Animal Use Protocol

General Information

AUP: A19027

(1) Project Title

Title: Neurobiology of Resilience

1b. AUP Application Type

Select the AUP application type
New Submission

1c. Project Category

Select all that apply
Research
Breeding Protocol

Not applicable

Start Date (DD-MM-YYYY):

18-06-2019

End Date (DD-MM-YYYY):

18-06-2022

(2) Personnel Data

2a.Principal investigator (PI) / Course Instructor

Mitra, Rusphi

2b.Emergency Contact(s)

Mitra, Rusphi
Suresh, Shruti

2c. Co-Investigator(s)

Not applicable

2d. Authors

Suresh, Shruti

2e. Research personnel

(Mustafa mahmoud) Helmy, Mohamed
Mitra, Rusphi
Suresh, Shruti

2f. Pre-Reviewer

Preiser, Peter

2g. Request Addition of a New User

If user(s) to be added is/are not available in selection lists, please provide the following details of user(s) to be added:

(3) Funding

3. Funding

Grant Type
AcRF Tier 3

Grant Title
Defining the brain circuitry defects that cause dementia

Other Funding Sources
AcRF Tier 1- Connectivity of ventromedial prefrontal cortex in simple and complex housing living environment.

Funding Status
Awarded

Start Date	Expiry Date
01-July-2018	30-June-2023

(4) Animal Requirements

Species

Mouse
Rat

Total number of animals

Species	Max
Mouse	1200
Rat	900

Mouse

4. Species Justification

4b. Animal Source

Are animals obtained from AVS-approved sources?
Yes

Select animal source location and enter address
Local
Invivos- NUS laboratory animal center, Singapore
Overseas
Charles River, Kingston, North America.

4c. Housing Location

Select the applicable housing location(s)
NTU ARF
LKC ARF

4d. Other Descriptions

State any other information relevant to animals allocated

4e. Breeding Plan

Determine and describe the number of breeding pairs you need to supply sufficient mice for your experiments

Attach any supporting documents, e.g. tables and charts, via the "Attachments" tab located at the top and bottom of this page.

4a. Animal Characteristics

Strain(s) and Sex
C57BL/6 Balb c Genetic mutants on this background

Age at Acquisition

Weight at Acquisition (metric)

Number Housed at Any One Time

Rat

4. Species Justification

4b. Animal Source

Are animals obtained from AVA-approved sources?
Yes

Select animal source location and enter address
Local
Invivos- NUS laboratory animal center, Singapore
Overseas
Charles River, Kingston, North America.

4c. Housing Location

Select the applicable housing location(s)
NTU ARF
LKC ARF

4d. Other Descriptions

State any other information relevant to animals allocated
Quantity required - 900

4e. Breeding Plan

Determine and describe the number of breeding pairs you need to supply sufficient mice for your experiments

Attach any supporting documents, e.g. tables and charts, via the "Attachments" tab located at the top and bottom of this page.

We will need 8 breeding pairs. Naive male and female breeders of 7-8 weeks of age will be procured and allowed to acclimatize for at least five days before breeding is set up. All breeders will be housed in the corner of the room away from the door and high traffic areas. Bedding will be changed once a week, with gentle handling of the females, in case of pregnancy. Ten days after the breeding pairs are set up, females will be checked once or twice a week for signs of pregnancy (swollen abdomen). Once pregnancy is confirmed (approx. 2 weeks), males will be removed.

4a. Animal Characteristics

Strain(s) and Sex
Wistar Male and female

Age at Acquisition
2- 3 months

Weight at Acquisition (metric)
200-350g

Number Housed at Any One Time
40-60 rats in 20-30 cages staggered, at any one time, to accommodate the limited space in the holding room.

(5) Lay Description

5. Lay Description

(i) Specific Objectives of the Project

List in point form

Serves as an ideal animal model To delineate brain circuits and interacting hormones that mediate behaviors in rodents. Study anxiety/depression/memory behaviour Study neuronal changes in structure and function that relates to behaviour Study molecular and epigenetic changes Study physiological changes, hormones, weights, locomotion etc Study other aspects of gut microbiome and proteomics

(ii) Project Abstract

***250 words or less**, using **simple language**, avoiding the use of scientific/medical jargon*

Neurobiology of resilience is the focus area of our research. We aim to investigate and design a framework for successful coping against psychological disorders throughout adulthood and aging. The need for management of stress-related psychological conditions like anxiety, depression and dementia is towering; specially in aging societies. This is borne out by high prevalence rates, huge economic costs and age-dependence of these disorders. Yet, there is significant gap between need and treatment options. We seek to understand the neurobiological and molecular substrates of psychological resilience and subsequently use this knowledge to search for possible bio-markers and novel therapeutics against stress disorders. We will be using animal models of stress and resilience. Large body of previous literature on stress is heavily dependent on rats and mice as animal models. This is based on the fact that brain areas that drive behavior is conserved between different animal species, including rat, mouse and human. Additionally, rats and mice has been well validated as an optimal animal for experimental purpose, due to it's size and ease of biological manipulation. Thus, we will be using rats and mice as our experimental animal and employ different groups, namely stress and resilient to pursue our research. We aim to carry out comprehensive understanding of brain mechanisms of resilience and identify some of the core factors that can be further developed into therapeutics against stress-disorders. Additionally we will look into unique brain pathways for individual variation that makes some resilient and others not. This knowledge will make treatment options more specified and personalized based on an individual's life history, which forms the core of successful treatment of psychological disorders.

(iii) Attach a Flowchart of Experimental Procedures and Timelines

The flowchart should give a clear overview of the experimental plan for the animals from entry into the project to proposal's endpoint, with timelines clearly indicated.

Attach the flowchart(s) via the Attachments tab located at the top and bottom of this page.

I have attached a flowchart

(iv) Significance of Expected Benefits to Humans or Animals
Since there is no good treatment option for neuropsychiatric disorders, we need to address the treatment gap fast. We thus need to know exact brain mechanism that underlies anxiety/depression/phobia so we can improve upon treatment significantly. Since we cannot directly work on humans to know micro-level brain mechanisms, we test animal models that are most suitable. Thus we apply for an animal protocol. We test stress-related behavior like anxiety and depression in animal models. Rats and mice have specific advantages as below: 1. Emotional circuit of brain/limbic regions are well conserved between rodents and human making it perfect model to study human diseases related to stress/emotion 2. Rats and mice have been historically used and most widely published in the field of stress-research, thus making our study and results in line and replicable with all other studies from all over the world. 3. Rats and mice are easiest to handle, compared to guinea pigs/small primates 4. Behavioral studies are most optimal in rats since they are more social and behaviorally active for most of the studies. Many time they can be tested in isolation too for control groups. In addition to providing detailed insight into rat behavior, animal model such as Wistar rats and some strains of mice provide the most near-perfect model to test human condition from behaviour, through brain network, neuronal structure and function, up to changes in molecules. Thus translational prospect of human neuropsychiatric situations is best addressed.

(6) Animal Use and 3Rs

Not applicable

Search Data

Search conducted	Searched from	Searched to	DB Title	# of hits	Keywords searched

Not applicable

6a. Additional Questionnaire

Based on search results obtained, how will the experiments outlined in this protocol be tailored to avoid duplication of earlier work done?
Being a scientist, one always need to be completely up-to-date in terms of knowledge related to our field, i.e., what are the publications in our field and our lab already worked upon. We do not initiate any project unless it is sufficiently novel, first because it will not be published since it cannot add any new information to the field, second, do not need/plan to use animals just to check something already published and replicated before. Thus when we initiate a project the maximum value is in its novelty where most of the work is likely first time, with some replicability options. Since our work is about animal models of psychopathology, measure of behavioural output is crucial. However the final read-out of projects also critically involve other important measures of physiology, protein and gene regulation. So there is minimal overlap between the main questions of different projects, hence minimising duplication of earlier work.

6b. Alternative non-animal methods

Are there any alternative non-animal methods used by other investigators for the type of work proposed in this AUP (e.g. tissue cultures, in vitro monoclonal antibody, computer model, etc)?
No
In this project we aim to study and understand behaviour and brain pathways of stress and resilience. We will be using behavior as the output and main criteria to identify stress or resilience. Thus it is essential to use awake behaving rodents. There is no vitro system suitable for the proposed experiments and computer models cannot produce/simulate the behavioral and physiological changes we expect to observe.

6c. Why must animals be used in these experiments?

Select all that apply
This is a study of animal behaviour
This phenomenon under study cannot be reproduced in vitro

6d. Characteristics of Species

What characteristics of this/these species make them appropriate for the proposed study?
These might include structural, behavioural, physiological, biochemical, or other features or considerations (such as availability of species-specific reagents, or the use of well-established model) which make the model compatible with the research objectives. Cost is not a primary consideration.

Wistar rats and mice provide the most near-perfect model to test human condition from behaviour, through brain network, neuronal structure and function, up to changes in molecules. Thus translational prospect of human neuropsychiatric situations is best addressed.

6e. 3Rs

(i) Reduction: Describe how you have attempted to reduce the number of animals.
Since I have been researching on the same species, Wistar rats and different strains of mice from various sources, across different countries over 20 years now, I have a good approximation of the number of animals needed for optimal behavioural output. Thus, many different experiments yielded optimal data based on approximately same number of animals per group of treatment. This have been replicated several times over the years. Additionally, for new experiments we calculated approximate number of animal required through statistical power analysis.

(ii) Replacement: Have you considered statistical models in vitro work, instead of using animals etc.? Please describe.
Our main objective and output of study is animal behaviour. Thus, there is no scope of in-vitro alternative.

(iii) Refinement: Have you considered pilot studies, how have you refined any surgical procedures or other manipulations? Please describe.
The surgeries have been optimised and refined over several experiments over the years to use anaesthetics that causes minimal physiological disturbance. The surgical procedures have also been refined, such that minimal number of animals are needed. For example, if we can use same animals for bilateral surgery, we typically do that instead of using two separate animals for two hemispheres. However, we also make sure our final behavioural output is not compromised so as to restart whole experiment again.

(7) Pain Distress

7a. Classification of Pain and Distress

Pain Classification

To add rows/columns to the summary tables, right click to edit the table.

Classification 1

NO pain, distress, or use of pain-relieving drugs. Routine procedures (e.g. injections, tattooing, blood sampling) should be reported with this classification.

Species	Strain	YEAR 1	YEAR 2	YEAR 3	Remarks
Rat	Wistar	150	150	150	Anaetheticis will be used during sacrifice sacrifice.
Mice	C57BL/6	-	150	150	
	Balb c	-	150	150	

Classification 2

Accompanying pain or distress to the animals for which appropriate anaesthetics, analgesics, or tranquillising drugs will be used.

Species	Strain	YEAR 1	YEAR 2	YEAR 3	Remarks		
Rat	Wistar	150	150	150		The main procedure where anaesthesia is needed is surgical implantation of	cannula inside brain.
Mice	C57BL/6	-	150	150			
	Balb c	-	150	150			

Total number of animals requested

Complete the table below to indicate the total number of animals requested per year. This should be a sum of the annual number requested under the selected pain categories:

Species	Strain	YEAR 1	YEAR 2	YEAR 3	Remarks
Rat	Wistar	300	300	300	
Mice	C57BL/6	-	300	300	
	Balb c	-	300	300	

7b. (only for Classification 3)

Explain the procedures producing pain or distress in these animals and justify why appropriate anaesthetic, analgesic or tranquilising drugs cannot be used.
Not applicable

7c. Please justify the number of animals used as stated in section 7.a

Provide a breakdown of how the total numbers are arrived at and explain the distribution of animal numbers across different procedures or experimental groups.
If statistics are used (e.g. χ^2 test, Fisher's Exact test, Student's t-test, ANOVA), describe the statistical analysis.
Based on previous results and pilot data we will need about 24-30 animals in each group to achieve required statistical power. Most of experiments have two and some have three/four experimental groups. So we will need between 100-120 rats and mice per experiment. We will strive to minimize number of animals used by using same animals for behavior and a subset of histology. We would need around 5-7 separate set of experiments, because not all histology experiments have similar requirements of tissue samples. We estimate to use a total of 300-400 animals. Approximately, we will be using 200-300 animals for behavior, 50-60 for surgery and same number for controls. Some of the surgery animals will also be used for behavior. Since we are in year 2 of this project currently, we need to request a total of 600 mice for year 2 and year 3 of the study respectively.

7d. Are pregnant animals used?

If pregnant animals are used, describe how you will take care of the offspring.
Pregnant animals will not be used for experimental producers.

(8) Study Overview

(8) Study Overview	Species
Neurobiology of resilience	Rat,Mouse

Neurobiology of resilience

Species to be used for the study

Mouse
Rat

Animal number calculation for experimental part Neurobiology of resilience

Rat		
	Max	Factor
	900	
	900	**Rat**

Mouse		
	Max	Factor
	1200	
	1200	**Mouse**

List of Non-Surgical Procedures

Description	Species
Blood Withdrawal	Rat,Mouse
Aversion to cat odours	Rat,Mouse
Field exploration task	Rat,Mouse
Stress treatment	Rat,Mouse
Enriched environment	Rat,Mouse
Sub-cutaneous injection of corticosterone and testosterone	Rat,Mouse
7. Fear Conditioning	Rat,Mouse
Porsolt test for anti-depressants	Rat,Mouse
Sacrifice: Trans-cardial perfusion	Rat,Mouse
Sacrifice: decapitation under anesthesia	Rat,Mouse
Sacrifice: decapitation without anesthesia	Rat,Mouse
Metabolic Cage	Rat,Mouse
Social Interaction Test	Rat,Mouse

Description of Non-Surgical Procedures

This procedure aims to collect a small volume of blood (~100 microlitre), through a small nick in dorsal tail vein, without producing significant stress in animal. This method does not produce stress, as evident by low levels of stress hormone, corticosterone, in blood, even after repeated sequential sampling. Moreover, restraint of animal is not essential. This makes this procedure ideal to collect small blood sample, where hormones can be measured without interference from stress endocrine response. Additionally, this allows intra-animal comparison, minimizing need of separate animals for blood collection at different time-points. Animals will be handled for two days before blood collection. Each handling session will last two minutes. Rat and mouse will be placed on a clean towel and towel will lightly wrapped around body to provide a dark cavity in which rat could hide. On the day of experiment, animal will be placed on towel and held gently without restraint. Animals keep quiet in this small and dark environment. A small 2mm incision will be made in dorsal tail vein, 15 cm from end of tail. Generally, no avoidance response like tail flicking is seen at this stage according to published literature. A drop of blood will be collected from nick. Tail vein will not be squeezed (a practice refereed as “milking”), as this pains the animal and also results in vasoconstriction. Bleeding usually stops spontaneously, if tail vein is not stimulated. Animals will be placed in a cage with fresh bedding for 5 minutes and then placed back in home cage. Collection of 100-150 microlitre blood from this technique takes no more than 45-60 seconds, from placing the animal on towel to collection of blood drop. Personnel involved have prior experience with this technique. This procedure does not result in avoidance response or secretion of stress hormones. If multiple blood collection is indicated, at least two days pass between subsequent blood samples. Maximum of four samples will be collected from each animal. New incision will be made 2-3 mm away from first incision. Previous work suggests that repeated sampling does not result in stress hormone secretion in this paradigm. Animals will be monitored two times a day throughout the collection regime.

Endpoint: This procedure aims to collect small volume of blood (~ 100 microlitre), through a small nick in dorsal tail vein, without producing significant stress in animal

Aversion/preference of predator or non-predator species will be tested by allowing exploration in presence of cat or rabbit odor. A rectangular arena, (76cm x 10cm) will be used and the area will be divided into 2 compartments with one having the cat odor (drops of bobcat urine, cloth impregnated with cat smell or worn cat collar) and the other with sham odor (rabbit urine, cloth impregnated with rabbit smell or an unworn collar). Animal will be habituated to testing apparatus for up to 3 sessions during which no odour will be presented during these sessions. Animals will be placed individually in the arena and location tracked during a 10-20 minutes trial. This experiment will be modified to include a T-maze (the long arm is 76cm x 10cm and the short arm is 46cm x 10cm) which has the added facility of an extra compartment for the experimental animal, for its refuge (short arm). Rest of the setup will be as described above. Behavioral test apparatus are cleaned with 70% alcohol to prevent the spread of infectious agents

Endpoint: To measure aversion to predator odors

This is for measuring anxiety in animals. There are 2 tests a. Elevated Plus Maze (EPM) and Open Field (OF). For EPM, animals are allowed to explore a plus maze elevated from ground. The maze has 2 open arms and 2 close arms. Anxiety is measured as percentage open arm exploration. In OF, animals are allowed to explore an well-lit open arena. Anxiety is measured as percentage time the animal spends in center of the arena.
Endpoint: This will test anxiety behavior in rats.

Animals will be immobilized in plastic restraint bags for 2 hours a day, for 10 successive days. Procedure will be conducted after 9AM and before 2PM. During the restraint, animals will be kept under constant observation. Restrained animals will be kept on a cloth pad to avoid inhaling home cage bedding. Body weights will be measured alternate days. Other form of stress that will be induced is maternal separation, wherein the pups on PND 2 to PND 14 will be separated from the dam for 3 hours everyday, placed on a heating pad in a novel room. The pups will be closely monitored during this period.
Endpoint: This will induce stress

Rats will be exposed to enriched environment 24 hours per day (that is continuously monitored) for < 21 days. Animals are kept in large rabbit cages, 3-4 animals per cage. The cages are lined all over with an extra layer of stiff net (small squared net with each square dimension as 4mm length and 4mm). This extra net protection prevents any rat to escape from the cage; and also forms a climbing surface animals need for physical enrichment. The cage contains objects with different textures (colorful toys, new bedding and wooden planks for climbing) and odors, in addition to tubes and boxes in and on to which the animals can climb. Every 2-3 days, objects in will be replaced with novel set to retain novelty. Control animals will be kept in single cages under standard dimensions.
Endpoint: This will induce resilience

Animals will be injected with corticosterone or testosterone subcutaneously (s.c.). Corticosterone/testosterone will be dissolved in sterile peanut oil. A set of control animals will receive sterile peanut oil only. Doses to be used are 10 mg/kg body weight for corticosterone and <1mg/kg body weight for testosterone. These doses are derived from previous literature and my previous work. Only a single injection will be delivered. Animals will be retained for < 12 days post.
Endpoint: This experiment will test the effects of endocrine changes on defensive and reproductive behaviors

This is for measuring conditioned fear. Animals are trained to fear a foot-chock associated with a tone. Fear is measured as percentage freezing with tone alone after footshock-tone pairing.
Endpoint: This will test conditioned fear in rats.

The animals are exposed initially to forced swim stress in a 15-min swim session on day one in a 28-30L cylindrical tank filled to 75% of total volume with water (25 °C). The animals are then placed in clean cages under warm lights for 15 min and then returned to their home cages. The animals are subjected 24 hours later to 5min under identical swim conditions.

Endpoint: To measure learned helplessness as proxy for depression.

The animals must be sacrificed in order to harvest tissues, like brain, liver, heart, nasal epithelium and testes for histology. This will be done under anesthesia with a ketamine (75mg/kg) and xylazine (10mg/kg) cocktail to be administered intraperitoneally and gaseous isoflurane (inhalation) as needed. Trans-cardial perfusion involves replacing whole blood from the body and injecting paraformaldehyde (PFA) from left ventricle with the right atrium cut so the blood does not circulate and is washed out. First an incision will be made from the neck down the chest cavity to gain access to the heart. Then the left and right diaphragms are cut so the lungs shrink to better expose the heart. The butterfly needle is inserted into left ventricle and the right atrium is cut. With the butterfly needle, first ~100ml PBS (pH7.4) and ~500ml 4% PFA in PBS are perfused. Finally the desired tissue is isolated and incubated in 4% PFA.
Endpoint: Once animal tissues are harvested ready for processing for histology.

Another method of sacrifice involves decapitation of the animal with guillotine. Anesthesia is done with a ketamine (75mg/kg) and xylazine (10mg/kg) cocktail to be administered intraperitoneally and gaseous isoflurane (inhalation) as needed, which helps to minimize pain and discomfort. The animal is transported to the guillotine where decapitation is done.
Endpoint: Once animal tissues are harvested ready for processing for histology.

The lack of use of anesthesia for decapitation is important in experiments (described in part 11) where mRNA levels are measured/ visualized. The animal when presented an odor, will process the sensory information in specific areas of the brain that will elicit a change at the gene expression level- either turning on some genes or turning off other genes. Thus, the use of anesthesia can be a confounding factor as this will also elicit its own changes at the gene expression level. Hence, it would be difficult to separate the effect of the odor and anesthesia. To reduce pain and discomfort during this procedure, Harvard restraint bags will be used to hold down the animal and a quick and smooth decapitation (taking less than a minute that also serves to reduce discomfort) with the guillotine will be done.
Endpoint: Once animal tissues are harvested ready for processing for histology.

This metabolic cage enables collection of blood, urine and feces providing information on behavioral and metabolism. The use of metabolic cage allows collection of urine from the animals in a safe and efficient manner. The dimension of the cage (290mm x 450mm x 430mm) is suitable so that the animal does not feel stressed affecting its behavior and metabolism. In addition, the animal can be fed and provided liquid which can be monitored to ensure that the animal is comfortable in its surroundings. Also, no restraints will be used and the animals will be in the cage for a maximum of 4-5 hours.
Endpoint: Collection of urine samples.

The social interaction test is carried out in a rectangular, three-chambered box (a centre 20 × 35 × 35 cm; a left and a right compartment 30 × 35 × 35 cm). Dividing walls had retractable doorways allowing access to each chamber. Left and right compartment are separated from the central compartment with the help of transparent plexiglas with small holes. A social (unfamiliar rat of similar weight) or a non-social stimulus (yellow plastic box) is placed in the left and right compartments. The plexiglas permits visual, tactile, auditory and olfactory communication. The animal (subject of test) is first habituated to the three-chambered apparatus by placing them individually in the central compartment for 10 min during the 3 consecutive days preceding the social test. The doorways into the two side chambers are closed during this habituation phase. On the testing day, the unfamiliar rat (~100g lower than the subject of test) is placed in one of the side chambers and the object is placed on the other side. The subject of test is placed in the middle chamber and allowed to explore both the ends for 5min. The location of the juvenile and the object in the left vs. right side chamber is counterbalanced. The entire apparatus is cleaned with 70% ethanol solution and dried properly between each test.

8. Non-Surgical Procedures

Will food/fluid or both be restricted for the animal(s) used?
No

Will the animal be restrained conscious for a continuous period exceeding 15 minutes?
No

Surgical Procedures

8. Surgical Procedures

Surgical procedures overview

Title of procedure	Description of procedure	Pain classification, if applicable	Describe relevant anaesthesia / analgesia administered. *Also state dosage and routes.*	Number of animals affected	Personnel performing procedure	Relevant experience
Stereotaxic surgery	This procedure will be carried out in order to allow implantation of intra-cranial cannula for infusions. This procedure will be carried out under anesthesia with a ketamine, xylazine cocktail. Surgical site will be prepared by first clipping the fur on the top of the head. This will be followed by scrubbing the skin with a 2% chlorhexidine solution, followed by 70% alcohol solution. This is repeated three times before incision. Care will be taken not to wet the animal to prevent heat loss due to evaporation of alcohol. Ophthalmic ointment (Lacrilube) will be applied to both eyes to prevent corneal desiccation. Animals are treated with prophylactic antibiotic and peripherally acting analgesia 15 minutes prior to surgery by injection of 5mg/kg Baytril (5mg/kg, SC) and Carprofen (5 mg/kg, SC), respectively.	Category D	Induction: Ketamine75mg/kg Xylazine 10mg/kg Intraperitoneal Maintenance: Isoflurane. Evaporated. Using anesthesia machine; flow rate 3-4% mixed in oxygen flowing at 1 liter/min. Inhalation. Analgesia:	50/ year	Rupshi Mitra Shruti Suresh Mohammed Helmy	Rupshi Mitra has more than 5 years of experience with surgery. She will be training Shruti Suresh under close supervision

	Animal is secured in the stereotaxic frame with their body on a warming blanket. Prior to commencement of surgical procedures, depth of anesthesia is verified by loss of toe-pinch reflex. A midsagittal incision is made to expose the cranium and bone surface cleared and dried with sterile cotton tips. A bore hole (approximately 2mm in diameter) is drilled through the skull in the location for each region of interest according to appropriate stereotaxic coordinates. Guide cannula (supported by internal dummy cannula) or electrodes are inserted and encased with cyanoacrylate gel to bond the assembly to the skull. The incision is sutured with around the guide cannulas or electrode and sealed with dental cement. Following completion of the surgical procedures, each animal receives 0.9% physiological saline (5mls, SC) and is placed in a warmed environment until recovery of righting reflex. Animals receive 5 – 7 days standard post-op care (soft bedding, soft wet diet, body weight monitoring, analgesic, lacrilube) with no further experimental procedures of at least 7 days or until pre-op body weight is achieved. Sutures are removed after 7 – 10 days. All surgical instruments will be sterilized prior to surgery by autoclaving. Instrument will be disinfected between each animal by dipping in a hot glass bead sterilizer for approximately 30 seconds after use and allowed time to cool down prior to use on the next animal. Regular breathing and heart rate (by feeling chest with fingers) will be monitored during the surgery. Mucus membranes will be monitored pre- and post-operatively. Post-operatively, animals will be placed in sternal recumbence on a paper-lined cage without bedding, housed separately till they are fully ambulatory. Animals are usually ambulatory within a few minutes in case of inhalation anesthetics. In addition, they will be monitored daily for at least one week post-surgery for need of additional wound care and analgesic. If an animal requires additional care, this animal will be monitored twice daily, at the beginning and the end of day. Thisprocedurewillbe carriedoutin orderto allowa)implantation ofintra-cranialcannulafor dyeinfusion;b)implantation ofelectrodesinthe brainformonitoring ofbrainwaves,inan anesthetizedrat.Thisprocedurewillbecarried outunderanesthesia withaketamine andxylazine cocktail.		Buprenex. Active ingredient: Buprenorphine. 0.01-0.05 mg /kg. Subcutaneous (SQ). Additional doses as needed at 8-12 hour intervals.			
-						

Nature of surgical procedure
Survival

Will multiple surgeries be carried out on animal(s)?
No

Complete this table for those with insufficient / no surgical experience on the relevant animal species:

Name of Trainee(s)	Name and Experience of Trainer
Shruti Suresh	Rupshi Mitra >5 years of experience
Mohammed Helmy	Rupshi Mitra >5 years of experience

Surgical considerations:
(i) How are surgical instruments sterilised and how is the sterility maintained?
All surgical instruments will be sterilized prior to surgery by autoclaving. Instrument will be disinfected between each animal by dipping in a hot glass bead sterilizer for approximately 30 seconds after use and allowed time to cool down prior to use on the next animal
(ii) Has adequate consideration been given to asepsis? Describe the method(s) for sterilising the surgical site and instruments?
Surgical site will be prepared by first clipping the fur on the top of the head. This will be followed by scrubbing the skin with a 2% chlorhexidine solution, followed by 70% alcohol solution. This is repeated three times before incision. Care will be taken not to wet the animal to prevent heat loss due to evaporation of alcohol. Ophthalmic ointment (Lacrilube) will be applied to both eyes to prevent corneal desiccation. Animals are treated with prophylactic antibiotic and peripherally acting analgesia 15 minutes prior to surgery by injection of 5mg/kg Baytril (5mg/kg, SC) and Carprofen (5 mg/kg, SC), respectively.
(iii) How is the site closed (including all layers)? Describe sutures used and suture method.
Internal layer during Ovariectomy and Orchiectomy will be closed using an absorbable material and simple interrupted closure. Cannulation site during stereotaxic surgery will closed using dental cement. Skin layer will be closed using wound clips in an interrupted pattern with clips about 5-8 mm apart.
(iv) Where will the procedure be conducted?
Animal facilities in NTU campus and in LKC Campus at Novena
(v) If clips or non-absorbable sutures are used, when will they be removed?
Non-absorbable suture and wound clips will be removed within 14 days, once the incision has healed.
(vi) How will the animals be monitored over the 72 hours post-surgery? How will records of this monitoring be carried out?
Post-operatively, animals will be placed in sternal recumbency on a paper-lined cage without bedding, housed separately till they are fully ambulatory. Animals are usually ambulatory within a few minutes in case of inhalation anesthetics. In addition, they will be monitored daily for at least one week post-surgery for need of additional wound care and analgesic. If an animal requires additional care, this animal will be monitored twice daily, at the beginning and end of day.

General Documentation

Required General AUP Training

RCULA Practical Certification
RCULA Theory Certification

Status

	(Mustafa mahmoud) Helmy, Mohamed	Mitra, Rusphi	Suresh, Shruti
RCULA Practical Certification	YES	YES	YES
RCULA Theory Certification	YES	YES	YES

8. General Documentation _ Tentative Training and Vaccination Dates

Select the vaccinations applicable to this study
Tetanus (compulsory for work with rodents)
Hepatitis B (compulsory for work with rodents)

All personnel listed must have undergone RCULA training and have the necessary vaccinations. If RCULA training or vaccination are not complete, state tentative completion dates:

RCULA Training

Vaccination

Personnel	Vaccination	Tentative vaccination date (dd/mm/yyyy)
Rupshi Mitra	Tetanus, Hepatitis B	01-05-2019

(9) Experimental Endpoints

9a.Experimental animal use endpoint

Describe when (use time line, clinical sign, etc) the EXPERIMENTAL ENDPOINT for EACH group of animals will be.
All animals (as described in Section 7) must be covered by an experimental endpoint. (not to be confused with humane endpoints).
Animals will be sacrificed 6-8 weeks after exposure to the treatment. In animals undergoing surgical interventions, animals will be sacrificed 6 to 10 weeks after surgery.

9b. Death as an experimental endpoint

Will death be the experimental endpoint for any group of animals?
No

(10) Humane Endpoints

9c. Indicate any clinical conditions or abnormalities expected or that could arise as a result of the proposed study or teaching exercise

Indicate any clinical conditions or abnormalities expected or that could arise as a result of the proposed study or teaching exercise

e.g. behavioural changes such as increased grooming, vocalization or postural changes, or physical abnormalities such as anorexia, dehydration, diarrhoea, etc.

The stressed rats may experience some weight loss. Post-surgery complications might include: 1. Edema and blood loss from brain surgery (cannula/ infusion). 2. Infection from wound of surgery site, due to inflammation or reopening of suture/ wound during recovery.

9d.

In terms of species-specific behavioural changes and physiological signs, list the criteria that will be used to trigger the decision to remove an animal from the teaching exercise or experiment, or to terminate the teaching exercise or experiment.

If necessary, consult the Attending Veterinarian for further advice.

Any animal showing weight loss of more than 20% during a one-week period will be excluded. We have not observed this occurrence in our prior work.

10c. Humane Endpoints

Select all the humane endpoints that apply:
Weight loss (more than 20% of bodyweight over 1 week OR no more than 10% over 24 hrs)
Clinical symptoms (e.g. lameness, ruffled fur/coat, dyspnea, vomiting, edema, discharges)
Wound infection/dehiscence(breakdown)
Severe bleeding

10d. Animal monitoring

Indicate frequency of observation and person(s) in charge:
Once a day for 10 days after surgery. At least two times a week for all other times. Shruti Suresh will be monitoring the animals.

10e. Treatment of animals at humane endpoint

State how animals that have arrived at humane endpoints will be treated or will they always be euthanized?
Animals at humane endpoints will be euthanized. This is because reaching this endpoint is rare and abnormal occurrence in context of this study.

10f. Other comments

(11) Animal Disposal

11a. Indicate how carcasses are to be disposed of after completion of the project/research.

Euthanasia [select preferred technique(s)]:
Decapitation
Animals will be deeply anesthetized using gaseous isoflurane. Then a rapid decapitation will be performed using rodent guillotine. The procedure will be conducted in designated procedures rooms in animal facilities at NTU campus and LKC Novena campus.
Others
Transcardial perfusion. The animals must be sacrificed in order to harvest tissues, like brain, liver, heart, nasal epithelium and testes for histology. This will be done under anesthesia with ketamine (75mg/kg) and xylazine (10mg/kg) cocktail to be administered intraperitoneally and an overdose of gaseous isoflurane (inhalation) if needed. Trans-cardial perfusion involves replacing whole blood from the body and injecting paraformaldehyde (PFA) from left ventricle with the right atrium cut so the blood does not circulate and is washed out. First an incision will be made from the neck down the chest cavity to gain access to the heart. Then the left and right diaphragms are cut so the lungs shrink to better expose the heart. The butterfly needle is inserted into left ventricle and the right atrium is cut. With the butterfly needle, first ~100ml PBS (pH7.4) and ~500ml 4% PFA in PBS are perfused. Finally the desired tissue is isolated and incubated in 4% PFA.

11b. Please specify the method of carcass disposal. (Include method of disposing contaminated organs/tissues):

We will place the carcass in two layers of plastic bag. We will label the bag with protocol number. Bags will be sealed and placed in designated containers. Animal carcasses must be autoclaved and then incinerated.

(12) Health & Safety

1) Potentially Hazardous Materials

12a. Biological Project Number (BPN)

Does this study have BPN approval?

Please contact the IBC Secretariat at IBC@ntu.edu.sg for queries related to Biological Project Number.

Not Applicable

12b. Genetically Modified Organisms (GMOs)

(i) Is Recombinant DNA or transgenic animal used?

GMAC approval must be obtained and attached to this section tab.

No

(ii) Is Lentiviral vector used?

Use of 3rd generation and higher Lentivirus vector does not require MOH approval.
Please select ABSL2 for biosafety level.
Please note that a maximum of 100ml of Lentivirus culture may be brought to NTU and that there should be no storage within the facility.
No

12c. Radioactive Substancess

(i) Are radioactive substances used?
No

Not applicable

D. Chemical Substances and New Chemical Entities

12d. Chemical Substances and New Chemical Entities

(i) Are chemical substances used?
Appropriate Safety Data Sheet (SDS) for the chemical(s) used must be included as well as SOP on waste management.
A copy of NTU's Cytotoxic and ABSL2 Waste Handling SOP can be requested from NTU-LKCMedicine ARF safety officer for this AUP's waste management consideration.

Note: ONLY WORKING VOLUME MAY BE BROUGHT INTO THE ANIMAL FACILITY. NO STORAGE OF CHEMICAL IS PERMITTED WITHIN THE FACILITY.
No

(ii) Are new Chemical Entity(ies) {NCE}/pharmaceutical substance(s) used
No

iii. Applicable ONLY if chemicals classified under the following categories are introduced or used in this AUP:

12e. Human Derived Materials

New restrictions are in place with regards to the use of human derived materials under the Human Biomedical Research Act. ***All use of human material in animals will require you to file for IRB approval. IRB approval letter must be included*** *in the AUP application or amendment. Restricted human biomedical research is defined in the Fourth Schedule of HBRA and these include:*

1. Human biomedical research involving human eggs or human embryos
2. Human biomedical research involving -
(a) the following types of human-animal combination embryos:
(i) cytoplasmic hybrid embryos;
(ii) human-animal combination embryos created by the incorporation of human stem cells (including induced pluripotent stem cells);
(iii) human-animal combination embryos created in-vitro by using —
(A) human gametes and animal gametes; or
(B) one human pronucleus and one animal pronucleus;
(b) the introduction of human stem cells (including induced pluripotent stem cells) into a prenatal animal foetus or animal embryo;
(c) the introduction of human pluripotent stem cells (including induced pluripotent stem cells) into a living postnatal animal;
(d) the introduction of human stem cells (including induced pluripotent stem cells) or human neural cells into the brain of a living postnatal animal; or
(e) any entity created as a result of any process referred to in sub paragraphs (b), (c) and (d)

For human-derived materials requiring IRB approval, please contact the Secretariat of the NTU Institutional Review Board (IRB) at (65) 65922495 or email at irb@ntu.edu.sg. The IRB approval letter obtained should include the use of human cells in animals.

Research involving the **use of human-derived commercial cell lines** (excluding embryonic and other stem cells) will **not** require NTU-IRB approval for exemption.

HBRA Regulatory Framework

FAQs on HBRA

(i) Are Materials Defined as per Items 2a-2e Used?

12f. Non Human Derived Materials

Are Other Biologically-Derived Materials Used?
No

G. Biological Agent and/or Toxins(s)

12g. Biological agent and/or toxins

(i) Are Biological Agent(s) or Toxin(s) Used?
No

12h. Other Inorganic or Organic Materials Used

Are Other Inorganic or Organic Materials Used?

e.g. microchip implantation, gel matrix implantation, etc.

No

12i. Overall Animal Biosafety Level

Overall Animal Biosafety Level and Relevant Chemical Safety Classification

*Only applicable if **GMOs** / **human derived materials** / **biological agents** / **toxins** are introduced or used in this AUP.*

ABSL2

12.2. Staff Health

Potential hazard to humans:
None

Describe potential health risks to animals or humans

Specify any special animal care required because of the hazard(s) involved, precautions to be taken by personnel and any special containment requirements (i.e. storage, waste/disposal requirements, etc).

The only available infection route from the animal material is through carnivory. Soiled cages used to hold animals must be autoclaved before cleaning. All personnel working with animals must use PPE that includes lab coats, gloves, hairnets and face masks. All personnel involved in sacrifice and surgery must also use eye protection.

Have you attached a Risk Assessment? (RA)

Access the Workplace Risk Assessment System (WRAS) to complete a RA.

Yes

12.3. Declaration of Biological material / animal products for use in animals (e.g. cell lines, antiserum, etc.).

To prevent the inadvertent infection of research animals and NTU colonies, declare the origin and sterility status of any biological materials/animal products you intend to use in this AUP:

Nature of Material (e.g. cell lines, antiserum, etc.)	**Animal Source**	**Supplied Sterile?** (Yes/No)	**Supplied Attenuated?** (Yes/No)
Not applicable			

The listed materials are PCR based or MAP/RAP/HAP/Mycoplasma tested:
*If derived from humans, materials **MUST** be tested for Mycoplasma. Such tests must be performed before this application is submitted. Contact NTU ARF for assistance.*

(13) Signatures

13. Signatures

- *Animals used in this research or teaching project will be cared for in accordance with the principles contained in Guide for the Care and Use of Laboratory Animals (8th Edition)*
- *You have considered alternative procedures that do not involve the use of living animals.*
- *You will use the minimum number of animals consistent with objectives of described research/teaching program.*
- *You have carefully selected the species that you propose to use.*
- *You will use techniques and facilities that are in accordance with the Guide for the Care and Use of Laboratory Animals (8th Edition)*
- *You will notify the IACUC of any revisions to this AUP.*
- *You will keep copies of all approved AUPs, revisions and amendments in an accessible file.*
- *This project has been reviewed for scientific merit.*
- *The consultant Attending Veterinarian has been consulted prior to AUP submission / consultation not considered necessary.*
- *Animals housed in other Satellite Rooms. Animals from external sources need to be quarantined or housed according to the revised guidelines on animal quarantine.*
- *Animals will be housed in Animal Facility*
- *Facility management has been consulted and has certified that indicated facility(ies) has(have) the resources to support this work*

Workflow History

File Version	Document Version	Status	Status Comment	Status set by	Status set at	Signature Summary
0.1.0	1.0	Draft		Suresh, Shruti	03-Apr-2019	
0.2.0	2.0	ICCO Administrative Review		---	17-Apr-2019	
0.3.0	3.0	For Revision (ICCO)	Please refer to Review tab of AUP for comments on changes to be made to this application. Kindly submit the revised application by end of day 24 April. Thank you.	Stanislaws, Anna Marina	22-Apr-2019	
0.4.0	4.0	ICCO Administrative Review		---	26-Apr-2019	
0.5.0	5.0	Designated Member Review	New application for May's review	---	30-Apr-2019	
0.6.0	6.0	ICCO Administrative Review	Status change initiated by IACUC Secretariat. Reviewer to continue discussion with PI on this application.	---	30-Apr-2019	
0.7.0	7.0	For Revision (ICCO)	Please follow up with Dr Fred to clarify changes to be made to this application. Thank you.	Stanislaws, Anna Marina	30-Apr-2019	
0.8.0	8.0	ICCO Administrative Review		---	06-May-2019	
0.9.0	9.0	Full Committee Review	For 10 May IACUC review. Training record and risk	---	08-May-2019	

			assessment are pending.			
0.10.0	10.0	ICCO Administrative Review	For editing the form.	---	09-May-2019	
0.11.0	11.0	For Revision (ICCO)	The changes requested are major so please assist in revising the protocol. Thank you.	Stanislaws, Anna Marina	09-May-2019	
0.12.0	12.0	ICCO Administrative Review		---	09-May-2019	
0.13.0	13.0	Full Committee Review	For review on 10th May. Additional non-surgical procedure has been added and formatting changes have been made.	---	09-May-2019	
0.14.0	14.0	ICCO Administrative Review	Changes requested.	---	10-May-2019	
0.15.0	15.0	For Revision (ICCO)	Changes to be made following IACUC review: - Use ket/xyl cocktail for induction for both intracranial operation and transcardial perfusion - Check space availability in animal facility and stagger animal use as required.	Stanislaws, Anna Marina	10-May-2019	
0.16.0	16.0	ICCO Administrative Review		---	14-May-2019	
0.17.0	17.0	For Revision (ICCO)	For revision of ket/xyl dosage.	Stanislaws, Anna Marina	15-May-2019	
0.18.0	18.0	ICCO Administrative Review	please review	---	12-Jun-2019	
0.19.0	19.0	Designated Member Review	Hi Dr Fred, please compare against document version 14. Changes to be made following IACUC review were: 1) Use ket/xyl cocktail for induction for both intracranial operation and transcardial perfusion 2) Revise number	---	12-Jun-2019	

			of animals housed at any one time according to facility space availability. Thank you.			
0.20.0	20.0	ICCO Administrative Review	Document reviewed offline by Dr Fred who has cleared document for approval. Status change initiated by IACUC Secretariat.	---	14-Jun-2019	
0.21.0	21.0	Designated Member Review	Dear Prof Ruedl, please compare against document version 14. The changes to be made following IACUC review were: - Use ket/xyl cocktail for induction for both intracranial operation and transcardial perfusion - Check space availability in animal facility and stagger animal use as required. ARF has shared that maximum 30 cages can be housed in RSB and the PI proposes staggering 20-30 cages at any one time. Dr Fred has reviewed the addition of ket/xyl mixture and recommended it for approval. Thank you.	---	14-Jun-2019	
0.22.0	22.0	ICCO Administrative Review		---	14-Jun-2019	
0.23.0	23.0	For Signature	For your signature please.	Lai, Chunying	18-Jun-2019	Ruedl, Christiane - 18-Jun-2019 11:26:50 AM
0.24.0	24.0	ICCO Administrative Review	Automatic status change triggered by tick@lab.	---	18-Jun-2019	
1.0.0	25.0	Approved	New protocol approval from 18 June 2019 to 18 June 2022	Lai, Chunying	18-Jun-2019	

1.1.0	26.0	Amendment		Suresh, Shruti	27-Jul-2020	
1.2.0	27.0	ICCO Administrative Review		---	30-Jul-2020	
1.3.0	28.0	For Revision (ICCO)	Please refer to the comment in the review tab for change to be made. Thank you.	Tan, Ying Shi	30-Jul-2020	
1.4.0	29.0	Withdrawn		Suresh, Shruti	30-Jul-2020	
1.5.0	30.0	Amendment		Suresh, Shruti	03-Aug-2020	
1.6.0	31.0	ICCO Administrative Review		---	05-Aug-2020	
2.0.0	32.0	Approved	Approval of addition of personnel and removal of co-I.	Tan, Ying Shi	06-Aug-2020	
2.1.0	33.0	Amendment		Suresh, Shruti	19-Aug-2020	
2.2.0	34.0	ICCO Administrative Review		---	21-Aug-2020	
2.3.0	35.0	Designated Member Review	emailed to Dr Fred on 25 Aug 2020.	---	26-Aug-2020	
2.4.0	36.0	ICCO Administrative Review	Returned to PI to revise the change in animal numbers	---	28-Aug-2020	
2.5.0	37.0	For Revision (ICCO)	Please bring down the increase in animal numbers to less than 20% of the current approved animal numbers, which is less than 180 mice to qualify as a minor amendment. Thanks.	Tan, Ying Shi	28-Aug-2020	