FOR IACUC USE

Protocol #_20190501YL00360-01_____

Approved for period (one year maximum)

Approved _5/1/2019_ to _4/30/2020_

Copy to PI _5/1/2019_____

Copy to Animal Care Worker _____

Protocol Approval Form

DHHS/NIH/OLAW ASSURANCE D16-00135 (#A-3216-01)
EFFECTIVE: *06/12/18-03/31/22*

UNIVERSITY of WYOMING
INSTITUTIONAL ANIMAL CARE and USE COMMITTEE
PROTOCOL APPROVAL FORM

Submit completed form electronically to the IACUC at IACUC@uwyo.edu.
RESEARCH SHALL NOT BEGIN UNTIL THIS FORM IS APPROVED.

New project _____x_____ Revised protocol _____ Date___11/30/2018_____
(Project will be approved for one year from date of IACUC approval. An annual update form must be submitted annually if project extends beyond one year.)

Title of project: In vivo calcium imaging at the frontal cortex in mouse models of brain disorders
Project leader(s): Yun Li
Department(s): Zoology and Physiology
Phone: 410-487-4608
Email: yli30@uwyo.edu

Proposal Category (The PI may choose more than one category).

****Note:** If live animals will only be observed in their natural habitat and the study will not involve an invasive procedure, harm to the animal, or materially alter the behavior of the animal, **an IACUC protocol is not required**. If live animals will only be used for **observational and/or non-invasive teaching**, please stop and complete the University Teaching Protocol Form.

B _____ Live animals will be bred or held for use in teaching, testing, experiments, research, or surgery, but not yet used for such purposes. **STOP**→ If this is all that is involved, please complete the University Breeding Colony Form.

C _____ Live animals will be involved in teaching, research, experiments, sample collection, and/or tests, but the procedures will only involve minimal, momentary, or no pain/distress to the animals and pain relieving drugs will not be used. Includes euthanasia via injection

D ___X__ Live animals will be involved in experiments, teaching, research, surgery or tests and either some or all of the procedures involve more than minimal and/or momentary pain or distress to the animal and appropriate anesthetics, analgesics, or tranquilizing drugs will be used.

E __ __ Live animals will be involved in experiments, teaching, research, surgery or tests and either some or all of the procedures involve pain or distress to the animal and anesthetics, analgesics, and/or tranquilizing drugs will not be used because of the adverse impact on the affected procedures or results.

Species Information

Animal species (genus, species, common name) (one species per protocol form-unless procedures are identical):Mus, or Mus musculus, or house mouse

Number to be used/year: 160 (Year1), 240 (Year2), 320 (Year3) Total animal days/year (# animals x #days): 120 x 270d + 40 x 365d = 47000 (Year1); 160 x 270d + 80 x 365d = 72400 (Year2); 260 x 270d + 60 x 365d = 92100 (Year3)

Number to be used/project: 720 Total animal days/project: 540 x 270d + 180 x 365d = 211500

Source of animals: National Institute on Drug Abuse (NIDA), The Jackson Laboratory, ***The Johns Hopkins University School of Medicine (JHUSOM), breeding colonies from the U of Wyoming (Breeding Protocol TBD)***

Location Information

Location of animal room: Building/Room number BS 511 and BS 519

Location of animal care log book/medical records: Building/Room number BS 511 and BS 519

Duration of project: Begin Jan 2019 End Dec 2021

Funding Information- **(Please note that this section is mandatory)**

Source of Funding (Government agency, Grant, Departmental Funds, etc.): Start-up package

UW Budget ID/Project Grant number (if applicable): 1004027 13291 1

Name person(s) and/or unit responsible for animal care:

Name:	Phone:	Email:
Yun Li	410-487-4608	yli30@uwyo.edu

1) a. Purpose (*in lay terms*)

[REVISION]
Our brain is the control center of our body. The average human brain has about 100 billion nerve cells. These nerve cells communicate and coordinate each other to control everything from our senses to the muscles throughout our body. Brain disorders refer to conditions or disabilities that affect our brain. For instance, mental illnesses affect our behavior patterns, and neurodegenerative disorders (diseases destroy our brain cells) cause problems in our memory, moving ability, behavior and even personality. Unfortunately, there is no cure for most of brain disorders.

I will use mouse model as an experimental system to study human brain disorders. Mouse brain shares a high similarity with human brain at levels of both the structure and connectivity. We can readily perform functional invasive tests inside of the mouse brain (which could be harmful and cannot be done with human subjects).

I have the following two specific purposes:
1) <u>Study nerve cell activity in the forebrains of normal mice</u>
In order to eventually cure brain disorders, we need to first understand how nerve cells normally work together to guide our behavior. I will take a direct approach to study the relationship between nerve cells' activity and animal's behavior, by simultaneously recording a large multitude of nerve cells' activity from the intact brain of behaving mice to capture the dynamic pictures of brain in action.
2) <u>Study nerve cell activity in the forebrains of diseased mouse models</u>
Many mouse models have been developed as valuable tools to study human brain disorders. These mouse models mostly are genetically modified mice either carrying mutant human genes or with disrupted/inactive gene expressions, corresponding to genetic links for certain human brain disorders. These transgenic mice typically display some (if not all) symptoms closely resembling the corresponding human brain disorders. Therefore, they are powerful tools to study the pathogenic mechanisms for the corresponding human brain disorders. I will take a similar approach to study nerve cells' activity together with the animal's behavior in different mouse models of brain disorders. By comparing these models to normal mouse brain, I will study what have been changed in nerve cells of these brain disorders. I will then try to identify the potential therapeutic strategy for these brain disorders.

The scope of this animal protocol covers in vivo imaging studies on mouse models of several related brain disorders, including neuropsychiatric disorders such as autism, schizophrenia, depression, and neurodegenerative disorders such as frontotemporal dementia and amyotrophic lateral sclerosis. These brain disorders all have dysfunctions in the forebrain and display behavioral deficits.

b. Scientific objective(s)

[REVISION]
The frontal cortex plays an essential role in behavior control. For instance, the prefrontal cortex (PFC) regulates complex cognitive processes through reciprocal connections with most cortical and subcortical brain regions, while the motor cortex control voluntary movements through the connection with the spinal cord. Disruptions or dysfunctions of the frontal cortex in a variety of neuropsychiatric and neurodegenerative disorders, are associated with behavioral abnormalities.

Deficit in social behavior is a common feature of many neuropsychiatric disorders, including autism spectrum disorder (ASD), schizophrenia and depression. Some neurodegenerative disorders, such as frontotemporal

dementia (FTD), also display abnormal social behaviors. Interestingly, a seemingly different neurodegenerative disorder, amyotrophic lateral sclerosis (ALS, characterized by the loss of control over voluntary movement due to motor neuron degeneration in the brain and the spinal cord), displays a significant level of comorbidity with FTD.

Previously, I have applied a custom miniScope recording system in the mouse PFC to study neural encoding mechanism of mouse social behavior. We demonstrated that principle neurons in the PFC formed ON and OFF neural ensembles to code social exploration. ON neurons increased activity and OFF neurons decreased activity during specific behavior, and their dysfunctions were associated with abnormal social exploration.

Here I propose to study the pathogenic mechanisms of these related brain disorders through applying miniScope recording system in the frontal cortex of diseased mouse models, and compare to that of wildtype mice. I have the following two specific objectives:

<u>Objective 1: To illustrate normal neural circuit during normal social behavior</u>
I will simultaneously label excitatory and inhibitory neurons with different fluorescent calcium indicators, or distinguish projection specific neural subgroups with different fluorescent calcium indicators through retrograde labeling and perform in vivo calcium imaging at the prefrontal cortex of the wild type mice during normal social behavior test. I will compare the neural circuitry and neural coding mechanism in the prelimbic and infralimbic cortex. The goal is to understand the input and output connectivity of the ON and OFF neural ensembles in the prefrontal cortex.

<u>Objective 2: To explore neural circuit in diseased mouse models during abnormal behaviors</u>
I will perform in vivo calcium imaging on several mouse models of related brain disorders. The goal is to identify any dysfunctional neural circuitry in mouse models of brain disorders that cause abnormal behaviors. I will have THREE sub-objectives:

<u>2A: Prelimbic and infralimbic cortex in several neuropsychiatric disorders</u>
I will perform in vivo calcium imaging recording from various subgroups of neurons at the prefrontal cortex of mouse models of ASD, schizophrenia, and depression during mouse social exploration test. I will compare the neural circuitry and neural coding mechanism in the prelimbic and infralimbic cortex of these brain disorders to wildtype mice.

<u>2B: Prelimbic and infralimbic cortex in the neurodegenerative disorder FTD</u>
I will perform in vivo calcium imaging recording from various subgroups of neurons at the prefrontal cortex of mouse models of FTD during mouse social exploration test. I will compare the neural circuitry and neural coding mechanism in the prelimbic and infralimbic cortex of FTD models to that of wildtype mice .

<u>2C: Primary motor cortex in the neurodegenerative disorder ALS</u>
The upper motor neurons are located within the layer 5B of the motor cortex but spatially intermingled with non-corticospinal neurons. I will label specifically those upper motor neurons with fluorescent calcium indicator through retrograde labeling and perform a longitudinal in vivo calcium imaging study at the motor cortex of mouse models for ALS during open field test and rotorad test. The goal is to determine if there is any abnormal neural activity and dysfunctional neural circuitry prior to neuronal death in the motor cortex. I will also explore if ALS and FTD display any similar dysfunctions of the connectivity and network within local neural circuitry.

c. Potential for use of in vitro systems or computerized models instead of live animals

i. Elaborate on current availability of animal data that could be used to predict outcomes
Currently there is no existing animal data to be used to predict outcomes.

ii. Elaborate on the uniqueness of the study such that the requirement for live animal research is necessary

Due to ethical reasons, the proposed mechanistic studies cannot be done with humans. The present studies require live animals. This is because it is not feasible to study neural circuit mechanisms underlying social behavior and motor activity in either tissue or cell cultures, or computer models.

2) Describe all procedures: *Description should allow the IACUC to understand the experimental course of an animal from its entry into the experiment to the endpoint of the study, including euthanasia and animal disposal*.

a. Overview of procedures

All experimental mice will receive two survival surgeries around age of 3 months, firstly an intracranial or intraspinal AAV viral injection surgery, and secondly an intracranial gradient-index (GRIN) lens implantation surgery two week after the viral injection. Mice after the intracranial GRIN lens implantation surgery will be housed individually to avoid damage to surgical site from group housing and animal fighting. Mice will be allowed to recover for at least 21 days before the beginning of in vivo calcium imaging experiments. To secure the miniScope on the mouse head, a detachable base part will be attached to the mouse skull permanently with dental cement while mouse is under anesthesia with isoflurane. The miniScope body will be connected to the mouse's head ONLY during the experimental test. The miniscope body will be attached to its base part while mouse is under anesthesia with isoflurane. The relay of images from GRIN lens will be achieved via optical relay, by matching the focus point between GRIN lens and the primary imaging lens of miniScope. At the end of the in vivo calcium imaging, the miniScope body will be detached from its base part while mouse is under anesthesia with isoflurane. For studies with mouse models of neuropsychiatric disorders, mice will be tested every other week for 3-6 months. For the longitudinal studies with mouse models of neurodegenerative disorders, mice will be tested monthly for 6-12 months. Mice will be euthanized and brain tissues will be collected at the end of the whole experiments.

Various adeno-associated viruses (AAV) expressing Cre-On or Cre-Off fluorescent calcium indicator or their combinations will be used in our studies as indicated in the below Table.

Virus Name	Purpose
AAV1.CamKII.GCaMP6f	label excitatory neurons with green fluorescent calcium indicator
AAV1.Syn.GCaMP6f	labeling all local neurons with green fluorescent calcium indicator
AAV1.CAG.FLEX.GCaMP6f	label specific neural populations with Cre-On green fluorescent calcium indicator
AAV1.CAG.DIO.jRGECO1a	label specific neural populations with Cre-On red fluorescent calcium indicator
AAV1.CAG.DO.jRGECO1a	label specific neural populations with Cre-Off red fluorescent calcium indicator
AAV2/9.CaMKII.Cre	Retrograde label projection specific neurons

(***Reference citations for b-e***)

b. Type and duration of restraint

Mouse will be anesthetized and mounted on a stereotaxic stage through bite plate/nose cone and blunted ear bars. There will be a 37°C heating pad underneath the mouse body to keep its body temperature. The time it takes for viral injection surgery varies with how many injection sites are being performed. Unilateral injection typically

takes about 30-40 minutes, bilateral or multiple-site injection takes 1-2 hours. The time it takes for GRIN lens implantation surgery is typically 2-3 hours.

c. Name and dose of anesthesia and/or tranquilizer (contact attending veterinarian)

Drug Name	**Procedure**	**Dose (mg/kg)**	**Route**	**Duration**	**Concentration and Volume**
Isoflurane (scavenging method: charcoal filtration)	Intracranial or intraspinal virus injection	5% (with O_2 1000ml/min)	Inhale in induction chamber	2-3min	5% (with O_2 1liter/min)
Isoflurane (scavenging method: charcoal filtration)	Intracranial intraspinal virus injection	1% (with O_2 200ml/min)	Inhale through nosecone	During the whole procedure	1% (with O_2 200ml/min)
Ketamine and Xylazine	GRIN lens implantation,	Ketamine: 100 mg/kg; Xylazine: 15 mg/kg ***Amount: 3mg Ketamine and 0.45mg Xylazine for a 30g mouse***	i.p. ***30-G needle***	once	Ketamine (10 mg/ml) and Xylazine (1.5 mg/ml), 0.1mL/10g of body weight ***Amount: 0.3mL for a 30g mouse***
Ketamine	GRIN lens implantation,	Ketamine: 50 mg/kg; ***Amount: 1.5 mg Ketamine for a 30g mouse***	i.p. ***30-G needle***	As need during surgery following initial injection	Ketamine (10mg/ml), 0.05mL/10g of body weight ***Amount: 0.15mL for a 30g mouse***
Isoflurane (scavenging method: charcoal filtration)	Secure the base of mini-scope to mouse skull with dental cement	5% (with O2 1liter/min)	Inhale in induction chamber	2-3 min	5% (with O2 1liter/min)

Isoflurane (scavenging method: charcoal filtration)	Secure the base of mini-scope to mouse skull with dental cement	1% (with O_2 200ml/min)	Inhale through nosecone	During the whole procedure	1% (with O_2 200ml/min)
isoflurane (scavenging method: charcoal filtration)	Connect/disconnect the mini-scope body to/from its base	5% (with O2 1liter/min)	Inhale in induction chamber	2-3 min	5% (with O2 1liter/min)]
Ketamine and Xylazine	Cardiac perfusion	Ketamine: 250 mg/kg; Xylazine: 37.5 mg/kg	i.p. ***30-G needle***	once	Ketamine (10mg/ml) and Xylazine (1.5 mg/ml), 0.25mL/10g of body weight

[REVISION]
Information for needle gauges:

Needle gauges	***Purpose***
30-G needle	***i.p. injection***
30-G needle	***s.c. injection***

d. Surgical procedures

i. pre-operative procedures

The subject mouse will be first weighed and anesthetized with either isoflurane or Ketamine/Xylazine as indicated above. Mouse hair will be removed with a clipper. Mouse will then be mounted on a stereotaxic stage. Ophthalmic ointment will be applied on both eyes of the mouse to keep moist during surgery. The skin will be disinfected with surgical iodine followed by 70% ethanol swabs.

[REVISION]
Pre-surgical pain relief

Drug Name	***Procedure***	***Dose (mg/kg)***	***Route***	***Frequency or Schedule***	***Concentration or Volume***
Xylocaine (Local)	***Intracranial/intraspinal viral injection and GRIN lens implantation***	***2mg/kg*** ***Amount: 1mg locally***	***Locally injected under the skin of the brain***	***once before surgery***	***2%; 0.05mL***

			surgical area, with 30-G needle		

ii. aseptic methods to be used: surgical attire

Aseptic techniques will be used for all surgical procedures. Surgical instruments are cleaned by soaking them in a Cidex Plus solution prior to surgery (overnight), rinsed in sterile distilled water, and then sterilized by inserting them into a Fine Science Instrument Sterilizer for 20 seconds prior to use. In between each surgery, instruments are soaked in 70% ethanol for 20 minutes, rinsed in distilled water, and then sterilized by inserting them into a Fine Science Instrument Sterilizer for 20 seconds. GRIN lens will be soaked in 70% ethanol for 20 minutes, then soaked in sterilized saline for 15 minutes prior to implantation. The O_2 saturated ACSF perfusion buffer will be filtered through a sterile 0.22um pore-size PES membrane. All gauze and surgical drapes are autoclaved before surgery and a new pair of sterile gloves is used for each surgery.

[Revision]
All attires and solutions will be autoclaved prior to use.

iii. who will perform surgical procedures
Dr. Yun Li

iv. where will surgical procedures be performed
BS 425

v. non-survival/survival surgery

1) Intracranial or intraspinal virus injection:

Mice will be injected with AAV virus into specific brains region or spinal cord region. Mice will be anaesthetized through an isoflurane vaporizer with 1~5% isoflurane in oxygen at a flow rate of 0.4 liter/min and mounted on a stereotactic frame over a heating pad. Dexamethasone (2mg/kg dosage) will be intramuscular injected into mouse before surgery to minimize brain inflammation response**.** Surgery starts after the mouse fails to show the withdrawal reflex after pinching the rear toe (a procedure that is repeated several times during the surgery to ensure full anesthesia level). Sterile ocular lubricant ointment was applied to mouse corneas to prevent drying.

For unilateral intracranial viral injection, the surgical area (top of the skull) will be shaved and scrubbed with 70% alcohol and betadine for three times. The skin will be cut down along the skull's midline and separated from the skull with wound clips. Small amount (1-2 drops) of xylocaine will be applied locally to skull at this point,

[Revision]

a small amount (0.05mL) of 2% xylocaine will be applied locally (by injection under the skin, with a 30-G needle) onto the skull at this point,

and the skull area is cleaned with sterile saline and a small hole is drilled above the injection area using a 0.5-mm diameter round burr on a high-speed rotary micro drill. Next, using a stereotaxic instrument, the mice will receive unilateral injections of the viral construct into the brain via an injector connected to an infusion pump. The AAV vectors are suspended in artificial cerebrospinal fluid (ACSF) before

the intracranial injections. A total of 500 nl of virus solution will be injected at a rate of 25 nl/min using a micro pump and Micro4 controller. After injection, the injection needle will be remained in the parenchyma for additional 5 min before being slowly withdrawn.

For bilateral intracranial viral injection, all steps are same as described above, except that two symmetric holes will be drilled on both hemispheres using a stereotaxic instrument and 500 nl of virus solution will be injected at each site.

For intracranial viral injection involved the retrograde labeling, all steps are similar as described above, except that one hole will be drilled on the axon-projected region and the Cre virus will be injected on this site; while a second hole will be drilled on the neural cell body region and either Cre-On or Cre-Off virus expressing fluorescent calcium indicator will be injected.

For intraspinal viral injection, the mice back will be shaved from below the shoulder blades to the top of the neck and scrubbed with 70% alcohol and betadine for three times. A midline incision will be made and the trapezius muscles will be exposed. The trapezius muscle will be cut along the midline to expose the spine. A total of 500 nl of virus solution will be injected into the midline of C5-C7 vertebrae segment.

After viral injection surgery, the skin will be sutured and Neosporin antibiotic ointment will be applied onto the skin. Mice will be given 1 ml of warm saline (injected SC) to replace lost fluid during surgery. The analgesic buprenorphine (0.1 mg/kg, SC) is given after surgery. Ibuprophen in drinking water (5ml in 100ml water, or 1mg/ml) will also be given for three days post-surgery.

[Revision]
The Hamilton syringe with a 34-Gauge needle (1.5 inches long) will be used for intracranial viral injection.

2) Grin lens implantation:

Two week after viral injection, mice will be permanently implanted with GRIN lens. Mice are anesthetized using Ketamine & Xylazine at the dosage of 100 mg Ketamine and 10 mg of Xylazine per 1kg of body weight. Buprenorphine (s.c., 0.1 mg/kg of body weight) will be used as analgesics. Dexamethasone (2mg/kg dosage) will be intramuscular injected into mouse before surgery to minimize brain inflammation response**.** Surgery starts after the mouse fails to show the withdrawal reflex after pinching the rear toe (a procedure that is repeated several times during the surgery to ensure full anesthesia level).

At the targeted brain area, brain tissue is precisely removed using vacuum through a 30-Gauge blunted needle attached on a motorized stereotactic instrument, which was a custom-constructed three-axis motorized translation stage. MATLAB-based software was developed to control the movement of the stereotactic arm to remove the brain tissue automatically by pre-defined trajectory guidance. The GRIN lens above the mouse skull will be secured to skull with dental cement and an additional plastic cap (0.2-mL PCR tube tip) was glued on the skull to protect the imaging surface of the GRIN lens.

[Revision]
Once fully anesthetized with Ketamine & Xylazine, a small amount (0.05mL) of 2% xylocaine will be applied locally (injection under the skin, with a 30-G needle) onto the skull at this point. The skin will be removed along the skull edge sufficiently to expose Bregma and Lamda. A thin layer of superglue will be applied along the edge of the skin excision to bond the skin to skull. Next, the exposed skull area will be cleaned with a small surgical blade to peel and scrape periosteum tissue

from the skull. Using a stereotaxic instrument, a small hole will be drilled through the skull at the targeted area using a 1.2-mm diameter round burr on a high-speed rotary micro drill.

After thoroughly cleaning up of the bony debris around the skull hole, brain tissue will then be stereotaxic removed precisely using vacuum through a 30-Gauge blunted needle attached on a motorized stereotactic instrument, which was a custom-constructed three-axis motorized translation stage. MATLAB-based software was developed to control the movement of the stereotactic arm to remove the brain tissue automatically by pre-defined trajectory guidance to create a precise hole (with 1.1-mm diameter) for the GRIN lens implantation.

The GRIN lens (1-mm in diameter) will be dropped into the brain hole. The GRIN lens being exposed above the mouse skull will be secured to the skull with dental cement. An additional plastic cap (0.2-mL PCR tube tip) will be glued on the skull to protect the imaging surface of the GRIN lens.

We now have a published protocol with step-by-step details for the GRIN lens implantation. Zhang L, Liang B, Barbera G, Hawes S, Zhang Y, Stump K, Baum I, Yang Y, Li Y, and Lin DT. 2018. Miniscope GRIN lens system for calcium imaging of neuronal activity from deep brain structures in behaving animals. Curr Protoc Neurosci. e56. doi: 10.1002/cpns.56

After surgery, mice are given 1 ml of warm saline (injected SC, ***with a 30-G needle***) to replace lost fluid during surgery. The length of surgery is typically under 1-2 hours. The analgesic buprenorphine (0.1 mg/kg, SC, ***with a 30-G needle***) is given after surgery. Ibuprophen in drinking water (5ml in 100ml water, or 1mg/ml) will also be given for three days post-surgery.

vi. justification if more than one surgical procedure per animal (this includes any procedures performed during their lifetime, not just under this protocol)

Previously in NIH/NIDA, we have tried for several months to inject AAV virus into a specific brain region, immediately followed by GRIN lens implantation on the same survival surgery. We found that combining AAV injection and GRIN lens implantation into one surgery does not allow us to see much fluorescent signal with our custom developed miniature microscope. We therefore conclude that combining AAV injection with GRIN lens implantation into one surgery is not feasible for our proposed experiments, and that separating AAV injection and GRIN lens surgical implant is required for our experiments. Therefore, we have to perform two survival surgeries on an animal during this proposed study.

Timeline between surgical procedures:

Time (weeks)	Week1	Week 3	After Week 6
Surgical procedure	Intracranial or intraspinal AAV injection	GRIN lens implantation	in vivo imaging and behavior training

d. Post-surgical care

i. Recovery facility

There will be two sets of veterinary care units (an isothermal chamber with optimal temperature and moisture). One will be located in the experimental animal housing room (BS519), the other will be located in the surgery room (BS425).

Immediately after viral injection surgery, mice will be group housed with littermates in a new cage with a clean bedding. Water containing 5% ibuprofen and food will be provided. Mice in their new cages will be put into the veterinary care unit localized in BS425 for recovery and monitored until they are ambulatory. Mice typically stay inside of the veterinary care unit in BS425 for overnight and will be returned to BS519 on the regular shelf.

Immediately after GRIN lens implantation surgery, mice will be individually housed in a new cage with a clean bedding. Water containing 5% ibuprofen and food will be provided. Mice in their cages will be put into the veterinary care unit localized in BS425 for recovery and monitored until they are ambulatory. Mice typically stay inside of the veterinary care unit in BS425 for overnight and will be returned to the veterinary care unit localized in BS519 for several days before returning to the regular shelf.

[Revision]

Post-surgical monitoring

During recovery period (i.e., in the warmed veterinary care units at BS425), experimental mice will be closely monitored until they have recovered from anesthesia sufficiently to right themselves and move about the cage. Then they will be returned to the animal room (BS519), there they will be placed again into the warmed veterinary care units for several days. The experimental mice will be examined periodically (i.e., at least once a day) during the next few days to assure that incisions are healing properly, no signs of distress (e.g., eyelid partially closed, rough hair coat, hunched posture, unresponsive), and that normal hydration is being maintained. Typically after 2-3 days, mice will be returned to the regular shelf. After three days, we will replace Ibuprophen containing water with normal drinking water.

A surgery log and a surgery card will be generated right after the surgery, including the information of 1) what type of surgery; 2) surgery date; 3) personnel's name who performed surgery; 4) mouse recovery situation for three post-surgical days. The surgery card will be put behind the regular cage card. The surgery log will be kept in Room BS425. All experimental mice will be monitored periodically for their health state during the entire experimental period. Signs of distress will be watched for, including eyelid partially closed, rough hair coat, hunched posture, unresponsiveness etc.

ii. Name, dose, route of administration and regimen for analgesia; (investigate literature for pain management for species used; consult attending veterinarian)

Drug Name	**Procedure**	**Dose (mg/kg)**	**Route**	**Frequency or Schedule**	**Concentration or Volume**
Xylocaine (Local)	Intracranial/intraspinal viral injection and	1-2 drops	local	once before surgery	2%; 1-2 drops

	GRIN lens implantation				
Buprenorphine*	Intracranial/intraspinal viral injection and GRIN lens implantation	0.1 mg/kg	s.c.	Post-surgery	0.02 mg/ml; 0.05mL/10g body weight

*Buprenorphine is in general suggested to be used in a dose of 0.05 ~ 0.1 mg/kg. I proposed here to subcutaneously apply Buprenorphine in a dose of 0.1 mg/kg. Based on my previous experience in National Institute on Drug Abuse (NIDA), acute administration of buprenorphine (up to 0.2 mg/kg) works well on mice and there is no known side-effect on mice.

[Revision]

Drug Name	***Procedure***	***Dose (mg/kg)***	***Route***	***Frequency or Schedule***	***Concentration or Volume***
Xylocaine (Local)	***Intracranial/intraspinal viral injection and GRIN lens implantation***	***2mg/kg*** ***Amount: 1mg locally***	***Locally injected under the skin of the brain surgical area***	***once before surgery***	***2%; 0.05mL***
Buprenorphine*	***Intracranial/intraspinal viral injection and GRIN lens implantation***	***0.1 mg/kg*** ***Amount: 0.003mg for a 30g mouse***	***s.c.***	***Post-surgery***	***0.02 mg/ml; 0.05mL/10g body weight*** ***Amount: 0.15mL for a 30g mouse***

3) Justification for species chosen (lowest possible species on phylogenetic scale)

Mouse is an ideal model organism for studies of human disease by virtue of their well-characterized genome and the availability of sophisticated techniques for genetic manipulations. In addition, it is particularly important for neural circuitry study, that lots of human brain regions share homology with corresponding mouse brain regions. Retrograde viral tools can label neural subgroups based on their specific axonal projections. In addition, transgenic mice that express Cre recombinase in specific population of brain neurons can be used to label specific subset of neurons in the brain for in vivo imaging purpose. Mouse has relatively short life span and it is an ideal species to study neurodegenerative disorder. Conditional knockout mice and transgenic mice carrying human genetic mutations are useful models to study pathogenic mechanisms in neuropsychiatric and neurodegenerative disorders.

4) ***Statistical*** justification for the specified number of animals (assistance to determine the appropriate number of animals per treatment)

a. Justification for number of animals per experiment including any statistical and/or power analyses

Based on my previous experience working with in vivo calcium imaging in the prefrontal cortex, there are only 5-10% of neurons whose activity are tuned to (i.e., correlated to) specific behavior from the entire imaged neural population. In order to be able to identify at least 2 behaviorally associated neurons from each mouse brain, we typically use a threshold of ≥ 50 active neurons (i.e., with detectable calcium dynamics) per mouse to determine if the in vivo recording is "good" enough to pursue the subsequent data analysis. This way will ensure sufficient statistical power to distinguish the potential differences between experimental groups and corresponding control groups.

Due to the variability of viral expression efficiency, as well as, the variability of scar tissue growth around the implanted GRIN lens in each individual mouse, there are only 20 ~ 40 % successful rate to obtain "good" quality in vivo calcium imaging with sufficient active neural numbers for meaningful data analysis. I therefore plan to have 30 male mice initiated with the proposed surgeries for each experimental group.

[Revision]
Statistics: the nonparametric Mann-Whitney U test was previously used for statistic analysis. I will continue use this method to compare diseased mice with their wildtype controls.

Please see detailed experimental groups for a plan for 3 years as listed below:

Group#	**Mouse strain**	**Objective #**	**Diseased Model**	**Targeted brain region**	**Virus Injection**	**Purpose**	**Mouse Number**
1	GAD2-Cre	1	Wild Type	Prelimbic cortex	CamKII.GCamp6f + DIO.jRGECO1a	Simultaneously labelling excitatory and inhibitory neurons	30
2	GAD2-Cre	1	Wild Type	Infralimbic cortex	CamKII.GCamp6f + DIO.jRGECO1a	Simultaneously labelling excitatory and inhibitory neurons	30
3	C57Bl6J	1	Wild Type	Prelimbic cortex	CAG.Cre + FLEX.GCamp6f + DO.jRGECO1a	Retrograde label Amygdala projecting cells	30
4	C57Bl6J	1	Wild Type	Prelimbic cortex	CAG.Cre + FLEX.GCamp6f + DO.jRGECO1a	Retrograde label NAc projecting cells	30
5	C57Bl6J	1	Wild Type	Prelimbic cortex	CAG.Cre + FLEX.GCamp6f + DO.jRGECO1a	Retrograde label VTA projecting cells	30
6	C57Bl6J	1	Wild Type	Infralimbic cortex	CAG.Cre + FLEX.GCamp6f + DO.jRGECO1a	Retrograde label Amygdala projecting cells	30

7	C57Bl6J	1	Wild Type	Infralimbic cortex	CAG.Cre + FLEX.GCamp6f + DO.jRGECO1a	Retrograde label NAc projecting cells	30
8	C57Bl6J	1	Wild Type	Infralimbic cortex	CAG.Cre + FLEX.GCamp6f + DO.jRGECO1a	Retrograde label VTA projecting cells	30
9	Shank3	***2A***	ASD	Prelimbic cortex	CamKII.GCamp6f	Abnormal social behavior	30
10	Shank3	***2A***	ASD	Infralimbic cortex	CamKII.GCamp6f	Abnormal social behavior	30
11	16p11.2	***2A***	ASD	Prelimbic cortex	CamKII.GCamp6f	Abnormal social behavior	30
12	16p11.2	***2A***	ASD	Infralimbic cortex	CamKII.GCamp6f	Abnormal social behavior	30
13	C57Bl6J	***2A***	Wild Type	Prelimbic cortex	CamKII.GCamp6f	Control	30
14	C57Bl6J	***2A***	Wild Type	Infralimbic cortex	CamKII.GCamp6f	Control	30
15	BDNF flox/flox	***2A***	Depression	Prelimbic cortex	CamKII.GCamp6f	Control	30
16	BDNF flox/flox	***2A***	Depression	Prelimbic cortex	CamKII.Cre + FLEX.GCamp6f	social behavior	30
17	BDNF flox/flox	***2A***	Depression	Infralimbic cortex	CamKII.GCamp6f	Control	30
18	BDNF flox/flox	***2A***	Depression	Infralimbic cortex	CamKII.Cre + FLEX.GCamp6f	Abnormal social behavior	30
19	TDP43 flox/flox	***2B***	FTD/ALS	Prelimbic cortex	CamKII.GCamp6f	Control	30
20	TDP43 flox/flox	***2B***	FTD/ALS	Prelimbic cortex	CamKII.Cre + FLEX.GCamp6f	TDP knockout	30
21	C57Bl6J	***2C***	Wild type	Motor cortex	CamKII.Cre + FLEX.GCamp6f	Control	30
22	TDP43 flox/flox	***2C***	FTD/ALS	Motor cortex	CamKII.Cre + FLEX.GCamp6f	TDP knockout	30
23	mtUBQLN2	***2C***	FTD/ALS with expression of human mutant UBQLN2	Motor cortex	CamKII.Cre + FLEX.GCamp6f	Degenerative motor neurons	30
24	hUBQLN2	***2C***	Tg animals with expression of human wildtype UBQLN2	Motor cortex	CamKII.Cre + FLEX.GCamp6f	Control	30

b. Justification for number of experiments per year (as stated on page 2)
I will gradually increase experiments each year, since more graduate students will be recruited and trained to perform experiments over years.

c. Literature cited/reviewed for justification of number of animals proposed
For calcium imaging purpose only, at least n = 5 mice has been used in the literature. For behavior purpose only, n > 10 mice has been in general used in literature. Given that 20-40% success rate is needed for obtaining good quality image, we will start surgery with 30 mice, in order to eventually achieve 6-12 mice for both imaging and behavior analysis per experimental group.

5) Will animals be subjected to euthanasia?

a. Method of euthanasia

[Revision]
Only some experimental mice (i.e., mice displaying good calcium images and being pursued with in vivo calcium imaging recordings) will be perfused with 4% PFA and brain tissues will be collected. These mice will be i.p. injected with overdosed Ketamine/Xylazine (250mg/kg and 37.5mg/kg, with a 30-G needle). For instance, a 30gram mouse will receive 7.5mg Katemine and 1.125mg Xylazine before perfusion.

For the rest of experimental mice (i.e., mice being performed with brain surgeries but not meeting the standard of image quality and not being used for in vivo calcium recordings) will be directly euthanized with cervical dislocation. No perfusion will be performed for these mice.

Dr. Yun Li will perform cervical dislocation. Dr. Li's trainings and experiences working with mouse is attached to this protocol.

b. Drug and dosage
Ketamine and Xylazine, in a dose of 250 mg/kg and 37.5mg/kg, respectively

[Revision]
Amount: A 30gram mouse will receive 7.5mg Katemine and 1.125mg Xylazine before perfusion.

c. If using drugs for euthanasia, describe disposal of animal remains.
After brain harvesting, the rest of animal bodies will be put into plastic bags and disposed into -20°C freezer.

d. If animals will not be euthanized, describe plan for future use or other dispersal.
All experimental mice will be euthanized at the end.

[Revision]
All experimental mice will be either perfused with 4% PFA and harvested brain tissues or being euthanized with cervical dislocation at the end.

6) If the proposal category checked is D or E, then the experimental procedures may cause more than momentary or slight pain or distress and the PI must address the following: Provide a written narrative description, including methods and sources used in search, of how it was determined that alternatives to potentially painful or distressful procedures are not available. The Narrative should include at a minimum the following:

a. A list of the databases (two or more) searched (see below);
b. The terms used to search for alternatives to **each** painful or distressful procedure;
c. Whether any alternatives were found and if so a description of each alternative; **and**

d. If alternatives were found, an explanation of why the alternatives can't be used in this study.

Note: *The purpose of this search is NOT to explain why the research does not duplicate other work. The purpose of this search is to show that there are no alternatives to the potentially painful or distressful procedures outlined in this protocol.*

Including i: Literature cited; database references must include name of databases searched, the date of the search, period covered, and keywords used. For assistance with literature searches please see: http://libguides.uwyo.edu/AWA (which includes a video from a representative of the USDA) or contact the following University of Wyoming Librarians: Kaijsa Calkins at: kcalkins@uwyo.edu or David Kruger at: tseliot@uwyo.edu .

And/Or ii: personal communications

e. **A minimum of two databases must be searched.**

i. Database 1:PubMed
ii. Database 2: Web of Science
iii. Please add additional databases as necessary

Please refer to Animal Welfare Act 9CFR Section 2.31 (d) (1) (ii)

Narrative: 1) Searches for alternatives of viral microinjection
In order to effectively transfect neurons in the adult mouse brain with fluorescent calcium indicator, viral vector will be delivered directly into the targeted brain region through microinjection.

Database	Date of search	Period covered	Keyword search	Non-invasive alternatives	Explanations
PubMed	Dec 4th, 2018	1960-2018	Genetically encoded calcium indicators	Using transgenic mice expressing calcium indicator	Transgenic mice do not have the specificity for labelling projection specific neurons to study neural circuitry. The current transgenic mouse lines cannot simultaneously label excitatory and inhibitory neurons with different fluorescent colors.
Web of Science	Dec 4th, 2018	1960-2018	Genetically encoded calcium indicators	Using transgenic mice expressing calcium indicator	Transgenic mice do not have the specificity for labelling projection specific neurons to study neural circuitry. The current transgenic mouse lines cannot simultaneously label excitatory and inhibitory neurons with different fluorescent colors.
PubMed	Dec 4th, 2018	1960-2018	Vascular viral delivery	Inject virus into mouse tail blood vessel	This method lacks of specificity for viral expression into the projection-specific neural subgroups. The current viral vectors cannot simultaneously

					label excitatory and inhibitory neurons with different color.
Web of Science	Dec 4th, 2018	1960-2018	Vascular viral delivery	Inject virus into mouse tail blood vessel	This method lacks of specificity for viral expression into the projection-specific neural subgroups. The current viral vectors cannot simultaneously label excitatory and inhibitory neurons with different color.

2) Searches for alternatives of GRIN lend implantation
Deep brain calcium imaging requires an implantation of a miniature GRIN lens into the targeted brain region. Since the brain tissue is light scattering, any non-invasive approach will not be able to collect optical signals from the medial prefrontal cortex (Note: prelimbic cortex is 2mm deep and infra limbic cortex is 3 mm deep from the brain surface). There is no alternative approach for GRIN lens implantation.

Database	**Date of search**	**Period covered**	**Keyword search**	**Non-invasive alternatives**	**Explanations**
PubMed	Jan 14th, 2019	1960-2019	GRIN lens implantation alternatives	No	The literature search indicates that alternative experimental procedures are not available.
Web of Science	Jan 14th, 2019	1960-2019	GRIN lens implantation alternatives	No	The literature search indicates that alternative experimental procedures are not available.

3) Explain why this research does not involve unnecessary duplication of previous research or experiments

For assistance with literature searches please see: http://libguides.uwyo.edu/AWA (which includes a video from a representative of the USDA) or contact the following University of Wyoming Librarians: Kaijsa Calkins at: kcalkins@uwyo.edu or David Kruger at: tseliot@uwyo.edu .

a. Please indicate range of search (i.e. 4/4/2010 - 4/4/2018), name of databases, keywords used, and number of responses. **A minimum of two databases must be searched.**

   i. Database 1:
   ii. Database 2:
   iii. Please add additional databases as necessary

b. Discuss relevant literature to justify why unnecessary duplication of previous research is not involved. The written narrative in this section should include at a minimum the following information:
   i. A list of the databases (two or more) searched (see above);
   ii. The terms used to search;
   iii. Whether any similar research was found and if so a description of that research; and
   iv. If similar research was found, an explanation of why this research is so different or why additional research is needed on the same topic that this research does not unnecessarily duplicate research that has already been done.

Please refer to Animal Welfare Act 9CFR Section 2.31 (d) (1) (iii)

Database	Date of search	Period covered	Keyword search	Explanations
PubMed	Jan 14th, 2019	1960-2019	1) "Dual-color miniScope" 2) "neural code" AND "prefrontal cortex" AND "brain disorders"	The literature search indicates that the proposed study is not an unnecessary duplication of previous research.
Web of Science	Jan 14th, 2019	1960-2019	1) "Dual-color miniScope" 2) "neural code" AND "prefrontal cortex" AND "brain disorders"	The literature search indicates that the proposed study is not an unnecessary duplication of previous research.

4) Training/experience documentation: Federal regulations require appropriate training and experience for all personnel involved in the care and use of animals. An up-to-date "Verification of Training for Animal Work" form must be on file in the Research Office for *each* person, including the P.I., involved in the care and use of animals to be used in this protocol.

Please list specific experience and/or qualifications of each animal care worker necessary to perform the specific techniques and procedures described in this protocol (such as surgery and euthanasia) on the following "Verification of Training for Animal Work" form:

UNIVERSITY OF WYOMING
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

VERIFICATION OF TRAINING FOR ANIMAL WORK

(Submit completed form to IACUC@uwyo.edu or the IACUC, Office of Research, Room 308, Old Main)

Date: 10/8/2018

Name: Yun Li

Position: Assistant Professor

Department: Zoology and Physiology **Phone:** 410-487-4608 **email:** yli30@uwyo.edu

Supervisor: Dr. Merav Ben-David **email:** BenDavid@uwyo.edu

Date employed: 8/22/2018

Species to be used: mouse

Procedures conducted with animals (i.e. types of surgery, routine husbandry, feeding trials, euthanasia, etc.)

1) Surgeries including AAV viral injection and GRIN lens implantation; 2) miniScope mounting; 3) various behavior tests (social behavior, novel object recognition, open field, treadmill etc); 4) in vivo calcium imaging and simultaneous behavior test; 4) I.P. injection; 5) chronic stress tests including chronic restraint stress and social defeat stress; 6) routine mouse handling/restraining, sexing adult or new born mice; ear tagging/notching and tail tipping; 7) routine husbandry (group house and single house) 8) routine feeding trials; 9) CO_2 euthanasia; 10) perfusion and harvest brains 11) cervical dislocation

Formal training in animal care and management for species indicated: 1) Johns Hopkins University; 2) The Jackson Laboratory; 3) NIH/NIDA/IRP

Informal or on-the-job training in animal care and management for species indicated:

PLEASE attach copies, if any, of training certificates or other documentation of formal animal care training completed and forward copies of training certificates or documentation completed in the future.

I certify that animals under my care will be cared for according to applicable animal husbandry practices, the NIH Guide for Care and Uses of Laboratory Animals, and the Animal Welfare Act. Animals used for research and instruction will be cared for as dictated in the animal care and use protocol approved by the Institutional Animal Care and Use Committee and according to the Program of Veterinary Care on file. ***I WILL INFORM THE ATTENDING VETERINARIAN (DAVID EVERTSON 745-7341) OR BACKUP VETERINARIAN ON STAFF AT ALPINE ANIMAL VETERINARY CLINIC IMMEDIATELY IF ANY PROBLEMS OCCUR, INCLUDING UNANTICIPATED PAIN OR DISTRESS, INJURY, MORBIDITY OR MORTALITY.***

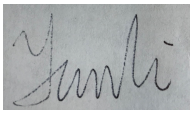
Signature of Employee — Date: 10/8/2018

Signature of Supervisor — Date: 10/30/18

Updated June 2015

Signature of Department Head 10/30/18
Date

Principal Investigator Assurance: *"I have received a copy of the NIH Guide for the Care and Use of Laboratory Animals and/or The Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching and will provide for the care, use and treatment of the animals used for the purpose described above accordingly. I will use procedures which will avoid or minimize discomfort, distress and pain to animals used in my research. I have considered alternatives to procedures that may cause more than momentary slight pain or distress to the animals. These studies do not unnecessarily duplicate previous experiments.* ***I WILL INFORM THE ATTENDING VETERINARIAN (DAVID EVERTSON 745-7341) OR BACKUP VETERINARIAN ON STAFF AT ALPINE ANIMAL VETERINARY CLINIC IMMEDIATELY IF ANY PROBLEMS OCCUR, INCLUDING UNANTICIPATED PAIN OR DISTRESS, INJURY, MORBIDITY OR MORTALITY.*** *I will submit a revised protocol for IACUC approval before making any significant deviations from the approved project procedures occurs. I will submit an annual update for IACUC approval for continuation if this project extends beyond one year. If it is decided to end the study, I will inform the IACUC of that decision using the Closure Form. I assure the IACUC that all persons involved in the care and use of animals related to this protocol have received the appropriate training and are qualified to perform the procedures described above."*



12/04/2018
Principal Investigator …….Date

Attending Vet …….. Date

ACTION BY THE ANIMAL CARE AND USE COMMITTEE: APPROVED X DISAPPROVED ☐

Occupational Health Program Information

As a researcher named on an animal protocol and to ensure your health and welfare while conducting research at the University of Wyoming, you are being offered the opportunity to participate in the University of Wyoming's Occupational Health Program. The University has contracted with occupational health specialists of University of Colorado Health Occupational Health to review the health evaluations for a cost of $55. If you would like to take advantage of this opportunity please complete the ***Baseline Health Questionnaire found at***

http://www.uwyo.edu/research/compliance/animal-care/ under the "Additional Resources" heading to fill out the baseline health questionnaire and risk assessment.

Once completed, submit to Occupational Health Services, UCHealth by email OHSNorth@uchealth.org, or Fax: 970-297-6598.

They will then provide you and the Safety Office with a Physician Evaluation Report. The Physician Evaluation Report lets you and the Safety Office know whether there are any medical conditions that may affect you in your current position. The report does not provide any medical information to UW, just whether follow-up is recommended. If the report recommends further medical follow-up, you will be referred to a physician.

Should you have questions on this program or process please contact one of the Safety Specialists at either 307-766-3203 or 766-2723. A common question asked has been whether UW personnel must complete the form with date of birth, social security number, and mother's first name. UCHealth Occupational Health must have the date of your birth on the form. Your social security number and mother's name helps confirm identity in the medical record, but are optional if UW personnel are uncomfortable supplying this information to them.