PI :	Dmochowski, Jacek P.
Protocol #	2024-1170
Status :	Approved (w/o Stipulation)
Approved :	12/23/2024
Expires :	12/23/2027
Title :	Ultrasonic Neuromodulation of the Rat Brain with Fiber Photometry

Table of Contents

Protocol Introduction

Tips on Navigation:

Browsers that support Cayuse: Safari, Google Chrome and Firefox. Cayuse utilizes pop-up windows, be sure to allow them for your Cayuse site.

In order to submit the protocol for IACUC review, please ensure that a green checkmark appears next to each section.

Please not that only the PI can officially submit for IACUC review. The PI will receive an email indicating the protocol is in a draft format and ready for the PI's review and submission.

To navigate between sections, you may use the navigation arrows at the bottom of each section when the section is complete. You can also click the section title in the Table of Contents. Some sections have attachment grids at the bottom of the section. Please feel free to attach any relevant documentation to those sections.

- **Preview Protocol:** To preview your protocol or to save a PDF, please click Preview Protocol from the Table of Contents on the lower left side of the screen.
- **Attachments:** Please click the Attachments section on the lower left of the Table of Contents to check the list of attachments.
- **Cancel Draft Protocol:** If the study is no longer active, or submission is no longer required, you can cancel this submission by going to the Cancel button in the Table of Contents on the left side of the section.

The selections below will populate the protocol form. Please feel free to return to the Options section to update selections as needed.

If you have any questions, please contact the IACUC Office at iacuc@ccny.cuny.edu.
Federal/Foundation funded?

☑

Internally funded?

☐

Private/Commercially funded?

☐

Other funding source?

☐

Will you use tissues or cell lines from an outside source?

Yes ○ No ◉

Will you be collaborating with an outside institution?

Yes ○ No ◉

Will any live animal research be conducted off campus?

Yes ○ No ◉

Will animals be housed outside central housing facilities for more than 12 hours?

Yes ○ No ◉

Will animals be moved through public access areas?

Yes ○ No ◉

Will field studies be conducted?

Yes ○ No ◉

Protocol Species Grid

A separate protocol applicaiton is required for each species.

To add a species, click the "ADD" button on the bottom of the grid, select the species from the picklist then place checkmarks in each column as applicable. Save the species selection by clicking the **Save** icon on the bottom of the grid. Then save the section by clicking the **Save Changes** button below the grid.

Species	Breeding?	Procedures?	Restraint?	Surgery?	Vet Drugs?	Test Agents?	Euthanize?
Rat (Rattus norvegicus)	No	Yes	Yes	Yes	No	No	Yes
Mouse (Mus musculus)	No	Yes	Yes	Yes	No	No	Yes

Protocol Overview

Note that you may click and drag the bottom right corner of any text box to resize it.

Enter title for this Protocol

Ultrasonic Neuromodulation of the Rat Brain with Fiber Photometry

Describe in non-technical terms the objectives of the work to be conducted on animals. Define all abbreviations.

The objective of this work is to test a new form of non-invasive brain stimulation using ultrasound waves. We hope to show that brain activity can be modulated by applying safe levels of ultrasonic stimulation from outside of the skull. If successful, this research could lead to new treatments for disorders marked by aberrant brain activity, such as Schizophrenia, Parkinson's Disease, and Epilepsy. The technique is being tested in animals to permit mechanistic and cellular-level insights into how low-intensity ultrasonic stimulation alters neuronal activity.

Briefly explain the relevance this work will have to human or animal health, the advancement of knowledge or the good of society. Define all abbreviations.

Aberrant neural activity is a hallmark of a myriad of neurological and psychiatric disorders. Pharmacological technique are inherently non-focal and often produce undesirable side effects. Non-invasive neuromodulation with ultrasound has the potential to rectify abnormal neural dynamics in a focal manner, and unlike conventional electromagnetic techniques such as TMS, it can penetrate deep subcortical regions. The findings of research in ultrasonic neuromodulation have the potential to transform how disorders of the brain are approached.

Please provide a brief summary of the proposed experiments. The summary should allow the committee to understand what combination of experimental interventions/procedures will be used for all animals, or groups of animals requested on the protocol. Please do not include specific experimental details here. These will be provided in the methodology section. The goal of this section is to capture the rationale and scope of the work to be conducted.

Experimental Summary

Experiments will be conducted in head-fixed awake rats. The experiments will combine transcranial ultrasonic stimulation (i.e., a transducer coupled to the animal's skull) with fiber photometry, which is an invasive technique that captures neurotransmitter activity by exciting brain tissue with light using an optical fiber inserted into the brain. Several weeks before any experimentation begins, the brain region of interest is injected with a virus (AAV) that causes the tissue to express a sensor for the neurotransmitter of interest (i.e, either glutamate of GABA). By recording glutamate and GABA levels before, during, and after application of the ultrasonic stimulation, we will be able to identify the effect of ultrasound on local excitability,

Protocol Federal/Foundation Funding List

The PI may select "Add From My List of Funds' to view the funding source options on previously approved protocols. If the funding source is not on the list, click "Cancel" to return to the main page for Federal/Foundation Funding. Then click on "Add Funding Source" to select from a predefined list and complete the funding information.

More than one funding source is allowed per protocol.

Please attach a copy of the approved grant per OLAW requirements.

Fund Source	Grant Title	Funded?	End Date	Grant #
National Institutes Of Health- Nigms	Explaining the variability in focused ultrasound neuromodulation	Yes	06/30/2026	R16GM145496

If the funding source is not available in the drop list, contact the IACUC office to have it added.

Fund Source

National Institutes Of Health- Nigms

Grant Title

Explaining the variability in focused ultrasound neuromodulation

Currently Funded?

☑

Grant Number

R16GM145496

PI on Grant (if different than PI on Protocol)

Proposed End Date

06/30/2026

Type of Animal Use

Identify all types of animal use for this protocol.
Your choices here and on subsequent pages will determine the correct USDA pain category.

Which of the following describes the type of animal use proposed in this application?

Research Type

Research

Other?

☐

Mouse (Mus musculus)

Species Information

Complete the required fields for the study animals in the protocol.

Age range

8-12 weeks

Target weight range

30 g

Maximum number of cages housed at one time.

4

What is the average length of time housed (weeks)?

16 weeks

Will the animals be Specific Pathogen Free?

Yes ◉ No ○

Identification Method

Type	Advantages	Disadvantages
Cage Cards	• Pertinent information is readily available	• Card can be lost or misplaced

Dye (Non-toxic, waterproof)	• Easy to apply • Non-invasive • Identifies individual animals	• Temporary
Ear Punch / Notch	• Easy to perform • Pattern can identify individual animals	• ID lost if ear is injured or mutilated • Punch / notch can become less readable over time • Animals can become indistinguishable if mixed between cages
Ear Tags	• Easy to apply • Permanent • Identifies individual animals	• Tags can be lost • Animal usually must be restrained to read tag
Tattoo	• Permanent • Identifies individual animals	• Anesthesia recommended • Animal may need to be restrained to read tattoo • Equipment can be expensive • Equipment must be disinfected between use • Training required to operate the tattoo instrument
Microchip	• Easy to apply • Permanent • Identifies individual animals • Not necessary to restrain animal to read	• Expensive • Microchip may migrate within the body
Toe Clip (requires scientific justification)	• Permanent • Pattern can identify individual animals	• Requires amputation of a body part • Requires anesthesia • Usually must restrain animal to read

Please enter animal identification methods to be utilized.
Examples:

- **Cage Cards**
- **Marker-Temporary**
- **Ear Punch/Notch**
- **Ear Tags**
- **Tattoo**
- **Microchip**
- **Toe Clip (neonates 5-7 days of age ONLY)**

Cage cards

Justification for Choice of Species

Justify the choice of species by stating why a species lower on the phylogenic scale is not appropriate.

Please indicate why the species selected is most appropriate for this study.

Large database exists for this species which allows comparisons with previous data

If you have selected Other, please describe your species justification below. If this does not apply, enter NA

NA

Enrichment and/or Exercise

The Animal Welfare Act requires facilities to provide exercise for dogs and programs to promote the psychological well-being of non-human primates, while the U.S. Public Health Service Guide to the Care and Use of Laboratory Animals encourages "enriching the environment as appropriate to the species....".

Will animals be group housed?

Yes ◉ No ○

Will environment enrichment be provided?

Yes ◉ No ○

Mice & Rats

NylaBones

☑

Nesting Material

☑

Plexiglas Tubes

☐

Cardboard Huts or Tubes

☑

Rabbit

Because rabbits are herbivores and have a large cecum, the regular feeding of items such as grass hay (cubes or loose), carrots, leafy greens, and other vegetables is beneficial to their digestion. In addition, since rabbit incisors as well as molars grow continuously, chewing allows teeth to wear down.

Food and Treats

☐

Large Plastic Balls

☐

Bells and Bar Bells

☐

Chew Sticks Which May Be Either Edible or Made of Wood

☐

Hay

☐

Food and Treats

☐

Ferret

Ferret Balls

☐

Nyla Bones or Other Chew Toys

☐

Food and Treats

☐

PVC Pipes for Tunneling

☐

Use Locations

Indicate all of the locations where surgeries, procedures and/or euthanasia will be performed.

Click "Add Use Locations" and complete location information. The PI may select "Add From My List of Locations" to view locations from previously approved protocols.

More than one option my be included.

Location/Building	Room	Type
Steinman	572C	USE

Location/Room

Steinman 572C

Location Type

USE

Strain Information

Click "Add Strain" and complete strain information. The PI may select "Add From My List of Strains" to view the strains from previously approved protocols.

More than one option my be included.

Strain	Age	Weight	Phenotype	Edited
C57BL/6J	8-12 weeks	30 g	wild-type, healthy	Edited

Hit the spacebar and choose a strain from the list. If the strain you want is not on the list, enter it in the same field.

Species Strain

C57BL/6J

Age

8-12 weeks

Weight

30 g

Phenotype

wild-type, healthy

Non-Surgical Procedures

Click "Add Procedure" and complete procedure information. The PI may select "Add From My List of Procedures" to view the procedures from previously approved protocols.

More than one option may be included.

Procedure Name
Drug Administration
Other
Training

Procedure Name

Drug Administration

Describe the procedure, giving enough detail so that another individual could carry it out (e.g. volume, dose, duration, frequency, ...)

Stereotaxic Surgery and Viral Vector Administration

Mice will undergo stereotaxic surgery under general anesthesia (e.g., isoflurane 1–3% in oxygen) for intracranial injection of an adeno-associated virus (AAV) encoding a genetically encoded fluorescent sensor suitable for fiber photometry (e.g., dLight1.3b for dopamine, or GCaMP variants for calcium). Animals will be placed in a stereotaxic frame, and the scalp will be shaved and sterilized. A small craniotomy will be made over the target brain region (e.g., dorsal striatum, VTA, etc.), and a pulled glass micropipette or 33-gauge Hamilton syringe will be used to deliver the viral vector. A total volume of ~300–500 nL will be injected at a rate of 50–100 nL/min using a microinjection pump. Following injection, the needle will remain in place for 5 minutes to minimize backflow, then be slowly withdrawn.

The surgical site will be closed with tissue adhesive or sutures as appropriate. Animals will receive perioperative analgesia (e.g., carprofen 5 mg/kg subcutaneously, pre- and post-operatively) and be monitored until full recovery from anesthesia. Animals will be allowed to recover for at least 2–3 weeks post-surgery to allow for viral expression before any behavioral testing or fiber photometry recordings.

Please describe any special precautions you have taken or will take to assure that the procedure can be conducted safely for animals and humans

Animal Safety:

- Controlled Injection Volume and Rate: The AAV solution will be injected slowly (e.g., 50-100 nL/min) to reduce pressure on surrounding brain tissue, minimizing potential damage and inflammation. After the injection, the syringe will be left in place for 5 minutes before withdrawal to prevent reflux of the virus and ensure safe distribution.
- Aseptic Technique: Aseptic conditions will be maintained throughout the procedure to prevent infection. Sterile gloves, instruments, and injection syringes will be used, and the animal's head will be disinfected with betadine or chlorhexidine.

Human Safety:
- Personal Protective Equipment (PPE): Researchers handling AAV will wear appropriate PPE, including lab coats, gloves, and safety goggles, to avoid accidental exposure to the virus.
- Designated Work Area: The AAV injection preparation and handling will occur in a designated biosafety cabinet or designated lab area, adhering to biosafety level 1 guidelines for AAV.
- Sharps Disposal: All syringes and needles used for the injection will be immediately disposed of in a designated sharps container after use to prevent accidental needle sticks or contamination.
- Virus Containment: Only trained personnel will handle the viral preparation. Any spills will be immediately disinfected with 70% ethanol or an appropriate virucidal agent to ensure the lab remains free of contamination.

Post-Procedure Protocols:
- Animal Monitoring: Animals will be closely monitored post-injection for any signs of adverse reactions, such as inflammation, distress, or abnormal behavior.
- Waste Disposal: All waste generated from the injection procedure, including PPE, syringes, and wipes, will be treated as biohazardous waste and disposed of according to institutional guidelines to prevent environmental contamination.

Procedure Name

Other

Describe the procedure, giving enough detail so that another individual could carry it out (e.g. volume, dose, duration, frequency, ...)

Transcranial ultrasonic neuromodulation

A small circular transducer (Sonic Concepts SU-132) will be coupled to the animal's skull via ultrasound gel. The skull coordinates relative to bregma/lambda will be selected so that the beam is focused on the CA3 region of the right hippocampus (obtained from a stereotaxic atlas). Ultrasonic stimulation parameters (intensity, pulse repetition frequency, pulse duration) will be selected from the list of waveforms that are to be assessed in the experiments. Intensity is limited to 550 kPa, a value that has been demonstrated by our group and others to be safe. Sonication is brief, spanning a duration of approximately 1 second, and is repeated across N=30-50 trials per session, with at least 10 seconds between sonications to ensure that any tissue heating has dissipated.

Fiber photometry

Equipment: Doric Lenses Fiber Photometry Console, Fluorescence Mini Cube, Mono Fiberoptic Patchcord, Mono Fiberoptic Cannula

The Fiber Photometry Console is connected to the Fluorescence Mini Cube. The Mini Cube integrates two photodetectors and three LEDs with specific excitation and emission filters for neurotransmitter sensors (e.g., iGluSnFR or GABASnFR). We then attach a Mono Fiberoptic Patchcord (400/430/1100-0.57) between the Fluorescence Mini Cube and the implanted optical cannula in the animal's hippocampus. The fiber optic cannula and patchcord ensure efficient light delivery and fluorescence collection. The excitation wavelength is set on the console to 460-493 nm for iGluSnFR or other green-fluorescing indicators. This LED intensity should be calibrated to approximately 20 µW at the fiber tip to minimize photobleaching and prevent tissue heating. Once the excitation light is applied, the system will capture the fluorescence emitted in the 500-540 nm range, corresponding to glutamate release in the brain as detected by iGluSnFR. Fluorescence signals are recorded through the two photodetectors integrated into the Mini Cube. Data acquisition software (provided by Doric Lenses) will capture and display real-time fluorescence intensity changes, indicating neurotransmitter dynamics. Each experimental session lasts up to 20 minutes, with photometry and ultrasonic stimulation applied in intervals. Rats are subjected to a maximum of three sessions per week, ensuring adequate recovery and avoiding prolonged exposure to excitation light.

Please describe any special precautions you have taken or will take to assure that the procedure can be conducted safely for animals and humans

Intensities are limited to values that have been shown by our group and others to be safe and painless (< 500 kPa). There is an interval of at least 10 seconds between consecutive sonications to allow any tissue heating to dissipate.

Procedure Name

Training

Describe the procedure, giving enough detail so that another individual could carry it out (e.g. volume, dose, duration, frequency, ...)

Training Procedure Description (Operant Chamber and Lever Task):

Mice will be trained in a standard operant conditioning chamber equipped with a lever and a food pellet dispenser. The goal of the training is to condition the animals to press the lever in order to receive a food reward, which will be used in conjunction with fiber photometry recordings and ultrasonic neuromodulation. Animals will undergo daily training sessions (approximately 30–60 minutes) for 5–10 consecutive days or until task acquisition criteria are met (e.g., consistent lever pressing for reward). Mice will be food-restricted to 85–90% of their baseline body weight during the training period to increase motivation, in accordance with institutional and AVMA guidelines. Water will be provided ad libitum at all times. Training will take place during the light cycle in a quiet behavioral testing room to minimize external stressors. Habituation to the operant chamber will occur on Day 1, followed by shaping procedures that reinforce lever-pressing behavior. All procedures will be conducted by trained personnel familiar with behavioral training in rodents.

Please describe any special precautions you have taken or will take to assure that the procedure can be conducted safely for animals and humans

Operant chambers will be cleaned and inspected between sessions to maintain hygiene and minimize transmission of pathogens. Animals will be closely monitored during each training session for signs of stress, dehydration, or weight loss. Any animal showing signs of distress or failing to maintain minimum body weight will be removed from food restriction and monitored closely.

For personnel, all individuals performing training will be trained in rodent handling and behavioral techniques. Food restriction and reward schedules will be documented and reviewed regularly. Personal protective equipment (PPE) including gloves and lab coats will be worn at all times, and all animal work will occur in designated vivarium or behavioral rooms approved for animal use. Any equipment involving live animals will be inspected routinely to ensure proper functioning and safety.

Restraint Types

This section applies to any method of restraint lasting 10 minutes or longer.

Click "Add Restraint" and complete restraint information. The PI may select "Add From My List of Restraints" to view the restraints from previously approved protocols.

More than one option may be included.

Restraint Type	Duration	Rationale	Edited
Routine (manual Or Restraint Device < 10minutes)	< 30 seconds	Brief restraint is required to connect the fiber optic patch cable to the implanted cannula.	Edited

Restraint Type

Routine (manual Or Restraint Device < 10minutes)

Rationale for this type of restraint

Brief restraint is required to connect the fiber optic patch cable to the implanted cannula.

Duration of restraint

< 30 seconds

How is animal acclimated to the restraint device?

Habituation during training sessions.

Surgical Procedures

Important Instructions: Items highlighted in red require editing. Please select the entry and click Edit Surgery.

Click "Add Surgery" and complete surgery information. If you are the PI, you may click "Add From My List of Surgeries" which will narrow the options to surgeries previously approved on your protocols. You may add more than one source.

Surgery Type	Other Surgery Name	MSS?	Edited	Recovery Type
Stereotaxic Surgery		No	Edited	Recovery

Surgery Type

Stereotaxic Surgery

If Other, provide Surgery Type below (otherwise enter N/A)

Recovery Type

Recovery

Describe pre-op procedures including acclimation period, fasting, procuring baseline information such as body weight, body temp, blood values, etc. as relevant.

Acclimation Period: Mice will undergo a three-week acclimation period after arrival to minimize stress and ensure they are in good health prior to surgery.

Baseline Measurements: Body weight will be recorded for each animal one day prior to surgery. Daily health checks, grooming behavior, and food intake will be monitored throughout the acclimation period.

Fasting: Fasting is not required for rodents as they are unable to vomit. Animals will have access to food and water up until the time of anesthesia.

Body Temperature: Baseline body temperature will be recorded immediately before surgery using a digital rectal thermometer. Body temperature will continue to be monitored and maintained within a physiological range throughout the procedure using a heated surgical pad.

Describe the surgical attire to be used and surgical site preparation (e.g. incision site).

Surgical Attire: Personnel performing the surgery will wear sterile surgical gloves, a surgical gown, mask, and cap to maintain aseptic conditions.

Surgical Site Preparation: The surgical area on the animal's head will be shaved, cleaned, and disinfected with a series of betadine or chlorhexidine scrubs, followed by a final rinse with 70% ethanol. The incision site will be covered with a sterile drape to ensure aseptic conditions throughout the surgery.

Describe any support and/or anesthetic monitoring equipment that will be used for surgeries.

Anesthesia: Isoflurane anesthesia will be used, starting with an induction dose of 3% in an induction chamber and maintained at 1.5-2% via a nose cone during surgery.

Monitoring Equipment:
- Temperature: A digital rectal thermometer and a heating pad will be used to monitor and maintain body temperature.
- Respiration: Respiratory rate will be visually monitored throughout the procedure to ensure that the animal maintains a stable breathing pattern.
- Oxygen Saturation: A pulse oximeter (Kent Scientific; paw pulse ox) will be used if available to monitor oxygen saturation.
- Additional Support: Saline (0.9% NaCl, subcutaneously) will be administered pre-operatively to maintain hydration.

Will any sedatives, anti-cholinergics, or other medications be administered prior to anesthetic induction? If so, describe here.

No

Will paralytics be used for this procedure? If yes please justify the use and describe your plan for monitoring.

No

Anesthetic Depth Measurement

Toe Pinch Reflex: The anesthetic depth will be assessed by the absence of the toe pinch reflex prior to making the incision and periodically during the procedure.

Respiratory Monitoring: The respiration rate will also be monitored, as slowing of respiratory rate and regular, stable breathing patterns indicate an appropriate level of anesthesia.

Include a plan for monitoring frequency, duration and intervals of post-op analgesia/antibiotics, post-operative monitoring, nursing care, etc.

Post-Operative Monitoring: Animals will be monitored every 15 minutes for the first hour post-surgery until they regain full consciousness. Following this, animals will be checked twice daily for the first 72 hours and once daily for the remainder of the first week.

Analgesia: Buprenorphine (0.05 mg/kg SC) will be administered every 8-12 hours for the first 48 hours following surgery to manage post-operative pain. If signs of discomfort persist, analgesia may be extended based on veterinary guidance.

Antibiotics: A topical antibiotic ointment (triple antibiotic or equivalent) will be applied to the surgical site once daily for 5-7 days to prevent infection.

Nursing Care: Rats will be placed in padded cages with access to soft food and hydration supplements (e.g., hydrogel) to encourage feeding and hydration while they recover. Bedding will be changed daily to maintain a clean environment.

When will sutures, wound clips, or staples be removed?

7-10 days post surgery or once the incision has healed.

Clinical parameters to be monitored during 1st week after surgery and contingency plans for post-surgical complications (e.g body weights)

Parameters Monitored:
- Body Weight: Body weight will be recorded daily during the first week post-surgery to detect any significant weight loss. Any weight loss exceeding 10% of the pre-surgical weight will prompt evaluation by veterinary staff.
- Grooming and Activity Levels: Animals will be observed for normal grooming behavior and activity.
- Incision Site: The surgical site will be inspected daily for signs of redness, swelling, or discharge. If any of these signs are noted, the animal will be evaluated by veterinary staff, and additional antibiotics or interventions will be administered as needed.

Contingency Plans:
- If an animal shows signs of distress, infection at the incision site, or significant weight loss, it will receive immediate veterinary attention. Additional supportive care, analgesics, or antibiotics will be administered based on veterinary recommendations. If complications are severe or unmanageable, the animal may be humanely euthanized to prevent suffering.

Will Analgesics be withheld?

Yes ○ No ◉

Surgical Locations

004:572C

Multiple Survival Surgeries

If you are conducting Multiple Survival Surgeries on this Species, please fill in the required fields.

Will multiple surgical survival procedures be performed on an individual animal (separate anesthetic events)?

Yes ○ No ◉

Euthanasia Method Information

AVMA Guidelines for the Euthanasia of Animals: 2020 Edition (https://www.avma.org/resources-tools/avma-policies/avma-guidelines-euthanasia-animals)

Euthansia Method
Carbon Dioxide Inhalation

Euthanasia Method

Carbon Dioxide Inhalation

AVMA Classification

Acceptable

If method is not AVMA acceptable, please provide justification

State the secondary method of euthanasia or assurance of death.

Thoracotomy

Animal Numbers

*The USDA categories marked as required (with a red *) below were determined to be appropriate based on the procedures reported.*

Please enter the number of animals requested for each of the required categories. The total of the required fields may not exceed the total requested number.

The USDA Categories are defined as follows:
B = animal held for breeding and/or not yet used in research
C = no pain or distress
D = alleviated pain or distress
E = unalleviated pain or distress

Please enter the number of animals you plan on utilizing in the three years of the protocol.

USDA Category	# of Animals
B	0
C	0
D	30
E	0

Justify the number of animals to be used.

- **Describe the statistical method (or other method) used to justify the number of animals per group.**
  Federal guidance states that statistical methods must be used in order to justify the number of animals requested.
- **Be sure to include breeding colony numbers (production, maintenance, undesired genotypes).**
- **Please provide a description of all animal procedures, surgical and non-surgical.**
- **To justify the number of animals that will be used, provide a statistical analysis, such as power analysis, and include the results of that analysis in the description. With the description, please insert a table that includes group, treatment, number of animals, pain category, and whether the animal will be euthanized at the end of the experiment.**
- **The numbers provided in this section must also match the total amount in the '# of Animals' column above.**

We request approval for n=10 animals per year, for a total of 30 animals. This number reflects our expectation that each animal will undergo ~15 sessions. Given the effect size in our previous electrophysiological study (Cohen's d = 0.3), we expect to obtain an alpha of 0.05 and a power of 0.8 with a sample size of n=175 (value obtained from statistical power table). The study has two separate dependent variables (glutamate and GABA; we do not have the capability for concurrent recording of glutamate and GABA), necessitating 175 * 2 = 350 samples. Our expected sample of 450 sessions (30 animals * 15 sessions per animal) accounts for the fact that the procedure is new to the laboratory, we expect that some of the sessions or animals will not yield valid data. Therefore, we are overpowering the study to accommodate potential pitfalls in fiber photometry.

Methodology

*For each species, **describe in narrative form all experimental or instructional procedures to be performed on the animals** (e.g. blood collection, surgery, behavioral training, administration of substances or test compounds, breeding, tumor induction, etc.). Describe the procedures in the order in which they will be performed, including time frames and intervals. Include a description of procedures performed on anesthetized animals. All procedures checked in the procedures section should be described below.*

Include the rationale for use of tissues in vitro. Do not describe in vitro procedures performed on tissues taken from animals or procedures performed on animals after they are euthanized.

*Please list the interventions/procedures in chronological order. Indicate the time interval between each procedure, and the final disposition of the animals at the end of the experiment. **All procedures in the table on the procedures section must be included.***

Flowcharts or other graphical representations of the methodology can be very helpful. Tables, pictures, and flowcharts can be created in the Procedure Description text box within Cayuse. The information can also be pasted into the text box. It is preferred that all tables, charts, and pictures be inserted in the text box but you may attach the files below if needed. Please contact a member of the IACUC or veterinary staff should you require guidance regarding the information and level of detail that should be provided here.

Procedure Description

Order of procedures

1. Stereotaxic Surgery
2. Drug Administration (AAV injection during surgery)
3. Training for operant conditioning task and fiber photometry
4. Fiber photometry + transcranial ultrasound during operant conditioning

1. Stereotaxic surgery

All instruments will be sterilized via an autoclave machine before the start of survival surgery. All gloves, gowns worn by surgeons will be new and sterile. The surgical area will be covered by a sterile surgical table cover. A glass bead sterilizer will be used during the surgery to ensure continuous sterilization of the instruments.

The duration of the surgical procedures for virus injection and cannula implantation will be less than 3 hours. To ensure the stability of the cannula, the animal must be of a certain age and size before surgery. The animal should be in their adult stage (>10 weeks) and weigh at least 30 g.

During the entirety of this survival surgery, the animal will be anesthetized with isoflurane via inhalation. First, the animal will be pre-anaesthetized with 3% isoflurane in the induction chamber with oxygen at 2 L/min. Once anesthetized, isoflurane will be lowered to 2%. This level will be maintained for head shaving, stereotaxic fixation, craniotomy, duratomy, AAV injection, cannula implantation, and finally, dental cement application.

During head shaving, the animal is placed on a sterile pad with 2% isoflurane and oxygen delivered via a nose cone. Once head shaving is complete, the animal is transferred to the stereotaxic frame (Kopf) which is covered by another sterile pad, and all parts making contact with the animal (ear bars, head holder, etc) have been autoclaved. The animal head is fixed via ear bars with lidocaine as a local analgesic into the ear canal to mitigate pain. Eye lubricating gel is placed onto the animal's eyes to help protect and prevent drying. Betadine is used to clean the surgical area (area shaved). At this point, 1 ml of lactated ringers dextrose is given (SC) to the animal to help fortify them for the upcoming surgery.

Next, bupivacaine solution (0.1ml:0.3ml, bupivacaine:saline cocktail) is injected into the area of incision over the rat's head. A short amount of time is given to allow the analgesic to take effect, then an incision is made to create an opening to expose the animal's dorsal skull. Once the skull is cleared of ligaments and connective tissues and bregma-lambda lines are visible to the eye, measurements of the bregma-lambda length is taken. Coordinates are determined on the skull and marked for a 1 mm^2 craniotomy. A dental drill is used to perform the craniotomy on the skull, followed by a duratomy to expose the brain in the craniotomy window.

2. AAV Injection

Once the craniotomy has been made over the target brain region a Hamilton syringe will be used to deliver the AAV expressing either iGluSnFR or GABASnFR into the hippocampus. The AAV solution will be injected slowly (100-200 nL/min) with a total volume of 1-2 µL, and the syringe will be held in place for 5 minutes post-injection to prevent reflux.

Following virus injection, an optical cannula will be lowered to the targeted brain area to permit subsequent photometry excitation and recording. The cannula will be fixed in place using dental cement to ensure stability during future experiments. The scalp will be sutured (4-0 gauge, Ethicon brand) around the implant, leaving the cannula head exposed for future attachment to fiber optic cables.

In a soft-padded cage (with food and water), the animal will be placed on a heating pad covered by a layer of sterile pad. The surgeon will wait for the animal to awaken. Once awake, the surgeon will monitor the animal for excessive stress for a period of 2 hours. The animal can be returned to the vivarium room if no overt signs of pain and distress are observed.

Please note that when fiber photometry was first developed, it was common to perform the AAV injection in a first session, have the animal recover, and then perform a second procedure to implant the optical cannula. Nowadays, researchers have been combining the virus injection and cannula implantation into a single session, as this reduces the number of required surgeries, stress and recovery time for the animals.

3. Training for operant conditioning task and fiber photometry

Mice will be trained in a standard operant conditioning chamber equipped with a lever and a food pellet dispenser. The goal of the training is to condition the animals to press the lever in order to receive a food reward, which will be used in conjunction with fiber photometry recordings and ultrasonic neuromodulation. Animals will undergo daily training sessions (approximately 30–60 minutes) for 5–10 consecutive days or until task acquisition criteria are met (e.g., consistent lever pressing for reward). Mice will be food-restricted to 85–90% of their baseline body weight during the training period to increase motivation, in accordance with institutional and AVMA guidelines. Water will be provided ad libitum at all times. Training will take place during the light cycle in a quiet behavioral testing room to minimize external stressors. Habituation to the operant chamber will occur on Day 1, followed by shaping procedures that reinforce lever-pressing behavior. All procedures will be conducted by trained personnel familiar with behavioral training in rodents.

4. Ultrasonic Stimulation and Fiber Photometry

Ultrasonic Stimulation: A thin layer of gel is applied to the skull to promote coupling with the transducer. A light cylindrical transducer (Sonic Concepts SU-132) is placed on the layer of gel. The transducer is connected to the control box provided by Sonic Concepts (TPO-201). The controller sends short pulses to the transducer which then emits pressure waves into the animal's brain. Stimulation intensity is limited to previously established safe levels (550 kPA), and the duration of sonication is limited to 1 sec. Each sonication consists of a train of pulses with the pulse repetition frequency ranging from 1 Hz to 100 Hz. The duty cycle ranges from 30 - 50 %, and allows any temperature increase to dissipate. Please note that heating is not expected at these low intensities, and the stimulation is widely believed to act mechanically, not thermally.

Fiber Photometry: An optical fiber will be connected to the implanted cannula, allowing excitation of the iGluSnFR or GABASnFR sensor and collection of emitted fluorescence. The intensity of the incident light is extremely low (approximately 20 uW), and is delivered with LEDs. Fiber photometry with these sensors and excitation levels is extensively used and widely believed to be safe.

Timeframe and Frequency: Each session will last up to 20 minutes, with a maximum of three sessions per week, ensuring adequate rest between sessions.

Unrelieved Pain & Distress

Will animals be subjected to procedures involving unrelieved pain or distress (category E)?

Yes ○ No ◉

Reduce, Refine, Replace

REDUCTION of animal use (i.e., modifying the experimental paradigm or performing statistical analysis to allow the use of fewer animals to obtain the needed information).

Please justify the number of animals requested is the minimum needed in order to obtain valid scientific conclusions.

We request approval for n=10 animals per year, for a total of 30 animals. This number reflects our expectation that each animal will undergo ~15 sessions. Given the effect size in our previous electrophysiological study (Cohen's d = 0.3), we expect to obtain an alpha of 0.05 and a power of 0.8 with a sample size of n=175 (value obtained from statistical power table). The study has two separate dependent variables (glutamate and GABA; we do not have the capability for concurrent recording of glutamate and GABA), necessitating 175 * 2 = 350 samples. Our expected sample of 450 sessions (30 animals * 15 sessions per animal) accounts for the fact that the procedure is new to the laboratory, we expect that some of the sessions or animals will not yield valid data. Therefore, we are overpowering the study to accommodate potential pitfalls in fiber photometry.

REFINEMENT of animal use such as modifying manipulations or measurement techniques to reduce the pain and/or distress experienced by the animals as compared to prior techniques, or using less sentient species (e.g., frog instead of mouse, mouse instead of dog).

Please describe why less invasive procedures or procedures which may cause less pain or distress cannot be used.

Less invasive procedures include scalp EEG or animal fMRI. Unfortunately, neither of these techniques provide access to neurotransmitter dynamics. The EEG is limited to recording only superficial areas of cortex and the signal reflects the coherence of post synaptic potentials (i.e., not glutatame or GABA levels). fMRI can capture deep brain regions but the readout is hemodynamic and reflects neurovascular coupling, which is not relatable to neurotransmission. In particular, it is widely believed that the fMRI signal cannot disentangle excitatory and inhibitory signaling.

REPLACEMENT of animals with non-animal techniques (e.g., using tissue culture, computer simulations, etc.).

Please describe why non animal models or invertebrate models will not suffice for this study.

Non-animal models or in vitro models are inadequate for evaluating transcranial stimulation techniques.

Adverse Reactions

Are there any adverse reactions expected in this study that have not already been described?

Yes ○ No ◉

Exemptions

Are you requesting any exceptions to the Guide or to IACUC policies?

Yes ○ No ◉

Will Animals be subject to Food and/or Water Restriction?

Yes ◉ No ○

If yes, please provide scientific justification and describe in detail, including monitoring to ensure that nutritional needs are met (e.g., body weight monitoring periods, maintenance of written record).

Scientific Justification:
Food restriction is necessary to motivate operant responding for food rewards during behavioral training. Mild caloric restriction enhances learning and task engagement without compromising animal welfare. This approach is standard and well-established in behavioral neuroscience, particularly when using food-reinforced tasks in mice.

Restriction Protocol:
Mice will be gradually food-restricted to 85?90% of their baseline free-feeding body weight, determined by taking the average weight over 3 consecutive days prior to restriction. Animals will be housed individually during the restriction period to allow precise monitoring of food intake and body weight.

Monitoring Procedures:
Body weight will be measured and recorded daily during food restriction and training. Mice will receive a measured daily food ration sufficient to maintain them within the 85?90% weight window. Animals showing more than 15% loss from baseline, signs of dehydration, poor grooming, or any other indicators of compromised welfare will be removed from restriction, provided free access to food, and evaluated by veterinary staff. Water will be available ad libitum at all times. A written log of body weight, food allotment, and training performance will be maintained for each animal throughout the food restriction and training period.

Will there be any special husbandry requirements?

Yes ○ No ◉

Rat (Rattus norvegicus)

Species Information

Complete the required fields for the study animals in the protocol.

Age range

> 10 weeks

Target weight range

350 - 450 g

Maximum number of cages housed at one time.

4

What is the average length of time housed (weeks)?

12 weeks

Will the animals be Specific Pathogen Free?

Yes ◉ No ○

Identification Method

Type	Advantages	Disadvantages
Cage Cards	• Pertinent information is readily available	• Card can be lost or misplaced
Dye (Non-toxic, waterproof)	• Easy to apply • Non-invasive • Identifies individual animals	• Temporary
Ear Punch / Notch	• Easy to perform • Pattern can identify individual animals	• ID lost if ear is injured or mutilated • Punch / notch can become less readable over time • Animals can become indistinguishable if mixed between cages
Ear Tags	• Easy to apply • Permanent • Identifies individual animals	• Tags can be lost • Animal usually must be restrained to read tag
Tattoo	• Permanent • Identifies individual animals	• Anesthesia recommended • Animal may need to be restrained to read tattoo • Equipment can be expensive • Equipment must be disinfected between use • Training required to operate the tattoo instrument
Microchip	• Easy to apply • Permanent • Identifies individual animals • Not necessary to restrain animal to read	• Expensive • Microchip may migrate within the body
Toe Clip (requires scientific justification)	• Permanent • Pattern can identify individual animals	• Requires amputation of a body part • Requires anesthesia • Usually must restrain animal to read

Please enter animal identification methods to be utilized.
Examples:

- **Cage Cards**
- **Marker-Temporary**
- **Ear Punch/Notch**
- **Ear Tags**
- **Tattoo**
- **Microchip**
- **Toe Clip (neonates 5-7 days of age ONLY)**

Cage cards

Justification for Choice of Species

Justify the choice of species by stating why a species lower on the phylogenic scale is not appropriate.

Please indicate why the species selected is most appropriate for this study.

Large database exists for this species which allows comparisons with previous data

If you have selected Other, please describe your species justification below. If this does not apply, enter NA

The rat is the most widely used model species for studying brain oscillations, and in particular those in the hippocampus (the target of this research). It provides a large enough brain for simultaneous application of focused ultrasound and fiber photometry, and generalizes well in the nature of the brain activity to the human brain.

Enrichment and/or Exercise

The Animal Welfare Act requires facilities to provide exercise for dogs and programs to promote the psychological well-being of non-human primates, while the U.S. Public Health Service Guide to the Care and Use of Laboratory Animals encourages "enriching the environment as appropriate to the species....".

Will animals be group housed?

Yes ○ No ◉

If not, what is the justification?

Once an animal has received the optical fiber implant, they are vulnerable to attack from cage mates.

Will environment enrichment be provided?

Yes ◉ No ○

Mice & Rats

NylaBones

☐

Nesting Material

☑

Plexiglas Tubes

☐

Cardboard Huts or Tubes

☑

Rabbit

Because rabbits are herbivores and have a large cecum, the regular feeding of items such as grass hay (cubes or loose), carrots, leafy greens, and other vegetables is beneficial to their digestion. In addition, since rabbit incisors as well as molars grow continuously, chewing allows teeth to wear down.

Food and Treats

☐

Large Plastic Balls

☐

Bells and Bar Bells

☐

Chew Sticks Which May Be Either Edible or Made of Wood

☐

Hay

☐

Food and Treats

☐

Ferret

Ferret Balls

☐

Nyla Bones or Other Chew Toys

☐

Food and Treats

☐

PVC Pipes for Tunneling

☐

Use Locations

Indicate all of the locations where surgeries, procedures and/or euthanasia will be performed.

Click "Add Use Locations" and complete location information. The PI may select "Add From My List of Locations" to view locations from previously approved protocols.

More than one option my be included.

Location/Building	Room	Type
Steinman	572C	USE

Location/Room

Steinman 572C

Location Type

USE

Strain Information

Click "Add Strain" and complete strain information. The PI may select "Add From My List of Strains" to view the strains from previously approved protocols.

More than one option my be included.

Strain	Age	Weight	Phenotype	Edited
LE- Long Evan	> 10 weeks	> 350 grams		Edited

Hit the spacebar and choose a strain from the list. If the strain you want is not on the list, enter it in the same field.

Species Strain

LE- Long Evan

Age

> 10 weeks

Weight

> 350 grams

Phenotype

Non-Surgical Procedures

Click "Add Procedure" and complete procedure information. The PI may select "Add From My List of Procedures" to view the procedures from previously approved protocols.

More than one option may be included.

Procedure Name
Drug Administration
Other
Training

Procedure Name

Drug Administration

Describe the procedure, giving enough detail so that another individual could carry it out (e.g. volume, dose, duration, frequency, ...)

AAV injection into hippocampus

During stereotactic surgery, a Hamilton syringe will be used to inject AAV expressing iGluSnFR or GABASnFR into the target brain region (e.g., hippocampus) at a controlled rate (e.g., 100-200 nL/min) and volume (e.g., 1-2 µL). The injection needle will be held in place for an additional 5 minutes to allow for complete diffusion before slowly retracting.

Please describe any special precautions you have taken or will take to assure that the procedure can be conducted safely for animals and humans

Animal Safety:
- Precise Stereotactic Positioning: The stereotactic apparatus will be calibrated and verified before each procedure to ensure accurate targeting of the hippocampus, minimizing the risk of unintended brain damage or excessive tissue disruption.
- Controlled Injection Volume and Rate: The AAV solution will be injected slowly (e.g., 100-200 nL/min) to reduce pressure on surrounding brain tissue, minimizing potential damage and inflammation. After the injection, the syringe will be left in place for 5 minutes before withdrawal to prevent reflux of the virus and ensure safe distribution.
- Aseptic Technique: Aseptic conditions will be maintained throughout the procedure to prevent infection. Sterile gloves, instruments, and injection syringes will be used, and the animal's head will be disinfected with betadine or chlorhexidine.

Human Safety:
- Personal Protective Equipment (PPE): Researchers handling AAV will wear appropriate PPE, including lab coats, gloves, and safety goggles, to avoid accidental exposure to the virus.
- Designated Work Area: The AAV injection preparation and handling will occur in a designated biosafety cabinet or designated lab area, adhering to biosafety level 1 guidelines for AAV.
- Sharps Disposal: All syringes and needles used for the injection will be immediately disposed of in a designated sharps container after use to prevent accidental needle sticks or contamination.
- Virus Containment: Only trained personnel will handle the viral preparation. Any spills will be immediately disinfected with 70% ethanol or an appropriate virucidal agent to ensure the lab remains free of contamination.

Post-Procedure Protocols:
- Animal Monitoring: Animals will be closely monitored post-injection for any signs of adverse reactions, such as inflammation, distress, or abnormal behavior.
- Waste Disposal: All waste generated from the injection procedure, including PPE, syringes, and wipes, will be treated as biohazardous waste and disposed of according to institutional guidelines to prevent environmental contamination.

Procedure Name

Other

Describe the procedure, giving enough detail so that another individual could carry it out (e.g. volume, dose, duration, frequency, ...)

Transcranial ultrasonic neuromodulation

A small circular transducer (Sonic Concepts SU-132) will be coupled to the animal's skull via ultrasound gel. The skull coordinates relative to bregma/lambda will be selected so that the beam is focused on the CA3 region of the right hippocampus (obtained from a stereotaxic atlas). Ultrasonic stimulation parameters (intensity, pulse repetition frequency, pulse duration) will be selected from the list of waveforms that are to be assessed in the experiments. Intensity is limited to 550 kPa, a value that has been demonstrated by our group and others to be safe. Sonication is brief, spanning a duration of approximately 1 second, and is repeated across N=30-50 trials per session, with at least 10 seconds between sonications to ensure that any tissue heating has dissipated.

Fiber photometry

Equipment: Doric Lenses Fiber Photometry Console, Fluorescence Mini Cube, Mono Fiberoptic Patchcord, Mono Fiberoptic Cannula

The Fiber Photometry Console is connected to the Fluorescence Mini Cube. The Mini Cube integrates two photodetectors and three LEDs with specific excitation and emission filters for neurotransmitter sensors (e.g., iGluSnFR or GABASnFR). We then attach a Mono Fiberoptic Patchcord (400/430/1100-0.57) between the Fluorescence Mini Cube and the implanted optical cannula in the animal's hippocampus. The fiber optic cannula and patchcord ensure efficient light delivery and fluorescence collection. The excitation wavelength is set on the console to 460-493 nm for iGluSnFR or other green-fluorescing indicators. This LED intensity should be calibrated to approximately 20 µW at the fiber tip to minimize photobleaching and prevent tissue heating. Once the excitation light is applied, the system will capture the fluorescence emitted in the 500-540 nm range, corresponding to glutamate release in the brain as detected by iGluSnFR. Fluorescence signals are recorded through the two photodetectors integrated into the Mini Cube. Data acquisition software (provided by Doric Lenses) will capture and display real-time fluorescence intensity changes, indicating neurotransmitter dynamics. Each experimental session lasts up to 20 minutes, with photometry and ultrasonic stimulation applied in intervals. Rats are subjected to a maximum of three sessions per week, ensuring adequate recovery and avoiding prolonged exposure to excitation light.

Please describe any special precautions you have taken or will take to assure that the procedure can be conducted safely for animals and humans

Intensities are limited to values that have been shown by our group and others to be safe and painless (< 500 kPa). There is an interval of at least 10 seconds between consecutive sonications to allow any tissue heating to dissipate.

Procedure Name

Training

Describe the procedure, giving enough detail so that another individual could carry it out (e.g. volume, dose, duration, frequency, ...)

Training Rats to Receive Awake, Head-Fixed Stimulation

The goal of this training protocol is to acclimate rats to the head-fixed setup in an awake state, allowing for neural recording during ultrasonic stimulation without the confounding effects of anesthesia.

Training will involve a gradual, acclimatization approach to minimize stress. Rats will undergo incremental exposure to the head-fixed setup, accompanied by administration of midazolam during the early stages to aid in habituation.

In the experimental setup, the animal will be in a tube sock setup to help limit movement. The animal's head will be restrained in a safe head holder along with soft earbars to limit head rotation.

We will employ a progressive training protocol where animals begin with short bouts of exposure to the head restraint without the transducer (days 1-3), short bouts of exposure with the transducer touching but turned off (days 4-10), and then short bouts of exposure with the transducer turned on for 3-5 trials of 1 second (days 11-15). At the end of the training, animals will be acclimated to the head restraint, transducer coupling, and sonication.

Please describe any special precautions you have taken or will take to assure that the procedure can be conducted safely for animals and humans

In the early sessions, we will administer Midazolam (0.5 mg/kg administered intraperitoneally 15-20 minutes before each session for the first five sessions). We will reduce the dose by half (0.25 mg/kg) for sessions 6-10, and discontinue for remaining sessions.

Treats (e.g., small pieces of favored food) will be provided at the start and end of each session to reinforce positive association with the head-fixed environment.

Rats will be closely monitored for signs of distress, including excessive vocalizations, attempts to escape, or signs of agitation. If distress indicators are noted, the training session will be shortened, and the animal will be returned to its home cage.

Restraint Types

This section applies to any method of restraint lasting 10 minutes or longer.

Click "Add Restraint" and complete restraint information. The PI may select "Add From My List of Restraints" to view the restraints from previously approved protocols.

More than one option may be included.

Restraint Type	Duration	Rationale	Edited
Prolonged (> 10 Minutes)	< 30 minutes	In order to allow the experimental team to obtain neural recordings from animals in the awake state (anesthetics confound the interpretation of changes to neurotransmitter levels observed with ultrasonic neuromodulation), we will employ a minimal restraint system consisting of a tube sock and soft ear bars to allow us to stimulate the animal.	Edited

Restraint Type

Prolonged (> 10 Minutes)

Rationale for this type of restraint

In order to allow the experimental team to obtain neural recordings from animals in the awake state (anesthetics confound the interpretation of changes to neurotransmitter levels observed with ultrasonic neuromodulation), we will employ a minimal restraint system consisting of a tube sock and soft ear bars to allow us to stimulate the animal.

Duration of restraint

< 30 minutes

How is animal acclimated to the restraint device?

Please see "Procedures". We are employing a graduated training protocol with Midazolam at the outset of training to minimize stress to the animal.

Surgical Procedures

Important Instructions: Items highlighted in red require editing. Please select the entry and click Edit Surgery.

Click "Add Surgery" and complete surgery information. If you are the PI, you may click "Add From My List of Surgeries" which will narrow the options to surgeries previously approved on your protocols. You may add more than one source.

Surgery Type	Other Surgery Name	MSS?	Edited	Recovery Type
Stereotaxic Surgery		No	Edited	Recovery

Surgery Type

Stereotaxic Surgery

If Other, provide Surgery Type below (otherwise enter N/A)

Recovery Type

Recovery

Describe pre-op procedures including acclimation period, fasting, procuring baseline information such as body weight, body temp, blood values, etc. as relevant.

Acclimation Period: All rats will undergo a three-week acclimation period after arrival to minimize stress and ensure they are in good health prior to surgery.

Baseline Measurements: Baseline information, including body weight and body condition score, will be recorded for each animal one day prior to surgery. Daily health checks, grooming behavior, and food intake will be monitored throughout the acclimation period.

Fasting: Fasting is not required for rodents as they are unable to vomit. Animals will have access to food and water up until the time of anesthesia.

Body Temperature: Baseline body temperature will be recorded immediately before surgery using a digital rectal thermometer. Body temperature will continue to be monitored and maintained within a physiological range throughout the procedure using a heated surgical pad.

Describe the surgical attire to be used and surgical site preparation (e.g. incision site).

Surgical Attire: Personnel performing the surgery will wear sterile surgical gloves, a surgical gown, mask, and cap to maintain aseptic conditions.

Surgical Site Preparation: The surgical area on the animal's head will be shaved, cleaned, and disinfected with a series of betadine or chlorhexidine scrubs, followed by a final rinse with 70% ethanol. The incision site will be covered with a sterile drape to ensure aseptic conditions throughout the surgery.

Describe any support and/or anesthetic monitoring equipment that will be used for surgeries.

Anesthesia: Isoflurane anesthesia will be used, starting with an induction dose of 3% in an induction chamber and maintained at 1.5-2% via a nose cone during surgery.

Monitoring Equipment:
- Temperature: A digital rectal thermometer and a heating pad will be used to monitor and maintain body temperature.
- Respiration: Respiratory rate will be visually monitored throughout the procedure to ensure that the animal maintains a stable breathing pattern.
- Oxygen Saturation: A pulse oximeter (Kent Scientific; paw pulse ox) will be used if available to monitor oxygen saturation.
- Additional Support: Saline (0.9% NaCl, subcutaneously) will be administered pre-operatively to maintain hydration.

Will any sedatives, anti-cholinergics, or other medications be administered prior to anesthetic induction? If so, describe here.

No

Will paralytics be used for this procedure? If yes please justify the use and describe your plan for monitoring.

No

Anesthetic Depth Measurement

Toe Pinch Reflex: The anesthetic depth will be assessed by the absence of the toe pinch reflex prior to making the incision and periodically during the procedure.

Respiratory Monitoring: The respiration rate will also be monitored, as slowing of respiratory rate and regular, stable breathing patterns indicate an appropriate level of anesthesia.

Include a plan for monitoring frequency, duration and intervals of post-op analgesia/antibiotics, post-operative monitoring, nursing care, etc.

Post-Operative Monitoring: Animals will be monitored every 15 minutes for the first hour post-surgery until they regain full consciousness. Following this, animals will be checked twice daily for the first 72 hours and once daily for the remainder of the first week.

Analgesia: Buprenorphine (0.05 mg/kg SC) will be administered every 8-12 hours for the first 48 hours following surgery to manage post-operative pain. If signs of discomfort persist, analgesia may be extended based on veterinary guidance.

Antibiotics: A topical antibiotic ointment (triple antibiotic or equivalent) will be applied to the surgical site once daily for 5-7 days to prevent infection.

Nursing Care: Rats will be placed in padded cages with access to soft food and hydration supplements (e.g., hydrogel) to encourage feeding and hydration while they recover. Bedding will be changed daily to maintain a clean environment.

When will sutures, wound clips, or staples be removed?

7-10 days post surgery or once the incision has healed.

Clinical parameters to be monitored during 1st week after surgery and contingency plans for post-surgical complications (e.g body weights)

Parameters Monitored:
? Body Weight: Body weight will be recorded daily during the first week post-surgery to detect any significant weight loss. Any weight loss exceeding 10% of the pre-surgical weight will prompt evaluation by veterinary staff.
? Grooming and Activity Levels: Animals will be observed for normal grooming behavior and activity. A lack of grooming or lethargy may indicate pain or discomfort, and additional analgesia will be provided if necessary.
? Incision Site: The surgical site will be inspected daily for signs of redness, swelling, or discharge. If any of these signs are noted, the animal will be evaluated by veterinary staff, and additional antibiotics or interventions will be administered as needed.

Contingency Plans:
? If an animal shows signs of distress, infection at the incision site, or significant weight loss, it will receive immediate veterinary attention. Additional supportive care, analgesics, or antibiotics will be administered based on veterinary recommendations. If complications are severe or unmanageable, the animal may be humanely euthanized to prevent suffering.

Will Analgesics be withheld?

Yes ○ No ◉

Surgical Locations

004:572C

Multiple Survival Surgeries

If you are conducting Multiple Survival Surgeries on this Species, please fill in the required fields.

Will multiple surgical survival procedures be performed on an individual animal (separate anesthetic events)?

Yes ○ No ◉

Euthanasia Method Information

AVMA Guidelines for the Euthanasia of Animals: 2020 Edition (https://www.avma.org/resources-tools/avma-policies/avma-guidelines-euthanasia-animals)

Euthansia Method
Carbon Dioxide Inhalation

Euthanasia Method

Carbon Dioxide Inhalation

AVMA Classification

Acceptable

If method is not AVMA acceptable, please provide justification

State the secondary method of euthanasia or assurance of death.

Thoracotomy

Animal Numbers

*The USDA categories marked as required (with a red *) below were determined to be appropriate based on the procedures reported.*

Please enter the number of animals requested for each of the required categories. The total of the required fields may not exceed the total requested number.

The USDA Categories are defined as follows:
B = animal held for breeding and/or not yet used in research
C = no pain or distress
D = alleviated pain or distress
E = unalleviated pain or distress

Please enter the number of animals you plan on utilizing in the three years of the protocol.

USDA Category	# of Animals
B	0
C	0
D	30
E	0

Justify the number of animals to be used.

- **Describe the statistical method (or other method) used to justify the number of animals per group.**
  Federal guidance states that statistical methods must be used in order to justify the number of animals requested.
- **Be sure to include breeding colony numbers (production, maintenance, undesired genotypes).**
- **Please provide a description of all animal procedures, surgical and non-surgical.**
- **To justify the number of animals that will be used, provide a statistical analysis, such as power analysis, and include the results of that analysis in the description. With the description, please insert a table that includes group, treatment, number of animals, pain category, and whether the animal will be euthanized at the end of the experiment.**
- **The numbers provided in this section must also match the total amount in the '# of Animals' column above.**

*We request approval for n=10 animals per year, for a total of 30 animals. This number reflects our expectation that each animal will undergo ~15 sessions. Given the effect size in our previous electrophysiological study (Cohen's d = 0.3), we expect to obtain an alpha of 0.05 and a power of 0.8 with a sample size of n=175 (value obtained from statistical power table). The study has two separate dependent variables (glutamate and GABA; we do not have the capability for concurrent recording of glutamate and GABA), necessitating 175 * 2 = 350 samples. Our expected sample of 450 sessions (30 animals * 15 sessions per animal) accounts for the fact that the procedure is new to the laboratory, we expect that some of the sessions or animals will not yield valid data. Therefore, we are overpowering the study to accommodate potential pitfalls in fiber photometry.*

Methodology

*For each species, **describe in narrative form all experimental or instructional procedures to be performed on the animals** (e.g. blood collection, surgery, behavioral training, administration of substances or test compounds, breeding, tumor induction, etc.). Describe the procedures in the order in which they will be performed, including time frames and intervals. Include a description of procedures performed on anesthetized animals. All procedures checked in the procedures section should be described below.*

Include the rationale for use of tissues in vitro. Do not describe in vitro procedures performed on tissues taken from animals or procedures performed on animals after they are euthanized.

*Please list the interventions/procedures in chronological order. Indicate the time interval between each procedure, and the final disposition of the animals at the end of the experiment. **All procedures in the table on the procedures section must be included.***

Flowcharts or other graphical representations of the methodology can be very helpful. Tables, pictures, and flowcharts can be created in the Procedure Description text box within Cayuse. The information can also be pasted into the text box. It is preferred that all tables, charts, and pictures be inserted in the text box but you may attach the files below if needed. Please contact a member of the IACUC or veterinary staff should you require guidance regarding the information and level of detail that should be provided here.

Procedure Description

Order of procedures

1. Stereotaxic Surgery
2. Drug Administration (AAV injection during surgery)
3. Training for head-fixation
4. Restraint (Head-fixation during recording experiments)
5. Other (Transcranial ultrasound during recording experiments)

Stereotaxic surgery + AAV Injection

All instruments will be sterilized via an autoclave machine before the start of survival surgery. All gloves, gowns worn by surgeons will be new and sterile. The surgical area will be covered by a sterile surgical table cover. A glass bead sterilizer will be used during the surgery to ensure continuous sterilization of the instruments.

The duration of the surgical procedures for virus injection and cannula implantation will be less than 6 hours. To ensure the stability of the cannula, the animal must be of a certain age and size before surgery. The animal should be in their adult stage (>10 weeks) and weigh at least 350 g.

During the entirety of this survival surgery, the animal will be anesthetized with isoflurane via inhalation. First, the animal will be pre-anaesthetized with 3% isoflurane in the induction chamber with oxygen at 2 L/min. Once anesthetized, isoflurane will be lowered to 2%. This level will be maintained for head shaving, stereotaxic fixation, craniotomy, duratomy, AAV injection, cannula implantation, and finally, dental cement application.

During head shaving, the animal is placed on a sterile pad with 2% isoflurane and oxygen delivered via a nose cone. Once head shaving is complete, the animal is transferred to the stereotaxic frame (Kopf) which is covered by another sterile pad, and all parts making contact with the animal (ear bars, head holder, etc) have been autoclaved. The animal head is fixed via ear bars with lidocaine as a local analgesic into the ear canal to mitigate pain. Eye lubricating gel is placed onto the animal's eyes to help protect and prevent drying. Betadine is used to clean the surgical area (area shaved). At this point, 1 ml of lactated ringers dextrose is given (SC) to the animal to help fortify them for the upcoming surgery.

Next, bupivacaine solution (0.1ml:0.3ml, bupivacaine:saline cocktail) is injected into the area of incision over the rat's head. A short amount of time is given to allow the analgesic to take effect, then an incision is made to create an opening to expose the animal's dorsal skull. Once the skull is cleared of ligaments and connective tissues and bregma-lambda lines are visible to the eye, measurements of the bregma-lambda length is taken. Coordinates are determined on the skull and marked for a 2 mm^2 craniotomy. A dental drill is used to perform the craniotomy on the skull, followed by a duratomy to expose the brain in the craniotomy window.

Once the craniotomy has been made over the target brain region a Hamilton syringe will be used to deliver the AAV expressing either iGluSnFR or GABASnFR into the hippocampus. The AAV solution will be injected slowly (100-200 nL/min) with a total volume of 1-2 µL, and the syringe will be held in place for 5 minutes post-injection to prevent reflux.

Following virus injection, an optical cannula will be lowered to the hippocampus to permit subsequent photometry excitation and recording. The cannula will be fixed in place using dental cement and titanium screws to ensure stability during future experiments. The scalp will be sutured around the implant, leaving the cannula head exposed for future attachment to fiber optic cables.

We will a 4-0 gauge suture (common brand: Ethicon) that is absorbable and sterile.

At this point, the surgery is completed, and another 1 ml of lactated ringers dextrose is given (SC) to the animal to aid recovery. As the animal is still under anesthesia, the first dose of buprenorphine is given so that the animal will not be under excessive pain upon waking up. Eye protecting gel will be removed, and the animal will be removed from the stereotaxic frame.

In a soft-padded cage (with food and water), the animal will be placed on a heating pad covered by a layer of sterile pad. The surgeon will wait for the animal to awaken. Once awake, the surgeon will monitor the animal for excessive stress for a period of 2 hours. The animal can be returned to the vivarium room if no overt signs of pain and distress are observed.

Note: when fiber photometry was first developed, it was common to perform the AAV injection in a first session, have the animal recover, and then perform a second procedure to implant the optical cannula. Nowadays, researchers have been combining the virus injection and cannula implantation into a single session, as this reduces the number of required surgeries, stress and recovery time for the animals.

Training for head Fixation

The rationale for this training is to reduce stress and to ensure that the animals can tolerate head fixation during the subsequent recordings (experiments) where neural activity is recorded concurrently to ultrasonic stimulation with animals in the awake state. The recordings require the animal to remain relatively still for short periods.

Rats will undergo training to adapt to awake, head-fixed conditions for fiber photometry and ultrasonic stimulation. Training will take place over two weeks to minimize stress and ensure compliance with the head-fixed setup.

Animals will first be acclimated to handling and then to the head-fixed apparatus. During initial sessions (Days 1-5), each rat will be introduced to the training environment for 10-15 minutes daily. From Days 6-10, rats will undergo head fixation for progressively longer periods, beginning with brief fixation (10 seconds) and increasing by 5-10 seconds each day.

During the early stages of training (Days 1-10), 0.5 mg/kg midazolam (days 1-5) will be administered intraperitoneally (i.p.) to reduce stress, with the dose reduced by half (days 6-10) and discontinued by Day 10.

Interval Between Sessions: Sessions will be conducted daily or on alternating days as tolerated by the animal, with rest days as needed.

Restraint during recordings

Animals will be placed in a soft tube sock with the top of the head exposed. Soft earbars will be gently applied to promote stillness of the head. If animals demonstrate that they are able to remain relatively still, we will discontinue the use of the earbars. Each recording session will last approximately 10-15 minutes, with 2-3 sessions per week.

Ultrasonic Stimulation and Fiber Photometry

Ultrasonic neuromodulation will be applied while measuring neurotransmitter levels via fiber photometry in awake, restrained rats. Note that experiments will begin following successful restraint training in the animal.

Ultrasonic Stimulation: A thin layer of gel is applied to the skull to promote coupling with the transducer. A light cylindrical transducer (Sonic Concepts SU-132) is placed on the layer of gel. The transducer is connected to the control box provided by Sonic Concepts (TPO-201). The controller sends short pulses to the transducer which then emits pressure waves into the animal's brain. Stimulation intensity is limited to previously established safe levels (550 kPA), and the duration of sonication is limited to 1 sec. Each sonication consists of a train

of pulses with the pulse repetition frequency ranging from 1 Hz to 100 Hz. The duty cycle ranges from 30 - 50 %, and allows any temperature increase to dissipate. Please note that heating is not expected at these low intensities, and the stimulation is widely believed to act mechanically, not thermally.

Fiber Photometry: An optical fiber will be connected to the implanted cannula, allowing excitation of the iGluSnFR or GABASnFR sensor in the hippocampus and collection of emitted fluorescence. The intensity of the incident light is extremely low (approximately 20 uW), and is delivered with LEDs. Fiber photometry with these sensors and excitation levels is extensively used and widely believed to be safe.

Timeframe and Frequency: Each session will last up to 20 minutes, with a maximum of three sessions per week, ensuring adequate rest between sessions.

The rationale for the recording experiments is that fiber photometry enables real-time monitoring of neurotransmitter dynamics, while ultrasonic stimulation allows for the study of neuromodulation effects on neurotransmitter release in the hippocampus.

Unrelieved Pain & Distress

Will animals be subjected to procedures involving unrelieved pain or distress (category E)?

Yes ○ No ◉

Reduce, Refine, Replace

REDUCTION of animal use (i.e., modifying the experimental paradigm or performing statistical analysis to allow the use of fewer animals to obtain the needed information).

Please justify the number of animals requested is the minimum needed in order to obtain valid scientific conclusions.

We request approval for n=10 animals per year, for a total of 30 animals. This number reflects our expectation that each animal will undergo ~15 sessions. Given the effect size in our previous electrophysiological study (Cohen's d = 0.3), we expect to obtain an alpha of 0.05 and a power of 0.8 with a sample size of n=175 (value obtained from statistical power table). The study has two separate dependent variables (glutamate and GABA; we do not have the capability for concurrent recording of glutamate and GABA), necessitating 175 * 2 = 350 samples. Our expected sample of 450 sessions (30 animals * 15 sessions per animal) accounts for the fact that the procedure is new to the laboratory, we expect that some of the sessions or animals will not yield valid data. Therefore, we are overpowering the study to accommodate potential pitfalls in fiber photometry.

REFINEMENT of animal use such as modifying manipulations or measurement techniques to reduce the pain and/or distress experienced by the animals as compared to prior techniques, or using less sentient species (e.g., frog instead of mouse, mouse instead of dog).

Please describe why less invasive procedures or procedures which may cause less pain or distress cannot be used.

Less invasive procedures include scalp EEG or animal fMRI. Unfortunately, neither of these techniques provide access to neurotransmitter dynamics. The EEG is limited to recording only superficial areas of cortex and the signal reflects the coherence of post synaptic potentials (i.e., not glutatame or GABA levels). fMRI can capture deep brain regions but the readout is hemodynamic and reflects neurovascular coupling, which is not relatable to neurotransmission. In particular, it is widely believed that the fMRI signal cannot disentangle excitatory and inhibitory signaling.

REPLACEMENT of animals with non-animal techniques (e.g., using tissue culture, computer simulations, etc.).

Please describe why non animal models or invertebrate models will not suffice for this study.

Non-animal models or in vitro models are inadequate for evaluating transcranial stimulation techniques. The mouse is too small to accommodate the transducer, and the rat represents the lowest animal model that is adequate for studying non-invasive neuromodulation techniques.

Adverse Reactions

Are there any adverse reactions expected in this study that have not already been described?

Yes ○ No ◉

Exemptions

Are you requesting any exceptions to the Guide or to IACUC policies?

Yes ○ No ◉

Will Animals be subject to Food and/or Water Restriction?

Yes ○ No ◉

Will there be any special husbandry requirements?

Yes ○ No ◉

Personnel List

For new protocols, all names currently listed on this page must be selected and edited to address additional questions. To add new staff to this protocol, click Add Personnel.

Please attach signed Prerequisite sign-off form and CITI training certificate of Animal Care and Use Course.

Name	Business Role	Phone	Email	Organization	Department	Primary?	Requester?	Edited
Dmochowski, Jacek P.	Principal Investigator		jdmochowski@ccny.cuny.edu	President	Biomedical Eng.	No	No	Edited
Gnazzo, Federico	Researcher Staff Members		g7federico@aol.com	President	Biomedical Eng.	No	No	Edited
Edwards, Mila	Researcher Staff Members		milaeedwards@gmail.com	President	Biomedical Eng.	No	No	Edited

Business Role

Principal Investigator

Name

Dmochowski, Jacek P.

Organization Department

President Biomedical Eng.

Email

jdmochowski@ccny.cuny.edu

Office Phone

Cell Phone

Alternate Phone

Home Phone

4087690434

Primary Contact?

☐

Copy on all Emails

☐

Will person be handling animal species?

Yes ◉ No ○

Species Name	Type	Procedure Description
Rat (Rattus norvegicus)	Euthanasia	Carbon Dioxide Inhalation
Rat (Rattus norvegicus)	Procedures	Drug Administration
Rat (Rattus norvegicus)	Procedures	Other
Rat (Rattus norvegicus)	Procedures	Training
Rat (Rattus norvegicus)	Restraints	Prolonged (> 10 Minutes)
Rat (Rattus norvegicus)	Surgery	Stereotaxic Surgery

Degrees

PhD

Experience and Qualifications

7 years experience in stereotaxic surgery, electrophysiology, transcranial ultrasound stimulation obtained from this specific project. We have collected data from over 200 animals since 2017, when the first research grant was obtained. I have also received training in fiber photometry from Dr. Qi Wang, Associate Professor of Biomedical Engineering at Columbia University, who is an expert in the technique.

Number of years experience in this field?

7

Have you completed your Policy Orientation and Wet Labs training?

Yes ◉ No ○

Have you submitted and have a signed copy of your Animal Contact Health Surveillance Questionnaire (https://www.ccny.cuny.edu/sites/default/files/2023-01/Animal%20Contact%20Health%20Surveillance%20Questionnaire.doc)?

Yes ○ No ○

Business Role

Researcher Staff Members

Name

Gnazzo, Federico

Organization Department

President Biomedical Eng.

Email

g7federico@aol.com

Office Phone

Cell Phone

Alternate Phone

Home Phone

Primary Contact?

☐

Copy on all Emails

☐

Will person be handling animal species?

Yes ◉ No ○

Species Name	Type	Procedure Description
Rat (Rattus norvegicus)	Euthanasia	Carbon Dioxide Inhalation
Rat (Rattus norvegicus)	Procedures	Drug Administration
Rat (Rattus norvegicus)	Procedures	Other
Rat (Rattus norvegicus)	Surgery	Stereotaxic Surgery

Degrees

Experience and Qualifications

Several years of experience in stereotaxic surgeries in the lab of Jeff Beeler at Hunter College. Now at Regeneron where he works with animals.

Number of years experience in this field?

5

Have you completed your Policy Orientation and Wet Labs training?

Yes ○ No ◉

Have you submitted and have a signed copy of your Animal Contact Health Surveillance Questionnaire (https://www.ccny.cuny.edu/sites/default/files/2023-01/Animal%20Contact%20Health%20Surveillance%20Questionnaire.doc)?

Yes ○ No ○

Business Role

Researcher Staff Members

Name

Edwards, Mila

Organization Department

President Biomedical Eng.

Email

milaeedwards@gmail.com

Office Phone

Cell Phone

Alternate Phone

Home Phone

Primary Contact?

☐

Copy on all Emails

☐

Will person be handling animal species?

Yes ◉ No ○

Species Name	Type	Procedure Description
Rat (Rattus norvegicus)	Euthanasia	Carbon Dioxide Inhalation
Rat (Rattus norvegicus)	Procedures	Drug Administration
Rat (Rattus norvegicus)	Procedures	Other
Rat (Rattus norvegicus)	Procedures	Training
Rat (Rattus norvegicus)	Surgery	Stereotaxic Surgery

Degrees

Experience and Qualifications

Received training in animal handling and surgery at Columbia University via lab of Dr. Qi Wang.

Number of years experience in this field?

1

Have you completed your Policy Orientation and Wet Labs training?

Yes ○ No ◉

Have you submitted and have a signed copy of your Animal Contact Health Surveillance Questionnaire (https://www.ccny.cuny.edu/sites/default/files/2023-01/Animal%20Contact%20Health%20Surveillance%20Questionnaire.doc)?

Yes ○ No ○

Databases Searched

USDA Policies state that alternative searches are required for all animal use Protocols causing pain or distress to animal subjects.

Details of the search must include keywords used, years covered, and databases searched.

Did you record any animals in pain level D or E on the USDA Categories page?

Yes ◉ No ○

Databases searched (at least two required).

Pubmed, Google Scholar

Please list all keywords, Boolean operators and search strategies for each potentially painful procedure.

"Brain+oscillations+neuromodulation+non-animal model"

Consultation with colleagues (include name, qualifications, etc.).

If applicable, cite any journals used in search.

If applicable, list any relevant scientific meetings (seminars, focus groups, etc.) attended.

Based on your literature search, please describe the potential alternatives to painful procedures and state why they are not appropriate.

In a search for "non-animal" as an alternative to using rats, PubMed results in 0 hits. Google Scholar results in 48 citations. However, of these 48 citations, the majority are human neuromodulation studies, which is an inappropriate alternative to our research at this time. Working with human subjects is not an alternative to test our hypothesis, which requires the probing of the responses of individual neurons. Human neuroscience only permits studying the brain on a macroscopic level, with the exception of special clinical populations. There are a few citations involving the use of neurocomputational models. However, in order to measure the effects of an intervention on brain oscillations, it is required to work with brain tissue. Therefore, neurocomputational models are not biologically plausible (at this stage) and importantly, they do not allow for modeling the effects of a non-electromagnetic stimulus such as ultrasound.

Date the search was conducted.

11/01/2024

Number of years covered in search.

74

Endpoints & Assurances

List clinical parameters that would lead to early euthanasia in any of the procedures described in this protocol

If the animal dislodges the optical implant (cannula/fiber optic implant) from the skull, which is an unlikely event, we would immediately euthanize the animal.

The information in this protocol is true and accurate, and to the best of my knowledge, it conforms to the Institutional Animal Care and Use Committee (IACUC) and USDA policies on the use of animals in research.

☑

Protocol Attachments

The following is a list of all attachments listed on this Protocol

Page	File Name	Description	Original File Name
Personnel List	2024-1170_1_0001_citiCompletionReport_2289756_42220212.pdf (https://ccny-cunyr4.app.cayuse.com/attachment/2024-1170_1_0001_citiCompletionReport_2289756_42220212.pdf)	CITI IACUC	citiCompletionReport_2289756_42220212.pdf

Amendment Reason

Protocol Number

2024-1170

Protocol Year

3

Protocol Title

Ultrasonic Neuromodulation of the Rat Brain with Fiber Photometry

Approve Date

12/23/2024

Expiration Date

12/23/2027

Full Name

Dmochowski, Jacek P.

Reason for Change

Adding wild-type mice to the protocol