dixon et al., 1991; Portbury et al., 2016; Zhou et al., 2017), our procedure does not involve this more intensive surgical procedure. Rather, our procedure uses a small amount of anesthesia followed by a closed head injury. In previous experiments, our lab has found that mice have a time to righting of about 2 minutes, and a time to ambulation of no greater than 3 minutes, both of which are indicative of a mild injury. No distress of the animals has been found immediately following the injury or in subsequent hours/days. Using a method of rmTBI has the lowest mortality rate of 10% in the field. There are no viable alternatives to TBI on mice that could fully model both the behavioral and neurological effects of TBI. However, within our lab, using rmTBI with a dropping platform, we have experienced no mortality due to TBI. Our method is also repetitive because this technique closely imitates head injuries to humans. For example, soldiers and athletes often experience multiple injuries in a short amount of time. Many athletes attempt to play through injuries that they deem minimal. In addition, humans typically drop to the ground after receiving a TBI. This rmTBI method has proven to be effective in our laboratory.

Our alternatives to painful procedures search involved the assessment of the above-mentioned studies. PubMed and PsychInfo were searched using the following keywords “TBI and mortality and mice” “TBI and pain and mice” “mTBI method and mice” “rmTBI method and mice”. In addition, this method results in no unnecessary pain, as determined from clinical assessment in previous studies in our lab utilizing this method. Animals display no overt signs of pain (i.e., hunching, abnormal gait, porphyrin staining, lack of socializing).

If the research involves only a terminal procedure or there is no procedure that will cause pain, discomfort or distress (regardless of any planned use of anesthetics/analgesics), this should be stated.

B. Preparative Regimen. What is the preparative regimen? *Note: A description of the preparative regimen should be provided which includes, as applicable, description of the aseptic preparation of the surgical field; specification of any antibiotics or tranquilizers to be administered; ventilation procedures; IV lines and other preparations.*

TBIs will be administered in the [redacted] surgery room in the [redacted]. The Impactor tip of the Leica CCI device will be cleaned with 70% ethanol between mice, and the platform will be sanitized between each use. As this method of TBI does not involve a craniotomy or opening the skin to expose the skull and previous use of this device has not shown breaks to the skin, no antibiotics need to be administered.

If the research does not require use of a preparative regimen this should be stated.

C. Pain Control During Procedure. How will pain be controlled, as necessary, during performance of the procedures described in Section 10? *Note: In the description of pain control, identify the procedure requiring pain control and specify the initial and any supplemental anesthetics to be used. Do not describe postprocedure pain control in this section. The dose (e.g., mg/kg) and route (e.g., IM, IV) of administration of any drugs should be stated.*

To control pain and distress during the mTBI using the Leica controlled cortical impactor, the use of general anesthesia (isoflurane) will be used. The process by which anesthesia will be used can be found in section III.8 under **rmTBI.** Briefly, mice will be anesthetized with isoflurane until unresponsive to tail or paw pinch. While under anesthesia the mouse will be quickly positioned so that its head is directly in the path of the CCI device. Once placed under the apparatus, the mouse will be removed from anesthesia and 30 seconds post-anesthesia will be struck by the CCI device and rotate on to the foam pad after the stage falls.

If pain control is not necessary/applicable, this should be stated.

D. Estimation of Potential Postprocedure Pain, Discomfort, Distress or Morbidity. Identify **all** procedures and induced conditions that will potentially cause more than momentary, slight pain, discomfort, distress or morbidity. Estimate the magnitude and duration of any adverse effects the animals may experience during the postprocedure period. *Note: Estimation of potential adverse effects on the animal in advance is necessary in order to develop plans to prevent, monitor and relieve as much pain as possible during the postprocedure period. The American Physiological Society has defined stimuli as potentially painful to animals if those stimuli are detected as painful in humans; approach or exceed tissue damaging proportions; produce escape behavior in animals.*

Previous research has shown that animals are not expected to experience any postprocedure pain or discomfort (Kane et al., 2012). We have experienced no mortality with a mild injury within our laboratory using the currently proposed procedure. We believe that the frequent monitoring of mice after TBI will ensure that any mice experiencing postprocedure pain, discomfort, or stress will be found and humanely euthanized. They are monitored every half hour the first 2 hours after TBI (4 checks), once the following day, and once before the subsequent TBI. In addition, they are monitored for 3 days following the last TBI. However, mice in our previous TBI studies have not shown any postprocedure pain or discomfort, as assessed through the clinical scoring sheet.

If animals will not experience any adverse effects, i.e., postprocedure pain, discomfort, distress or morbidity in association with any specific procedure(s) or induced condition this should be stated and **explained** as necessary.

E. Postprocedure Monitoring. What is the frequency (specified times per day) and duration (number of days) over which postprocedure monitoring of the animals will be performed by the investigator(s)/technician(s)? *Note: All animals must be monitored at appropriate intervals which are dictated by the nature of the intervention(s), the degree of potential postprocedure pain, the likely duration of the pain and possible complications. For example, monitoring is often more frequent during the immediate postsurgical period (e.g., first 24 hours postoperatively) and during the latter stages of tumor induction or toxicology experiments that have a high degree of morbidity.*

Following rmTBI: Once the mouse has regained consciousness, can maintain normal posture, and shows no sign of neurological damage, the mouse is placed back into its home cage. Every 30 minutes for the next 2 hours, the mouse is observed. After two hours, the mouse is observed at least once daily until the end of the project.

If postprocedure monitoring is not necessary/applicable, this should be stated and explained as necessary.

F. Criteria for Monitoring Animal Pain/Well-being. What are the specific criteria that will be used to assess/monitor acute and chronic pain as well as general animal well-being during postprocedure monitoring? *Note: During monitoring, animals should be evaluated for the presence of pain, discomfort or distress. In assessing pain, the investigator should use behavioral signs based upon the normal behavioral pattern of the species, i.e., species-specific signs of pain. In some cases, physiological parameters should also be used. Animals should also be evaluated to determine their overall well-being by examining factors such as food and water intake, locomotion, body temperature (where applicable), integrity and general healing of surgical incisions (where applicable).*

Animals will be monitored daily and checked for any distress. This includes grooming and social habits, along with any behavioral changes which could be due to neurological damage. All animals will also be assessed using the clinical scoring sheet, which is used to monitor health after receiving TBI. The clinical scoring sheet will monitor the animals' physical appearance, natural movement, provoked movement, gait, head tilt or circling, paresis or paralysis, and body weight. Scores between 0-2 will be given for physical appearance, and head tilt and circling; scores between 0-3 will be given for natural movement, provoked movement, gait and body weight, and a score of either 0 or 3 will be given for paresis or paralysis. For a more detailed explanation of how an animal received a score see the clinical scoring sheet added as a separate file in IRBNet. Animals that score a 7-11 will received increased monitoring, and if an animal scores higher than an 11 the animal will be euthanized.

If postprocedure pain assessment is not necessary/applicable, this should be stated and explained as necessary.

G. Postprocedure Analgesic(s). If it is anticipated that an animal may be subjected to more than slight pain, discomfort or distress during the postprocedure period, specify the analgesic(s) which will be used to **prophylactically** treat the animal. Include dose (e.g., mg/kg), route (e.g., IM, IV), frequency and duration of administration of the analgesic(s). *Note: Administration of analgesics prophylactically is based upon the premise that where pain is concerned, the animal should usually be given the benefit of the doubt.*

If pain is unlikely but, nevertheless, possible and analgesic(s) will **not** be administered **routinely/prophylactically**, state the specific criteria (species-specific signs of pain) for administration of analgesic(s) should treatment of pain become necessary. In addition, specify the analgesic(s); include dose (e.g., mg/kg), route (e.g., IM, IV) and frequency of administration. *Note: If it is unlikely but, nevertheless, possible that animals may experience pain during the postprocedure period, analgesics should be administered immediately upon evidence of pain, using species-specific signs of pain.*

No post-procedure analgesics will be used, as it is not expected that the animal will experience any post-procedure pain or discomfort based on previous research with the method of rmTBI (Craven et al., 2018) and other rmTBI measures (Kane et al., 2012). If an animal is experiencing more than slight pain, discomfort, or distress, the attending veterinarian will be consulted and the animal will be euthanized. However, this is not expected, as we have not observed this in previous studies in our lab utilizing this method of rmTBI.

Strong justification for withholding analgesic(s) must be given if their use is indicated, but they are not to be administered. *Note: The withholding of analgesics normally required to treat pain must be based upon referenceable scientific fact or experimental*

data.

Post procedure analgesics will not be used as if the animal is shown to be experiencing more that slight discomfort they will be assessed by the veterinarian, animal care staff and the primary researcher, removed from the study and euthanized.

If the procedure is acute, this should be stated or if postprocedure analgesics are unnecessary, this should be explained.

H. Postprocedure Antibiotics. Specify any postprocedure antibiotic to be used. Include dose (e.g., mg/kg), route (e.g., IM, IV), frequency and duration of administration.

No antibiotics will be used during this study, as this is a closed head injury, no craniotomy or opening of the skin will occur and this procedure has not shown any breaking of the skin. As such, no infection is expected.

If antibiotics are not necessary/applicable, this should be stated

Principal Investigator:	Jane Flinn
Protocol # (if available):	
Date:	4/21/2021

APPENDIX A: BREEDING COLONIES

B1. JUSTIFICATION for Breeding On Site

Please check the factors that justify the breeding of animals at GMU. Mark an "X" in all applicable box(es):

X	**Animals are not available commercially or are not available in sufficient numbers.**		**The physiologic status of the mutant is too severely affected for it to survive shipment**
	Study requires that a large population of animals be available at all times.		**Captive populations reduce the need to replenish colony numbers from wild stock.**
	Transgenics require back-crossing or other management strategies to generate the correct type of research animals.		**Animals are needed at an age that would preclude safe shipping (i.e., embryos or neonates).**
	Other:		

These animals display or possess the following characteristics that make them useful for our supported research project: (Describe the genotype and/or phenotype that make them useful for this research. Include the genotype(s) specifically required for research purposes.)

This study will exclusively use the wildtype C57BL/6J which is the most commonly used mouse for research purposes.

B2. BREEDING METHODOLOGY and BREEDING COLONY MANAGEMENT

The colony will follow the management plan below to breed animals (mark an "X" in applicable box and provide additional information as requested):

	Monogamous pairs (single male permanently housed with single female)
	Single pairs (single male and single female in the breeding pairs, but rotate breeders; not permanently mated)

X	**Harem breeding (single male with multiple females)** Harem breeding strategies must follow requirements in the 8th Ed. of *The Guide 2011* (or most current version). Females should be separated out and housed singly after mating and before delivering pups. Describe ratio of males to females below.
For pair mating systems, animals will be bred using a:	
	Continuous mating system (male and female are housed together continuously, thereby taking advantage of the post-partem estrous)
X	**Interrupted mating system** (male and female are separated after mating but before pups are born)
Artificial insemination and/or chemical estrus synchronization:	
	Animals will be artificially inseminated. Describe procedures below. Include chemical estrus synchronization if used.
Additional colony management information:	
Breeding colony manager and contact information:	[redacted]
Specific colony management practices include [discuss length of time males left with females (be consistent with breeding system descriptions indicated above), weaning age of offspring, procedures for separating animals at weaning, retirement age of breeders, source of replacement breeders, methods for monitoring and maintaining genetic integrity of the colony, special procedures performed on suckling or weanling animals (if genotyping will be performed, state so here and describe in B3 below), disposition of retired breeders and animals not suitable for experimental use or as replacement breeders, etc.]:	
1 male mouse will be paired with 2 female mice for a period of two weeks. Two weeks prior to pairing, all breeders will receive 2 pellets each of "Love Mash" which is commercially available through Bio-Serv, a certified company that has developed diets and enrichment products for research animals. This is used as a supplement to the normal diet and was developed to help increase fertility and milk production in mothers and also decrease stress. Two pellets will be added to the food source every other day. Starting one week prior to pairing, bedding from the female cage will be added to the male cage and bedding from the male cage will be added to the female cage to introduce the scent of each of the breeding pairs. The female mice will all be housed together for 3 weeks during pregnancy and separated just prior to birthing to avoid cannibalization of pup, unintentional fostering and overcrowding with pups. Researcher will check for vaginal plug, the date when the plug is observed will be gestational day 0 to 0.5. However not all females will plug, thus, to ensure females are pregnant researcher will observe their body shape daily. If their abdomen has a bulge giving them a pear-shaped appearance, or enlarged nipples,	

they will be considered pregnant and separated out. Upon separation, female mice will be housed singly for the duration of their pregnancy until pups are weaned between p21-28. Once pups are weaned, females will be returned to their harem cages, until the next breeding cycle, which will occur NO SOONER than 2 weeks post weaning. Mice are weaned 3-4 weeks after birth. We will assess whether or not mice are ready for weaning based on activity level. The mice will have adult fur, teeth, and their eyes will be fully open before weaning is completed. Mice will be closely monitored for the first few days after weaning in order to be sure they are properly adjusting. All mice will be assessed daily for general well-being by the animal husbandry staff and/or secondary investigators. Mice will not be bred for more than three rounds in order to prevent genetic drift. As all breeding pairs are the same genotype, offspring will all be the C57BL/6J mice.
Recordkeeping/animal use tracking systems: Provide a comprehensive description of the colony recordkeeping system used for tracking breeding, production, and animal disposition.
All records of mice will be recorded in a notebook and then transferred into an online document. Dates of deliveries, acquisition period, Love Mash diet administration, bedding transfers, harem forming, separation of harems, dates of birth, dates of weaning and number of offspring will be recorded. A clinical scoring sheet will be kept for each TBI administration and injection monitoring the health of the animal. Weaning and ear punching will be recorded once offspring are old enough and the source animal of each mouse will be recorded on the notebook and on the notecard on the front of the mouse cage, as discussed above. Aggressive, measures of general health and attrition will also be recorded.
Are there any special health concerns, phenotypic abnormalities, etc., that may adversely impact the health and welfare of the animals?

	Yes. Describe below.
X	**No.**

B3. GENOTYPING (fin punch, ear punch, tail snip, etc.)

Animals will be genotyped at a <u>juvenile</u> stage Mark an "X" in applicable box **Note**: Juvenile = 10-21 days for mice/rats; list in **pain category C.**	
	Yes. Please describe genotyping procedure below including the type and use of anesthesia for this procedure.

X	**No.**
Animals will be genotyped at <u>adult</u> stage. Mark an "X" in applicable box **Note**: Rats/Mice > 21 days, use local or general anesthesia; rats > 35 days, general anesthesia is required; list in **pain category D.**	
	Yes. Please describe genotyping procedure below including the type and use of anesthesia for this procedure.
X	**No.**

B4. ANIMAL NUMBERS Add rows to table as necessary for additional crosses. Total number of breeding animals and offspring in this Appendix must be described and justified in Section III.9. of the main protocol form.

Species	Strains Crossed (if homogeneous breeding, please insert same strain name in each of the two boxes below)			# Breeding Males	# Breeding Females	Predicted # of Offspring (est. litter size X # litters)	Predicted # of Usable Offspring	# Offspring Euthanized w/o Being Used	Total # animals this cross (incl. breeding prs)
C57BL/6J Cohort 1: Experimental Mice	C57BL/6J	X	C57BL/6J	5	10	100 mice (10 pups per mom)	100	0 All offspring will be used	115
C57BL/6J Cohort 2: Experimental Mice	C57BL/6J	X	C57BL/6J	5 (same males as first cohort)	10 (same females as first cohort)	100 mice (10 pups per mom)	100	0	100 (breeding pairs are the same from cohort 1, no additional breeders will be used)

C57BL/6J Cohort 1: Donor Mice	C57BL/6J	X	C57BL/6J	8	16	160 mice (10 pups per mom)	160	0	184
C57BL/6J Cohort 2: Donor Mice	C57BL/6J	X	C57BL/6J	8 (same males as the first cohort)	16 (same males as the first cohort)	160 mice (10 pups per mom)	160	0	160 (breeding pairs are the same from the first cohort, no additional breeders will be used)
C57BL/6J Preliminary Work Mice	C57BL/6J	X	C57BL/6J	2	4	30 mice	30	0 Any mice over 30 produced will be used as donor mice	36
C57BL/6J Cohort 1: Additional Donor Mice	C57BL/6J	X	C57BL/6J	8	16	160 mice (10 pups per mom)	160	0	184
C57BL/6J Cohort 2: Additional Donor Mice	C57BL/6J	X	C57BL/6J	8 (same males as the first cohort)	16 (same males as the first cohort)	160 mice (10 pups per mom)	160	0	160 (breeding pairs are the same from the first cohort, no additional breeders

									will be used)
									Total Mice: 939
If heterozygote breeders must be used, explain why this is necessary (Heterozygote breeding systems result in 50% heterozygotic offspring that may not be useful for research purposes. Explain why this system must be used, e.g., homozygous males or females are sterile; homozygous males or females have a lethal mutation; etc.)**:** N/A									

Explain any animals euthanized without being used: All offspring will be used for this experiment