

VA Puget Sound Health Care System Research & Development Institutional Animal Care and Use Committee (IACUC)		VAPSHCS ACORP: MAIN BODY (Version 4.3)
Last Name of PI: ► Iliff	Protocol #: ► 0947	IRBNet ID: ► 1588099
Official Date of Approval: ► 08-04-2022		Date of Approval of Change: ► 08-22-2024

ANIMAL COMPONENT OF RESEARCH PROTOCOL (ACORP): Main Body

See [Instructions for Completion of the Animal Component of Research Protocol \(ACORP Instructions\)](#), for help in completing specific items.

A. ACORP Status.

1. Full Name of Principal Investigator(s)
► **Jeffrey J. Iliff**
2. VA Station Name (City) and 3-Digit Station Number
► **Seattle, 663**
3. Protocol Title
► **Defining the role of age-related glymphatic pathway impairment in amyloid beta plaque deposition**
4. Animal Species covered by this ACORP
► **Mouse**
5. Funding Source(s). Check each source that applies:
 - () Department of Veterans Affairs.
 - (X) US Public Health Service (e.g. NIH).
 - (X) Private or Charitable Foundation -- Identify the Foundation: **Alzheimer's Association**
 - () University Intramural Funds -- Identify the University and Funding Component:
 - () Private Company -- Identify the Company:
 - () Other -- Identify Other Source(s):
6. Related Documentation for IACUC reference.
 - () This ACORP is being submitted for its INITIAL REVIEW (Answer 6a and 6c, but do not answer 6b)
 - (X) This ACORP is being submitted for its THREE YEAR DE NOVO REVIEW (Answer 6a, 6b, and 6c)
 - a. If this protocol applies to an R&D project that has already been submitted to the R&D Committee for review, identify the project:
 - (1) Title of project
► **Defining the role of age-related glymphatic pathway impairment in amyloid beta plaque deposition**
 - (2) If approved by the R&D Committee, give the most recent date of approval and IRBNet ID:
► **9/12/2019**
 - b. Triennial review. If this protocol is being submitted for triennial *de novo* review, complete the following:
 - (1) Identify the studies described in the previously approved ACORP that have already been completed
► As proposed in the original version of this protocol, *5xFAD:Snta1^{-/-}* and *5xFAD:Aqp4^{-/-}* mouse lines were generated by crossing the *5xFAD* mouse line with each the *Snta1^{-/-}* and *Aqp4^{-/-}* lines. Both lines were aged 2 and 4 months before being perfused and brain tissue collected for histological analysis. This study is complete and the *5xFAD:Snta1^{-/-}* and *5xFAD:Aqp4^{-/-}* mouse lines have been terminated.

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We completed and published a methodology paper validating the use of the LI-COR Pearl live animal imaging system to visualize CSF tracer distribution over the cortical surface of the brain in real-time in wild type C57BL/6 mice. This study confirmed that this technique permits sensitive dynamic imaging of glymphatic function. It also confirmed that ketamine/xylazine (K/X) anesthesia allows for more normal distribution of tracer compared to isoflurane, which shows diminished glymphatic function by reduced CSF tracer distribution. This study optimized and validated the use of the LI-COR system for tracer distribution experiments in our hands and resulted in the development of the method for future experiments using the equipment. This study also resulted in the determination that we will only use K/X anesthesia going forward for all measures of glymphatic function.

C57BL/6, *Snta1*^{-/-}, and *Aqp4*^{-/-} mice were bred for MRI and LI-COR in vivo imaging experiments proposed in the protocol amendments approved on 9/29/2020. The LI-COR experiments, where tracers were injected into the cisterna magna and monitored for CSF distribution, were completed and tissue was collected for further analysis. After several attempts the MRI experiments were abandoned, due to high mortality rates of mice while inside the MRI equipment.

The initial study, which proposed to cross and use *5xFAD:Pad4* mice for CSF tracer distribution and behavioral testing has been completed. *5xFAD:Pad4* mice were successfully crossed with all genotypes surviving to weaning age. Tissue has been collected for analysis. The amendment for this study was approved by the IACUC on 3/15/2021. The next stages of this study are proposed below in section C.

The 5xFAD mouse and sleep fragmentation study to inform on the role of age-related glymphatic dysfunction on amyloid plaque deposition within the brain, approved by the IACUC on 5/6/2021, is partially complete, a proposal for the remaining animals required to complete the study is included below in section C.

- (2) Indicate the numbers of animals of each breed/strain/genotype that have already been used, and adjust the numbers shown in Item I accordingly

► Due to the COVID-19 pandemic, there was a period of a few months where we were unable to access the mouse colony. Breeding continued, but many litters born shortly prior to and during that time were of the wrong age for experiments by the time we were able to get back into the colony and a large number had to be euthanized.

For the transgenic colonies, breeding has proved problematic, especially during times of construction and other noise/vibrations, and many stillborn and small litters (2-4 pups) have resulted, as well as an increased number of unhealthy runt pups that needed to be euthanized. Often the knockout animals are the most likely to not survive to experimental age. With small litters and a lower proportion of knockout mice in litters, it has been difficult to assemble complete cohorts for experiments, therefore, many more animals had to be generated than were anticipated in the original proposed studies. Whenever possible, extra cages were offered to the ARF for training purposes or to other labs looking for extra mice. To minimize the waste of animals as much as possible, within the lab, extra mice were used for training students learning new procedures, or tissue was collected and banked for potential future use by our lab or collaborators.

The transgenic lines, not currently being used in studies (*Aqp4*^{-/-}, *Snta1*^{-/-} and *5xFAD* [once current study is complete]), continue to be maintained, as they are difficult to obtain commercially. Therefore, one to

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two breeder cages are always ongoing, and a few cages of offspring are kept on-hand as replacement breeders. Also, we often maintain a breeder cage of C57BL/6 mice to use for ease of backcrossing to transgenic lines to maintain health and be used as breeder mates for 5x*FAD* mice, which can only be bred to wildtype animals.

See table below for numbers of each background strain/transgene/genotype that have already been used on this protocol:

Background Strain	Transgene	Genotype						Ungenotyped	Total mice
C57BL/6J									311
		Wild type	FAD+						
C57BL/6J	5XFAD	180	191					47	418
		Wild type	HET	KO					
C57BL/6J	Aqp4-/-	46	113	34				46	239
		Wild type	HET	KO					
C57BL/6J	Snta1-/-	46	81	36				226	389
		Wild type	HET	KO					
C57BL/6J	Pad4-/-	9	19	5				31	64
		Wild type	FAD+/WT	WT/HET	FAD+/HET	WT/KO	FAD+/KO		
C57BL/6J	5XFAD:Aqp4-/-	16	35	37	31	15	12	116	262
		Wild type	FAD+/WT	WT/HET	FAD+/HET	WT/KO	FAD+/KO		
C57BL/6J	5XFAD:Snta1-/-	50	42	84	86	32	27	282	603
		Wild type	FAD+/WT	WT/HET	FAD+/HET	WT/KO	FAD+/KO		
C57BL/6J	5XFAD:Pad4-/-	32	35	69	69	27	27	89	348
Grand Total									2634

Due to COVID-19 staff shortages and lockdown, mis-timed (from aging breeders and transgenic lines that breed poorly) and small litters, litters that were from maintenance breeding, as well as animals that were lost after weaning but prior to genotyping, not all cages were genotyped, the ungenotyped numbers are noted in the table above.

(3) Describe any study results that have prompted changes to the protocol, and briefly summarize those changes, to guide the reviewers to the details documented in other Items below.

► The study to validate the use of the LI-COR Pearl system to evaluate glymphatic function (discussed above in 6.b.1), which showed that isoflurane anesthesia diminishes normal CSF tracer distribution compared to K/X, has informed the type of anesthesia to be used for all future studies. We will no longer use isoflurane for any measures of glymphatic function. This study also determined the experimental timing and optimal settings for all future studies using the LI-COR system.

c. List any other relevant previously approved animal use protocols (copy the lines below as needed for each protocol listed) For example, breeding protocols that produce animals for this protocol, or vice-versa.

- (1) Title of other protocol ► **Role of perivascular aquaporin-4 polarization in post-traumatic neurodegeneration**
- (2) IACUC approval number of other protocol ► **0949**

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Give the name of the VA station or other institution that approved it, if it was not approved by the IACUC that will review this ACORP ► **N/A**

7. Indicate the type(s) of animal use covered by this protocol (check all that apply):

- ☒ Research
- ☐ Teaching or Training
- ☐ Testing
- ☒ Breeding and colony management only; not for any specific research project
- ☐ Holding protocol (as specified by local requirements; not required by VA, PHS, or USDA)
- ☐ Other. Please specify ►

Proposal Overview

B. **Description of Relevance and Harm/Benefit Analysis.** Using non-technical (lay) language that a senior high school student would understand, briefly describe how this research project is intended to improve the health of people and/or other animals, or otherwise to serve the good of society, and explain how these benefits outweigh the pain or distress that may be caused in the animals that are to be used for this protocol.

► Advanced age is the strongest risk factor for the development of Alzheimer's disease (AD), with incidence increasing from 11% at the age of 65 to 32% of people aged 85 or older. Neurodegeneration, like that associated with AD, is the progressive loss of structure and function of neurons, including neuron death. We are interested in identifying what contributes to neurodegeneration in the aging brain. Our work has suggested that the close association between the brain's supportive cells, called glia, and the blood vessels of the brain is critical for maintaining basic neuron function by clearing out waste. We seek to understand how this relationship goes awry as the brain ages and how these processes might be targeted in the treatment of neurodegeneration.

We have demonstrated that the clearing of brain waste such as the protein, amyloid-beta (A β), via a brain-wide pathway surrounding the blood vessels that is termed the 'glymphatic' system, is slowed in the aged mouse brain. These changes may contribute to the build-up of A β into aggregates, called plaques, in the brain. These plaques have been associated with neurodegenerative conditions, such as AD. We propose to evaluate how changes in brain water transport contribute to the failure of these waste clearance pathways and additionally observe the impact of sleep disruption and inflammation in brain tissue on glymphatic function. In the aging human population, sleep quality decreases and an increased incidence of neuroinflammation associated with A β plaques is observed, therefore these two states are of interest in relation to AD and glymphatic clearance pathways that we intend to study in a mouse model.

The goal of the sleep fragmentation portion of this project is to better understand the role of age-related glymphatic system dysfunction on A β plaque deposition within the brain. In humans, this glymphatic dysfunction is thought to be partially caused by chronic sleep disruption. In fact, obstructive sleep apnea, which is characterized by frequent bouts of sleep disruption, is a significant risk factor for the development of neurodegenerative diseases including AD. Additionally, it has been found that sleep disruption leads to glymphatic dysfunction in mice. We intend to study how chronic sleep fragmentation impacts glymphatic function and A β deposition, as well as cognition (perception and learning).

Another study component in this protocol proposal relates to neuroinflammation. Neutrophils, the most abundant type of white blood cell, eject web-like structures of DNA and other cellular proteins, called neutrophil extracellular traps (NETs) when activated. While NETs contribute to the sequestration and killing of extracellular pathogens during infection, NET formation can promote inflammation, damage healthy tissue, and clog small blood vessels. In

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the context of AD, NETs are found around A β plaques in post-mortem human brain tissue, yet the relationship between NETs and amyloid is not known. We propose that NETs may clog the channels of the glymphatic pathway by physically blocking the movement of fluid and by promoting swelling and inflammation, resulting in decreased clearance of amyloid. Using a mouse model for AD, we will test whether NET degradation or impairment of NET formation improves glymphatic transport of A β and reduces cognitive impairment.

The harm, loss of life for these animals used, is offset by the possibility that the outcomes from this study could lead to a better understanding of waste clearance in the aging brain and new treatments to prevent or slow the progression of AD and other neurodegenerative diseases in humans.

C. Experimental Design.

1. **Lay Summary.** Using non-technical (lay) language that a senior high school student would understand, summarize the conceptual design of the experiment in no more than one or two paragraphs.

► **Experiment 1 – Sleep fragmentation** - The purpose of this study is to investigate how amyloid deposition increases with age, specifically focusing on known timepoints of disease progression in 2- and 4-month-old *5xFAD* animals (a transgenic mouse model of Alzheimer's Disease that develops amyloid protein build-up in the brain, as normal (wild type) mice do not spontaneously develop A β plaques on their own). In the study, we will additionally investigate if chronic sleep disruption (often experienced in humans prior to neurodegenerative disease diagnosis) will increase the amyloid plaque burden. To investigate the role sleep disruption might play in plaque deposition, we propose to use Lafayette Sleep Fragmentation Chambers. These chambers, built and developed to cause chronic sleep fragmentation like that seen in obstructive sleep apnea, serve as a home cage for the mice throughout experiment. Sleep fragmentation is performed in such a way as to disrupt but not prevent sleep using a sweeping rod every 2 minutes for the 12 daylight hours (the period of greatest sleep for mice) for 8 weeks. This paradigm does not result in absolute sleep deprivation, but instead increases sleep fragmentation and wakefulness during normal sleeping periods, while increasing sleep during normal nighttime waking periods (when the rod is inactive). We will then use cognitive behavioral testing and the evaluation of A β deposition, neuroinflammation, and Cerebrospinal fluid (CSF) tracer distribution in brain tissue to investigate the impacts of sleep disruption on glymphatic function and A β pathology.

► **Experiment 2 –NET formation and A β plaques** – In this study, we propose to use the same *5xFAD* mouse, and in some experiments the *5xFAD* will be crossed with the *Pad4*^{-/-} mouse. The *Pad4*^{-/-} line lacks the peptidyl arginine deiminase 4 (Pad4) gene which is required for the formation of NETs. By crossing the *Pad4*^{-/-} with the *5xFAD* mouse line (*5xFAD:Pad4*), we can use a genetic approach to investigate how impairment of NET formation impacts glymphatic clearance in an animal that develops A β plaques. Additionally, to complement the genetic approach, impact on glymphatic function will be tested by degrading NETs with intravenous (tail vein injection) enzyme treatment of bovine Deoxyribonuclease 1 (bovine DNase1) or recombinant human deoxyribonuclease 1 (rhDNase1) and evaluating the effect of Pad4 gene deletion (which prevents NET formation) in *5xFAD* mice. The movement of injected fluorescent tracers in the CSF will be assessed in these mice as a measure of glymphatic flow. We expect degradation of NETs and genetically targeting NET formation will improve glymphatic function and A β clearance. Cognitive function will be evaluated using behavioral testing and A β plaques and NETs will be measured in brain tissue and whole blood. We expect targeting NETs will reduce A β plaque burden in the brain and will improve behavioral test performance.

► **Experiment 3 – Investigating the effects of CSF from young, old, and Alzheimer's disease subjects on glymphatic and lymphatic function in mice** – This project aims to advance our understanding of how certain

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proteins in CSF can either improve or impair the waste removal processes in the brain. By studying these mechanisms in mice, we hope to find potential treatments for AD and other degenerative brain disorders, which are major health challenges in our aging society. This study focuses on two types of brain clearance systems: the glymphatic system (GS), which helps clear waste with the help of neuron support cells called astrocytes, and the lymphatic system (LS), which drains waste out of the brain. We are particularly interested in which proteins in young CSF can improve and which proteins in old CSF can impair clearance function these systems. Moreover, we will also examine how certain substances in CSF associated with AD, called biomarkers, contribute to the breakdown of these systems and lead to disease symptoms. It has been previously demonstrated that aged mice infused into the ventricles of their brain with CSF from young mice showed increased cognitive function and neuron support cell (oligodendrocyte) regeneration. We aim to expand on these findings from a waste clearance perspective to identify therapeutic or pathological mechanisms influenced by proteins in CSF.

2. **Complete description of the proposed use of animals.** Use the following outline to detail the proposed use of animals.

- a. **Summarize** the design of the experiment in terms of the specific groups of animals to be studied. Note: Details of animal numbers and experimental treatments are to be provided in C2c, below.

► **TRANSGENIC BREEDING**

5xFAD colony – These transgenic mice rapidly recapitulate major features of Alzheimer's disease amyloid pathology and are useful models of intraneuronal neurodegeneration and amyloid plaque formation. The rapid formation of amyloid plaques in these animals allows for us to conduct our experiments in younger animals (2-4 months of age, compared to 12 months). Amyloid deposition in the 5xFAD animals begins at 2 months of age, compared to other transgenic lines that do not show amyloid plaques until 13 months of age. 5xFAD mice must be crossed to a wild-type C57BL/6 (B6) for all breeding. Each generational cross rotates between a male and female B6 for ongoing colony health and effective propagation of the transgenes. 5xFAD animals develop Aβ plaques early in life which seems to impact breeding. Litter size and production decreases at ~4 months of age and as a result, new breeder pairs are set up regularly at approximately 2-month intervals to keep the colony healthy and productive. In our experience, litter sizes consist of approximately 5 pups with a 50/50 distribution of wildtype and 5xFAD positive animals.

5xFAD:Pad4^{-/-} colony - 5xFAD and Pad4^{-/-} have been successfully crossed in our lab and the F1 generation of this breeding produced a mixture of pups that were positive or negative for 5xFAD and heterozygous for Pad4. F1 progeny were bred to generate 5xFAD:Pad4^{-/-} and littermate 5xFAD:Pad4^{+/-} mice which are being used in proposed NET formation and Aβ plaque formation studies. 5xFAD⁺:Pad4^{-/-} pups show lower survival rates than other littermates (8% of pups have this genotype, compared to an expected 25%). It seems that the pups die in utero or shortly after birth.

The following transgenic lines will be maintained by the lab for future studies, for sharing with collaborators in other labs, and for tissue collection to send to other institutions. This is important, as transgenic mice are often difficult to obtain commercially. One to two breeder cages will always be maintained to keep each line going.

Aqp4^{-/-} mice: These mice globally lack the astroglial aquaporin-4 water channel. This makes it possible to study Aβ plaque deposition when no AQP4 channels are present and to reveal the importance of this channel in normal glymphatic function. Mice will be maintained with either a heterozygous to heterozygous cross or backcrossed as a heterozygote to a wild type B6. We intend to maintain this line by always keeping two cages in production as these animals are only commercially available from Riken in Japan and are very difficult to obtain. It is possible to get more

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live animals from Riken, if necessary, but previously it took almost one year to obtain two breeders.

Snta1^{-/-} mice: These knockout mice lack the adapter protein α-syntrophin (α-Syn), which binds AQP4 to the dystrophin-associated complex in astrocytic endfoot processes. These animals are utilized to test the hypothesis that loss of perivascular AQP4 localization slows interstitial Aβ clearance and promotes Aβ plaque deposition. Although, we are not currently proposing any studies with these mice in this protocol, we do intend to use them again for future studies. These animals are only available by cryo-recovery through Jackson Labs, and are therefore expensive and slow to obtain, therefore we propose to maintain the line in the Iliff lab’s mouse colony by keeping one to two cages always going. Mice will be maintained with either a heterozygous to heterozygous cross or backcrossed as a heterozygote to a wild type B6.

We also breed C57BL/6 mice in low quantities to have on-hand for backcrossing transgenic lines and to breed with the 5x*FAD* mice that can only be crossed to wild type B6 mice. We plan to bring in fresh B6 mice from Jax every few generations to avoid inducing genetic drift issues into all mouse lines.

► **EXPERIMENTS:**

Experiment 1 – Sleep fragmentation We propose to continue this experiment in which we induce chronic sleep fragmentation in male and female 5x*FAD* and wild type control mice. At 2 or 4 months of age, mice will be acclimated to the Lafayette Instruments sleep fragmentation chambers one week prior to beginning the sleep disruption paradigm to reduce any confounding stress from a new home cage. The chambers are similar to normal husbandry cages, but have a rod connected to the ceiling that sweeps the floor intermittently (a photo of the chambers is provided in section C.2.c). After one week of acclimation, the experimental period will begin. Enrichment that could obstruct the rod will be removed, the rod will be turned on, sweeping every 2 minutes for the 12 daylight hours (6am-6pm) and will be turned off and enrichment returned during normal dark hours (6pm-6am) each day. After 8 weeks of daily sleep disruption, the animals will undergo five consecutive days of behavioral testing.

Timeline for behavior testing:

- Day 1:** Barnes Maze Learning Trial Day 1
- Day 2:** Barnes Maze Learning Trial Day 2
- Day 3:** Barnes Maze Learning Trial Day 3
- Day 4:** Barnes Maze Learning Trial Day 4, Open Field Test
- Day 5:** Barnes Maze Prove Trial, Y-maze, Burrowing Test

Daily sleep disruption will be ongoing during the normal light period when animals are not undergoing behavioral tests. Once all days of behavioral testing are complete, mice will be anesthetized, receive a stereotaxic injection of CSF tracer into the cisterna magna, be imaged for tracer distribution in real-time using the LI-COR Pearl system and then perfused (terminal). Mice die during the perfusion procedure while phosphate buffered saline is flowing through the heart. Brain tissue will be evaluated for Aβ deposition, neuroinflammation, and changes in tracer distribution.

Experiment 2 –NET formation and Aβ plaques

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Experiment 2.a – Whole blood and brain tissue collection for analysis: Using a genetic approach, we will investigate the differences in protein expression in *5xFAD:Pad4^{-/-}* mice that develop Aβ plaques but don't produce NETs and *5xFAD:Pad4^{+/+}* mice. Brain tissue and whole blood is to be collected. Fixed brain tissue has previously been collected after fluorescent CSF tracer injection, to observe differences in glymphatic flow between genotypes, and is being analyzed presently. To investigate NET protein level differences in tissue using ELISA, non-fixed tissue is needed. Also, we need fixed brain tissue that doesn't contain fluorescent tracers, so that fluorescent antibodies can be used, post-mortem, to measure Aβ plaques and NET localization/levels. Therefore, a separate group of 4-month old *5xFAD:Pad4^{-/-}* and littermate *5xFAD:Pad4^{+/+}* mice will be used to collect whole blood via cardiac puncture and brain tissue with no injected tracers. Naïve mice will be anesthetized with isoflurane. The thorax will be opened and whole blood will be collected by cardiac puncture, immediately followed by perfusion with PBS only, and brains will be collected. Mice die during the cardiac puncture procedure due to exsanguination. One brain hemisphere will be fixed by submersion in 4% paraformaldehyde (PFA) and then paraffin embedded for tissue staining and the other hemisphere will be flash-frozen and reserved for ELISA protein assessment of NETs. Whole blood will be used to measure NETs levels.

Experiment 2.b – rhDNase1 NET degradation and glymphatic exchange:

Experiment 2.b.1 – In vivo enzyme treatment with bovine DNase1 or rhDNase1 will be used to degrade NETs to compliment the genetic approach above that used in gene deletion to prevent the formation of NETs. Originally, we proposed to use intravenous administration of recombinant human Deoxyribonuclease 1 (rhDNase1) from Genentech/Roche to degrade neutrophil extracellular traps (NETs) in mice. We now propose to use bovine DNase1 instead. RhDNase1 was first selected because it is pharmaceutical-grade, used clinically, and is FDA approved for use in human patients. However, the VA Office of Research and Development will not approve the materials transfer agreement to receive the rhDNase from Genentech. Furthermore, alternative sources of rhDNase1 are cost prohibitive for the dosage needed. Therefore, we propose to use bovine DNase1 diluted in saline, which has been widely used and published in similar experiments in mice, has been demonstrated to be safe and effective, and the enzyme is functionally the same. Dosage will remain the same, but the vehicle for bovine DNase1 is saline, not phosphate buffered saline (PBS). We will leave rhDNase1 in the protocol for now, in case it becomes available in the future.

In an initial pilot study, we will use 4-month old *5xFAD* mice, that develop Aβ plaques, but still have the *Pad4* gene and are able to form NETs. bovDNase1 or rhDNase1 (5-10 mg/kg) or vehicle (saline or PBS) will be injected into the tail vein of restrained, unanesthetized mice using a 30-gauge Hamilton syringe. 24 hours later, mice will be anesthetized with K/X injection, CSF tracers will be injected into the cisterna magna and recorded using the LI-COR live-imaging system to measure glymphatic influx. Immediately following LI-COR imaging, mice will have whole blood collected via cardiac puncture, be perfused, and brains will be collected for slice analysis and additional staining. Whole blood will be used to measure NETs and DNase1 levels in the circulation.

Experiment 2.b.2 – If CSF tracer distribution glymphatic changes are observed between the DNase1 and control group in the above pilot study (2.b.1), a follow up study will be conducted looking at the effects of rhDNase1 NET degradation on behavior. Because behavioral effects resulting from such treatments can be subtle and difficult to detect, we want to confirm first that DNase1 treatment shows measurable impact on glymphatic function. Once confirmed, treatment will be repeated in a second cohort of *5xFAD* mice (tail vein injection of 5-10 mg/kg DNase1), then behavior will be performed starting 24 hours post-DNase1 treatment. The behavioral paradigm for this study will be as follows:

Timeline for behavior testing:

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Day 1: Open Field, Y-maze, Burrowing, and Barnes Maze Learning Trial Day 1

Day 2: Barnes Maze Learning Trial Day 2

Day 3: Barnes Maze Learning Trial Day 3

Day 4: Barnes Maze Learning Trial Day 4

Day 5: Barnes Maze Prove Trial Day

Immediately following the final probe trial of Barnes Maze on Day 5, mice will be anesthetized with isoflurane, whole blood from cardiac puncture will be collected, and the mice will then be perfused, and brain tissue will be collected as follows: one brain hemisphere will be post-fixed in PFA then paraffin embedded for tissue staining and the other hemisphere will be flash-frozen and reserved for ELISA protein assessment of NETs and DNase1 levels. Whole blood will be used to measure NETs and DNase1 levels in the circulation.

Experiment 3 – Investigating the effects of CSF from young, old and Alzheimer’s disease subjects on glymphatic and lymphatic function in mice - This study integrates physiological data with CSF proteomic analyses to understand the changes in glymphatic and lymphatic system (GS/LS) functionality in mice that receive a 7-day, continuous, intracerebroventricular infusion of human CSF related to aging, AD-related biomarkers, and the AD phenotype.

We have access to human CSF samples through the MIRECC BNG Repository run by Dr. Elaine Peskind, MD. These samples have been characterized in terms of protein concentration, as well as measures of AD-related biomarkers including Amyloid beta (Aβ), Intracellular adhesion molecule (ICAM), and Vascular cell adhesion molecule (VCAM). From these samples we will obtain 3-6 different human donors per group that will be pooled together to fit the following criteria:

- [Y]: Young; Healthy female 20-30 years old
- [O]: Old; Healthy female 65-76 years old
- [B]: Old with AD-related biomarkers; Healthy female 65-76 years old
- [Aβ]: AD with Aβ without AD-related biomarkers (ICAM and VCAM); Cognitively impaired female 65-76 years old
- [AD]: AD with Aβ and AD-related biomarkers (ICAM and VCAM): Female 65-76 years old

We are using female human CSF following a similar study that was previously published. The rationale, is that AD has a higher incidence in females, suggesting that female CSF is more likely to contain proteins that disrupt the glymphatic and lymphatic systems. Currently, we do not plan to use human male CSF. We will be infusing CSF into the ventricles of both male and female mice. We have not standardized the CSF donor’s sex with the mice sex, as we do not expect the sex of the recipient mouse to make a difference in response.

In this experiment, human CSF intracerebroventricular (ICV) infusion via osmotic pump will be conducted in wild type C57BL/6 mice over a period of 7 days followed injection and imaging of fluorescent tracers in the brain (under anesthesia), then perfusion (which will end the animal’s life) and collection of brain tissue and deep cervical lymph nodes (cLNs).

Detailed Experimental Design: Each experiment (3.1 and 3.2) will undergo both stages (A and B)

Experiments

- **Experiment 3.1:** Measurement of GS/LS function
- **Experiment 3.2:** CSF and brain cell isolation

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Stages

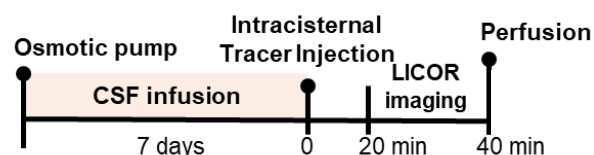
- **Stage A:** Examining the effects of human CSF in young mice (2 months of age)
- **Stage B:** Examining the effects of human CSF in aged mice (12, 14, 16, or 18 months old)

All experiments and stages will use 90 µl of pooled human CSF which will be infused ICV into the brain at a rate of 0.5 µl/hr for 7 days using an Alzet osmotic pump.

EXPERIMENTS:

- **Experiment 3.1: Measurement of GS/LS function** – In a single surgical procedure, mice will be implanted with an intracerebroventricular (ICV) cannula and subcutaneous osmotic pump under isoflurane anesthesia*. The pump will immediately begin infusing CSF upon implantation and 90 µl of artificial CSF (aCSF) or human CSF will be infused at a rate of 0.5 µl/hr for 7 days. Following the 7-day CSF infusion, under i.p. K/X anesthesia, as a terminal procedure, we will inject infrared and fluorescent tracers into the cisterna magna and use LI-COR imaging to visualize CSF dynamics/glymphatic system function over a maximum of 40-minutes post-injection, then perfuse the mice and examine the brain and cervical lymph nodes (cLNs) post-mortem following euthanasia.

These above processes are all conducted in the same mouse. However, for measuring the protein levels of astrocytes and lymphatic vessels by sectioning and immuno-staining, we will need an additional cohort of mice as the isolation method is different. During the brain sectioning process, the meninges must be cut, which means separate mice are needed to observe the lymphatic vessels in the meninges. Thus, a cohort of 11 mice per group will receive a tracer injection along with LI-COR in vivo imaging, and post-perfusion will have their brains isolated and sectioned to analyze the astrocytes within the brain and cLNs, assessing the glymphatic system. A second cohort of 11 mice per group will receive the tracer injection, without LI-COR in vivo imaging, followed by post-perfusion extraction of meningeal tissue to observe lymphatic vessel coverage and identify changes in the lymphatic vessels.



- **Experiment 3.2: CSF and Cell isolation** – As in Experiment 3.1, mice will be surgically implanted with an ICV cannula and osmotic pump*. Post 7-day CSF infusion, mice will be anesthetized with i.p. KX and CSF will be collected for proteomic analysis. Following CSF extraction, the mice will be perfused with ice-cold PBS, and the meninges and brain will be isolated to extract lymphatic endothelial cells (LECs) and astrocytes, respectively. These cells will then be analyzed for gene-level changes. Although different mice will be used in Experiment 3.2 from those in Experiment 3.1, a single mouse can undergo the procedures of CSF extraction, LEC isolation, and astrocyte isolation. This approach ensures efficient use of each mouse while allowing us to gather comprehensive data on the effects of human CSF infusion.

*The surgical procedure details for ICV cannula and subcutaneous osmotic pump implantation, CSF collection, and perfusion used in Experiments 3.1 and 3.2 are provided in section C.2.c and Appendix 5.

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EXPERIMENTAL STAGES:

- **Stage A: Comparative Young Mouse Infusion** - Young mice (2 months old) will be infused with human CSF (five CSF conditions: Young (Y), Old (O), B (AD-related biomarkers), Aβ (AD with Aβ without AD-related biomarkers), AD (AD with Aβ and AD-related biomarkers) or artificial CSF (aCSF) for 7 days. Functional changes in the glymphatic and lymphatic systems will be assessed, along with protein levels in CSF and gene-level changes in astrocytes and lymphatic endothelial cells.
 - **Experiment 3.1:** 2 sexes x 2 tissue collection groups (brain tissue vs meninges) x 6 treatment groups (aCSF, Y, O, B, Aβ, AD) x 11 mice = 264 mice
 - **Experiment 3.2:** 2 sexes x 6 treatment groups (aCSF, Y, O, B, Aβ, AD) x 40 mice = 480 mice
- **Stage B: Aged Mouse Differential Infusion** - Aged mice (12-18 months old) will be used to study functional changes in the glymphatic and lymphatic systems. First, we will identify the optimal age range (12, 14, 16, 18 months) for observing significant changes and then perform the same experiments as in Stage A using the selected age group (Age optimization group). For age optimization, we will conduct Experiment 3.1 using 12, 14, 16, and 18-month-old mice and total of 176 mice are required. We will conduct Experiment 3.1 to compare the integrity of the glymphatic system and identify the age group that shows the most significant difference between Young and Old CSF infused group. This stage is crucial because the extent of glymphatic system damage can vary widely with age. By treating the mice with Young and Old CSF, we can more accurately identify the age group that exhibits the most significant changes in glymphatic function. This approach allows us to pinpoint the age at which is most responsive to CSF conditions, thereby providing a clearer understanding of how CSF variability impacts brain waste clearance. Once the appropriate age is determined, we will proceed with Experiments 3.1 and 3.2 in the selected age group, following the same protocol as Stage A.
 - **Experiment 3.1 (Age optimization):** 2 sexes x 4 age groups (12, 14, 16, and 18 months) x 2 treatment groups (Y and O) x 11 mice = 176 mice
 - **Experiment 3.1:** 2 sexes x 2 tissue collection groups (brain tissue vs meninges) x 6 treatment groups (aCSF, Y, O, B, Aβ, AD) x 11 mice = 264 mice
 - **Experiment 3.2:** 2 sexes x 6 treatment groups (aCSF, Y, O, B, Aβ, AD) x 40 mice = 480 mice

Trial Phase: Before we begin the above experiments, we need to optimize the procedures and observe the effects of injecting human CSF into mice. It is possible that human CSF may trigger an immune response in the C57BL/6 mice, so we plan to do a trial phase first to both troubleshoot the experimental procedures and outputs, and monitor the results of the CSF infusion. For this, we will need 80 mice (2 sexes x 2 Experiments (3.1 and 3.2) x 2 treatments [aCSF, control vs human CSF] x 10 mice). If a severe immune reaction occurs in this trial, we would need to switch to an immuno-incompetent nude mouse line. In that event, we would seek approval from the IACUC for a modification which would include of the addition of these mice.

Additionally, the use of osmotic pumps for CSF infusion and dissection of cLNs and meninges are new procedures in our lab and some personnel have not performed mouse cannulation surgery before, therefore we will require practice animals before beginning the actual experiments and trial phase laid out above. We estimate that we will need 30 mice for practice and training purposes. The same mice can be used for both surgical and dissection practice. We plan to use extra mice that are no longer needed from other groups/projects whenever possible to minimize animal numbers.

All non-practice C57BL/6 mice for Experiment 3 will be purchased commercially.

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- Overall, the total number of mice required is as follows:
- C57BL/6 (2 months old): 854 mice
 - C57BL/6 (12-18 months old) mice: 920 mice

Experiment 3 Required Mouse Numbers: Total 1774

Stage	Expt.	GS/LS	aCSF	Y	O	B	Aβ	AD	Sex	C57BL/6 (2 months)	C57BL/6 (12-18 months)
Practice			30						Both	30 (any age)	
Trial	1		20	20					Both	40 (any age)	
	2		20	20					Both	40 (any age)	
Stage A	1	Brain	11	11	11	11	11	11	Both	264	
		Meninges	11	11	11	11	11	11	Both		
	2		40	40	40	40	40	40	Both	480	
Stage B: Age Optimization (4 ages*)	1	Brain		11 x 4 ages	11 x 4 ages				Both		176
Stage B	1	Brain	11	11	11	11	11	11	Both		264
		Meninges	11	11	11	11	11	11	Both		
	2		40	40	40	40	40	40	Both		480
*12, 14, 16, and 18 months of age									Total number	854	920

Experiment 4 (Schindler lab collaboration) - We have ongoing collaborations with the Schindler lab (blast trauma, alcohol, and aging) that involve transferring mice from the Schindler lab to the Iliff lab for tracer injection followed by glymphatic imaging and transcardial perfusion (terminal procedure). Wild type C57BL6/J animals for Aim 1 and 2 will be purchased on the Schindler protocol. For Aim 3, Aqp4^{-/-} mice and their wild type littermates will be bred on this protocol and then transferred to Schindler lab protocol #0917. Following blast trauma procedure and/or alcohol or prazosin treatments on #0917, mice will be transferred back to this protocol for stereotaxic cisterna magna glymphatic tracer microinjection (Category D) and LICOR tracer imaging, followed by transcardial perfusion (Category D), terminating the animals on this protocol. These procedures will be performed by experienced Iliff lab staff approved on this protocol. Animal numbers are requested and approved on the Schindler protocol and are as follows:

Table 1						
	Genotype/ Drug	Sham/blast age (months)	Start alcohol age (months)	Endpoint age (months)	groups	n total
Aim 1,2	WT	3	4	12	male/female; sham/blast	64
	WT	3	4	24	male/female; sham/blast	64
	WT	3	12	24	male/female; sham/blast	64
Aim 3	AQP4	x	4	12	male/female; WT/KO	64
	AQP4	x	4	24	male/female; WT/KO	64
	AQP4	x	12	24	male/female; WT/KO	64
	Prazosin	3	4	24	male/female; sham/blast; vehicle/drug	128
TOTAL						512

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b. **Justify the group sizes and the total numbers of animals requested.** A power analysis is strongly encouraged; see ACORP instructions.

► An n=10 is sufficient to detect differences in CSF protein tracer clearance experiments, but an n=15 is required to detect differences in behavioral test results for Experiment 1 and 2 due to the higher variability associated with this procedure. Sample sizes have been determined based upon preliminary and published studies carried out within this group, based on statistical power analysis of similar types of data from mice and rats, and using power analysis research calculators (<https://www.dssresearch.com/resources/calculators/>). Based upon reported effect sizes and variance, sample sizes between 8-15 are necessary to detect 10-20% differences with alpha=0.05 and power of 0.8.

Maintenance breeding of mouse lines* – We need to maintain the following 4 mouse lines:

- **C57BL6/J** – although commercially available we would like to have a couple cages on hand for readily mating with *5xFAD* animals and for back-crossing other lines. We will bring in fresh B6 mice every few generations to minimize genetic drift.
- ***Aqp4*^{-/-}** - These mice are only available from Japan and are very difficult to import
- ***Snta1*^{-/-}** - These mice are only available by cryo-recovery from Jackson Labs
- ***5xFAD*** – Once the sleep fragmentation study is complete, we would like to continue to maintain this line for studies that are currently in the planning stages. This line is a crucial tool in AD research and will be important in future studies.

We are requesting 1-2 breeder cages (one established as a productive breeder with one new one that has yet to be proven a successful breeder cage) at all times of each of the four above lines. We will only keep 2-3 cages of each line's offspring on-hand as potential future breeders. Breeders will need to be replaced 3-4 times per year, as some of the transgenic lines don't regularly produce litters past 4 months of age. Assuming a litter every 4 weeks, and with litter sizes of approximately 5-8 pups, we request mice 416 mice for colony maintenance.

4 lines x 8 pups per litter x 1 breeder cage x 13 litters/year = 416 mice per year

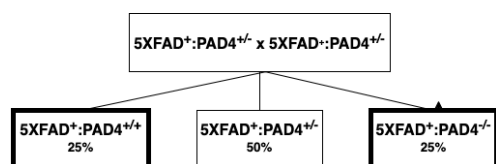
Of those 416 mice, 32 would become breeders to continue the lines. Every effort will be made to use the extra mice from these crosses within the lab for teaching/training purposes, donated to other approved protocols for tissue collection or training, or will be euthanized by a method listed in the table in section U as a last resort.

*Note: These lines, other than the *5xFAD*, are also used on our other protocol (ACORP 0949). We are currently planning studies and further amendments will be filed soon for experiments using these maintained lines on this protocol, therefore it is important to keep these lines going.

Experiment 1 - A portion of this experiment has been completed prior to submission of this renewal. We require an n=10 per group to achieve statistical significance. A remaining 6 mice per group (*5xFAD*⁻ and *5xFAD*⁺) are needed to complete this experiment, 12 mice total (6 female and 6 male). We have found that *5xFAD* litters display roughly 50/50 distribution for genotype. *5xFAD* litter sizes have averaged about 5 pups each and we need to ensure we are balanced for sex and genotype; therefore, we will require 5 more litters (estimated 25 mice) from 3 breeder pairs (6 mice) to complete this experiment.

Experiment 2.a - In this experiment we are requesting n=12 of each *5xFAD:Pad4*^{-/-} and *5xFAD:Pad4*^{+/-}, for a total of 24 experimental mice. N=10 per group has been shown to be sufficient for statistical significance, but we request an extra 2 mice per group (n=12) in case of poor quality of perfusion or other procedural issues. Since *5xFAD:Pad4* mice have to be crossed as a *5xFAD*⁻:*Pad4*^{+/-} to a *5xFAD*⁺:*Pad4*^{+/-} to maintain the *5xFAD* genes

appropriately, these crosses would theoretically produce only 25% $5xFAD^+;Pad4^{-/-}$ and 25% $5xFAD^+;Pad4^{+/+}$ pups out of each litter:



Our past breeding results have shown that although litters produce the roughly 25% expected of $5xFAD^+;Pad4^{+/+}$, litters only show closer to 8% of $5xFAD^+;Pad4^{-/-}$ pups surviving to weaning age. Therefore, to generate 12 of each useful genotype, we will require 150 offspring from the double cross. Litters sizes have been approximately 5 pups, so we would need an estimated 30 litters to complete this experiment from 12 breeder pairs (24 mice). The remaining $Pad4$ heterozygous and $5xFAD$ negative mice will be used for teaching and training, donation to ARF or other labs with approved protocols, or will be euthanized by a method listed in the table in section U as a last resort.

Experiment 2.b.1 - For this study, we also need $n=12$ per group (DNase1 treatment and vehicle control), for a total of 24 $5xFAD$ mice. This includes an extra 2 mice per group for failed tail vein injection, poor perfusion, or other procedural issues. Only $5xFAD^+$ mice will be used in this experiment, but to maintain the $5xFAD$ genes appropriately, these mice must be crossed with a B6 (wild type) mouse. Therefore, only 50% of offspring will be $5xFAD^+$ and useful in these studies. In our hands, $5xFAD$ breeding has shown roughly a 50/50 distribution of positive to wildtype mice surviving to weaning age. So, 48 mice will need to be generated to get the 24 experimental mice. The average litter size of $5xFAD$ mice has been 5 pups, meaning we will require 10 litters from 5 breeders (10 mice). The $5xFAD$ negative mice generated from these breeders are functionally wild type animals and can be used within the lab for teaching/training purposes, donated to ARF or other labs, or will be euthanized by a method listed in the table in section U as a last resort.

Experiment 2.b.2 - In this experiment, we need $n=17$ per group (DNase1 treatment and vehicle control), for a total of 34 $5xFAD$ mice. In our experience, 15 mice per group is sufficient to achieve statistical significance in behavioral studies, but we request an addition 2 mice per group (as in the experiments above) in case of procedural issues. Since we will only use $5xFAD^+$ mice, we will need to generate 68 offspring total, so 14 litters from 5 breeders (10 mice). Extra mice ($5xFAD$ negative) will be utilized as stated above.

Experiment 3 - To determine the appropriate sample size for Experiment 3.1, we used imaging data from our previous projects and performed a power analysis which determined 11 mice per group would be needed. For Experiment 3.2, we require more mice to extract cells. Lymphatic endothelial cells (LECs) are present in small portions in the meninges, making cell extraction challenging. Due to the low number of LECs in the meninges, pooling meninges from 10 mice is necessary to obtain a single sample for mRNA sequencing. Therefore, we will need 40 mice (10 per group x 4 groups) for LEC extraction in Experiment 3.2.

c. **Describe each procedure** to be performed on any animal on this protocol. (Use Appendix 9 to document any of these procedures that involve "departures" from the standards in the *Guide*. Consult the IACUC or the

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Attending Veterinarian for help in determining whether any “departures” are involved.)

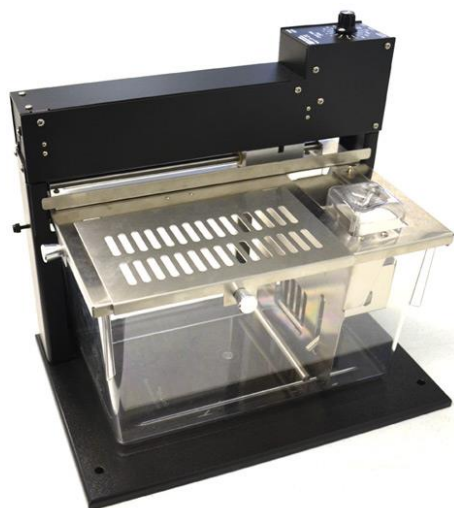
►

Breeding Colony Management – The mouse breeding colony management will be shared by lab personnel and ARF staff. Lab personnel will determine the most efficient breeding scheme to meet the requirements of the study based on litter size, frequency, and any issues with pup survival. Lab personnel will also establish the breeding pairs themselves. Breeder cages will contain one male and one female and one litter at a time, at most. ARF staff will maintain the breeding cages and wean mouse litters at 21 days of age. We expect an average litter size of 6 pups per litter for all colonies, with four to six litters per female per year on average. Depending on the mouse line, breeding longevity is between 3-8 months, therefore breeders will be switched out two to four times per year. Standard enrichment and diet will be provided for all cages. Breeders that are no longer producing regular, healthy litters will be euthanized by methods listed in the table in section U.

Genotyping – For all transgenic breeding strains, mice between the age of 21-35 days will be briefly anesthetized with isoflurane and marked for identification using a sharp ear punch tool and the ear punch tissue will be collected for genetic analysis of genotype. Mice receive no more than 2 x 2mm round ear punches per ear. Animals will be returned to the home cage and monitored until they fully recover from anesthesia. The ear punch procedure will be performed by lab personnel. Collected tissue will be sent out for commercial genotyping.

Chronic Sleep disruption: Socially housed mice (2-5 per chamber) will first be habituated to the Lafayette Sleep Fragmentation Chambers (see photo below). Mice always have ad libitum access to food and water in the chambers. Habituation consists of a one-week acclimation to the chambers to reduce any confounding stress from a new home cage, with the same food and enrichment that was available in the home cage, but the sweeping rod will not be turned on. This will familiarize the animals with the chambers and the motionless rod. After one week of habituation, the chambers will be turned on daily (7 days a week) during normal light hours (6am-6pm) and will be turned off during normal dark hours (6pm-6am) each day for a total of 8 weeks. Enrichment is removed during the hours that the rod is turned on (6am-6pm) as it will obstruct and jam the rod. Control chambers will also have enrichment removed daily from 6am-6pm and the rod will be turned on for 2 minutes in the morning to expose the controls to it, without it impacting their ability to sleep normally, but then the rod will remain off the rest of the 12-hour period. All animal enrichment devices will be returned to the chamber during the period the rod is not sweeping across the chamber floor. After the 8-week sleep fragmentation paradigm, all mice will undergo 1 week of cognitive behavioral testing, see “Behavioral Testing” procedure below. During this 5-day period, the 6am-6pm sleep chamber sleep fragmentation protocol will continue during the times the animals are not being tested. Following completion of behavioral testing, mice will be anesthetized with K/X and injected into the cisterna magna with fluorescent tracers during stereotaxic surgery, then live-imaged on the LI-COR Pearl system before being perfused (see “Stereotaxic microinjection of tracers”, “LI-COR imaging”, and “Perfusion” procedures below).

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Lafayette Sleep Fragmentation Chamber (Dimensions: 9.625"L x 8.25"W x 7.75"H)

Behavioral Testing: Animals will be tested at 2-4 months of age. On each testing day, animals are moved into the testing room 45-60 minutes prior to the start of testing, to allow them to acclimate to the testing room. On the day before the first testing day, animals are moved into the testing room to acclimate, then weighed and briefly handled by the experimenter to habituate them to transport and handling. All testing equipment is sprayed with ethanol and wiped clean with a paper towel in between testing each animal, to sanitize the equipment and remove any scent traces that influence behavior. At the end of each day of use, the behavior test equipment is wiped with a peroxide sani-wipe for disinfection. The behavior test schedule includes the following:

- **Barnes Maze** – The Barnes maze tests spatial learning and memory. Testing will be conducted during the animals' light phase, and the testing room will be brightly lit. The Barnes maze is a large circular platform made of white plastic, which has 20 small circular holes around the perimeter. One of the holes has a dark escape box located underneath it. Visual cues are placed around the maze, and the mouse must learn the location of the escape box based on spatial memory of the visual cues. Mice should be motivated to find and enter the escape box based on their natural dislike for open brightly lit spaces. On each training day, each mouse will receive four trials of 3 minutes each, during which time they are allowed to explore the maze freely. Each mouse will be placed at the center of the maze to start, and their movements will be recorded by a computer tracking system. Total locomotion will be measured, as well as the amount of time elapsed before entering the escape box. At the end of three minutes, if the mouse has not entered the escape box, it will be gently placed inside the box for 30 seconds before returning it to the home cage. The probe trial on day 5 is identical to the test trials, except that the escape box is removed.
- **Open Field Activity** – Each animal will be allowed to explore a 16 x 16-inch square field for 10 minutes, and total distance traveled will be recorded by a computer tracking system. In addition to general activity levels and speed travelled, the amount of time spent in the center versus the edges of the open field will also be recorded as a measure of anxiety-like behavior.

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- **Burrowing** - Burrowing is a normal spontaneous behavior in various rodent species, which is disturbed in various models of neurological disease. Animals are placed in individual cages, identical to the home cage but with the addition of a burrow partially filled with bedding material. The burrow is a plastic tube approximately 20cm long and 6cm in diameter, closed at one end. The open end is propped up 2cm off the cage floor by metal screws bolted to the tube. Normal animals will dig in the burrow and displace some percentage of the bedding. The amount remaining in the burrow after one hour will be measured in grams. Fresh bedding material is used for each animal tested.
- **Spontaneous Alternation Y-maze** – This test measures spatial working memory by assessing how many times the mouse moves into a new vs previously explored arm. A mouse is placed in the center of the Y-shaped maze and the dividing arms are removed at the start of the test. The mouse is allowed to explore the maze for 10 minutes and then removed back to its home cage.

Tail vein injection – This procedure will be performed as part of Experiment 2. Mice will be administered a systemic 100 µl bolus of DNase1 (5-10 mg/kg) or vehicle (saline) by tail vein injection using a 30-gauge Hamilton syringe. Injections will be administered using a tail vein injection platform from Braintree Scientific as a restraint device, pictured below. The animal is placed on the platform and the tail is gently pulled through the slot, securing the mouse for the injection. This will significantly reduce the number of animals wasted due to failed injections.



Braintree Tail Vein injection platform

Stereotaxic microinjection of tracers (terminal) – Mice 2 to 4 months of age will be anesthetized with intraperitoneal (i.p.) ketamine/xylazine. Ketamine must be used for these studies, as isoflurane has been shown to interact with the glymphatic waste clearance system that we are studying. The animal will be monitored for any sign of responsiveness or movement to mild noxious stimulation during the procedure and additional anesthesia given if needed. The surgical site is shaved to remove the fur. The skin is disinfected with betadine and 70% ethanol. The surgeon will wear clean gloves, and a lab coat. Mice will be positioned in a stereotaxic frame. The mouse is placed on a water-circulating warming pad during the procedure.

- **Intracortical injection** - A 1 cm rostral-caudal midline skin incision is made over the cranium with dissection of soft tissue away from the calvarium. A hole is drilled into the skull above the injection tract. 1-5 microliters of agents (fluorophore-conjugated dextran tracers or dyes) will be injected at 0.5 µl/min by using a 5-µl Hamilton syringe with a 30-gauge beveled needle attached to a syringe pump. Following agent delivery, the syringe will be left in place for 10 min before being withdrawn from the brain (this minimizes backflow of CSF and agents out of the skull).
- **Intracisternal injection** – A ~2 cm rostral-caudal midline skin incision is made over the cranium and muscles of the neck. The muscles of the neck are retracted with suture to expose the transparent membrane of the atlanto-occipital membrane. Care is taken to avoid the blood vessels of the cisterna magna. A 30-gauge needle with fine tubing attached to a 1 ml syringe is inserted through the membrane. 10 µl of tracer (or in the case of training, Evans Blue dye will be used to visualize the

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injection site) will be injected at a rate of 1 ul/min into the cisterna magna. using a syringe pump. Following agent delivery, the syringe will be left in place for 10 min and then withdrawn from the cisterna magna to minimize backflow of CSF and agents from the brain.

The animal's anesthetic plane will be maintained for a maximum of 120 minutes post-injection using ketamine (no xylazine). Some animals will be live-imaged in the LI-COR system during the post-injection period (see "LI-COR imaging" procedure below). After all stereotaxic injection surgical procedures, animals will be perfused for histological evaluation (see "Perfusion" procedure below). This is a terminal procedure therefore each animal will undergo the surgery only once. Perfusions will be performed under isoflurane anesthesia, as animals are usually beginning to recover from K/X at this point. Concern for isoflurane's impact on tracer distribution is no longer an issue, as the tracer distribution experiment terminates at perfusion. Post-perfusion, brains will be collected and evaluated for A β deposition, neuroinflammation, and changes in tracer distribution.

LI-COR imaging – Immediately following the above microinjection of tracers into the brain, some mice will be imaged using the LI-COR Pearl small animal imaging system (see photo below). The LI-COR instrument detects fluorescence from dyes injected in vivo and is designed specifically for rodents. Only one imaging procedure will be carried out on a single mouse. Tracers will be injected into the brain as part of the non-survival surgery procedure above. After the agents are injected, mice are imaged to visualize the movement of the tracer through the brain, in real time. Mice will be imaged for 30-40 minutes, anything longer would be measuring efflux of tracer, not the influx that we are interested in. The procedure is as follows: Remaining under K/X anesthesia, with ketamine bolus dosing as needed, mice will be removed from the stereotaxic frame and placed on the bed of the imager to record the real-time CSF distribution of tracer through the brain compartments. At the end of the imaging session, mice will be euthanized by perfusion and brains collected for further analysis. We will perform these experiments in C57BL/6, 5xFAD and 5xFAD:Pad4 mice to examine the effects of AD-like pathology on CSF production and movement. Examination of CSF dynamics in our AD mouse model and in mice that have received human CSF containing A β and AD biomarkers will inform how amyloid pathology influences CSF production and/or flow. Obtaining this physiological data will help us better understand the mechanism of impaired CSF clearance of soluble proteins like A β that is observed in AD.



LI-COR Pearl Small Animal Imaging System

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CSF collection (Intracisternal) – The surgeon will wear clean gloves, and a lab coat. The mouse will be anesthetized with intraperitoneal (i.p.) ketamine/xylazine (K/X) or by inhaled isoflurane. The fur will be removed from above the cisterna magna. The mouse will be placed into a stereotactic device. The animal will be monitored for any sign of responsiveness or movement to mild noxious stimulation during the procedure and anesthesia adjusted if needed. The skin is disinfected with betadine and 70% ethanol. The mouse is placed on a warming pad during the procedure. A ~2 cm rostral-caudal midline skin incision is made over the cranium and muscles of the neck. The muscles of the neck are retracted with suture to expose the transparent membrane of the atlanto-occipital membrane. Care is taken to avoid the blood vessels of the cisterna magna. A pulled glass pipette with fine tubing attached to a 1 ml syringe is inserted through the membrane. Negative pressure will be used to collect 10-15 µl of CSF, then the pipette will be retracted. Finally, while still under anesthesia, mice will be perfused as described below to obtain brain tissue for analysis. This procedure is part of a terminal surgery, animals will not recover from anesthesia.

Cardiac Puncture – As animals will be perfused immediately after this procedure, they will be maintained in an anesthetic plan under isoflurane (4-5%), delivered in 100% oxygen at 0.5-1.0 L/min via nasal cone. Once it is confirmed that the mouse is unresponsive, an incision using surgical scissors is made caudal to the xiphoid then two incisions are made along either side of the sternum, and the chest wall is retracted anteriorly to expose the heart and major vessels. At this time, blood will be collected from the heart (500-1000 µl) using a 25-gauge needle. Mice will then be perfused with PBS as detailed below which will ensure the mouse has expired.

Perfusion – In some cases, this procedure will be performed after the stereotaxic injection surgery. As the animal's anesthetic plane will begin to lessen by the end of the injection procedure, all mice will be moved to isoflurane for the terminal perfusion procedure. Inhaled isoflurane will be used at 4% for induction and 1.5-2% during the procedure, delivered in 100% oxygen at 0.5-1.0 L/min via nasal cone. In a fume hood, the animal is placed on its back and checked for complete absence of responsiveness by pinching of the extremities (withdrawal reflex) periodically throughout the procedure. Once it is confirmed that the mouse is unresponsive, an incision using surgical scissors is made caudal to the xiphoid then two incisions are made along either side of the sternum, and the chest wall is retracted anteriorly to expose the heart and major vessels. The right atrium is opened via sharp dissection to decompress the circulatory system, and infusion of 5 mL of phosphate buffer saline (PBS) is pushed through using a syringe at 1 to 2 mL/min via needle into the left ventricle, directed toward the ascending aorta. The mouse dies during the PBS infusion. Some animals will then be perfused with 10 mL of 4% paraformaldehyde (PFA) which is pushed through at the same rate making sure that no air is introduced during the switch of solutions. The PFA is administered after the animal has died and is not included in the list of test substances. The perfusion is continued until the effluent runs clear. Tissues are harvested. This procedure is terminal.

Intracerebroventricular cannulation and osmotic pump implantation surgery - This is a survival surgery and will be conducted under sterile conditions. the mouse is anesthetized with isoflurane and the surgical site is prepared. Mice receive 2-4 mg/kg Lidocaine and 1-2 mg/kg Bupivacaine (s.c.) at the incision site and will be positioned in a stereotaxic frame. A 1.5-2 cm rostral-caudal midline skin incision will be made to expose the skull, and a hole will be drilled at the precise coordinates (with the drill positioned above the bregma and zeroed at all three coordinates, from bregma, position cannula 1.1 mm lateral and 0.5 mm ventral) for cannula insertion. The sterile micro-osmotic pump (Alzet, Model 1007D) will then be positioned under the skin at the back of the skull, with the cannula inserted through the drilled hole for ICV infusion of human CSF. The cannula will be secured to the skull with Vetbond surgical glue and the skin closed using [either wound clips or suture \(4-0 nylon, non-absorbable\) covered with a small amount of Vetbond](#). The cannula, tubing, and pump are all fully subcutaneous and no device protrudes from the skin. Finally, the mouse will be removed from anesthesia, singly housed in a clean cage placed on top of a circulating heat pad, and observed until it has fully recovered from anesthesia. Following awakening from anesthesia, mice will be monitored every 10 minutes for 30 minutes and once daily for 1-3 days to track recovery. Mice will remain singly

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housed to protect the surgical site while they recover 7 days post-procedure before termination of the experiment. Wound clips [or suture](#) will remain in place for the duration of the 7 days. Mice will receive the analgesic, Meloxicam (5 mg/kg, s.c.) during the surgery and once every 24 hours for 3 days post-surgery. This technique will allow for controlled delivery of human CSF into the mouse brain. Approximately 90 µl of human CSF will be slowly infused at a rate of 0.5 µl/hr over a period of 7 days from the osmotic pump. This setup will provide a model for studying the effects of human CSF on mouse physiology.

D. **Species.** Justify the choice of species (as well as strains, mutants) for this protocol.
► Mouse models are well accepted in the AD field, largely because of the availability of an ever-expanding toolbox of transgenic animals to evaluate the biological bases of AD. Studying the relationship between the brain vasculature, surrounding astrocytes, the cerebrospinal fluid and interstitial fluid compartments requires study in a living brain that must be conducted over weeks and months through the aging process. For these reasons, we propose to utilize the mouse as the animal model in all studies.

We use a mouse model of AD, the *5xFAD* mouse, that spontaneously develops Aβ plaques, key hallmarks of human AD. Normal wild type mice do not spontaneously develop these plaques. The reason that Aβ builds up and begins to deposit in the human aging brain remains unclear. This mouse model develops these plaques very quickly in the life span of a mouse making it ideal for studying the impacts of Aβ on glymphatic function.

The *Pad4*^{-/-} line lacks the peptidyl arginine deiminase 4 (Pad4) gene which is required for the formation of NETs. By crossing the *Pad4*^{-/-} with the *5xFAD* mouse line (*5xFAD:Pad4*^{-/-}), we can investigate how impairment of NET formation impacts glymphatic clearance in an animal that develops Aβ plaques.

The *Aqp4*^{-/-} and *Snta1*^{-/-} animals are the simplest way to test the hypothesis that AQP4 presence and localization, respectively, supports interstitial Aβ clearance in the brain.

C57BL/6 mice are the most commonly used inbred mouse strain. This makes them an ideal model for genetic and biomedical research. All the transgenic models in this project are generated on a C57BL/6 background, reinforcing this strain’s use as a wild type model in our studies.

Personnel

E. **Current qualifications and training.** (For personnel who require further training, plans for additional training will be requested in Item F.)

1. PI

Name ► Jeffrey Iliff
Animal research experience ► Dr. Iliff has nearly 20 years of experience working with mice in a research setting. Specific to this project, he has 18+ years of experience performing mouse perfusion and stereotaxic surgery (intracortical and intracisternal injections) and has a great deal of experience handling mice.

Qualifications to perform specific procedures

Specific procedure(s) that the PI will perform personally	Experience with each procedure in the species described in this ACORP
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Back-up training and consulting only* - Perfusion	Dr. Iliff has 18+ years of experience performing perfusions in mice. He will only train and provide oversight; he will not perform perfusions himself.
Back-up training and consulting only* - Stereotaxic injection surgery	Dr. Iliff has 18+ years of experience performing stereotaxic injection surgery (intracortical and intracisternal) in mice. He will only train and provide oversight; he will not perform surgeries himself.

*Dr. Iliff will not perform the surgeries above, he will only supervise or consult personnel during their training period and, only in the event that no other trained personnel are available.

2. Other research personnel (copy the lines below for each individual)

Name ► Katherine Suchland

Animal research experience ► Ms. Suchland has worked with rodents in research for 20+ years and has a great deal of experience in handling animals and conducting surgical procedures.

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this ACORP
Ear punch	Ms. Suchland has 15+ years of experience identifying mice by ear punch and collecting tissue for genotyping purposes.
Behavioral Testing	Ms. Suchland has 15+ years of experience working with mice in behavioral paradigms.

Name ► Mathew Sevaio

Animal research experience ► Mr. Sevaio has worked with mice in research for 1+ years and has gained significant experience in handling animals and conducting surgical procedures.

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this ACORP
Ear punch	Mr. Sevaio has 1+ years of experience identifying mice by ear punch and collecting tissue for genotyping purposes.
Perfusion	Mr. Sevaio has 1+ years of experience performing perfusions in mice.
Stereotaxic microinjections surgery	Mr. Sevaio has a year of experience performing stereotaxic microinjections in mice.
Chronic cannula and osmotic pump implantation	Some experience with chronic cannulation surgery. To be further trained

Name ► Justyna Swierz

Animal research experience ► Ms. Swierz has completed the mouse handling course but is otherwise new to mouse handling and experimentation. The Iliff lab will ensure her complete training with the procedures below.

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this ACORP
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Ear punch	To be trained
Behavioral testing	To be trained
Perfusion	To be trained
Stereotaxic surgery	To be trained
CSF collection	To be trained
Cardiac puncture	To be trained

Name ► Jay Jang

Animal research experience ► Mr. Jang has completed the mouse handling course but is otherwise new to mouse handling and experimentation. The Iliff lab will ensure his complete training with the procedures below.

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this ACORP
Ear punch	To be trained
Behavioral testing	To be trained
Perfusion	To be trained
Stereotaxic surgery	To be trained
CSF collection	To be trained
Cardiac puncture	To be trained
Chronica cannula and osmotic pump implantation	To be trained

Name ► ~~Keith Sanderson~~

Animal research experience ► ~~Mr. Sanderson has completed the mouse handling course but is otherwise new to mouse handling and experimentation. The Iliff lab will ensure his complete training with the procedures below.~~

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this ACORP
Ear punch	To be trained
Behavioral testing	To be trained
Perfusion	To be trained
Stereotaxic surgery	To be trained
CSF collection	To be trained
Cardiac puncture	To be trained

Name ► Joshua Cho

Animal research experience ► Dr. Cho has 5 years of experience working with mice and has a great deal of experience in handling animals and conducting surgical procedures.

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this ACORP
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Ear punch	5 years of experience
Behavioral testing	5 years of experience
Perfusion	5 years of experience
Stereotaxic surgery	5 years of experience
CSF collection	To be trained
Cardiac puncture	To be trained
Sleep disruption	1 year of experience

Name ► Elizabeth Rhea

Animal research experience ► Dr. Rhea has >15 years of experience working with mice and has a great deal of experience in handling animals.

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this ACORP
Sleep disruption	No experience, will be trained by Joshua Cho and remotely by Dr. Samantha Keil

Name ► Riley Weaver

Animal research experience ► Riley Weaver has >2 years of experience working with mice and has a great deal of experience in handling animals.

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this ACORP
Sleep disruption	No experience, will be trained by Joshua Cho and remotely by Dr. Samantha Keil

Name ► Isabella Shafizadeh

Animal research experience ► Ms. Shafizadeh has completed the mouse handling course but is otherwise new to mouse handling and experimentation. The Iliff lab will ensure her complete training with the procedures below.

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this ACORP
Ear punch	To be trained
Behavioral testing	To be trained
Sleep disruption	To be trained
Perfusion	To be trained
Stereotaxic surgery	To be trained
CSF collection	To be trained
Cardiac puncture	To be trained
Sleep disruption	To be trained

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Name ► Mila Jolie Friesch

Animal research experience ► Ms. Friesch has completed the mouse handling course but is otherwise new to mouse handling and experimentation. The Iliff lab will ensure her complete training with the procedures below.

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this ACORP
Ear punch	To be trained
Behavioral testing	To be trained
Sleep disruption	To be trained
Perfusion	To be trained
Stereotaxic surgery	To be trained
CSF collection	To be trained
Cardiac puncture	To be trained
Sleep disruption	To be trained

Name ► William Zhang

Animal research experience ► Mr. Zhang has completed the mouse handling course but is otherwise new to mouse handling and experimentation. The Iliff lab will ensure his complete training with the procedures below.

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this ACORP
Ear punch	To be trained
Behavioral testing	To be trained
Sleep disruption	To be trained
Perfusion	To be trained
Stereotaxic surgery	To be trained
CSF collection	To be trained
Cardiac puncture	To be trained

Name ► Deidre Jansson

Animal research experience ► Dr. Jansson has 5 years of experience working with mice and has a great deal of experience in handling animals and conducting surgical procedures. She has worked with mice in the Iliff lab, previously, and has experience with many of the lab's techniques.

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this ACORP
Ear punch	5 years of experience
Behavioral testing	2 years of experience, previously trained by Iliff lab staff
Perfusion	5 years of experience
Stereotaxic surgery	2 years of experience
CSF collection	5 years of experience
Cardiac puncture	5 years of experience

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Sleep disruption	5 years of experience
Chronica cannula and osmotic pump implantation	2 years of experience in non-survival cannula implant surgery. To be trained in chronic implant surgery.

Name ► Thomas Ehler

Animal research experience ► Thomas has completed the mouse handling course but is otherwise new to mouse handling and experimentation. The Iliff lab will ensure his complete training with the procedures below.

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this ACORP
Ear punch	To be trained
Behavioral testing	To be trained
Sleep disruption	To be trained
Perfusion	To be trained
Stereotaxic surgery	To be trained
CSF collection	To be trained
Cardiac puncture	To be trained
Chronica cannula and osmotic pump implantation	To be trained

Name ► Jiyeon Ryu

Animal research experience ► Jiyeon has completed the mouse handling and rodent surgery courses but is otherwise new to mouse handling and experimentation. The Iliff lab will ensure her complete training with the procedures below.

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this ACORP
Behavioral testing	To be trained
Sleep disruption	To be trained
Perfusion	To be trained
Stereotaxic surgery	To be trained
Chronica cannula and osmotic pump implantation	To be trained

[Name ► Sarah Pei](#)

[Animal research experience ► Sarah has completed the mouse handling course but is otherwise new to mouse handling and experimentation. The Iliff lab will ensure her complete training with the procedures below.](#)

[Qualifications to perform specific procedures](#)

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this ACORP
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Behavioral testing	To be trained
Sleep disruption	To be trained
Ear punch	To be trained

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3. VMU animal care and veterinary support staff personnel (only for services provided beyond normal husbandry).
 Copy the lines below for each individual.

Name ►

Qualifications to perform specific support procedures in the animals on this protocol

Specific support procedure(s) assigned to this individual	Qualifications for performing each support procedure in the species described in this ACORP (e.g., AALAS certification, experience, or completion of special training)

4. For each of the research personnel listed in items 1 and 2 above, enter the most recent completion date for each course

Name of Individual	Working with the VA IACUC (CITI)	ORD web-based species specific course (CITI). Identify the species	Hands-On Training		Any other training required locally (Identify the training)
			Species	Completion Date (MM/DD/YY)	
Jeffrey Iliff	7/26/22	Mouse, 7/26/22	Mouse	8/15/19	
Katherine Suchland	6/16/22	Mouse, 5/2/23	Mouse	6/20/19	UW Rodent Surgery Lab, 1A and 2
Mathew Sevaio	11/29/23	Mouse, 11/29/23	Mouse	2/24/21	UW Rodent Surgery Lab, 1A and 2
Justyna Swierz	7/6/22	Mouse, 7/5/22	Mouse	7/8/22	UW Rodent Surgery Lab, 1A and 2, completed 11/17/22
Jay Jang	9/8/22	Mouse, 8/30/22	Mouse	9/22/22	UW Rodent Surgery Lab, 1A and 2, completed 12/19/22
Keith Sanderson	6/16/22	Mouse, 6/16/22	Mouse	9/26/22	UW Rodent Surgery Lab, 1A and 2, completed 11/30/22
Joshua Cho	2/1/23	Mouse, 1/31/23	Mouse	3/3/23	UW Rodent Surgery Lab, 1A and 2, completed 4/28/23
Elizabeth Rhea	1/6/22	Mouse, 1/6/22	Mouse	8/4/14	
Riley Weaver	6/8/23	Mouse, 6/8/23	Mouse	6/17/20	
Isabella Shafizadeh	4/4/23	Mouse, 3/31/23	Mouse	5/5/23	UW Rodent Surgery Lab, 1A and 2, completed 12/6/23
Mila Jolie Friesch	6/25/23	Mouse, 6/21/23	Mouse	8/25/23	UW Rodent Surgery Lab, 1A and 2, completed 12/6/23

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William Zhang	10/11/23	Mouse, 8/30/23	Mouse	11/13/23	UW Rodent Surgery Lab, 1A and 2, completed 5/6/24
Deidre Jansson	11/14/23	Mouse, 11/7/23	Mouse	8/29/19	UW Rodent Surgery Lab, 1A and 2, completed 12/4/19
Thomas Ehler	1/3/24	Mouse, 1/3/24	Mouse	1/29/24	UW Rodent Surgery Lab, 1A and 2, completed 4/26/24
Jiyeon Ryu	4/15/24	Mouse, 4/15/24	Mouse	5/22/24	UW Rodent Surgery Lab, 1A and 2, completed 5/31/24
Sarah Pei	1/16/24	Mouse, 8/23/23	Mouse	12/11/23	

F. Training to be provided. List here each procedure in Item E for which anyone is shown as “to be trained”, and describe the training. For each procedure, describe the type of training to be provided, and give the name(s), qualifications, and training experience of the person(s) who will provide it. If no further training is required for anyone listed in Item E, enter “N/A”

► Ear punch – Katherine Suchland and Joshua Cho are qualified to train, as they have extensive experience performing this simple procedure. Training consists of setting up, administering isoflurane anesthesia, collecting ear punch tissue, monitoring animals post-procedure, cleaning up. Individuals will observe the trainer performing the procedure first, and then attempt it themselves under supervision until such a time that the trainer feels they are proficient to perform the procedure independently.

► Behavioral testing – Mathew Sevaio and Joshua Cho are qualified to train, as they have extensive experience performing these tests. Individuals will observe the trainer performing the tests first, and then attempt it themselves under supervision until such a time that the trainer feels they are proficient to perform the testing independently. They will learn to transport the cages, set up, administer the tests, monitor the animals for well-being throughout the experiment, and clean the equipment between animals and at the end of the day.

► Perfusion - Mathew Sevaio and Joshua Cho are qualified to train, as they have extensive experience with this non-survival surgical procedure. Individuals to be trained will first observe the trainer performing the procedure several times, and then attempt it themselves under close supervision until such a time that the trainer feels they are proficient to perform the procedure independently. Training will consist of the procedure itself, as well as induction of anesthetic and monitoring of anesthesia plane.

► Stereotaxic surgery - Mathew Sevaio and Joshua Cho are qualified to train, as they have extensive experience with this surgical procedure. Individuals to be trained will first observe the trainer performing the procedure several times, and then attempt it themselves under close supervision until such a time that the trainer feels they are proficient to perform the procedure independently. Training will consist of the procedure itself, as well as induction of anesthesia and monitoring of anesthetic plane.

► CSF collection - Mathew Sevaio is qualified to train, as he has extensive experience with this procedure. Individuals to be trained will first observe the trainer performing the procedure several times, and then attempt it themselves under close supervision until such a time that the trainer feels they are proficient to perform the procedure independently. Training will consist of the non-survival procedure itself, as well as induction of anesthesia and monitoring of anesthetic plane.

► Cardiac puncture – Mathew Sevaio and Joshua Cho are qualified to train, as they have extensive experience with this procedure. Individuals to be trained will first observe the trainer performing the procedure several times, and

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then attempt it themselves under close supervision until such a time that the trainer feels they are proficient to perform the procedure independently. Training will consist of the non-survival procedure itself, as well as induction of anesthesia and monitoring of anesthetic plane.

► Sleep disruption - Josh Cho (1 year of experience) is qualified to train, as he has extensive experience with this procedure. Individuals to be trained will first observe the trainer setting up and turning the chambers on and off, monitoring the mice during the sleep disruption period, as well as, maintaining and cleaning the chambers. Trainees will then do the above under close supervision until such a time that the trainer feels they are proficient to use the chambers independently. Even though no longer with the VA, Dr. Samantha Keil will remain available by phone, email, or in-person for additional questions and troubleshooting.

► Chronic cannula and osmotic pump implantation - Josh Cho has experience with chronic cannula implantation and can assist with training. Deidre Jansson has experience performing cannula implantation to the ventricles in mice, however not chronically. Both Dr. Jansson and any other trainees will obtain training from UW surgery class and VA veterinarians on performing sterile techniques, chronic cannula implantation and placement of osmotic pumps. Trainees will perform practice surgeries on sacrificed mice until the veterinarians and Dr. Jansson are confident they are proficient to perform this procedure in live animals. If proficiency of the procedure proves too challenging after practicing on euthanized mice, Jiyeon Ryu and/or Dr. Jansson would consider visiting the lab, located in Palo Alto, where these studies have been previously conducted and published to observe and learn this cannulation procedure.

G. Occupational Health and Safety.

1. Complete one line in the table below for each of the personnel identified in Item E:

Name	Enrollment in OHSP		Declined optional services	Current on Interactions with OHSP? (yes/no)
	VA program	Equivalent Alternate Program – identify the program		
Jeffrey Iliff	(X)	()	()	Yes
Katherine Suchland	(X)	()	()	Yes
Mathew Sevaio	(X)	()	()	Yes
Justyna Swierz	(X)	()	()	Yes
Jay Jang	(X)	()	()	Yes
Keith Sanderson	(X)	()	()	Yes
Joshua Cho	(X)	()	()	Yes
Elizabeth Rhea	(X)	()	()	Yes
Riley Weaver	(X)	()	()	Yes
Isabella Shafizadeh	(X)	()	()	Yes
Mila Jolie Friesch	(X)	()	()	Yes
William Zhang	(X)	()	()	Yes
Deidre Jansson	(X)	()	()	Yes

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Thomas Ehler	(X)	()	()	Yes
Jiyeon Ryu	(X)	()	()	Yes
Sarah Pei	(X)	()	()	Yes

2. Are there any non-routine OHSP measures that would potentially benefit, or are otherwise required for, personnel participating in or supporting this protocol?

► () Yes. Describe them ►

► (X) No.

Animals Requested

H. **Animals to be Used.** Complete the following table, listing the animals on separate lines according to any specific features that are required for the study (see ACORP Instructions, for guidance, including specific terminology recommended for the “Health Status” column):

Description (include the species/strain and any other special features not shown elsewhere in this table)	Gender	Age/Size on Receipt	Source (e.g., Name of Vendor, Collaborator, or PI of local breeding colony)	Health Status
Mice/C57BL/6	Both	Birth or 6-8 weeks, 12, 14, 16, and 18 months old	Bred in house or Jackson Labs	SPF
Mice/ <i>Snta1</i> ^{-/-} and wild type littermates	Both	Birth or 6-8 weeks old	Bred in house or Jackson Labs, available by cryo-recovery only	SPF
Mice/ <i>Aqp4</i> ^{-/-} and wild type littermates	Both	Birth or 6-8 weeks old	Bred in house, also available from Riken (Japan)	SPF
Mice/ <i>5xFAD</i> mice and wild type littermates	Both	Birth or 6-8 weeks old	Bred in house or Jackson Labs	SPF
Mice/ <i>5xFAD:Pad4</i> ^{-/-} and wild type littermates	Both	Birth or 6-8 weeks old	Bred in house, or Jackson Labs	SPF
Mice/ <i>Pad4</i> ^{-/-} and wild type littermates	Both	Birth or 6-8 weeks old	Bred in house, or Jackson Labs	SPF

I. **Numbers of animals requested.** See ACORP Instructions, for descriptions of the categories and how to itemize the groups of animals.

USDA Category B

Procedures ► Colony maintenance/breeding						
Species / Experimental Group / Procedures(s)	Year 1	Year 2	Year 3	Year 4	Year 5	Category B TOTAL

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Mouse/C57BL6/J/Replacement breeders from Jackson Labs	2	2	2			6
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USDA Category C

Procedures► Colony maintenance/breeding						
Species / Experimental Group / Procedure(s)	Year 1	Year 2	Year 3	Year 4	Year 5	Category C TOTAL
Mouse/C57BL6/J/ Maintenance and 5xFAD cross breeders	8	8	8			24
Mouse/ <i>Aqp4</i> ^{-/-} /Maintenance Breeders	8	8	8			24
Mouse/ <i>Snta1</i> ^{-/-} / Maintenance Breeders	8	8	8			24
Mouse/5xFAD/ Maintenance Breeders	4	4	8			16
Mouse/5xFAD/Breeders/Exp. 1	3					3
Mouse/C57BL6/Breeders/Exp. 1	3					3
Mouse/5xFAD: <i>Pad4</i> /Breeders/Exp. 2.a	12	12				24
Mouse/5xFAD/Breeders/Exp. 2.b.1	5					5
Mouse/ C57BL6/Breeders/Exp. 2.b.1	5					5
Mouse/5xFAD/Breeders/Exp. 2.b.2	2	3				5
Mouse/ C57BL6/Breeders/Exp. 2.b.2	2	3				5

USDA Category D

Procedures► Stereotaxic injection, ICV cannulation and osmotic pump implantation surgery, CSF collection, cardiac puncture, perfusion						
Species / Experimental Group / Procedure(s)	Year 1	Year 2	Year 3	Year 4	Year 5	Category D TOTAL
Mouse/C57BL6/J/offspring not used as breeders/Training, tissue banking, or transfer to other groups*	88	93	96			277
Mouse/ <i>Aqp4</i> ^{-/-} /offspring not used as breeders/Training, tissue banking, or transfer to other groups*	96	96	96			288
Mouse/ <i>Snta1</i> ^{-/-} /offspring not used as breeders/Training, tissue banking, or transfer to other groups*	96	96	96			288
Mouse/5xFAD/offspring not used as breeders/Training, tissue banking, or transfer to other groups*	48	48	96			192
Mouse/5xFAD/Exp. 1/Sleep frag, behavior, stereotaxic injection, perfusion	25					25
Mouse/5xFAD:Pad4/Exp. 2.a/cardiac puncture, perfusion	75	75				150

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Mouse/5xFAD/Exp. 2.b.1/tail vein inj, stereotaxic injection, cardiac puncture, perfusion	48					48
Mouse/5xFAD/ /Exp. 2.b.2/tail vein injection, behavior, cardiac puncture, perfusion	24	44				68
Mouse/any sex, strain, genotype/Experiment 3 practice	30					30
Mouse/C57BL/6/Experiment 3 trial phase	80					80
Mouse/C57BL/6/2 months old/Exp 3.1, Stage A	132	132				264
Mouse/C57BL/6/2 months old/Exp 3.2, Stage A	240	240				480
Mouse/C57BL/6/12-18 months old/Female/Exp 3.1, Stage B/Age optimization	176					176
Mouse/C57BL/6/12-18 months old/Exp 3.1, Stage B		132	132			264
Mouse/C57BL/6/12-18 months old /Exp 3.2, Stage B		240	240			480
Mouse/C57BL6 wild type/male and female/Exp 4, Aim 1 and 2	64	64	64			192
Mouse/Aqp4 ^{-/-} /male and female/Exp 4, Aim 3	32	32	32			96
Mouse/C67BL6 wild type/male and female/Exp 4, Aim 3	75	75	74			224

*Note: These lines, other than the 5xFAD, are also used on our other protocol (ACORP 0949). It is also highly likely that amendments will be filed soon for experiments using these maintained lines on this protocol, therefore it is important to keep these lines, and, in that case, there will be fewer non-experimental animals than proposed here.

USDA Category E

Procedures ►						
Species / Experimental Group / Procedure(s)	Year 1	Year 2	Year 3	Year 4	Year 5	Category E TOTAL

TOTALS over all Categories

Species	Year 1	Year 2	Year 3	Year 4	Year 5	GRAND TOTAL
Mouse	1391	1415	960			3766

J. **Management of USDA Category D procedures.** Indicate which statement below applies, and provide the information requested.

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- () This protocol does NOT include any Category D procedures.
- (X) This protocol INCLUDES Category D procedures. List each Category D procedure and provide the information requested. (For surgical procedures described in Appendix 5, only identify the procedure(s) and enter “See Appendix 5 for details.”)

Procedure	Monitoring (indicate the method(s) to be used, and the frequency and duration of monitoring through post-procedure recovery)	Person(s) responsible for the monitoring	Method(s) by which pain or distress will be alleviated during or after the procedure (include the dose, route, and duration of effect of any agents to be administered)
Stereotaxic injection surgery	(See appendix 5 for details)	(See appendix 5 for details)	(See appendix 5 for details)
Perfusion	(See appendix 5 for details)	(See appendix 5 for details)	(See appendix 5 for details)
CSF Collection	(See appendix 5 for details)	(See appendix 5 for details)	(See appendix 5 for details)
Cardiac Puncture	(See appendix 5 for details)	(See appendix 5 for details)	(See appendix 5 for details)
Chronic cannula implantation	(See appendix 5 for details)	(See appendix 5 for details)	(See appendix 5 for details)
Osmotic pump implantation	(See appendix 5 for details)	(See appendix 5 for details)	(See appendix 5 for details)

K. **Justification of Category E procedures.** Indicate which statement below applies, and provide the information requested.

- (X) This protocol does NOT include any Category E procedures
- () This protocol INCLUDES Category E procedures. Identify each Category E procedure included in this ACORP and justify scientifically why the pain or distress cannot be relieved.
-

Veterinary Care and Husbandry

L. **Veterinary Support.**

- Identify the laboratory animal veterinarian who is responsible for ensuring that the animals on this protocol receive appropriate veterinary medical care.

Names► **Cindy Pekow, DVM and Leandra Mosca, DVM**
Institutional affiliation► **VA PSHCS - Seattle**
email contact► cpekow@uw.edu and Leandra.Mosca@va.gov

- Veterinary consultation during the planning of this protocol.

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Name of the laboratory animal veterinarian consulted ► Cynthia Pekow
Date of the veterinary consultation (meeting date, or date of written comments provided by the veterinarian to the PI) ► 7/1/22

M. **Husbandry.** As a reference for the animal husbandry staff, summarize here the husbandry requirements of the animals on this protocol. (Use Appendix 6 to justify the use of any special husbandry and to detail its effects on the animals. Use Appendix 9 to document any aspects of the husbandry that involve “departures” from the standards in the *Guide*. Consult the IACUC or the Attending Veterinarian for help in determining whether any “departures” are involved.)

1. Caging needs. Complete the table below to describe the housing that will have to be accommodated by the housing sites for this protocol:

a. Species	b. Type of housing* (Note: wire bottom cages for rodents are not standard at our station.)	c. Number of individuals per housing unit**	d. Is this housing consistent with the <i>Guide</i> and USDA regulations? (yes/no***)	e. Estimated maximum number of housing units needed at any one time
Mouse	Standard	2-5	Yes	75
Mouse	Breeding cages	2 adults plus 1 litter	Yes	15
Mouse	Lafayette sleep study chambers	2-5	Yes	8
Mouse	Standard (see SOP) or in boxes without wire lids***	1	No	30

***Wire lids may interfere with and dislodge cannulas of post-surgical mice.

*See ACORP Instructions, for guidance on describing the type of housing needed. If animals are to be housed according to a local Standard Operating Procedure (SOP), enter “standard (see SOP)” here, and enter the SOP into the table in Item Y. If the local standard housing is not described in a SOP, enter “standard, see below” in the table and describe the standard housing here:

► Standard (see SOP)

** The *Guide* states that social animals should generally be housed in stable pairs or groups. Provide a justification if any animals will be housed singly (if species is not considered “social”, then so note)

► After ICV cannulation and osmotic pump implantation surgery mice must be individually housed to protect the surgical site and wound clips or suture from being disturbed by other animals in the cage. We will provide extra enrichment and bedding material for all single housed mice. Animals will only be individually housed for a maximum of 7 days post-surgery before the completion of the experiment; therefore, the effects of social isolation should be minimal.

***Use Appendix 9 to document “departures” from the standards in the *Guide*.

2. Enrichment. Complete the table below to indicate whether “standard” exercise and environmental enrichment will be provided to the animals on this protocol, or whether any special supplements or restrictions will be

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required (See ACORP Instructions, for more information on enrichment requirements. Use Appendix 9 to document any enrichments requirements that represent “departures” from the standards in the *Guide*.):

a. Species	b. Description of Enrichment*	c. Frequency
Mouse	Standard	Standard

*If enrichment will be provided according to a local SOP, enter “standard (see SOP)” and enter the SOP into the table in Item Y. If the local standard enrichment is not described in a SOP, enter “standard, see below”, and describe the standard species-specific enrichment here.

► Standard (see SOP) for standard and breeder housing, although mice in sleep-fragmentation chambers will have limited nesting material and no other enrichment during the hours the chamber is in operation.

3. Customized routine husbandry. Check all of the statements below that apply to the animals on this protocol, and provide instructions to the animal husbandry staff with regard to any customized routine husbandry needed.

► (X) This ACORP INCLUDES genetically modified animals.

List each group of genetically modified animals, and describe for each any expected characteristic clinical signs or abnormal behavior related to the genotype and any customized routine husbandry required to address these. For genetic modifications that will be newly generated on or for this protocol, describe any special attention needed during routine husbandry to monitor for unexpected clinical signs or abnormal behavior that may require customized routine husbandry.

► *Aqp4*^{-/-} mice: The literature does not list any behavioral abnormalities associated with these mice at the ages we will be using them.

Snta1^{-/-} mice: Jackson Laboratories does not list any behavioral abnormalities associated with these mice at the ages we will be using them.

5xFAD: Jackson Laboratories does not list any behavioral abnormalities associated with these mice at the ages we intend to use them.

Pad4^{-/-} mice: Jackson Laboratories does not list any behavioral abnormalities associated with these mice at the ages we will be using them.

5xFAD:Pad4 mice: We have successfully crossed the *5xFAD* and *Pad4* lines already. The animals breed well, appear to be healthy, and display normal behavior. The only issue we have noted is that fewer *5xFAD⁺:Pad4^{-/-}* animals survive than other littermates. Instead of a predicted 25% distribution, only 8% of pups are that genotype at weaning.

► () Devices that extend chronically through the skin WILL be implanted into some or all animals on this protocol. Describe any customized routine husbandry to be provided by animal husbandry staff to minimize the chances of chronic infection where the device(s) penetrate the skin.

►

► (X) Some or all of the animals on this protocol WILL require other customized routine husbandry by the animal husbandry staff, beyond what has been described above. Describe the special husbandry needed. Note: (Briefly list here, noting that more detailed information is requested in ACORP item V. and Appendix 6)

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► Sleep study chambers (Lafayette Instruments) will be used for some experiments. Lab personnel will be responsible for turning the chambers on and off daily (on at 6am, off at 6pm), as well as, daily health checks of mice housed in the chambers, monitoring water and food availability, and for routine cleaning of the chambers. More detailed information is available in ACORP item V. and appendix 6.

► () This ACORP does NOT include use of any animals that will require customized routine husbandry.

N. **Housing Sites.** Document in the tables below each location where animals on this protocol may be housed.

► (X) Housing on VA property. Identify each location on VA property where animals on this protocol will be housed, and indicate whether or not each location is inside the VMU.

Building	Room number	Inside of VMU?	
		Yes	No
ARF – Building 34	151	(X)	()
ARF – Building 34 (Breeders)	165	(X)	()

► () Housing in non-VA facilities. Identify each location not on VA property where animals on this protocol will be housed, and provide the information requested in the table.

Name of Non-VA Facility	Is this facility accredited by AAALAC?		Building	Room Number
	Yes -- enter status*	No**		
	()	()**		
	()	()**		
	()	()**		

*See ACORP Instructions, for a list of AAALAC accreditation status options.

**For any facility listed above that is not accredited by AAALAC, attach documentation that a waiver has been granted by the CRADO.

Special Features

O. **Antibody Production.** Will any of animals on this protocol be used for the production of antibodies?

► () Some or all of the animals on this protocol WILL be used in the production and harvesting of antibodies. Check “Appendix 2” in Item Y, below, and complete and attach Appendix 2, “Antibody Production”.

► (X) NO animals on this protocol will be used in the production and harvesting of antibodies.

P. **Biosafety.** Will any substances (other than those used in routine husbandry or veterinary care) be administered to the animals on this protocol?

► (X) This protocol INVOLVES administration of substances to the animals other than those used in routine husbandry and veterinary care. Check “Appendix 3” in Item Y, below, and complete and attach Appendix 3, “Biosafety”.

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► () This protocol does NOT involve administration of any substances to the animals other than those used in routine husbandry and veterinary care.

Q. **Locations of procedures.** Complete the table below, listing the location(s), inside or outside of the animal facility, for each of the procedures to be performed on animals on this protocol.

Procedure	Surgical?		Bldg/Room Number	Requires transport through non-research areas?	
	Yes	No		Yes – describe method of discreet transport	No
Stereotaxic injection surgery	(X)	()	13/109	Cages will be transported on a cart completely covered by a drape	(X)
CSF collection	(X)	()	13/109	Cages will be transported on a cart completely covered by a drape	(X)
Behavioral Testing	()	(X)	11/7 and 34/120	Cages will be transported on a cart completely covered by a drape	(X)
Perfusion	(X)	()	13/109	Cages will be transported on a cart completely covered by a drape	(X)
Ear-punch for identification and tissue collection	()	(X)	34/151 or ARF procedure rooms		(X)
Cardiac Puncture	(X)	()	13/109	Cages will be transported on a cart completely covered by a drape	(X)
Breeding	()	(X)	34/165		(X)
Intracerebroventricular cannulation and osmotic pump implantation	(X)	()	13/109	Cages will be transported on a cart completely covered by a drape	(X)

R. **Body Fluid, Tissue, and Device Collection.** List each body fluid, tissue, or device to be collected, and complete the table below to indicate the nature of the collection. Check the relevant Appendices in Item Y, below, and complete and attach them, as shown in the column headings.

Body Fluid, Tissue, or Device to be Collected	Collected AFTER Euthanasia	Collected BEFORE Euthanasia		
		Blood Collection Associated with Antibody Production (Appendix 2, “Antibody Production”)	Collected as Part of a Surgical Procedure (Appendix 5, “Surgery”)	Other Collection from Live Animals (Appendix 4, “Antemortem Specimen Collection”)
Ear punch	()	()	()	(X)
Brain	(X)	()	()	()
CSF – as part of a terminal surgery	()	()	()	(X)

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Blood – as part of a terminal surgery	()	()	(X)	(X)
Meninges- as part of a terminal surgery	(X)	()	()	()
Deep cervical lymph nodes	(X)	()	()	()

S. **Surgery.** Does this protocol include any surgical procedure(s)?

► (X) Surgery WILL BE PERFORMED on some or all animals on this protocol. Check “Appendix 5” in Item Y, below, and complete and attach Appendix 5, “Surgery”.

► () NO animals on this protocol will undergo surgery.

T. **Endpoint criteria.** Describe the criteria that will be used to determine when animals will be removed from the protocol or euthanatized to prevent suffering. (Use Appendix 9 to document any “departures” from the standards in the *Guide* represented by these criteria. Consult the IACUC or the Attending Veterinarian for help in determining whether any “departures” are involved.)

► A veterinarian will be consulted if there is evidence of infection or other complications. Animals will be removed from the study if they display a body condition score below 2 (mouse is underconditioned: segmentation of vertebral column is evident and dorsal pelvic bones are readily palpable). Additionally, animals that develop post-surgical infection will be removed from the study and euthanized. In the studies that include ICV infusion of human CSF, we will monitor the mice closely for an adverse immune reaction. Indications of such a reaction may include head pressing, squinting, ataxia, immobility, and seizures. If any of these problems occur anytime during the 7-day infusion, the animals will be removed from the study and euthanized immediately. We will also halt the experiment and consider the alternative of an immune-incompetent mouse line. Occurrence of other animal-health related problems (i.e. malocclusion, dystocia, ocular/nasal discharge, roughened hair coat, respiratory distress, seizures, abnormal posture, abscess, swellings/masses, etc.) will immediately be brought to the attention of a veterinarian and will result in removal of the animal from the study. Animals that must be removed from the study for these reasons will be euthanized in the manner described in this protocol.

U. **Termination or removal from the protocol.** Complete each of the following that applies:

► () Some or all animals will NOT be euthanatized on this protocol. Describe the disposition of these animals. (Use Appendix 9 to document any “departures” from the standards in the *Guide* represented by these methods of disposition. Consult the IACUC or the Attending Veterinarian for help in determining whether any “departures” are involved.)



► (X) Some or all animals MAY be euthanatized as part of the planned studies. Complete the table below to describe the exact method(s) of euthanasia to be used. (Use Appendix 9 to document any departures from the standards in the *Guide* represented by these methods. Consult the IACUC or the Attending Veterinarian for help in determining whether any “departures” are involved.)

Check each	Method of Euthanasia	Species	AVMA Classification
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method that may be used on this protocol			Acceptable	Conditionally Acceptable	Unacceptable
(X)	CO ₂ from a compressed gas tank Duration of exposure after apparent clinical death ► Minimum of 1 minute Method for verifying death ► Lack of visible respiration Secondary physical method ► Decapitation with scissors	Mouse	()	(X)	()
()	Anesthetic overdose Agent ► Dose ► Route of administration ►		()	()	()
()	Decapitation under anesthesia Agent ► Dose ► Route of administration ►		()	()	()
()	Exsanguination under anesthesia Agent ► Dose ► Route of administration ►		()	()	()
(X)	Other (Describe) ► Terminal surgery will involve perfusion under deep anesthesia Agent – Isoflurane Dose – 4-5% Route of administration - inhalation		(X)	()	()
(X)	Other (Describe) ► Terminal surgery will involve perfusion under deep anesthesia Agent – Ketamine/Xylazine Dose – Ketamine 80-100 mg/kg, Xylazine 5-10 mg/kg, 0.2 ml/100 grams body weight Route of administration – intraperitoneal		(X)	()	()

1. For each of the methods above that is designated as “Conditionally Acceptable” by the AVMA, describe how the conditions for acceptability will be met. Note: CO₂ is conditionally acceptable. We meet conditions by use of compressed gas source at flow rates recommended by the AVMA.

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► CO₂ from compressed tank – without pre-charging the chamber, the animals will be placed in the chamber and a regulator will be used to displace 30 to 70% of the chamber/cage volume/min (or current AVMA recommendations). Expected time to unconsciousness is usually within 2 to 3 minutes. Animals will be observed for lack of respiration. CO₂ flow will be maintained for a minimum of 1 minute after respiration ceases. Decapitation will assure death.

2. For each of the methods above that is designated as “Unacceptable” by the AVMA, give the scientific reason(s) that justify this deviation from the AVMA Guidelines:
 - N/A
3. Identify all research personnel who will perform euthanasia on animals on this protocol and describe their training and experience with the methods of euthanasia they are to use in the species indicated.
 - Katherine Suchland will perform CO₂ euthanasia. She has over 20 years of experience performing euthanasia with CO₂ from a compressed tank.
 - Jeffrey Iliff may perform CO₂ euthanasia. He has 20 years of experience performing euthanasia with CO₂ from a compressed tank.
 - Mathew Sevaio will perform CO₂ euthanasia and by perfusion under deep anesthesia. He has more than a year of experience performing euthanasia with CO₂ from a compressed tank. Mr. Sevaio has a year of experience euthanizing mice by performing perfusions under deep inhaled and injected anesthetics.
 - Justyna Swierz will perform CO₂ euthanasia. She received experience during the mouse handling course and will be further trained by Mathew Sevaio to performing euthanasia with CO₂ from a compressed tank.
 - Jay Jang will perform CO₂ euthanasia. He received experience during the mouse handling course and will be further trained by Mathew Sevaio to performing euthanasia with CO₂ from a compressed tank.
 - ~~► Keith Sanderson will perform CO₂ euthanasia. He received experience during the mouse handling course and will be further trained by Mathew Sevaio to performing euthanasia with CO₂ from a compressed tank.~~
 - Joshua Cho will perform CO₂ euthanasia and by perfusion under deep anesthesia. He has over 5 years of experience performing euthanasia with CO₂ from a compressed tank. Dr. Cho has 5 years of experience euthanizing mice by performing perfusions under deep inhaled and injected anesthetics.
 - Isabella Shafizadeh will perform CO₂ euthanasia. She received experience during the mouse handling course and will be further trained by Mathew Sevaio and Joshua Cho to performing euthanasia with CO₂ from a compressed tank.
 - Mila Jolie Friesch will perform CO₂ euthanasia. She received experience during the mouse handling course and will be further trained by Mathew Sevaio and Joshua Cho to performing euthanasia with CO₂ from a compressed tank.
 - William Zhang will perform CO₂ euthanasia. He received experience during the mouse handling course and will be further trained by Mathew Sevaio and Joshua Cho to performing euthanasia with CO₂ from a compressed tank.
 - Deidre Jansson will perform CO₂ euthanasia and by perfusion under deep anesthesia. She has over 5 years of experience performing euthanasia with CO₂ from a compressed tank. Dr. Jansson has 5 years of experience euthanizing mice by performing perfusions under deep inhaled and injected anesthetics.
 - Thomas Ehler will perform CO₂ euthanasia and by perfusion under deep anesthesia. He received experience during the mouse handling course and will be further trained by Katherine Suchland, Deidre Jansson, Mathew Sevaio, and Joshua Cho to performing euthanasia with CO₂ from a compressed tank.
 - Jiyeon Ryu will perform CO₂ euthanasia and by perfusion under deep anesthesia. She received experience during the mouse handling course and will be further trained by Katherine Suchland, Deidre Jansson, Mathew Sevaio, Thomas Ehler, and Joshua Cho to performing euthanasia with CO₂ from a compressed tank.

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► [Sarah Pei will perform CO₂ euthanasia and by perfusion under deep anesthesia. She received experience during the mouse handling course and will be further trained by Katherine Suchland, Deidre Jansson, Mathew Sevaio, Thomas Ehler, and Joshua Cho to performing euthanasia with CO₂ from a compressed tank.](#)

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4. Instructions for the animal care staff in case an animal is found dead.
- a. Describe the disposition of the carcass, including any special safety instructions. If disposition is to be handled according to a local SOP, enter “according to local SOP” and enter the information requested about the SOP into the table in Item Y.
- If an animal is found dead, please inform in a Katherine Suchland by A-tune notification system. The carcass does not need to be saved.
- b. Describe how the PI’s staff should be contacted.
- (X) Please contact a member of the PI’s staff immediately. (Copy the lines below for each individual who may be contacted)
- Name ► Katherine Suchland
Contact Information ► Contact information is on file in the ARF
- Name ► Joshua Cho
Contact Information ► Contact information is on file in the ARF
- Name ► Mathew Sevaio
Contact Information ► Contact information is on file in the ARF
- Name ► Jiyeon Ryu
Contact Information ► Contact information is on file in the ARF
- Name ► Deidre Jansson
Contact Information ► Contact information is on file in the ARF
- () There is no need to contact the PI’s staff immediately. Describe the routine notification procedures that will be followed. If the routine notification procedures are described in a local SOP, enter “according to local SOP” and enter the information requested about the SOP into the table in Item Y.
-

V. **Special Procedures.** List each special procedure (including special husbandry not already covered in item M, and other special procedures such as behavioral studies) that is a part of this protocol, and specify where the details of the procedure are documented. See ACORP Instructions, for examples.

Name of Procedure	Identify Where the Details of the Procedure are Documented		
	SOP (title or ID number)*	Other Items in this ACORP -- specify the Item letter(s)	Appendix 6
Sleep fragmentation		Items: C.1, C.2.a, C.2.b	(X)**
Behavioral Testing		Items: C.2.a, C.2.b	(X)**

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*If any special procedure is detailed in a SOP, identify the SOP and enter the information requested about the SOP in the table in Item Y.

**If any special procedure is detailed in Appendix 6, check “Appendix 6” in Item Y, below, and complete and attach Appendix 6.

(Use Appendix 9 to document any “departures” from the standards in the *Guide* represented by these procedures. Consult the IACUC or the Attending Veterinarian for help in determining whether any “departures” are involved.)

W. **Consideration of Alternatives and Prevention of Unnecessary Duplication.** These are important to minimizing the harm/benefit to be derived from the work.

- Document the database searches conducted.
List each of the potentially painful or distressing procedures included in this protocol. Refer to Item J for Category D or E procedures. Use these items to direct your search terms for alternatives. In the table, the 4th column asking about the painful or distressing procedures is not for a “Yes or No” response; list here which procedure is being addressed by the search.
► Chronic Sleep disruption, Stereotaxic injection surgery, Perfusion, CSF collection, Tail Vein Injection, Cardiac Puncture

Then complete the table below to document how the database search(es) you conduct to answer Items W.2 through W.5 below address(es) each of the potentially painful or distressing procedures.

Name of the database	Date of search	Period of years covered by the search	Potentially painful or distressing procedures addressed	Key words and/or search strategy used	Indicate which mandate each search addressed			
					Replacement of animals (item W.2)	Reduction in numbers of animals used (item W.3)	Refinement to minimize pain or distress (item W.4)	Lack of unnecessary duplication (item W.5)
Pubmed	6/1/2022	1990-Present	Chronic Sleep Disruption	Sleep fragmentation, sleep disruption, obstructive sleep apnea	(X)	(X)	(X)	(X)
Pubmed	6/10/22	1980-Present	Stereotaxic injection surgery	Mice, stereotaxic, injection, Alzheimer’s disease	(X)	(X)	(X)	(X)
Pubmed	6/10/22	1980-Present	Perfusion	Alzheimer’s disease, rodents, amyloid plaques, perfusion	(X)	(X)	(X)	(X)
Pubmed	5/25/22	1990 - Present	CSF	CSF collection, mouse,	(X)	(X)	(X)	(X)

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			collection	handling, anesthesia				
Pubmed	6/10/22	1980-Present	Tail vein injection	DNase1, bolus, tail vein, Neutrophil, NETs	(X)	(X)	(X)	(X)
Pubmed	6/7/22	1990-present	Cardiac puncture under isoflurane	puncture, blood sample collection from mice	(X)	(X)	(X)	(X)
Pubmed	6/11/22	1980-Present	Alternatives to generating genetic crosses	Pad4 KO, cl-amidine, DNase1, MPO KO, 5xFAD, NETs, neutrophils	(X)	(X)	(X)	(X)
Pubmed	5/15/24	2019-present	intracerebrov entricular osmotic pump	Antisense oligonucleotides, Rodent, cannulation, infusion, brain, osmotic pump	(X)	(X)	(X)	(X)
Pubmed	7/2/2024	2019-present	CSF infusion	CSF infusion, mouse, intracerebroventricula r	(X)	(X)	(X)	(X)

2. Replacement. Describe the replacements that have been incorporated into this work, the replacements that have been considered but cannot be used, and the reason(s) that further replacements are not acceptable.
► These studies use mouse models of aging that allow us to study the interaction between brain blood vessels and glial cells in an intact, living organism. There is no alternative to an in vivo model to study this complex multi-cellular system and how it breaks down during aging, sleep disruption, and AD. Primary cortical cultures can't model this complex integrated system since we are trying to understand how numerous cell types interact with the brain vasculature. Because the overall goal of this project is to study changes in A β deposition, these studies must be carried out in mice that develop these plaques. We have replaced a transgenic line that developed A β plaques at 13 months (Tg2576 AD model), with the 5xFAD line that develops deposits beginning at 2 months of age. This will speed up the studies conducted and hopefully remove variability and aging distress to the mice. A computer model is not a viable replacement alternative because it is unclear what effect transgenic mutations will have on A β plaques deposition and AD pathology. Non-mammalian systems do not have the glymphatic system we are studying.
3. Reduction. Describe how the number of animals to be used has been minimized in this protocol and explain why further reduction would disproportionately compromise the value of the data.
► The number of animals outlined in this proposal have been determined based on statistical power analysis of similar types of data from mice and rats. Based upon reported effect sizes and variance, sample sizes between 8-15 are necessary to detect 10-20% differences with alpha=0.05 and power of 0.8. Also, we plan to conduct a functional experiment, ending with tissue collection so that we do not need to use two different groups to arrive at these two outputs. We aim to collect as many different measurements and tissues as possible from each mouse to reduce the total number of mice required. We are donating excess animals produced to other researchers with approved protocols that can use the mice for training or tissue collection.
4. Refinement. Describe the refinements that have been incorporated into this work and explain why no further refinements are feasible.

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► These studies are deemed the most feasible way to assess how sleep disruption and inflammation are associated with the deposition of A β in the brain in an animal model of AD. Animals will be euthanized prior to the age at which they experience deficits from the progression of neurological disease. We will use anesthesia during the terminal procedures of tracer stereotaxic injection and perfusion to prevent the animals from experiencing pain or distress associated with the procedure. The study employs minimal stress handling techniques, optimal anesthesia, analgesia protocols, and dynamic imaging methods to refine procedures. Further refinements are not feasible without compromising the study's core objectives and data accuracy.

5. Describe how it was determined that the proposed work does not unnecessarily duplicate work already documented in the literature.

► Thorough literature searches have been performed to assure that the work in this project is novel. Our group regularly attends scientific conferences to keep up with the ongoing research being conducted in the field. In 2012, our group first described a brain-wide paravascular pathway, termed the 'glymphatic system', that is an important route for the clearance of proteins from the brain interstitium. We are highly involved in, and familiar with, related work that is being conducting in the field.

X. Other Regulatory Considerations.

1. Controlled drugs.

- a. Complete the table below for each drug that is used in animals on this protocol and that is classified as a controlled substance by the DEA. See ACORP Instructions, for explanations about the information requested.

Controlled substances	Storage		Personnel Authorized to Access	Location for Use		Procurement	
	Double-locked	Not Double-locked*		VA Property	Not on VA Property	VA Pharmacy	Non-VA
Ketamine	(X)	()*	Jeffrey Iliff, Katherine Suchland, Joshua Cho, Mathew Sevaio, **Deidre Jansson, Jiyeon Ryu, Jay Jang	(X)	()	(X)	()

**Deidre Jansson has previously had Omnicell access, but needs to have access added again.

*For any controlled substance that will NOT be stored under double lock, with limited access, describe how it will be stored, and explain why this is necessary.

► N/A

- b. Check each statement below that applies, to confirm that all controlled substances used on this protocol will be procured according to VA pharmacy policies:

► (X) Some controlled substances will used on VA property, and all of these will be obtained through the local VA pharmacy.

► () Some controlled substances will not be obtained through the local VA pharmacy, but none of these will be used on VA property. See the ACORP Instructions, for further information.

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► () Other. Explain ►

2. **Human patient care equipment or procedural areas.** Does this protocol involve use of any human patient care equipment or procedural areas?

► () Yes, some human patient care equipment or procedural area(s) will be used for the animal studies on this protocol. Check "Appendix 7" in Item Y, below, and complete and attach Appendix 7, "Use of Patient Procedural Areas for Animal Studies".

► (X) No human patient care equipment or procedural areas will be used for the animal studies on this protocol.

3. **Explosive agents.** Does this protocol involve use of any explosive agent?

► () Yes, some explosive agent(s) will be used on this protocol. Check "Appendix 3" and "Appendix 8" in Item Y, below, and complete and attach Appendix 8, "Use of Explosive Agent(s) within the Animal Facility or in Animals", as well as Appendix 3, "Biosafety".

► (X) No explosive agent(s) will be used as part of this protocol.

- Y. **Summary of Attachments.** To assist the reviewers, summarize here which of the following apply to this ACORP.

Appendices. Indicate which of the Appendices are required and have been completed and attached to this protocol. Do not check off or attach any appendices that are not applicable to this ACORP.

- () Appendix 1, "Additional Local Information"
- () Appendix 2, "Antibody Production"
- (X) Appendix 3, "Biosafety"
- (X) Appendix 4, "Ante-mortem Specimen Collection"
- (X) Appendix 5, "Surgery"
- (X) Appendix 6, "Special Husbandry and Procedures"
- () Appendix 7, "Use of Patient Care Equipment or Areas for Animal Studies"
- () Appendix 8, "Use of Explosive Agent(s) within the VMU or in Animals"
- () Appendix 9, "Departures from "Must" and "Should" Standards in the *Guide*"

Standard Operating Procedures (SOPs). List in the table below, each of the SOPs referred to in this protocol, providing the information requested for each one. The approved SOPs must be included when the approved ACORP and Appendices are submitted for Just-in-Time processing before release of VA funding support.

Item	SOP		Approval Date
	Title	ID	
C.2.c	N/A		
M.1	Husbandry SOP with date of most recent December .		December 2023
M.2	Enrichment SOP with date of most recent December .		December 2023
U.4.a	N/A		
U.4.b	N/A		

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V			

- Z. **Signatures and Certifications.**
Once all items above have been answered and addressed, attach and sign the VAPSHCS ACORP: MAIN BODY Signature Page.

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ACORP APPENDIX 3: Biosafety

See [ACORP App. 3 Instructions](#), for more detailed explanations of the information requested.

- Summary of All Materials Administered to Animals on this Protocol.** Complete the table below for all materials to be administered to any animal on this protocol, indicating the nature of the material by marking EVERY box that applies, and indicating the BSL number for any infectious agents:

Material* (Identify the specific agent, device, strain, construct, isotope, etc.)	Source (Identify the vendor or colleague, or specify which animals on this protocol will serve as donors)	Nature of Material						
		Toxic Agent (Item 4)	Infectious Agent (Item 5) -- Enter the CDC Biosafety Level (BSL 1, 2, 3, or 4)	Biological Agent (Item 6)	Radioactive Agent (Item 7)	Contains Recombinant Nucleic Acid (Item 8)	Routine Anesthetic Agent (Pre- or Post- Procedural Drug)	Euthanasia agent
Isoflurane	VedCo	(X)	() BSL_	()	()	()	(X)	()
Ketamine	Fort Dodge	()	() BSL_	()	()	()	(X)	()
Xylazine	VedCo	()	() BSL_	()	()	()	(X)	()
Saline	Hospira	()	() BSL_	()	()	()	(X)	()
Artificial CSF (aCSF)	Tocris	()	() BSL_	()	()	()	(X)	()
Dextran, Texas Red, 3000 MW, Lysine Fixable Tracer	ThermoFisher Scientific	()	() BSL_	()	()	()	()	()
Dextran, Oregon Green, 70,000 MW, Lysine Fixable Tracer	ThermoFisher Scientific	()	() BSL_	()	()	()	()	()
Dextran, 10-70 kDa	Sigma	()	() BSL_	()	()	()	()	()
IRDye, LI-COR Biosciences	LI-COR Biosciences	()	() BSL_	()	()	()	()	()
Evans Blue	Fisher Scientific	()	() BSL_	()	()	()	()	()
Phosphate Buffered Saline	Millipore Sigma	()	() BSL_	()	()	()	()	()
Recombinant Human Deoxyribonuclease I (rhDNase1)	Genentech Inc.	()	() BSL_	()	()	()	()	()

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DNase1 (bovine)	Roche	()	()BSL_	()	()	()	()	()
VetBond	3M	()	()BSL_	()	()	()	()	()
Human CSF (pooled)	MIRECC BNG Repository (Dr. Elaine Peskind, MD)	()	()BSL 2	(X)	()	()	()	()

* A list of commonly used materials and the “Nature of Material” information is provided. There is no need to list CO2 used for euthanasia, O2 used for anesthesia, surgical scrub (iodine or chlorhexidine), coupling gel, ophthalmic lubricant, supplemental diet treats (Diet Gel, Mini-Treats).

Material	Nature of Material
Acepromazine:	Routine Pre-or Post-Procedural Drug or Anesthetic Agent
Artificial CSF:	Routine Pre-or Post-Procedural Drug or Anesthetic Agent
Atipamezole:	Routine Pre-or Post-Procedural Drug or Anesthetic Agent
Bromodeoxyuridine (BrdU):	Toxic agent
Bupivacaine:	Routine Pre-or Post-Procedural Drug or Anesthetic Agent
Buprenorphine Sustained Release:	Routine Pre-or Post-Procedural Drug or Anesthetic Agent
Buprenorphine:	Routine Pre-or Post-Procedural Drug or Anesthetic Agent
Carprofen:	Routine Pre-or Post-Procedural Drug or Anesthetic Agent
Depilatory Cream (Nair):	Routine Pre-or Post-Procedural Drug or Anesthetic Agent
Enrofloxacin (Baytril) :	Routine Pre-or Post-Procedural Drug or Anesthetic Agent
Ethanol:	Routine Pre-or Post-Procedural Drug or Anesthetic Agent
Euthanasia Solution:	Euthanasia agent
Glucose:	Routine Pre-or Post-Procedural Drug or Anesthetic Agent
Heparin:	Routine Pre-or Post-Procedural Drug or Anesthetic Agent
High Fat Rodent Diet:	Routine Pre-or Post-Procedural Drug or Anesthetic Agent
Insulin pellets:	Routine Pre-or Post-Procedural Drug or Anesthetic Agent
Insulin:	Routine Pre-or Post-Procedural Drug or Anesthetic Agent
Isoflurane:	Toxic agent
Ketamine:	Routine Pre-or Post-Procedural Drug or Anesthetic Agent
Ketoprofen:	Routine Pre-or Post-Procedural Drug or Anesthetic Agent
Lactated Ringer’s Solution:	Routine Pre-or Post-Procedural Drug or Anesthetic Agent
Lidocaine/Prilocaine cream:	Routine Pre-or Post-Procedural Drug or Anesthetic Agent
Lidocaine:	Routine Pre-or Post-Procedural Drug or Anesthetic Agent
Meloxicam:	Routine Pre-or Post-Procedural Drug or Anesthetic Agent
Midazolam:	Routine Pre-or Post-Procedural Drug or Anesthetic Agent
Nicotine:	Toxic agent
Pentobarbital:	Routine Pre-or Post-Procedural Drug or Anesthetic Agent
Propofol:	Routine Pre-or Post-Procedural Drug or Anesthetic Agent
Red Blood Cells:	Biological agent
Saline:	Routine Pre-or Post-Procedural Drug or Anesthetic Agent
Streptozotocin:	Toxic agent

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Tiletamine/Zolazepam (Telazol) :	Routine Pre-or Post-Procedural Drug or Anesthetic Agent
Urethane:	Toxic agent
Xylazine:	Routine Pre-or Post-Procedural Drug or Anesthetic Agent

2. **Summary of How Materials will be Administered.** Complete the table below for each of the materials shown in the table in Item 1 above:

Material* (Identify the specific agent, device, strain, construct, isotope, etc.)	Dose (e.g., mg/kg, CFU, PFU, number of cells, mCi) and Volume (ml)	Diluent* or Vehicle*	Reasons for use of non-pharmaceutical grade substances.**	Route of admin	Frequency or duration of admin	Reason for Administration and Expected Effects	Location of Further Details in this ACORP (specify “ Main Body” or “ App #” , and identify the Item)	Administration Under Anesthesia ,
Isoflurane	1.5-5%	Oxygen gas	Pharm-grade to be used	inhalation		Anesthetic, temporary sedation and insensitivity to pain	Main body, Items A.6.b.(1) and (3), C.2.a, C.2.c, U, Appendix 4 and 5	N

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Ketamine	Induction dose: 80-100 mg/kg, 0.2 ml/100 grams, Re-dosing: 40-50 mg/kg ketamine alone	Saline	Pharm-grade to be used	IP injection	Once and re-dosed with a partial dose as needed to maintain anesthetic plane for the duration of tracer injection and incubation	Anesthetic, temporary sedation and insensitivity to pain	Main body, Items A.6.b.(1), C.2.a, C.2.c, U, X, Appendix 4 and 5	N
Xylazine	5-10 mg/kg, 0.2 ml/100 grams	Saline	Pharm-grade to be used	IP injection	Once, xylazine will not be given for redosing	Anesthetic, temporary sedation and insensitivity to pain	Main body, Items C.2.a, C.2.c, U, Appendix 4 and 5	N
Saline	As diluent, as needed	Diluent	Pharm-grade to be used	IV, IP injection		Diluent of injectable agents and anesthetics, no effects expected		N
*Artificial CSF (aCSF) in mM: 120 NaCl, 2.5 KCl, 2.5 CaCl ₂ , 1.3 MgSO ₄ , 1 NaH ₂ PO ₄ , 25 NaHCO ₃ , 10 glucose	As diluent, as needed	Diluent	#3	Intracisterna magna	Once with tracer/dye	Diluent of tracers and dyes injected into the brain compartment, no side effects as aCSF is formulated to mimic the normal conditions of the fluid in the brain	Appendix 5	Y
*Dextran, Texas Red, 3000 MW, Lysine Fixable Tracer	1-5 ug, 1-5 ul	aCSF (diluent)	#1	Intracisterna magna	Once: 2-3 minutes	Monitor protein distribution in CSF, no expected side effects	Main body, Items C.2.a, C.2.c, Appendix 5	Y

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*Dextran, Oregon Green, 70,000 MW, Lysine Fixable Tracer	1-5 ug, 1-5 ul	aCSF (diluent)	#1	Intracisterna magna	Once: 2-3 minutes	Monitor protein distribution in CSF, no expected side effects	Main body, Items C.2.a, C.2.c, Appendix 5	Y
*Dextran, 10-70 kDa	0.5% Mixed with Irdye	aCSF (diluent)	#1	Intracisterna magna	Once: 2-3 minutes	Used to detect movement of CSF from brain compartments. No effects expected.	Main body, Items C.2.a, C.2.c, Appendix 5	Y
*Irdye, LI-COR Biosciences	10uM (10ul)	aCSF (diluent)	#1	Intracisterna magna	Once: 2-3 minutes	Used to measure movement of CSF from brain compartments. No effects expected	Main body, Items C.2.a, C.2.c, Appendix 5	Y
*Evans Blue	4% in aCSF (2-5 ul)	aCSF (diluent)	#1	Intracisterna magna	Once: 2-3 minutes	Used to confirm injection sites. No effects expected	Appendix 5	Y
*Phosphate Buffered Saline	5 ml at 1-2 ml/min	Water	#1	Transcardiac perfusion	Once: 3-5 minutes	Flush blood from circulatory system	Main body, Items C.2.a, C.2.c, Appendix 5	Y
rhDNase1	5-10 mg/kg (0.1 ml)	Phosphate Buffered Saline	Pharm-grade to be used	Tail vein injection	Once	Degradation of NETs (neutrophil extracellular traps)	Main body, Items C.2.a, C.2.c	N
DNase1* (bovine)	5mg/kg-10mg/kg, 0.2 mL	saline	#3	intravenous	once	To degrade NETs		N
VetBond	1-3 drops	Comes from manufacturer, ready for application	Pharm-grade to be used	Topical (skull)	Once	To close hole made in cisterna magna for tracer injection	Main body Item C.2.c and Appendix 5	Y

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Human CSF (pooled)	90 ul	None	*#1 see below	Via subcutaneous Alzet osmotic pump	0.5 ul/hr for 7 days	To observe the effects of young, aged, and AD human CSF on the glymphatic and lymphatic systems of different aged mice	Main body Item C.2.c and Appendix 5	N
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* Each material, diluent, or vehicle that is listed as FDA approved or is labeled "USP" is pharmaceutical grade. Check on-line for formulations that are FDA approved for administration to humans

(<http://www.fda.gov/Drugs/InformationOnDrugs/ucm129662.htm>) or animals

(<http://www.fda.gov/AnimalVeterinary/Products/ApprovedAnimalDrugProducts/UCM042847>). Designate with a *

each material and each diluent or vehicle to be used that is not pharmaceutical grade. For each of these, explain here why the use of a non-pharmaceutical grade formulation is necessary, and describe how it will be ensured that the material is suitable for use. (See ACORP App. 3 Instructions, for specifics about the level of detail required.)

► Artificial CSF, the dextrans, IRDye, Evan's Blue, and phosphate buffered saline are not readily available in pharmaceutical grade, but we are purchasing from reputable vendors and using agents that are specifically manufactured for animal research purposes. We have previously used all the above reagents, but for rhDNase1, which will be a pharm-grade reagent. For bovine DNase 1, the product documentation states that it is suitable for use in mammalian studies.

► *Human CSF is not pharmaceutical. It was obtained during lumbar punctures from subjects in a clinical setting under sterile conditions.

** List the reason number as appropriate for each substance marked as non-pharmaceutical grade in the column:

#1. This substance is not available in pharmaceutical grade. The suitability and safety of the substance (grade, purity, sterility, pH, pyrogenicity, osmolality, stability, formulation, and pharmacokinetics) are assured to the best of our ability.

#2. This substance is not available in pharmaceutical grade in an appropriate concentration. The suitability and safety of the substance (grade, purity, sterility, pH, pyrogenicity, osmolality, stability, formulation, and pharmacokinetics) are assured to the best of our ability.

#3. This substance is not available in pharmaceutical grade in a pure or appropriate formulation that meets our research needs. The suitability and safety of the substance (grade, purity, sterility, pH, pyrogenicity, osmolality, stability, formulation, and pharmacokinetics) are assured to the best of our ability.

3. Anesthesia, Sedation, or Tranquilization. Complete 3.a. and 3.b. below:

- a. For each material with "Y" entered in the last column of the table in Item 2 above, describe the anesthesia, sedation, or tranquilization to be used, identifying the anesthetic, sedative, or chemical tranquilizer, and detailing the dose, volume, and route of administration (Make sure that these agents are also included in Item 1 of this appendix, as materials to be administered): ►

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- For all the following agents:
 - aCSF (diluent)
 - Dextran, Texas Red, 3000 MW, Lysine Fixable Tracer
 - Dextran, Oregon Green, 70,000 MW, Lysine Fixable Tracer
 - Dextran, 10-70 kDa
 - IRDye
 - Evans Blue

Anesthesia: Ketamine/xylazine, ketamine (80-100 mg/kg) and xylazine (5-10 mg/kg) and saline diluent, 0.2ml/100grams body weight, intraperitoneal injection. The administration of this tracer must be done under ketamine anesthesia as isoflurane interacts with the lymphatic waste clearance pathway that we are studying.

- Phosphate buffered saline, Vetbond

Anesthesia: Isoflurane, inhalation, 4% isoflurane for induction followed by 1.5-2% isoflurane during the procedure, delivered in 100% oxygen via nose cone at a rate of 1.5-2 liters/minute OR ketamine/xylazine, ketamine (80-100 mg/kg) and xylazine (5-10 mg/kg) and saline diluent, 0.2ml/100grams body weight, intraperitoneal injection

- b. For each material with "N" entered in the last column of the table in Item 2 above, explain why no anesthesia, sedation, or tranquilization is necessary, or can be provided, and describe any alternate methods of restraint that will be used.

► Isoflurane, Ketamine, Xylazine are anesthetics, themselves.

- Isoflurane - animals will be placed in an induction chamber to begin inhalation of the substance and therefore will experience minimal distress.
- Ketamine and Xylazine - animals will be lightly restrained momentarily in one hand to expose the abdomen of the mouse for i.p. injection

► Saline – Saline is the diluent for the mixture of ketamine and xylazine and will be administered as part of the injection described above.

► DNase1 – this will be administered via tail vein injection. Mice are awake during the procedure and are in a restrainer designed for tail vein injections.

► Human CSF – this will be administered via an implanted subdermal osmotic pump. The pump is implanted under anesthesia, but then the animal awakens, and the infusion of CSF continues for 7 days. The infusion causes no discomfort and is intended to administer a constant level of CSF while the animal continues its normal routine.

4. **Toxic Agents.** Complete the table below for each of the materials listed as a "toxic agent" in the table in Item 1 above, checking the all of the properties that apply (see ACORP App. 3 Instructions, for details).

Name of Toxic Agent	a. M. L.	b. Car.	c. Ter.	d. Select Agent?	e. Other - specify toxic properties
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				Not a Select Agent	Select Agent Used in Sub-threshold Quantities	Select Agent that Requires Registration/Approval	
Isoflurane	()	()	()	(X)	()	()*	(X) ► May have effects on the following systems: cardiovascular, respiratory, gastrointestinal, and nervous. May affect fertility or unborn child.

*For each “select agent” that requires registration/approval (copy the lines below for each agent):

Name of agent ►

Registered with CDC or USDA ►

Registration Number ►

Registration Date ►

Expiration Date of Registration ►

Name of official who granted approval on behalf of VACO ►

Date of approval ►

5. **Infectious Agents.** Complete the table below for each of the materials listed as an “infectious agent” in the table in Item 1 above (see ACORP App. 3 Instructions, for details).

Name and BSL Number of Infectious Agent	a. ABSL Number*	b. Drug Sensitivity Panel Available? (Describe)	c. Select Agent?		
			Not a Select Agent	Select Agent used in Sub-threshold quantities	Select Agent that Requires Registration/Approval
Human CSF	ABSL-2	(Yes/No) No	(X)	()	()**
		(Yes/No)	()	()	()**
		(Yes/No)	()	()	()**

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	(Yes/No)	()	()	()**
	(Yes/No)	()	()	()**
	(Yes/No)	()	()	()**

*Complete the following for each agent for which the ABSL Number given is less than the BSL Number shown (copy the lines below for each agent. If more space is needed please add additional agents on an attached page):

Name of agent ►

Justification for applying ABSL measures that are less protective than those recommended ►

**For each “select agent” that requires registration/approval (copy the lines below for each agent):

Name of agent ►

Registered with CDC or USDA ►

Registration Number ►

Registration Date ►

Expiration Date of Registration ►

Name of official who granted approval on behalf of VACO ►

Date of approval ►

6. **Biological Agents.** Complete the table below for each of the materials listed as a “biological agent” in the table in Item 1 above (see ACORP App. 3 Instructions, for details).

Name of Biological Agent	Screening for Infectious Agents
Human CSF	CSF is collected in a sterile clinical setting. CSF is not screened for infectious agents.

7. **Radioactive Agents.** Complete the table below for each of the agents listed as a “radioactive agent” in the table in Item 1 above (see ACORP App. 3 Instructions, for details).

Name of Radioactive Agent (specify the isotope)	Authorized Individual	Approving Committee or Official

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8. **Agents Containing Recombinant Nucleic Acid.** For each of the materials checked in the table in Item 1, above, as “contains recombinant nucleic acid”, indicate which of the conditions applies (see ACORP App. 3 Instructions, for details).

Name of Agent that Contains Recombinant Nucleic Acid	Subject to the <i>NIH Guidelines for Research Involving Recombinant DNA Molecules</i>	Exempt
	()	()
	()	()
	()	()
	()	()
	()	()

9. **Potential for Pain or Distress.** Complete the table below for each of the agents listed in Item 1, above, that is expected to have potentially painful or distressing effects on the animals (see ACORP App. 3 Instructions, for details).

Name of Agent	Nature of Potential Pain/Distress	Measures to Alleviate Pain/Distress
Human CSF	Immune reaction to human CSF infusion into mouse brain	We will monitor the mice closely for 30 minutes post-surgery and once daily for 7 days for an adverse immune reaction. Indications of such a reaction may include head pressing, squinting, ataxia, immobility, and seizures. If any of these problems occur anytime during the 7-day infusion, the animals will be removed from the study and euthanized immediately. We will also halt the experiment and consider the alternative of an immune-incompetent mouse line.

10. **Protection of Animal Facility Staff from Hazardous Materials.** Complete Items 10.a and 10.b, below, for each of the agents listed in the table in Item 1, above, as “toxic”, “infectious”, “biological”, “radioactive”, or “contains recombinant nucleic acid” (detailed in Items 4 – 8). This item specifically addresses members of the animal facility

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VAPSHCS ACORP: Appendix 3

Biosafety (Version 4.3)

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staff; protection of the research staff from each of these agents must be addressed in Item G of the main body of the ACORP. See ACORP App.3 Instructions, for details.

a. Complete the table below.

Name of Hazardous Agent	Approving Committee or Official	Institution (VA or affiliate)	Names of Animal Facility Staff Members at Risk
Isoflurane	RSS	VAPSHCS	N/A
Human CSF	RSS	VAPSHCS	N/A

b. Detail how the individuals listed in the table above (Item 10.a.) have been (or will be) informed of the possible risks of exposure, and have been (or will be) trained to avoid exposure to these agents.

► CSF will be loaded into pump apparatus in a biosafety cabinet. Research staff will wear a face mask and face shield when implanting the pumps. Cages will be marked as BSL2. Animals will be placed in a fresh cage after implantation surgery so that no cage changes are necessary after the procedure.

11. **Once all of items above have been answered, attach and sign the VAPSHCS ACORP: Appendix 3 – Signature Page.**

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ACORP Appendix 4: ANTEMORTEM SPECIMEN COLLECTION

See [ACORP App. 4 Instructions](#), for more detailed explanations of the information requested.

- Summary.** Complete the table below for each specimen to be collected from a live animal on this protocol (see ACORP App. 4 Instructions, for details).

Specimen Collected	Site and Method of Collection	Anesthesia (Yes/No)	Amount Collected Each Time	Volume Replacement (Yes/No/N/A)	Total Number of Collections per Animal	Time Intervals Between Successive Collections
Ear punches	One or both ears, sharp ear punch tool	Yes	1-4, 2 mm rounds	N/A	1	N/A
Whole blood	Heart via terminal cardiac puncture with sharp needle	Yes	Total blood volume (500-1000 ul)	No	1	N/A
Cerebrospinal Fluid (CSF)	Cranium/Dorsal Neck/Cisterna magna, by stretched glass micropipette	Yes	10-15 µl	No	1	N/A

- Use of Anesthetics, Tranquilizers, or Analgesics.**

- For each specimen described in Item 1, above, as being collected WITHOUT anesthesia, complete Items 2.a(1) and 2.a(2), below:

(1) Explain why no measures will be taken to prevent pain (e.g., because of scientific requirements described here, or because the collection method involves no more than minor or momentary pain).



(2) Completely describe any method of physical restraint that may be used.



- For each specimen described in Item 1, above, as being collected WITH anesthesia, complete the following table:

Anesthetic, tranquilizer, or analgesic agent	Dose (mg/kg) and volume (ml)	Route of administration	Frequency of administration
--	------------------------------	-------------------------	-----------------------------

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Ear punch: <u>Isoflurane</u>	Isoflurane: 2-3% in ½ L O ₂ /min	inhalation	Once, briefly, until animal is immobile for the momentary procedure
Whole Blood: <u>Isoflurane</u>	Isoflurane (4-5%, 1.0L/min oxygen) to fully anesthetize, then 1.5-2% to maintain anesthesia	inhalation	Once, as need during procedure to maintain surgical plane
CSF: Ketamine/Xylazine	Ketamine (80-100 mg/kg) and xylazine (5- 10 mg/kg), 0.2ml/100grams body weight	intraperitoneal	Once and as need during procedure to maintain surgical plane
CSF: Isoflurane	Isoflurane (4-5%, 1.0L/min oxygen) to fully anesthetize, then 1.5-2% to maintain anesthesia	inhalation	Once, as need during procedure to maintain surgical plane

3. Volume Replacement for Fluid Collections.

- a. For each fluid specimen described in Item 1, above, for which NO volume replacement will be provided, explain why not.

► Cardiac puncture: This is a terminal procedure, blood will be collected immediately before perfusion, the animal will not recover after procedure.

► CSF collection: This is a terminal procedure, conducted immediately before perfusion, the animal will not recover after procedure.

- b. For each fluid specimen described in Item 1, above, for which volume replacement WILL be provided, describe the replacement fluids that will be administered (including their composition, volume, and route of administration).

►

4. Monitoring the animals. Detail how the animals will be monitored after collection of specimens to ensure that they recover appropriately (see ACORP App. 4 Instructions, for details).

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<p>▶ Ear punch: Mice will be returned to the home cage and monitored immediately after tissue collection until fully recovered from anesthesia.</p> <p>▶ Cardiac puncture: This is a terminal procedure; the animal will not recover.</p> <p>▶ CSF Collection: This is a terminal procedure; the animal will not recover.</p>			

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ACORP Appendix 5: SURGERY

See [ACORP App. 5 Instructions](#), for more detailed explanations of the information requested.

1. **Surgery Classification.** Complete the table below for each surgery included in this protocol, and indicate how it is classified (terminal, minor survival, major survival, one of multiple survival). See ACORP App. 5 Instructions, for details.

#	Surgery Description (specify the species, if ACORP covers more than one)	Terminal	Survival		
			Minor	Major	One of Multiple*
1	Stereotaxic injection of fluorescent tracers (intracisternal or intracortical)	(X)	()	()	()*
2	CSF collection (intracisternal)	(X)	()	()	()*
3	Perfusion	(X)	()	()	()*
4	Cardiac Puncture	(X)	()	()	()*
5	Intracerebroventricular cannulation and osmotic pump implantation	()	()	(X)	()*

*If survival surgery (including major surgeries and any minor surgeries that may induce substantial post-procedural pain or impairment) will be performed as part of this protocol in addition to any other such surgery (on this or another protocol) on the same individual animal, complete items 1.a and 1.b, below:

a. Provide a complete scientific justification for performing the multiple survival surgeries on an individual animal:
► N/A

b. Give the interval(s) between successive surgeries, and the rationale for choosing the interval(s):
► N/A

2. **Description of Surgeries.** Describe each surgery listed in Item 1, providing enough detail to make it clear what the effects on the animal will be. (Pre-operative preparation, anesthesia, and post-operative recovery will be covered in items 5, 6, and 7, below.)

Surgery 1 ► **Stereotaxic microinjection of tracers (terminal)** – Mice 2 to 6 months of age will be anesthetized with intraperitoneal (i.p.) ketamine/xylazine. Ketamine must be used for these studies, as isoflurane has been shown to interact with the glymphatic waste clearance system that we are studying. The animal will be monitored for any sign of responsiveness or movement to mild noxious stimulation during the procedure, and additional ketamine anesthesia given as needed. The surgical site is shaved to remove the fur. The skin is disinfected with betadine and 70% ethanol. The surgeon will wear clean gloves, and a lab coat. Surgical gloves will be available to the surgeon, but the surgical site will only be touched by sterile instruments, so normal nitrile gloves may also be worn for these acute terminal procedures. Mice will be positioned in a stereotaxic frame. The mouse is placed on a water-circulating warming pad during the procedure to maintain body temperature.

Intracortical injection - A 1 cm rostral-caudal midline skin incision is made over the cranium with dissection of soft tissue away from the calvarium. A hole is drilled into the skull above the injection tract. 1-5 microliters of agents (fluorophore-conjugated dextran tracers or dyes) will be injected at 0.5 µl/min

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by using a 5- μ l Hamilton syringe with a 30-gauge beveled needle attached to a syringe pump. Following agent delivery, the syringe will be left in place for 10 min before being withdrawn from the brain (this minimizes backflow of CSF and agents out of the skull).

Intracisternal injection – A ~2 cm rostral-caudal midline skin incision is made over the cranium and muscles of the neck. The muscles of the neck are retracted with suture to expose the transparent membrane of the atlanto-occipital membrane. Care is taken to avoid the blood vessels of the cisterna magna. A 30-gauge needle with fine tubing attached to a 1 ml syringe is inserted through the membrane. 1-5 microliters of agent (fluorophore-conjugated dextran tracers of 3kD and 70kD) will be injected at 0.5 μ l/min by using a syringe pump. Following agent delivery, the syringe will be left in place for 10 min and then withdrawn from the cisterna magna to minimize backflow of CSF and agents from the brain.

The animal's anesthetic plane will be maintained for a maximum of 120 minutes post-injection using ketamine (no xylazine). Some animals will be live-imaged in the LI-COR system during the post-injection period. After all stereotaxic injection surgical procedures, animals will be perfused for histological evaluation. This is a terminal procedure. Perfusions will be performed under isoflurane anesthesia, as animals are usually beginning to recover from K/X at this point. Concern for isoflurane's impact on tracer distribution is no longer an issue, as the tracer distribution experiment terminates at perfusion. Post-perfusion, brains will be collected and evaluated for A β deposition, neuroinflammation, and changes in tracer distribution.

Surgery 2 ► CSF collection (intracisternal) – The surgeon will wear clean gloves, and a lab coat. The mouse will be anesthetized with intraperitoneal (i.p.) ketamine/xylazine (K/X) or by inhaled isoflurane. The fur will be removed from the skin above the cisterna magna. The mouse will be placed into a stereotaxic device. The animal will be monitored for any sign of responsiveness or movement to mild noxious stimulation during the procedure and additional ketamine given if needed to maintain a surgical plane of anesthesia. The skin is disinfected with betadine and 70% ethanol. The mouse is placed on a circulating-water warming pad during the procedure to maintain body temperature. A ~2 cm rostral-caudal midline skin incision is made over the cranium and muscles of the neck. The muscles of the neck are retracted with suture to expose the transparent membrane of the atlanto-occipital membrane. Care is taken to avoid the blood vessels of the cisterna magna. A pulled glass pipette with fine tubing attached to a 1 ml syringe is inserted through the membrane. Negative pressure will be used to collect 10-15 μ l of CSF, then the pipette will be retracted. Finally, while still under anesthesia, mice will be perfused as described below to obtain brain tissue for analysis. This procedure is part of a terminal surgery, animals will not recover from anesthesia.

Surgery 3 ► Perfusion – In some cases, this procedure will be performed after the stereotaxic injection surgery. As the animal's anesthetic plane will begin to lessen by the end of the injection procedure, all mice will be moved to isoflurane for the terminal perfusion procedure. Inhaled isoflurane will be used at 4% for induction and 1.5-2% during the procedure, delivered in 100% oxygen at 0.5-1.0 L/min via nose cone. In a fume hood, the animal is placed on its back and checked for complete absence of responsiveness by pinching of the extremities (withdrawal reflex) periodically throughout the procedure. Once it is confirmed that the mouse is unresponsive, an incision using surgical scissors is made caudal to the xiphoid then two incisions are made along either side of the sternum, and the chest wall is retracted anteriorly to expose the heart and major vessels. The right atrium is opened via sharp dissection to decompress the circulatory system, and infusion of 5 mL of phosphate buffered saline (PBS) is pushed through using a syringe at 1 to 2 mL/min via needle into the left ventricle, directed toward the ascending aorta. The mouse dies during the PBS infusion. Then, in some mice, 10 mL of 4% paraformaldehyde (PFA) is pushed through at the same rate making sure that no air is introduced during the switch of solutions. The PFA is administered after the

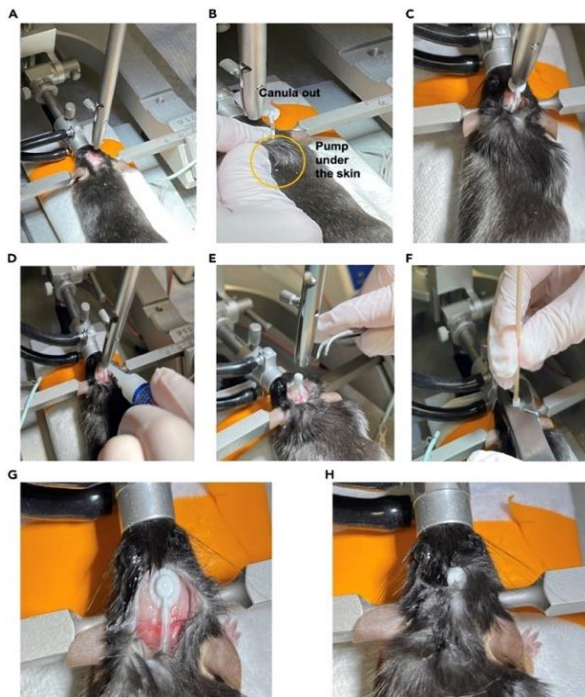
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animal has died and is not included in the list of test substances. The perfusion is continued until the effluent runs clear. Tissues are harvested. This procedure is terminal.

Surgery 4 ► Cardiac Puncture (terminal and as a part of Surgery 3) – As animals will be perfused immediately after this procedure, they will be maintained in an anesthetic plan under isoflurane (1.5-2%). Once it is confirmed that the mouse is unresponsive, an incision using surgical scissors is made caudal to the xiphoid then two incisions are made along either side of the sternum, and the chest wall is retracted anteriorly to expose the heart and major vessels. At this time, blood will be collected from the heart (500-1000 ul) using a 25-gauge needle. Mice will then be perfused with PBS as above in Surgery 3.

Surgery 5 ► Intracerebroventricular cannulation and osmotic pump implantation - This is a survival surgery and will be conducted under sterile conditions. the mouse is anesthetized by inhaled isoflurane (4-5%, 1L/min O₂ then maintenance at 2-3%). Once the animal is confirmed to be anesthetized by toe pinch and observation of slow and steady respiration, ophthalmic ointment is applied and the fur above the skull is shaved. The mouse's head is positioned in a stereotaxic frame. The mouse will be placed on a circulating-water warming pad during the duration of procedure to maintain body temperature. The skin is disinfected with 3 times alternating betadine and 70% ethanol. Mice receive 2-4 mg/kg Lidocaine and 1-2 mg/kg Bupivacaine (s.c.) at the incision site and will be positioned in a stereotaxic frame. Lidocaine has a fast onset (within 5 min) with a duration of up to 1-2 hours, while bupivacaine has a longer onset (20 mins) with longer duration, up to 4 to 6 hours in most species. The combination of the two analgesics should ensure that animals that are allowed to awaken from isoflurane are being appropriately treated to relieve post-procedural pain. A 1.5-2 cm rostral-caudal midline skin incision will be made to expose the skull, and a hole will be drilled at the precise coordinates (with the drill positioned above the bregma and zeroed at all three coordinates, from bregma, position cannula 1.1 mm lateral and 0.5 mm ventral) for cannula insertion. We will use the Alzet brain infusion kit containing the pre-sterilized cannula with PE-60 tubing to connect to the osmotic pump. A subcutaneous pocket will be bluntly dissected slightly further back from the skull above the neck and back muscles to allow room for the pump to sit and move naturally with the animal. The sterile micro-osmotic pump (Alzet, Model 1007D) will then be positioned in the pocket and the cannula inserted through the drilled hole for ICV infusion of human CSF. See figure below.

The cannula will be secured to the skull with Vetbond surgical glue or dental cement, and the skin closed using [wound clips or suture \(4-0 nylon, non-absorbable\) covered with a small amount of Vetbond](#). The cannula, tubing, and pump are all fully subcutaneous and no device protrudes from the skin post-surgery. Once the skin is closed, the mouse will be removed from anesthesia, singly housed in a clean cage placed on top of a circulating heat pad, and observed until it has fully recovered from anesthesia. Following awakening from anesthesia, mice will be monitored every 10 minutes for 30 minutes and once daily for 1-3 days to track recovery. Mice will remain singly housed to protect the cannula and surgical site while they recover 7 days post-procedure before termination of the experiment. Wound clips [or suture](#) will remain in place for the duration of the 7 days. [If needed, toenails will be trimmed if the surgical area is irritated due to scratching](#). Mice will receive the analgesic, Meloxicam (5 mg/kg, s.c.) during the surgery and once every 24 hours for 3 days post-surgery. This technique will allow for controlled delivery of human CSF into the mouse brain. Approximately 90 µl of human CSF will be slowly infused at a rate of 0.5 µl/hr over a period of 7 days using an osmotic pump. This setup will provide a model for studying the effects of human CSF on mouse physiology.



3. **Personnel.** Complete the table below for each individual who will be involved in any of the surgeries on this protocol.

Name	Surgery # (s) (see Item 1)	Role in Surgery			
		Surgeon	Assistant	Manage Anesthesia	Other (describe)
Katherine Suchland	1 and 3	(X)	(X)	(X)	()
Mathew Sevaio	1,2,3,4, 5	(X)	(X)	(X)	()
Justyna Swierz	1,2,3,4	(X)	(X)	(X)	()
Jay Jang	1,2,3,4, 5	(X)	(X)	(X)	()

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Surgery (Version 4.3)

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Keith Sanderson	1,2,3,4	(X)	(X)	(X)	()
Joshua Cho	1,2,3,4,5	(X)	(X)	(X)	()
Isabella Shafizadeh	1,2,3,4	(X)	(X)	(X)	()
Mila Friesch	1,2,3,4	(X)	(X)	(X)	()
Deidre Jansson	1,2,3,4,5	(X)	(X)	(X)	()
Thomas Ehler	1,2,3,4,5	(X)	(X)	(X)	()
William Zhang	1,2,3,4	(X)	(X)	(X)	()
Jiyeon Ryu	1,2,3,4,5	(X)	(X)	(X)	()

4. **Location of surgery.** Complete the table below for each location where surgery on this protocol will be performed.

Building	Room Number	Surgery #s (see Item 1)	Type of Space		
			Dedicated Surgical Facility	Other Dedicated Surgical Space	Other Space not Dedicated to Surgery
13	109	1,2,3,4,5	()	(X)*	()*

*For each space that is not in a dedicated surgical facility, provide the justification for using this space for surgery on this protocol

►Surgery in rodents does not require a dedicated surgical facility. Although outside of the ARF facility, the room where surgical procedures are conducted is separate from other lab procedures. It is designated for mouse procedures only.

5. **Pre-operative protocol.**

a. **Pre-operative procedures.** Complete the table below for each pre-operative procedure that will be performed to prepare the animal(s) for surgery.

Surgery #s (see Item 1)	Fast (Specify Duration)	Withhold Water (Specify Duration)	Place Intravenous Catheter(s) (Specify Site(s))	Other – Describe
1	() --	() --	() --	() --
2	() --	() --	() --	() --
3	() --	() --	() --	() --
4	() --	() --	() --	() --

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b. **Pre-operative medications.** Complete the table below. Include agent(s) for induction of anesthesia, as well as any other pre-treatments that will be administered prior to preparation of the surgical site on the animal.

Agent	Surgery # (see Item 1)	Dose (mg/kg) & volume (ml)	Route of administration	Frequency of administration (e.g., times/day)	Pre-operative period of treatment (e.g., immediate, or # of days)
Ketamine/Xylazine	1,2	Ketamine (80-100 mg/kg) and xylazine (5-10 mg/kg), 0.2ml/100grams body weight	intraperitoneal	Once	Immediate
Ketamine	1,2	Ketamine (80-100 mg/kg), 0.2ml/100grams body weight	intraperitoneal	*Supplemental dosing, as need during procedure to maintain surgical plane	Immediate
Isoflurane	2,3,4,5	3-4% in 0.5-1.0 L O ₂ /min	inhalation	Once	Immediate
Lidocaine	5	Bolus: 2-4 mg/kg, 0.02-0.04 ml/100 grams	s.c. at surgical site	One bolus dose at the time of surgery	immediate
Bupivacaine	5	Bolus: 1-2 mg/kg, 0.04-0.08 ml/100 grams	s.c. at surgical site	One bolus dose at the time of surgery	immediate

*The half-life of ketamine in mice is approximately 13 minutes and xylazine is approximately 1.5 hours. To avoid overdosing animals, we redose using only ketamine, since it is likely that xylazine is still present in the animal’s system.

c. **Pre-operative preparation of the surgical site.** For each surgery, identify each surgical site on the animals, and describe how it will be prepared prior to surgery.

- Surgery 1 ► Skull or atlanto-occipital membrane - The surgical site (top of the head) is shaved to remove the fur. The skin is disinfected with betadine and 70% ethanol. This is a terminal procedure.
- Surgery 2 ► Atlanto-occipital membrane - The surgical site (top of the head) is shaved to remove the fur. The skin is disinfected with betadine and 70% ethanol. This is a terminal procedure.
- Surgery 3 ► Thorax – This procedure is terminal. No special preparation of surgical site is made.

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Surgery 4 ► Thorax – This procedure is terminal. No special preparation of surgical site is made.

Surgery 5 ► Skull - Ophthalmic ointment will be applied to both eyes. The scalp will be shaved using small clippers and betadine will be applied with clean gauze in a circular fashion starting at the surgical incision site and rotating outward. Betadine solution will be alternated with 70% ethanol and this process will be repeated 3 times, discarding the gauze after each use. 2-4 mg/kg lidocaine and 1-2 mg/kg bupivacaine will be administered s.c. at the incision site.

6. Intra-operative management.

- a. **Intra-operative medications.** Complete the table below for each agent that will be administered to the animal during surgery.

Agent	Paralytic*	Surgery #(s) (see Item 1)	Dose (mg/kg) & volume (ml)	Route of administration	Frequency of dosing
Ketamine	()*	1,2	80-100 mg/kg, 0.2 ml/100 grams	intraperitoneal	As needed to maintain anesthesia throughout the procedure
Xylazine	()*	1,2	5-10 mg/kg, 0.2 ml/100 grams	Intraperitoneal	As needed to maintain anesthesia throughout the procedure
Isoflurane	()*	2,3,4	2-3% in ½ L O2/min	inhalation	As needed to maintain anesthesia throughout the procedure
IRDye	()*	1	10uM in aCSF, 2-5 ul	Intracisternal or intraventricular	Once
Dextran (10-70 kDa)	()*	1	0.5% in aCSF, 2- 5 ul	Intracisternal or intraventricular	Once

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Dextran, Texas Red, 3000 MW, Lysine Fixable Tracer	()*	1	1-5 ug in aCSF, 1-5 ul	Intracisternal or intraventricular	Once
Dextran, Oregon Green, 70,000 MW, Lysine Fixable Tracer	()*	1	1-5 ug in aCSF, 1-5 ul	Intracisternal or intraventricular	Once
Evans Blue	()*	1	4% in aCSF, 2-5 ul	Intracisternal or intraventricular	Once
Phosphate Buffered Saline	()*	3	5 ml at 1-2 ml/min	Transcardiac perfusion	Once

* For each agent shown above as a paralytic, explain why its use is necessary, and describe how the animals will be monitored to ensure that the depth of anesthesia is sufficient to prevent pain.

► N/A

- b. **Intra-operative physical support.** For each surgery, describe any physical support that will be provided for the animals during surgery (e.g., warming, cushioning, etc.).
- Surgery 1: The animal will rest on a circulating water heating pad during the procedure.
- Surgery 2: The animal will rest on a circulating water heating pad during the procedure.
- Surgery 3: This is a brief terminal procedure, and the animal will be maintained on a deep anesthetic plane to keep the animal unresponsive until it is deceased. No other physical support is necessary.
- Surgery 4: This is a brief terminal procedure, and the animal will be maintained on a deep anesthetic plane to keep the animal unresponsive until it is deceased. No other physical support is necessary.
- Surgery 5: Animal temperature will be maintained by a circulating-water warming pad during surgery.
- c. **Intra-operative monitoring.** Describe the methods that will be used to monitor and respond to changes in the state of anesthesia and the general well-being of the animal during surgery.
- The animal’s level of anesthesia will be determined by toe-pinch during the procedure, the animal will be monitored for any twitching of limbs, vibrissae, respiratory rate change or color that would indicate that the level of anesthesia has lessened or become too deep.
- In the case of surgery 5 (recovery surgery), the mouse’s temperature will be monitored by rectal probe.

7. **Survival surgery considerations.** For each survival surgical procedure indicated in Item 1 and described in Item 2, complete Items 7.a. – 7.g.
- a. Complete the table below for each survival surgery listed in Item 1, above.

	Survival Period	Measures for Maintaining Sterility
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Surgery # (see Item 1)		Sterile Instruments	Surgical Cap	Sterile** Gloves	Surgical Scrub	Sterile Drapes	Sterile Gown	Face Mask	Other*
5	7 days	(X)	()	()	(X)	()	()	(X)	()*
		()	()	()	()	()	()	()	()*
		()	()	()	()	()	()	()	()*
		()	()	()	()	()	()	()	()*

* Describe any "other" measures to be taken to maintain sterility during surgery.

**Justification is required here if sterile gloves are not use for survival surgery:

► A clean lab coat will be used in place of a sterile gown.

► Sterile gloves will be made available for the surgeon if they prefer to use them. To properly identify landmarks on the skull, stereotaxic surgeries require the surgeon to adjust the arms and knobs of the stereotaxic frame and surgical microscope during the procedure. Therefore, it is nearly impossible to keep surgical gloves sterile throughout the entire procedure and replacing gloves multiple times throughout the procedure increases the length of the procedure and the time the animal is under anesthesia. During the surgical procedure, the surgeon will never touch the surgical area of the scalp or skull with anything other than the tips of sterilized instruments once the surgical site has been cleansed. This will keep the surgical area of the mouse aseptic.

b. For each surgery, describe the immediate post-operative support to be provided to the animals.

Surgery 5 ► Following awakening from anesthesia, mice will be monitored every 10 mins for 30 mins. Followed by once daily for 3 days.

c. Post-operative analgesia. Complete the table below for each surgery listed in item 1, above.

Surgery # (see Item 1)	Agent*	Dose (mg/kg) & Volume (ml)	Route of Administration	Frequency of Dosing (e.g., times/day)	Period of treatment (e.g. days)
5	Meloxicam	Bolus: 5 mg/kg, 0.1 ml/100grams	s.c. injection	Once at surgery and once every 24 hours for 3 days post- surgery	Once 24 hours post- surgery

*For each surgery for which NO post-operative analgesic will be provided, enter "none" in the "Agent" column, and explain here why this is justified:

►

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- d. Other post-operative medications. Complete the following table to describe all other medications that will be administered as part of post-operative care.

Surgery # (see Item 1)	Medication	Dose (mg/kg) & Volume (ml)	Route of Administration	Frequency of dosing (e.g. times/day)	Period of treatment (e.g. days)

- e. Post-operative monitoring. After-hours contact information for the personnel listed must be provided to the veterinary staff for use in case of an emergency.

(1) Immediate post-operative monitoring

Surgery # (see Item 1)	Frequency of Monitoring	Duration at this Frequency	Name(s) of Responsible Individual(s)
5	Every 10 mins	30 min	Mathew Sevaio, Joshua Cho, Jay Jang, Deidre Jansson, Jiyeon Ryu

(2) Post-operative monitoring after the immediate post-operative period

Surgery # (see Item 1)	Frequency of Monitoring	Duration at this Frequency	Name(s) of Responsible Individual(s)
5	1x/day	3 days	Mathew Sevaio, Joshua Cho, Justyna Swierz, Jay Jang, Keith Sanderson , Isabella Shafizadeh, Mila Jolie Friesch, Deidre Jansson, Isabella Shafizadeh, William Zhang, Justyna Swierz, Katherine Suchland, Keith Sanderson

- f. Post-operative consequences and complications.

- (1) For each surgery, describe any common or expected post-operative consequences or complications that may arise and what will be done to address them.

Surgery 5 ► Mice will be monitored every day for 3 days post-procedurally. Any minor complications arising post-operatively, such as minor dehydration or minor injuries from fighting, can be addressed. For dehydration, we will put hydrogel in the cage. If necessary, 0.2-0.5 mL of subcutaneous warmed fluids may be given and the mouse will be monitored for improvement. If a mouse presents with severe dehydration (lethargy, severe skin tenting, sunken eyes), a veterinarian will be contacted. Mice will be

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single housed post-surgery to protect the surgical site and cannula. See below for more serious complications.

(2) List the criteria for euthanasia related specifically to post-operative complications:

Surgery 5 ►

- Altered respiratory rate
- Altered behavior: head tilt, eyes held closed/squinting, unwillingness to move/rear/stretch, abnormal posture, seizures, ruffled fur/poor grooming, dehydration
- Weight loss: animals with a body condition score below 2 will be removed from the study and euthanized.
- Infection at the incision site
- Animals showing signs of distress will be removed from the study and will be euthanized.

(3) In case an emergency medical situation arises and none of the research personnel on the ACORP can be reached, identify any drugs or classes of drugs that should be avoided because of the scientific requirements of the project. (If the condition of the animal requires one of these drugs, the animal will be euthanized instead.)

► No special requirements

g. Maintenance of post-surgical medical records. Complete the table below for each surgery, specifying where the records will held, and identifying at least one individual who will be assigned to maintain accurate, daily, written post-surgical medical records. Indicate whether the named individuals are research personnel involved in this project, or members of the veterinary staff.

Surgery # (see Item 1)	Location of Records	Name(s) of Individual(s) Responsible for Maintaining Written Records	Research Personnel	Veterinary Staff
5	Building 13, room 109 or Building 101, room 3W60	Jiyeon Ryu, Deidre Jansson, Joshua Cho, Mathew Sevaio, Jay Jang	(X)	()

8. Signature(s) and Certification.

Once all of the above items have been answered, the PI must attach and sign the certification statement on the Appendix 5 signature page.

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ACORP APPENDIX 6: SPECIAL HUSBANDRY AND PROCEDURES

See [ACORP App. 6 Instructions](#), for more detailed explanations of the information requested.

- Description of Procedures.** Complete the table below for each procedure listed in Item V of the main body of the ACORP that is not detailed in a SOP or in another item or Appendix of the ACORP. For each special procedure, check all features that apply.

Special Procedure		Features							
Number	Brief Description	Husbandry	Restraint	Noxious Stimuli	Exercise	Behavioral Conditioning	Irradiation	Imaging	Other**
1	Sleep Fragmentation: housing in Lafayette sleep disruption, rod-sweep chambers	(X)	()	(X)	()	()	()	()	(X)
2	Behavioral Testing	()	()	()	()	(X)	()	()	()

*Husbandry refers to all aspects of care related to the maintenance of the animals, including (but not limited to) provision of an appropriate diet, access to water, control of environmental conditions, and the selection of primary and secondary enclosures.

**Describe any “Other” features that are involved.

► Animals will be habituated to the sleep study chambers for one week prior to sleep experiments. These sleep chambers have an attachment that fits on top of the cage with a rod that sweeps the bottom of the chamber at specified short intervals to keep the animals from sleeping. This feature will not be powered until the time of the experiment; however, it will be there to get the animals used to its presence.

- Provide a complete description of each special procedure listed above, including the duration of the procedure, how frequently it will be repeated in any one animal, and any effects it is expected to have on the animal:

Special Procedure 1 ► **Sleep Fragmentation:** Lafayette Sleep Fragmentation Chambers were built and developed to recapitulate the chronic sleep fragmentation experienced by humans with obstructive sleep apnea and serve as a home cage for the mice throughout experimentation. Sleep fragmentation is performed in such a way as to disrupt but not prevent sleep through the utilization of a sweeping rod every 2 minutes for the 12 daylight hours (the period of greatest sleep for mice). This paradigm does not result in absolute sleep deprivation, but instead increases sleep fragmentation and wakefulness during normal sleeping periods, while increasing sleep during normal nighttime waking periods (when the rod is inactive). Sleep disruption will be conducted for one 8-week period and then for an additional week while the animals undergo daily behavioral testing. During the time that the bar is turned on, enrichment will be removed from the chambers to keep from jamming the rod. A control group will be housed in chambers for the 8-week period as well. Control chambers will have the rod turned on for 2 minutes in the morning to expose the controls to it, without it impacting their ability to sleep normally, but then the rod will remain off the rest of the 12-hour period.

This will be performed one time (no repeat procedures) from 2- months of age to 4- months of age, enabling a direct comparison to known timepoints of neuropathologic development in the *5xFAD* animals. Enrichment that

would prevent the rod from sweeping across the chamber floor will be removed each day for the 12 hours during which the sleep disruption is in action; enrichment will be returned to the chamber for the other 12 hours each night.

Animals in groups of two to four will be relocated into sleep study chambers (Lafayette Instruments). They will be habituated to these chambers for one week prior to experiments.

The dimensions of each chamber are: 9.625”L x 8.25”W x 7.75”H. The chambers will be cleaned between use by hand (effectiveness of sanitization will be assessed regularly as part of the ARF program to monitor hand-cleaned items, described in the ARF SOPs). Sleep fragmentation chambers will be housed in ARF building 34 room 151 and are not expected to produce noise levels above that of the rack ventilators etc. These chambers have been used successfully by our lab and by Dr. Abigail Schindler without adverse events (e.g., no harm to the mice). In this protocol and device, several factors contribute to a reduction of distress. First, the animals are group housed in essentially a standard housing environment, including adequate bedding and a bit of nestlet (a whole nestlet is too much and can “jam” the rod. A partial nestlet works well and ends up in a “corner” of the shoebox). Second, there is ad lib access to food and water, so animals are not calorically restricted.

Special Procedure 2 ► Behavioral Testing: Animals will be tested at 2-4 months of age. On each testing day, animals are moved into the testing room 45-60 minutes prior to the start of testing, to allow them to acclimate to the testing room. On the day before the first testing day, animals are moved into the testing room to acclimate, then weighed and briefly handled by the experimenter to habituate them to transport and handling. All testing equipment is sprayed with ethanol and wiped clean with a paper towel in between testing each animal, to sanitize the equipment and remove any scent traces that influence behavior. At the end of each day of use, the behavior test equipment is wiped with a peroxide sani-wipe for disinfection. The chambers are part of the ARF sanitization effectiveness monitoring program and will be screened regularly for effectiveness of the cleaning procedure. The behavior test paradigm is as follows:

- a) **Barnes Maze** – The Barnes maze tests spatial learning and memory. Testing will be conducted during the animals’ light phase, and the testing room will be brightly lit. The Barnes maze is a large circular platform made of white plastic, which has 20 small circular holes around the perimeter. One of the holes has a dark escape box located underneath it. Visual cues are placed around the maze, and the mouse must learn the location of the escape box based on spatial memory of the visual cues. Mice should be motivated to find and enter the escape box based on their natural dislike for open brightly lit spaces. On each training day, each mouse will receive four trials of 3 minutes each, during which time they are allowed to explore the maze freely. Each mouse will be placed at the center of the maze to start, and their movements will be recorded by a computer tracking system. Total locomotion will be measured, as well as the amount of time elapsed before entering the escape box. At the end of three minutes, if the mouse has not entered the escape box, it will be gently placed inside the box for 30 seconds before returning it to the home cage. The probe trial on day 5 is identical to the test trials, except that the escape box is removed.
- b) **Open Field Activity** – Each animal will be allowed to explore a 16 x 16-inch square field for 10 minutes, and total distance traveled will be recorded by a computer tracking system. In addition to general activity levels and speed travelled, the amount of time spent in the center versus the edges of the open field will also be recorded as a measure of anxiety-like behavior.
- c) **Spontaneous Alternation Y-maze** – This test measures spatial working memory by assessing how many times the mouse moves into a new vs previously explored arm. A mouse is placed in the center of the Y-shaped maze and the dividing arms are removed at the start of the test. The mouse is allowed to explore the maze for 10 minutes and then removed back to its home cage.

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- d) **Burrowing** - Burrowing is a normal spontaneous behavior in various rodent species, which is disturbed in various models of neurological disease. Animals are placed in individual cages, identical to the home cage but with the addition of a burrow partially filled with bedding material. The burrow is a plastic tube approximately 20cm long and 6cm in diameter, closed at one end. The open end is propped up 2cm off the cage floor by metal screws bolted to the tube. Normal animals will dig in the burrow and displace some percentage of the bedding. The amount remaining in the burrow after one hour will be measured in grams. Fresh bedding material is used for each animal tested.

Timeline for behavior testing:

- Day 1:** Barnes Maze Learning Trial Day 1
- Day 2:** Barnes Maze Learning Trial Day 2
- Day 3:** Barnes Maze Learning Trial Day 3
- Day 4:** Barnes Maze Learning Trial Day 4, Open Field
- Day 5:** Barnes Maze Prove Trial, Y-maze, Burrowing

These behavioral tests have been conducted regularly by many labs at the VA Puget Sound and are not expected to have any negative impact on the animals. The above timeline will only be conducted once per animal over the course of five days.

b. **Explain why each of these special procedures is necessary:**

Special Procedure 1 ► Sleep disruption is known to increase glymphatic dysregulation, and to investigate the role chronic sleep disruption (similar to that experienced in humans) has on amyloid plaque deposition we will induce chronic sleep fragmentation in mice and directly compare to animals which do not undergo sleep alterations. The chambers are necessary to study the effects of sleep, we must have a condition of waking, but also waking in the normal sleeping time to dissociate this regulation from normal circadian rhythms.

Relocating animals to sleep study chambers immediately prior to sleep experiments may introduce unnecessary stress and confound any results obtained. Allowing a week to adjust will reduce this factor.

Special Procedure 2 ► We propose testing cognitive and motor abilities of wild type, *5xFAD*, and *5xFAD/Pad4^{-/-}* mice to determine potential deficits due to these genetic manipulations resulting in the deposition of beta-amyloid. As a model for Alzheimer's Disease and it is necessary to test anxiety, learning, memory, motor ability, and the ability to complete normal activities of daily living, to understand if experimental interventions worsen or improve these abilities.

2. **Personnel.** Complete the table below for each special procedure listed in Item 1, above. Identify the individual(s) who will be responsible for carrying out the procedures, and those who will be responsible for monitoring the condition of the animals during and after the procedures. After-hours contact information for the personnel listed must be provided to the veterinary staff for use in case of an emergency.

Procedure Number (see Item 1)	Responsible Individual(s)	
	Carrying Out Procedure	Monitoring the Animals

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1	Joshua Cho, Elizabeth Rhea, Riley Weaver, Isabella Shafizadeh, Mila Jolie Friesch, William Zhang, Deidre Jansson, Thomas Ehler, Jiyeon Ryu, Sarah Pei	Justyna Swierz, Jay Jang, Keith Sanderson , Joshua Cho, Elizabeth Rhea, Riley Weaver, Isabella Shafizadeh, Mila Jolie Friesch, William Zhang, Deidre Jansson, Thomas Ehler, Jiyeon Ryu, Sarah Pei
2	Mathew Sevaio, Justyna Swierz, Jay Jang, Keith Sanderson , Joshua Cho, Isabella Shafizadeh, Mila Jolie Friesch, William Zhang, Deidre Jansson, Thomas Ehler, Jiyeon Ryu, Sarah Pei	Mathew Sevaio, Justyna Swierz, Jay Jang, Keith Sanderson , Joshua Cho, Isabella Shafizadeh, Mila Jolie Friesch, William Zhang, Deidre Jansson, Thomas Ehler, Jiyeon Ryu, Sarah Pei

3. **Potential Pain or Distress.** Complete the table below for each special procedure identified in Item 1, above, indicating for each procedure, whether potential pain and/or distress is expected, and, if so, describing the potential pain and/or distress and indicating whether any measures are to be taken to prevent or alleviate it.

Procedure Number (see Item 1)	Expected Potential Pain and/or Distress			
	No	Yes		
		Description	To Be Relieved	Not to Be Relieved
1	()	Chronic sleep fragmentation does induce minimal stress in the animal	() ^a	(X) ^b
2	(X)	Behavioral Testing	() ^a	() ^b

- a. For each procedure for which potential pain and/or distress is expected, but WILL be prevented or alleviated by administration of the analgesic(s) or stress-relieving agents, complete the table below:

Procedure Number (see Item 1)	Agent	Dose (mg/kg) & vol (ml)	Route of admin	Freq of admin (times/day)	Duration of admin (days post-procedure)
1					
2					
3					
4					

Describe any non-pharmacological measures to be taken to address the potential pain and/or distress:

Special Procedure 1 ►

Special Procedure 2 ►

- b. For each procedure for which potential pain and/or distress is expected and will NOT be prevented or alleviated, provide the scientific justification for this:

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Special Procedure 1 ► Alleviation of stress caused by chronic sleep fragmentation would include removing the sleep fragmentation paradigm and allowing for full nights rest with no additional fragmentation, thereby making the experiment itself ineffectual.

Special Procedure 2 ►

4. **Monitoring.** Describe how the condition of the animals will be monitored during and after each of the special procedures, and list the criteria that will be used to determine when individual animals will be removed from groups undergoing these procedures, because of pain or distress (see ACORP App. 6 Instructions, for details):

Procedure Number (see Item 1)	Monitoring Methods	Endpoint Criteria
1	Visual inspection: Animals will be monitored every single day over 2 months. While we do expect some mild stress expressions, any conditions that exhibit pain or extreme stress in the animals will be discussed with the Veterinarians.	If animals show observable signs of weight loss, abnormal posture, abnormal lethargy, or signs of lack of grooming they will be removed from the study. Otherwise, all animals will be maintained under the sleep deprivation study for 2 months, at which point they will be perfused, brains removed and sectioned for immunofluorescent studies.
2	Visual Inspection	Any animal that exhibits other signs of illness (hunched posture, scrawny appearance, lethargy, severe fight wounds, dermatitis etc.) will be excluded from the experiment and euthanized.

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ADMINISTRATIVE USE ONLY:
ACORP Summary of Changes

Date of Approval	Change Summary
08-04-2022	Three-Year Renewal
09-28-2022	Addition of Staff: Justyna Swierz
10-05-2022	Addition of Staff: Jay Jang, Keith Sanderson
01-12-2023	Update to existing staff: Adding Justyna Swierz, Jay Jang, and Keith Sanderson to surgical procedures.
02-23-2023	Change to previously approved procedures: Use of bovine DNase1 or rhDNase1 for Experiment 2.b. Addition of test substances: DNase1 (bovine). (Significant Change – VVC)
03-13-2023	Addition of Staff: Joshua Cho
04-06-2023	Addition of Staff: Elizabeth Rhea, Riley Weaver
05-18-2023	Addition of Staff: Isabella Shafizadeh. Removal of Staff: Sanjana Agarwal. Update to existing staff: Addition of surgery experience information for Joshua Cho.
09-13-2023	Removal of Staff: Samantha Keil, Molly Braun, Taylor Pedersen, Ryan O’Boyle, Ron Vered.
10-12-2023	Addition of Staff: Mila Jolie Friesch
11-21-2023	Addition of Staff: William Zhang
01-04-2024	Update to existing staff: Adding Isabella Shafizadeh and Mila Jolie Friesch to surgical procedures.
01-16-2024	Addition of Staff: Deidre Jansson
01-31-2024	Addition of Staff: Thomas Ehler
05-07-2024	Update to existing staff: Adding Thomas Ehler to surgical procedures.
05-20-2024	Update to existing staff: Adding William Zhang to surgical procedures.
06-17-2024	Addition of Staff: Jiyeon Ryu
08-12-2024	Addition of animals: 1774 C57BL/6 mice. Addition of test substances: VetBond, Human CSF. Addition of surgery: ICV cannulation and osmotic pump implantation. Removal of tail snips from antemortem specimen collection (Appendix 4). (Significant Change)
08-22-2024	Addition of animals: 512 mice. (Significant Change – VVC)