ANIMAL COMPONENT OF RESEARCH PROTOCOL (ACORP)

Main Body

VERSION 4 v2 6-17-2015

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

A. **ACORP Status.**

1. Full Name of Principal Investigator(s)► [redacted] **Ph.D.**
2. VA Station Name (City) and 3-Digit Station Number► **VA Greater Los Angeles 691**
3. Protocol Title► **Resolution of the Mechanisms Responsible for Atonia during REM Sleep**
4. Animal Species covered by this ACORP► **Cat**
5. Funding Source(s). Check each source that applies:

   ►( ) Department of Veterans Affairs.
   ►(x) US Public Health Service (e.g. NIH).
   ►( ) Private or Charitable Foundation -- Identify the Foundation:
   ►( ) University Intramural Funds – Identify the University and Funding Component:
   ►( ) Private Company – Identify the Company:
   ►( ) Other – Identify Other Source(s):

6. Related Documentation for IACUC reference.

   a. If this protocol applies to a project that has already been submitted to the R&D Committee for review: ► **NO ( ) -- GO TO Item #7**
   Else, identify the project:
   (1) Title of project► **Resolution of the Mechanisms Responsible for Atonia during REM sleep**
   (2) If approved by the R&D Committee, give the date of approval► **May 27, 2015**

NOTE TO REVIEWERS: Part 6b differs from version 3 ACORP. This is basically a progress report. Reviewers should consider if this project has been inactive and flag that for discussion by committee.

   b. Triennial review. If this protocol is being submitted for triennial *de novo* review, complete the following:
   (1) Identify the studies described in the previously approved ACORP that have already been completed
   ► **During the last triennial (May 27, 2015 to May 27, 2017), we conducted electrophysiological experiments for the approved studies. Over this period, we have achieved the following objectives:**

In reviewing our intracellular data of hypoglossal motoneuron's firing behavior during naturally-occurring REM sleep in the chronically implanted cat, we found electrophysiological evidence (membrane hyperpolarization, increased rheobasic

current, chloride-dependent IPSP synaptic potentials, and cessation of spontaneous firing) that demonstrates that postsynaptic inhibition was crucial for the hypoglossal motoneuron suppression/atonia of the tongue muscles under normal REM sleep condition. The literature of extracellular studies in rats, however, suggested that a withdrawal of excitatory input from monoamine (norepinephrine and serotonin) premotor interneurons could result in REM sleep atonia of the hypoglossal motoneuron-genioglossus muscle. It was important to note that these latter studies include experimental procedures such as vagotomy in order to raise the baseline monoaminergic drives to the hypoglossal system prior to REM sleep state. An elevated excitatory drive to the hypoglossal system also occurred during hypoxia. Consequently, disfacilitation could occur when the monoaminergic inputs cease at the onset of REM sleep. In view of these findings, we hypothesized that, in the chronic cat model, under hypoxic REM sleep condition, both disfacilitation as well as postsynaptic inhibition could operate together to bring about the atonia of the genioglossus muscles.

To test this hypothesis, we developed successfully a new technique applicable for chronic cat intracellular studies to evaluate the significance of postsynaptic inhibitory versus disfacilitatory mechanisms towards the hypoglossal atonia during normoxic versus hypoxic REM sleep conditions. Specifically, a breathing mask designed for cats was positioned in close proximity to the animal's nose and mouth. The animal was subjected to breathing under a hypoxic condition by replacing the normal air supply (21% oxygen in air; oxygen saturation in blood or SpO2 = 100%) with one that contains a lower oxygen level (10% oxygen in air; oxygen saturation in blood or SpO2 = 75%). The SpO2 levels were continuously monitored using the SurgiVet Pulse oximeter with the sensor attached to the ear of the animal. The hypoxic condition last for approximately 10-15 minutes during REM sleep and normal air was re-introduced to the animal once the animal awoke. In addition, the animal, after adaptation, exhibited spontaneous periods of REM sleep and non-REM sleep under hypoxic conditions with no change in sleep pattern. The present study demonstrates the feasibility of studying the effects of hypoxia in chronic unanesthetized cats that exhibit naturally-occurring states of sleep and wakefulness. We believe that this in vivo animal model of hypoxia, combined with intracellular recording techniques, is a novel way to study the synaptic mechanisms and neurotransmitters that control the hypoglossal motoneuron activity during REM sleep under normal as well as pathological (hypoxic) conditions, such as OSA.

1.

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

► **One**

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

► **In this triennial renewal, we eliminated several less important neurotransmitter candidates (acetylcholine, GABA, and glutamate) in order to free up time and effort so we can concentrate on more detail delineation of three high-priority neurotransmitters that control the postsynaptic inhibition (glycine) and disfacilitation (norepinephrine, serotonin) processes that result in REM sleep-related hypoglossal motoneuron-genioglossus muscle atonia. With this change, we reduce the usage of cats (down to 2**

per year). This reduction of drugs studies will also provide us a more realistic timeline in achieving our main goal in resolving the neurotransmitter mechanisms vis-à-vis REM sleep atonia under normal as compared to hypoxic condition.

c. List any other relevant previously approved animal use protocols (copy the lines below as needed for each protocol listed).
(NOTE TO REVIEWERS: This is section would only be filled out under specific circumstances. This section should normally be left blank)
(1) Title of other protocol ►
(2) IACUC approval number of other protocol ►
Give the name of the VA station or other institution that approved it, if it was not approved by the IACUC that will review this ACORP ►

7. Indicate the type(s) of animal use covered by this protocol (check all that apply):
►(x) Research
►( ) Teaching or Training
►( ) Testing
►( ) Breeding and colony management only; not for any specific research project
►( ) Holding protocol (as specified by local requirements; not required by VA, PHS, or USDA)
►( ) Other. Please specify►

Proposal Overview

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

(NOTE TO REVIEWERS: Please check that benefits to the veteran population is specifically addressed in this section)

► In the central nervous system, special neurons control how open the throat is for airflow to the lungs. During sleep these neurons become less active and the throat becomes less open. In about 5% of the population, the throat becomes so narrow that sometimes it closes during sleep. This condition is known as Obstructive Sleep Apnea or OSA. In some people this happens many times over the course of the night. Each time they stop breathing the oxygen level falls, causing a condition called hypoxia (low oxygen). This hypoxia can cause brain cells to die and may play a role in conditions such as Parkinson's Disease and Alzheimer's disease. Veterans are four times more likely than other Americans to suffer from sleep apnea, with about 20% of veterans affected (Max Hirshkowitz, director of the Sleep Disorder Center at the Houston Veterans Affairs Medical Center, http://usatoday30.usatoday.com/news/health/2010-06-07-apnea_N.htm).

The results of this research will help us develop better treatment for OSA by helping us learn how these neurons normally function. In particular, our results may point to drugs that can make these neurons more active during sleep so they will keep the throat open and prevent OSA.

The proposed research cannot be done in a human clinical study for a number of reasons. First, the experiments involve surgical procedures, including the permanent placement of

electrodes inside the brain. In addition, we need to obtain brain tissue to examine under the microscope after the sleep study is completed. Therefore, this research cannot be carried out as part of a human clinical study. It is also not possible to use human clinical pathology specimens because we first need to collect data from living cells, which is not possible with this kind of specimen.

The cat is the most appropriate animal for the proposed studies because the cat has relatively long periods of REM sleep compared with those in the mouse, rat or guinea pig; therefore, when cells are penetrated, they can be studied for relatively long periods of time during this state in this species. The duration of REM sleep episodes in rats and mice is extremely short, especially compared with the cat (Lydic et al., 1987; Sterman et al., 1965; Vivaldi et. al., 1994; Fenik et al., 2013; Xi et al., 2001a, 2002). Cats are the lowest animal that can be successfully used for this study.

C. **Experimental Design.**

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

► **There are three kinds of experiments.**

Experiment 1: Under surgical anesthesia and sterile techniques, electrodes will be implanted to monitor both activity in the neurons controlling the throat muscles and also electrodes to monitor for rapid-eye-movement (REM) sleep and neck muscle activity. Animals will be given post-operative pain-killers for 3 days and then have a one-two week recovery from surgery. We will spend a lot of time handling and playing with these animals so they are very tame and comfortable in our laboratory. Three days a week for four to eight weeks we will bring the animals to the laboratory where they will fall asleep naturally. We will record their sleep for 4 hrs and then feed them Vital Essentials Cat Freeze- Dried Chicken Breast treats and play with them for a while before returning them to their home cages. During the sleep recording, we will occasionally administer tiny amounts of drugs directly to the neurons we are studying to see if they make the neurons more or less active.

Experiment 2: Using the identical procedures as described in Experiment 1 above, and when the animal goes to REM sleep (that lasts for 15 minutes), it will be subjected to the experimental (hypoxic) condition by breathing air that contains a lower oxygen level than the normal air (as in Experiment 1). Upon awaking, the air supply will be switched back to normal oxygen content. The recording sessions will be the same as in Experiment 1, i.e., 4 hrs per day, three days per week for four to eight weeks. After the recording session, the animal will be given Vital Essentials Cat freeze-dried chicken breast treats and its relaxation and play-time before we return them to their home cages. During the sleep recording, we will occasionally administer tiny amounts of drugs directly to the neurons we are studying to see if they make the neurons more or less active.

Experiment 3: The animals will be anesthetized for five hours during which we will give them a drug that reproduces REM sleep for up to an hour and try to record from as many neurons as possible. We will also administer tiny amounts of drug to the neurons just like the Experiment 1. The point of this experiment is since the animal is anesthetized and holding perfectly still the whole time, we can study far more neurons at once than we can in Experiment 1, where REM sleep lasts only up to 15 minutes. However, we still need Experiment 1 in order to study truly natural sleep.

Once the experiments are done, the animals will be painlessly euthanized with the same drug veterinarians use, and we will collect their brains and examine them under the microscope.

2. **Complete description of the proposed use of animals. (NOTE TO REVIEWERS: This section is similar but not exact to ACORP Version 3. Please note the new layout).**

Use the following outline to detail the proposed use of animals.

a. **Summarize** the design of the experiment in terms of the specific groups of animals to be studied.

▶ Our overall intent is to understand the control of cells (neurons) in the brainstem during REM sleep and wakefulness and the mechanisms that are utilized to control motor activity during those states. For this purpose, we will use *in vivo* preparations in the cat in conjunction with coordinated intracellular, iontophoretic, electrophysiological, pharmacological, and immunocytochemical investigations combined with microscopic analyses. Chronic cat preparations (survival surgery) and acute cat preparations (non-survival surgery) will be employed. We are utilizing the cat since it is a species in which it is possible to conduct the preceding and simultaneously record the intracellular activity of hypoglossal motoneuron studies that involve an animal that is awake and asleep. It is not possible to utilize other animals, such as the mouse or rat, because the duration of REM sleep episodes in these animals is extremely short in comparison with the cat.

An overview of the experiments to be performed and individual anticipated results post-drug are contained in Table I (see below). At the end of each study, as noted in the ACORP Appendix 5, Section 2, the phenotype of cells in the region of recording will be examined with standard histological processes. The data from cells that exhibit similar electrophysiological profiles in closely delineated anatomical sites will be aggregated to determine whether the data are scientifically valid, as has been carried out in numerous previously published studies.

TABLE I

Chronic Preparation: Naturally Occurring Sleep and Wakefulness[1]						
Areas Recorded	*Type of Recording*	*Data Recorded*	*Behavioral State*	*Test Substances Injected onto Hypoglossal Neurons*	*Anticipated Results*	*Importance of Data Obtained*
Hypoglossal nucleus, hypoglossal nerve, genioglossal muscle	Intracellular and Extracellular	Membrane potentials and membrane properties (e.g, afterhyperpolarization, AHP) from hypoglossal motoneurons. Compound nerve action potentials from hypoglossal nerve. EMG from genioglossal muscle.	Wakefulness, NREM Sleep,and REM Sleep under normoxic and hypoxic conditions	Glycine (Gly) antagonist (strychnine).	Membrane hyperpolarization during REM sleep will be reduced by blocking Gly. AHP will be unchanged. Increase in activities (amplitude) in hypoglossal nerve and genioglossal muscle.	Depending on the changes of the AHP and membrane potential, the mechanisms of action of the test substances can be deduced, which indicates whether postsynaptic or disfacilitation mechanisms control the REM sleep atonia of hypoglossal motoneurons.
Same as above	Same as above	Same as above	Same as above	Norepinephrine (NE) antagonist (prazocin); serotonin (5HT) antagonist (methysergide).	Membrane hyperpolarization during REM sleep will increase. Decrease in the AHP and in activities (amplitude) in hypoglossal nerve and genioglossal muscle.	Same as above

Acute Preparation: Carbachol-induced REM Sleep[2]						
Areas Recorded	*Type of Recording*	*Data Recorded*	*Behavioral State*	*Test Substances Injected onto hypoglossal motoneurons*	*Anticipated Results*	*Importance of Data Obtained*
Hypoglossal nucleus, hypoglossal nerve, genioglossal muscle	Intracellular and Extracellular	Membrane potentials and membrane properties (e.g, afterhyperpolarization, AHP) from hypoglossal motoneurons. Compound nerve action potentials from hypoglossal nerve. EMG from genioglossal muscle.	AS-Carbachol (REM Sleep). The animals are anesthesitized and artificially ventilated. Hypoxia (75% SpO2) is induced by ventilating the cat with low oxygen air supply. This is maintained for 10 to 15 minutes prior to normoxic ventilation.	Gly antagonist (strychnine).	Membrane hyperpolarization during REM sleep will be reduced by blocking Gly. AHP will be unchanged. Increase in activities (amplitude) in hypoglossal nerve and genioglossal muscle.	Depending on the changes of the AHP and membrane potential, the mechanisms of action of the test substances can be deduced, which indicates whether postsynaptic or disfacilitation mechanisms control the AS-carbachol-induced REM sleep atonia of hypoglossal motoneurons.
Same as above	Same as above	Same as above	Same as above	NE antagonist (prazocin); 5HT antagonist (methysergide).	Membrane hyperpolarization during REM sleep will increase. Decrease in the AHP and in activities (amplitude) in hypoglossal nerve and genioglossal muscle.	Same as above

[1]The development of the chronic preparation for recording during naturally occurring states of sleep and wakefulness is described in the ACORP in Appendix 5, Section 2. At the end of the experiments, animals are euthanized as described in the ACORP in Section U and Appendix 5, Section 2.

[2]The AS-carbachol preparation is one in which REM sleep is induced by injecting carbachol into the brainstem. Additional data are presented in the ACORP in Appendix 5, Section 2. At the end of the experiment, each animal is euthanized as described in the ACORP in Section U and Appendix 5, Section 2.

The chronic preparation will allow us to obtain data during natural sleep behaviors. Each chronic cat will be prepared using aseptic surgical techniques while the cat is anesthetized with Isoflurane. Stainless steel recording electrodes will be attached to the skull and embedded in sterile acrylic resin. A small hole will be made in the calvarium so that microelectrode recordings and injections can be carried out. The hole is sealed and protected between recording sessions by a stainless steel recording chamber fitted with a screw cap. Recovery from surgery will be monitored, as described in Appendix 5.

The experiment consists of intracellular recordings from identified hypoglossal motoneurons during normal and pharmacologically (carbachol)-induced REM sleep, as well as NREM sleep and wakefulness. In addition, we will modify the activity of cells by injecting small amounts of neurotransmitter antagonists onto cells from which we are recording. The chronic cat will be head-restrained during recording sessions, but the animal must not be in any physical discomfort for the experiment either to be of value and/or be relevant to the normal functioning of the nervous system during naturally occuring states of sleep and wakefulness.

Cats will also be used for acute studies (non-survival surgery), which will enable us to obtain statistically significant results in less time using fewer animals. The electrophysiological measurements made in the acute preparation are the same as those in the chronic preparation except that the cat will be anesthetized throughout the entire study session (and euthanized at the end of the experiment).

Cats are the optimal species that can be utilized when the objective is to record intracellularly during states of sleep and wakefulness and compare these data with the wealth of existing information obtained in the anesthetized condition. It is not possible to utilize other animals, such as the mouse or rat, because the duration of REM sleep episodes in these animals is extremely short in comparison with the cat. In addition, the size of the rodent's brain is too small to simultaneously monitor the activity of multiple sites.

The following experimental procedures and paradigms are employed in all studies (chronic in vivo, acute in vivo). The principal differences are in the behavioral states wherein the data are obtained. Where pertinent, additional distinctions are noted.

All studies are designed to determine the effects of those key neurotransmitters glycine (Gly), norepinephrine (NE), and serotonin (5HT) (via blocking their receptors with corresponding antagonists), on the electrical activity and electrophysiological properties of hypoglossal motoneurons during different behavioral states, e.g., sleep, wakefulness, anesthesia, and hypoxia. For each study, intracellular and extracellular records are obtained from hypoglossal motoneurons prior to, during, and following the injection of antagonists next to cells. Each experiment and the location wherein it will be conducted, are indicated below.

A. Survival (chronic) normoxic studies [redacted] Building [redacted], Room [redacted] and Building [redacted], Room [redacted])

1) Cats are prepared for chronic recordings according to procedures that are described, in detail, in Morales and Chase,1981 (Brain Res 225:279-95), Soja et al.,1991 (J Neurosci 9:2804-11), Fung and Chase 2015 (Sleep 38:139-45).
2) After a 2-week period, the animals are placed in a recording apparatus in order to adapt them to the experimental conditions.
3) The adaptation period lasts 1-2 months, and is completed when animals exhibit multiple spontaneous cycles of sleep and wakefulness during each recording sessions (3 days per week).
4) Once adaptation is achieved, chronically-implanted animals are recorded on alternate days that last up to four hours.
5) Intracellular recordings are obtained from hypoglossal motoneurons during naturally-occurring states of sleep and wakefulness.
6) Determinations of the following variables are obtained prior to and following the injection next to cells of various neurotransmitter antagonists (see Appendix 3: Biosafety):

Resting Membrane Potential	Input Resistance
Spike Amplitude and Duration	Rheobase
AHP Amplitude, Duration and Half-width	Discharge Rate and Synaptic Activity

7) Polygraphic, extracellular hypoglossal nerve and genioglossal EMG records are obtained in parallel with recordings from hypoglossal motoneurons.
8) At the completion of the experiment, the animal is euthanised and perfused intracardially with 10% formalin. The brain is removed for histological analysis.

B. Survival (chronic) hypoxic studies ([redacted] Building [redacted], Room [redacted] and Building [redacted], Room [redacted])

1) Cats are prepared for chronic recordings according to procedures that are described, in detail, in Morales and Chase,1981 (Brain Res 225:279-95), Soja et al.,1991 (J Neurosci 9:2804-11), Fung and Chase 2015 (Sleep 38:139-45).

2) After a 2-week period, the animals are placed in a recording apparatus in order to adapt them to the experimental conditions.
3) A breathing mask designed for cats will be positioned in close proximity to the animal's nose and mouth. During REM sleep, the animal will be subjected to breathing under a hypoxic condition by replacing the normal air supply (21% oxygen in air; oxygen saturation in blood or SpO_2 = 100%) with one that contains a lower oxygen level (10% oxygen in air; oxygen saturation in blood or SpO_2 = 75%). The episodes of hypoxia will lasts approximately 10-15 min before normal air will be re-introduced to the animal.
4) The adaptation period lasts 1-2 months, and is completed when animals exhibit multiple spontaneous cycles of sleep and wakefulness during individual recording sessions (3 days per week).
5) After adaptation, chronically-implanted animals are recorded on alternate days that last four hours.
6) Intracellular recordings are obtained from hypoglossal motoneurons during naturally-occurring states of sleep and wakefulness.
7) Determinations of the following variables are obtained prior to and following the injection next to cells of various neurotransmitter antagonists (see Appendix 3: Biosafety):

Resting Membrane Potential	Input Resistance
Spike Amplitude and Duration	Rheobase
AHP Amplitude, Duration and Half-width	Discharge Rate and Synaptic Activity

8) Polygraphic, extracellular hypoglossal nerve and genioglossal EMG records are obtained in parallel with recordings from hypoglossal motoneurons.
9) At the completion of the experiment, the animal is euthanised and perfused intracardially with 10% formalin. The brain is removed for histological analysis.

C. Non-survival (acute) studies [redacted] Building [redacted], Room [redacted] and Building [redacted], Room [redacted])

1) Cats are anesthetized.
2) Intracellular recordings are obtained from hypoglossal motoneurons during anesthesia, and during carbachol-induced REM sleep in conjunction with applications of various neurotransmitter antagonists (see Appendix 3: Biosafety).
3) Polygraphic, extracellular hypoglossal nerve and genioglossal EMG records are obtained in parallel with intracellular recordings from neurons.
4) Determinations of the following variables are obtained prior to and following the injection next to cells of various substances:

Resting Membrane Potential	Input Resistance
Spike Amplitude and Duration	Rheobase
AHP Amplitude, Duration and Half-width	Discharge Rate, Synaptic Activity

5) At the completion of the experiment, the animal is euthanised and perfused intracardially with 10% formalin. The brain is removed for histological analysis.

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

▶ The basic plan for the statistical analysis of data is a 2x2 ANOVA design. Factor A has two levels (control and experimental) while factor B has two levels (control and the effect of a single drug). This basic design will be used to examine the effect of several drugs. This design achieves 80% power for an overall F-test at a 5% significance level when at least one mean is different by 0.5 SD. Since more than one neuron per animal will be studied, a repeated measures ANOVA may be required which will include a random effect for animal. Depending on the within-animal correlation, the power may be somewhat lower than 80%.

c. **Describe each procedure** to be performed on any animal on this protocol. (Use Appendix 9 to document any of these procedures that involve "departures" from the standards in the *Guide*. Consult the IACUC or the Attending Veterinarian for help in determining whether any "departures" are involved.) Describe each procedure in a few words or just one sentence, and then write "see appendix X for details". e.g. stereotaxic surgery – see appendix 5 for details, Morris water maze – see appendix 6 for details.

(NOTE TO REVIEWERS: This section is a significant change in the previous ACORP. Note that *departures from the Guide* are to be listed here and in appendix 9. This section will likely require significantly more attention from the VMO to identify departures from the GUIDE.

► Procedure 1 is multiple survival surgery for chronic experiment (see Appendix 5 for details). Procedure 2 is acute electrophysiological study surgery (see Appendix 5 for surgical details).
Procedure 3 is sleep recording +/- hypoxia for chronic animals (see Appendix 6 for details).
Procedure 4 is euthanasia, as a nonsurvival surgery (see Appendix 5 for details).
Procedure 5 is acute electrophysiological recording (see Appendix 6 for details).
Significance of histology: Microscopical examination of the brain sections can reveal the gliosis/scar formation that indicates the placement of microelectrode tracks. Locations of the glial scar can be correlated to the stereotaxic coordinates of the intracellular recording sites within the hypoglossal nucleus. This histological evidence adds credence to the fact that the intracellular data are recorded from individual hypoglossal motoneurons that are electrophysiologically identified by antidromic stimulation of the ipsilateral hypoglossal nerve.

D. **Species.** Justify the choice of species for this protocol.

► The cat is the most appropriate animal for the proposed studies because the objective is to record intracellularly during naturally-occurring states of sleep and wakefulness. The cat has relatively long periods of REM sleep compared with those in the mouse, rat or guinea pig; therefore, when cells are penetrated, they can be studied for relatively long periods of time during this state in this species. The duration of REM sleep episodes in rats and mice is extremely short, especially compared with the cat (Lydic et al., 1987; Sterman et al., 1965; Vivaldi et. al., 1994; Fenik et al., 2013; Xi et al., 2001a, 2002). To the best of our knowledge, there have been no published accounts of intracellular recordings from hypoglossal or other motoneurons during naturally-occurring REM sleep in the chronic rat or mouse. In addition, the implantation device for chronic recording in the unanesthetized animal can be bonded more securely to the calvarium of the cat than of other commonly used experimental species. Finally, the gold standard for animal studies is data obtained during spontaneously-occurring states of sleep and wakefulness, since these directly inform us about the natural functioning of the nervous system. The cat is the only species that anyone has been able to do this kind of work in.

Personnel

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

1. PI
Name► [redacted] PhD.
Animal research experience ► Dr. [redacted] has been conducting animal research for over 35 years and has dealt with all aspects of the experiments that are described in this protocol.

Qualifications to perform specific procedures

Specific procedure(s) that the PI will perform personally	Experience with each procedure in the species described in this ACORP
Oversee experimental design, data analysis and manuscript preparation.	Dr. [redacted] will oversee the planning, development and execution of research at the VMU GLAHS. [redacted] extensive experience in studies utilizing the cat as an animal model make [redacted] an ideal candidate for determining the proper scientific approach and technique.
Chronic cat surgeries	Dr. [redacted] has performed chronic cat surgeries since 1980 and published 9 original research articles (from 1982 to present).
Acute cat surgeries	Dr. [redacted] has published 45 papers on acute cat studies since 1975, with 5 papers specifically dealing with carbachol-induced REM sleep in cats which is the same technique to be used in this current triennial study.
Intracellular studies in chronic (conscious but head-restrained) cats	Dr. [redacted] has performed these electrophysiological studies in chronic cats since his postdoctoral training in 1980. [redacted] has published 5 original papers in this area, including the 2015 paper entitled "Postsynaptic inhibition of hypoglossal motoneurons produces atonia of the genioglossal muscle during rapid eye movement sleep" ([redacted] and [redacted], *Sleep* 38:139-146).
Euthanasia of cats	Dr [redacted] has performed the euthanasia procedure on cats since 1975.

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

(NOTE TO REVIEWERS: Each person on the ACORP should be discussed here. Qualifications should likely be described in general terms—[euthanasia, surgery, feeding, etc, rather than specifics, but the IACUC committee should decide level of experience. Likely, more complex procedures/techniques will require more specific descriptions.)

Name▶ [redacted], PhD
Animal research experience ▶ Dr [redacted] has been conducting animal research for over 30 years. [redacted] is an expert in carrying out experiments pertaining to this protocol.

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this ACORP
Chronic and acute cat surgeries, data handling, and research publication	Dr. [redacted] has performing both survival and non-survival surgeries and running experiments pertaining to this protocol. [redacted] is the first author on numerous articles in peer-reviewed publications that deal with findings in the acute and chronic cat preparations.
Electrophysiological recording from anesthetized, awake and sleeping cats	Dr. [redacted] has performed these kinds of recordings in cats for over 20 years.
Euthanizing the cats	Dr. [redacted] has performed this euthanasia procedure on cats for over 20 yrs.

Name▶ [redacted], PhD

Animal research experience ▶ Dr. [redacted] has been conducting animal research for over 20 years. [redacted] has abroad background in biophysics and physiology, with specific training and expertise in the neurobiology of sleep and breathing which are required for the proposed project.

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this ACORP
Microinjection of drugs to hypoglossal nucleus	Dr. [redacted] has performed both survival (in rats) and non-survival surgeries (in both cat and rats) and running experiments pertaining to this protocol. [redacted] has a strong record of productive research studying mechanisms of the state-dependent control of hypoglossal motoneurons that have high relevance with respect to the development of a pharmacological treatment of Obstructive Sleep Apnea. [redacted] also has expertise and experience in examining processes that control hypoglossal motor activity during REM sleep.

Name▶ [redacted] PhD
Animal research experience ▶ Dr. [redacted] has been conducting animal research for over 40 years. [redacted] s an expert in carrying out experiments pertaining to this protocol.

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this ACORP
Reverse dialysis of drugs to hypoglossal nucleus	Dr. [redacted] has extensive experience with this technique over the last 16 years. [redacted] lab is the first to combine reverse dialysis with extracellular unit recording to quantify the responsiveness of neurons during sleep and waking states (Alam et al., 1999).

3. VMU animal care and veterinary support staff personnel (copy the lines below for each individual)
Name▶ **To be determined by VMO (NOTE TO REVIEWERS: This should always be "TBD BY VMO) and will be pre-populated)**

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

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

4. For each of the research personnel listed in items 1 and 2 above, enter the most recent completion date for each course. **(PI must fill it out after consultation with Dr. [redacted], IACUC coordinator)**

Name of Individual	Working with the VA IACUC	ORD web-based species specific course (Identify the species)	Any other training required locally (Identify the training)
[redacted]	7/20/17	7/20/2017 (Cat)	
[redacted]	12/20/17	9/17/2017 (Cat)	
[redacted]	4/7/16	9/26/2017 (Cat)	
[redacted]	9/2/2017	4/6/2016 (Cat)	

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

G. **Occupational Health and Safety.**

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

Name	Enrollment in Occupational Health and Safety Program		Declined optional services	Current on Interactions with OHSP? (yes/no)
	VA program	Equivalent Alternate Program – identify the program		
[redacted]	x			yes
[redacted]	x			yes
[redacted]	x			yes
[redacted]	x			yes

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

► ( ) Yes. Describe them ►

► (x) No.

Animals Requested
(NOTE TO REVIEWERS: This section is basically the same as previous version of the ACORP)

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

Description (include the species and any other special features not shown elsewhere in this table)	Gender	Age/Size on Receipt	Source (e.g., Name of Vendor, Collaborator, or PI of local breeding colony)	Health Status
cat	Male/female	1-3 yrs of age	[redacted] Inc.	Normal/healthy

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

USDA Category B

Procedures▶						
Species / strain	**Year 1**	**Year 2**	**Year 3**	**Year 4**	**Year 5**	**Category B TOTAL**

USDA Category C

Procedures▶						
Species / strain	**Year 1**	**Year 2**	**Year 3**	**Year 4**	**Year 5**	**Category C TOTAL**

USDA Category D

Procedures▶ Surgery/restraint/recording						
Species / strain	**Year 1**	**Year 2**	**Year 3**	**Year 4**	**Year 5**	**Category D TOTAL**
Cat/each cat serves as both predrug control and postdrug treated groups/ survival and non-survival surgeries	**2**	**2**	**2**			**6**

USDA Category E

Procedures▶

Species / strain	Year 1	Year 2	Year 3	Year 4	Year 5	Category E TOTAL

TOTALS over all Categories

Species / strain	Year 1	Year 2	Year 3	Year 4	Year 5	GRAND TOTAL
cat	**2**	**2**	**2**			**6**

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

► ( ) This protocol does NOT include any Category D procedures.

► (x) This protocol INCLUDES Category D procedures. List each Category D procedure and provide the information requested. (For surgical procedures described in Appendix 5, only identify the procedure(s) and enter "See Appendix 5 for details.)

Procedure	Monitoring (indicate the method(s) to be used, and the frequency and duration of monitoring through post-procedure recovery)	Person(s) responsible for the monitoring	Method(s) by which pain or distress will be alleviated during or after the procedure (include the dose, route, and duration of effect of any agents to be administered)

Survival surgery for implanting chronic electrodes (See Appendix 5 for details)	Check every 5 minutes for vital signs (core temperature, heart and breathing rate). We also check for depth of anesthesia by checking changes in heart/ breathing rate and withdrawal reflex in response to toe-pinch	Veterinarian and veterinarian technicians, as well as Dr. [redacted] and Dr. [redacted]	Prior to surgery, these injectable drugs are given once: Xylazine, 2 mg/kg i.m., Ketamine, 8 mg/kg i.m., Carprofen 5 mg/kg, s.c., dexamethasone, 0.5 mg/kg, i.m.; atropine sulfate, 0.04 mg/kg, i.m.; Buprenorphine, 0.02 mg/kg s.c. Anesthetic: Isoflurane, 2-4%, inhalation via tracheal intubation, throughout the surgery. Postoperation analgesic: Buprenorphine, 0.02 mg/kg s.c., every 12 hours, for 3 days; Carprofen 5 mg/kg, s.c., once daily for 3 days; Antibiotic: Baytril 2.27%, 0.11 ml/kg, s.c., once daily for 3 days.
Acute electrophysiological study (See Appendix 5 for details)	Check every 15 minutes for vital signs (core temperature, heart and breathing rate). We also check for depth of anesthesia by checking changesin heart/ breathing rate and withdrawal reflex in response to toe-pinch	Dr. [redacted] and Dr. [redacted]	Anesthetic: Isoflurane, 2-4%, inhalation, throughout surgery; Alpha Chloralose, 120 mg/kg loading, 60 mg/kg maintenance, i.v., throughout the experiment.
Non-survival surgery: euthanasia	Once the cat is euthanized using Fatal-Plus, we will verify death by cessation of breathing and heart beat prior to performing the thoracotomy for intracardiac perfusion.	Dr. [redacted] and Dr. [redacted]	Euthanasia: Pentobarbital, 100mg/kg, i.v.

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

► (x) This protocol does NOT include any Category E procedures

► ( ) This protocol INCLUDES Category E procedures. Identify each Category E procedure included in this ACORP and justify scientifically why the pain or distress cannot be relieved.
►

Veterinary Care and Husbandry

L. **Veterinary Support**.

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

   (NOTE TO REVIEWERS: This section will be prepopulated)
   Dr. [redacted], DVM,
   VMO, GLAHS
   [redacted]@va.gov

2. Veterinary consultation during the planning of this protocol.

   Name of the laboratory animal veterinarian consulted► **Dr. [redacted] DVM**
   Date of the veterinary consultation (meeting date, or date of written comments provided by the veterinarian to the PI) ► **April 6, 2018.**

M. **Husbandry.** As a reference for the animal husbandry staff, summarize here the husbandry requirements of the animals on this protocol. (Use Appendix 6 to justify the use of any special husbandry and to detail its effects on the animals. Use Appendix 9 to document any aspects of the husbandry that involve "departures" from the standards in the *Guide*. Consult the IACUC or the Attending Veterinarian for help in determining whether any "departures" are involved.) **(NOTE TO REVIEWERS: This is a potentially confusing section that is a significant departure from previous ACORPs. Please be aware of non-standard housing and *departures from the GUIDE*. The VMO should provide extra attention to this section. Areas that may be important in this section are housing of breeding pairs, weaning of babies, and housing of animals singly for experimental reasons.)**

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

a. Species	b. Type of housing*		c. Number of individuals per housing unit**	d. Is this housing consistent with the *Guide* and USDA regulations? (yes/no***)		e. Estimated maximum number of housing units needed at any one time
Cat	x	**Standard**	TBD by VMO	x	**Yes**	2
		Departures from the Guide			**No**	

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

► (x) **Standard (See SOP)—Enter SOP in the table in Item Y.**
► ( ) **Standard (not covered by a SOP)**
► **Describe:** Animals will be housed under standard SPF sterile conditions (cages with micro-isolator tops, sterile bedding, food, and water).

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

► (x) **N/A: Animals will be housed in stable pairs or groups .**
► ( ) Animals will be housed singly:
►Provide justification:

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

2. Enrichment. Complete the table below to indicate whether "standard" exercise and environmental enrichment will be provided to the animals on this protocol, or whether any special supplements or restrictions will be required (See ACORP Instructions, for more information on enrichment requirements. Use Appendix 9 to document any enrichments requirements that represent "departures" from the standards in the *Guide*.):

(NOTE TO REVIEWERS: This section will be prepopulated, as the VMO will determine appropriate enrichment)

a. Species	b. Description of Enrichment*		c. Frequency	
cat		**TBD By VMO**	x	**TBD By VMO**
		Non-standard enrichment (describe and justify below)		**Other**

*If enrichment will be provided according to a local SOP, enter "standard (see SOP)" and enter the SOP into the table in Item Y. If the local standard enrichment is not described in a SOP, enter "standard, see below", and describe the standard species-specific enrichment here. **(NOTE TO REVIEWERS: In those cases where experimental design or other factors preclude use of standard enrichment, the PI may need to fill out the "non-standard box").**

► (x) **Standard (TBD by VMO)**
► ( ) **Non-standard**
Description on non-standard enrichment and justification:

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

► ( ) This ACORP INCLUDES genetically modified animals.

List each group of genetically modified animals, and describe for each any expected characteristic clinical signs or abnormal behavior related to the genotype and any customized

routine husbandry required to address these. For genetic modifications that will be newly generated on or for this protocol, describe any special attention needed during routine husbandry to monitor for unexpected clinical signs or abnormal behavior that may require customized routine husbandry.

►

► ( ) This ACORP does NOT include use of any animals that will require customized routine husbandry. If checked, go to item N.

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

► ( ) **N/A**
► (x) **Describe:** The marginal surgical areas will be cleaned according to procedures requested by Dr [redacted], D.V.M. by using chlorhexedine as follows: On a routine daily basis, the marginal area surrounding the cranial implant will be cleaned with chlorhexedine. The surrounding area will first be moistened using a swab (Q-tip, or gauze) that contains chlorhexedine. Once the area is moist, a Q-tip containing chlorhexidine is used, a section at a time, to remove dead tissue. Dead tissue that is hard to remove with the Q-tip is removed with a forcep. Once the area is thoroughly cleaned, gauze is used to completely dry the surface.

► ( ) Some or all of the animals on this protocol WILL require other customized routine husbandry by the animal husbandry staff, beyond what has been described above. Describe the special husbandry needed.

► (x) **N/A**
► ( ) **Describe:**

N. **Housing Sites**. Document in the tables below each location where animals on this protocol may be housed. **(NOTE TO REVIEWERS: All VMU space should be noted as TBD BY VMO.)**

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

Building	Room number	Inside of VMU?	
		Yes	No
[redacted] VMU	TBD by VMO	x	

► ( ) Housing in non-VA facilities. Identify each location not on VA property where animals on this protocol will be housed, and provide the information requested in the table. **(NOTE TO REVIEWERS: This space is for NON VA Facilities, like [redacted] and [redacted]. Dr. [redacted] can assist on AAALAC status).**

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

		( )**		
		( )**		
		( )**		

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

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

Special Features

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

► ( ) Some or all of the animals on this protocol WILL be used in the production and harvesting of antibodies. Check "Appendix 2" in Item Y, below, and complete and attach Appendix 2, "Antibody Production".
► (x) NO animals on this protocol will be used in the production and harvesting of antibodies.

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

► (x) This protocol INVOLVES administration of substances to the animals other than those used in routine husbandry and veterinary care. Check "Appendix 3" in Item Y, below, and complete and attach Appendix 3, "Biosafety".
► ( ) This protocol does NOT involve administration of any substances to the animals other than those used in routine husbandry and veterinary care.

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

Procedure	Surgical?		Bldg/Room Number	Requires transport between the VMU and the laboratory, or transport between laboratories?	
	Yes	No		No	If Yes – describe method of discreet transport
head electrode implant	x		VMU operating room	X	
hypoglossal nerve cuff implant	x		VMU operating room	X	
Genioglossal EMG electrode implant	x		VMU operating room	x	
Non-survival surgery (for carbachol study)	x		Bldg [redacted]/Rm [redacted] & Bldg [redacted]/Rm [redacted]		Animal will be transported in a pet carrying case covered with a drape.

Polygraphic, intracellular, hypoglossal nerve and genioglossal EMG recordings in conjunction with iontophoresis, microinjections, and reverse dialysis of neurotransmitter antagonists		X	Bldg [redacted]/Rm [redacted] & Bldg [redacted]/Rm [redacted]		Animal will be transported in a pet carrying case covered with a drape.
Intracardiac perfusion with fixative		x	[redacted]/necropsy room [redacted]		Animal will be transported in a pet carrying case covered with a drape.

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

Body Fluid, Tissue, or Device to be Collected	Collected AFTER Euthanasia	Collected BEFORE Euthanasia		
		Blood Collection Associated with Antibody Production (Appendix 2, "Antibody Production")	Collected as Part of a Surgical Procedure (Appendix 5, "Surgery")	Other Collection from Live Animals (Appendix 4, "Antemortem Specimen Collection")
Brain (medulla region containing the hypoglossal nucleus)	x			

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

▶ (x) Surgery WILL BE PERFORMED on some or all animals on this protocol. Check "Appendix 5" in Item Y, below, and complete and attach Appendix 5, "Surgery".
▶ ( ) NO animals on this protocol will undergo surgery.

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

In addition, specify how often the animals will be weighed to be sure weight loss does not exceed 10%.

(NOTE TO REVIEWERS: This section is important and should be carefully reviewed to ensure humane and safe endpoint criteria. The VMO should spend particular attention to this section as well. Departures from the Guide should be noted as described in Appendix 9).

► For chronic cats: continual depression, loss of appetite, significant weight loss (over 10%), aggression, neurological signs (abnormal gait, cerebellar signs of motor deficit (e.g., ataxia and dysmetria), lethargy), abnormal phase-switching of sleep-wake cycles, failure to groom, illness refractory to veterinary intervention, wound dehiscence, and dislodgement of head implant.

For acute cats: during the experiment, deterioration of an animal's health under anesthesia when occurs will be detected by appearance of subnormal vital signs (of heart rate, core temperature, and blood oxygenation level).

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

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

►

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

Check each method that may be used on this protocol	Method of Euthanasia	Species	AVMA Classification		
			Acceptable	Conditionally Acceptable	Unacceptable
	CO_2 from a compressed gas tank Duration of exposure after apparent clinical death► Method for verifying death► Secondary physical method►				
x	Anesthetic overdose Agent► Pentobarbital Dose► 100 mg/kg Route of administration► i.v. Method for verifying death► Lack of breathing and heartbeat	cat	x		

	Decapitation under anesthesia Agent▶ Dose▶ Route of administration▶				
	Exsanguination under anesthesia Agent▶ Dose▶ Route of administration▶ Method for verifying death▶				
	Other (Describe) ▶ Method for verifying death▶				
	Other (Describe) ▶ Method for verifying death▶				

1. For each of the methods above that is designated as "Conditionally Acceptable" by the AVMA, describe how the conditions for acceptability will be met:

   ▶ (x) **N/A**
   ▶ ( ) **Justification:**

2. For each of the methods above that is designated as "Unacceptable" by the AVMA, give the scientific reason(s) that justify this deviation from the AVMA Guidelines:

   ▶ (x) **N/A**
   ▶ ( ) **Justification:**

3. Identify all research personnel who will perform euthanasia on animals on this protocol and describe their training and experience with the methods of euthanasia they are to use in the species indicated.

   ▶ ( ) **N/A**
   ▶ Dr. [redacted] has over 30 yrs of experience with this method of euthanasia of cats. Dr. [redacted] has 20 yrs of experience with this method of euthanasia of cats.

4. Instructions for the animal care staff in case an animal is found dead.
   a. Describe the disposition of the carcass, including any special safety instructions. If disposition is to be handled according to a local SOP, enter "according to local SOP" and enter the information requested about the SOP into the table in Item Y.
   (NOTE TO REVIEWERS: The VMU has a specific SOP (The Biocontainment SOP). Normally, this SOP would be the one used in this section. Only if some other SPECIFIC alternative procedure is used would the other box be checked.

   ► (x) **According to Biocontainment SOP.**
   ► ( ) **Not according to Biocontainment SOP:**
   ►**Justification and description (must review first with VMO):**

   b. Describe how the PI's staff should be contacted.

   ► (x) Please contact a member of the PI's staff immediately. (Copy the lines below for each individual who may be contacted)
   Name► Dr. [redacted]
   Contact Information► [redacted] office), [redacted] (cell phone);
   email [redacted]

   ► ( ) There is no need to contact the PI's staff immediately. Describe the routine notification procedures that will be followed. If the routine notification procedures are described in a local SOP, enter "according to local SOP" and enter the information requested about the SOP into the table in Item Y.
   ►

   Name►
   Contact Information►

V. **Special Procedures.** List each special procedure (including special husbandry and other special procedures) that is a part of this protocol, and specify where the details of the procedure are documented. See ACORP Instructions, for examples.

(NOTE TO REVIEWERS: This is a confusing section to understand and may require some additional discussion and review as to what special procedures should be listed here—but may generally include things like diet/fasting, restraint, etc).

Name of Procedure	Identify Where the Details of the Procedure are Documented		
	SOP (title or ID number)*	Other Items in this ACORP -- specify the Item letter(s)	Appendix 6
Electrophysiology recording in sleeping cats		Items:	(x) **

Head restraint conditioning		Items:	(x) **
		Items:	( ) **
		Items:	( ) **

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

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

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

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

(NOTE TO REVIEWERS: This new section is slightly modified from previous ACORP and focuses on replacement/refinement/reduction).

1. Document the database searches conducted.
   List each of the potentially painful or distressing procedures included in this protocol.
   - ► ( ) **N/A**
   - ► (x) **Painful or distressing procedures:**

     ► Having chronic electrodes implanted to the cranium.
     Head-restraint for sleep recording for 4 hours per day, 3 days per week.

   Then complete the table below to document how the database search(es) you conduct to answer Items W.2 through W.5 below address(es) each of the potentially painful or distressing procedures.
   PI should run at least one search on the ALTBIB website for animal use alternatives. Please use the link http://toxnet.nlm.nih.gov/altbib.html

Name of the database	Date of search	Period of years covered by the search	Potentially painful or distressing procedures addressed	Key words and/or search strategy used	Indicate which mandate each search addressed			
					Replacement of animals (item W.2)	Reduction in numbers of animals used (item W.3)	Refinement to minimize pain or distress (item W.4)	Lack of unnecessary duplication (item W.5)
PubMed	3-15-18	2000-present	N/A	Hypoglossal motoneuron, sleep,	( )	()	( )	(x)

				neurotransmitter, atonia ***8 items found***				
PubMed	3-15-18	2000-present	N/A	Hypoglossal, sleep, glycine, atonia ***5 items found***	( )	()	( )	(x)
PubMed	3-15-18	2000-present	N/A	Hypoglossal, sleep, serotonin, atonia ***6 items found***	( )	()	( )	(x)
PubMed	5-10-18	2000-present	N/A	"hypoglossal", "intracellular recording", chronic, (rat OR mouse OR guinea pig OR rabbit) ***No items found***	( )	()	( )	(x)
PubMed using ALTBIB animal alternatives search strategy	3-15-18	2000-present	Restraint for sleep recording from intracellular electrodes	sleep, "intracellular recording" ***No items found***	(x)	(x)	(x)	( )
PubMed using ALTBIB animal alternatives search strategy	3-15-18	2000-present	Restraint for sleep recording from intracellular electrodes	"head restraint", "intracellular recording" ***No items found***	(x)	(x)	(x)	( )
PubMed using ALTBIB animal alternatives search strategy	3-15-18	2000-present	Restraint for sleep recording from intracellular electrodes	"restraint bag", "intracellular recording" ***No items found***	(x)	(x)	(x)	( )
AGRICOLA (USDA alternatives website)	3-15-18	2000-present	Restraint for sleep recording from intracellular electrodes	sleep, "intracellular recording" ***No items found***	(x)	(x)	(x)	( )
AGRICOLA (USDA alternatives website)	3-15-18	2000-present	Restraint for sleep recording from intracellular electrodes	"head restraint", "intracellular recording" ***No items found***	(x)	(x)	(x)	( )
AGRICOLA (USDA alternatives	3-15-18	2000-present	Restraint for sleep recording	"restraint bag", "intracellular	(x)	(x)	(x)	( )

website)			from intracellular electrodes	recording" ***No items found***				
Go3R Web	3-15-18	2000-present	Restraint for sleep recording from intracellular electrodes	"sleep", "intracellular recording" ***No items found***	(x)	(x)	(x)	( )
Go3R Web	3-15-18	2000-present	Restraint for sleep recording from intracellular electrodes	"head restraint", "intracellular recording" ***No items found***	(x)	(x)	(x)	( )
Go3R Web	3-15-18	2000-present	Restraint for sleep recording from intracellular electrodes	"restraint bag", "intracellular recording" ***No items found***	(x)	(x)	(x)	( )
Education Resources Information Center (ERIC)	3-15-18	2000-present	Restraint for sleep recording from intracellular electrodes	sleep, "intracellular recording" ***No items found***	(x)	(x)	(x)	( )
Education Resources Information Center (ERIC)	3-15-18	2000-present	Restraint for sleep recording from intracellular electrodes	"head restraint", "intracellular recording" ***No items found***	(x)	(x)	(x)	( )
Education Resources Information Center (ERIC)	3-15-18	2000-present	Restraint for sleep recording from intracellular electrodes	"restraint bag", "intracellular recording" ***No items found***	(x)	(x)	(x)	( )

2. Replacement. Describe the replacements that have been incorporated into this work, the replacements that have been considered but cannot be used, and the reason(s) that further replacements are not acceptable.

► Computer models have been considered, but literature searches do not indicate that they can replace those methods described in this protocol because there are no alternatives that provide data related to the normal behaviors of sleep and wakefulness. There are insufficient data that describe the activity of hypoglossal motoneurons and how they discharge during sleep and wakefulness to be able to develop a model to investigate. Therefore, computer models could replace none of the animal procedures described in the ACORP.

Though in vitro (brain slice) study is a possibility but this reduced preparation typically transects all brain input pathways to the target neurons of interest. Importantly, it is impossible to induce sleep and waking states and in vitro models lack standard correlative indices of REM sleep such as polygraphic (EEG) changes and motor/EMG atonia. Therefore this preparation lacks the physiological significance, i.e., state-dependency of data collected from such studies.

As can be seen above, we did extensive literature searches on a number of animal use alternative websites for suitable alternatives and found no papers.

3. Reduction. Describe how the number of animals to be used has been minimized in this protocol and explain why further reduction would disproportionately compromise the value of the data.

   ▶ Based upon our past experience in chronic cat studies, we estimated an average total of 2 cats per year will be minimally required for harvesting meaningful scientific data for our proposed chronic and acute studies. Each cat serves its own control (pre-drug) as well as treatment (post-drug) measurements. Therefore, the group size needed for the control and the treatment groups are effectively reduced. To maximize the amount of data collected from each chronic cat, we will acquire data on multiple days over the viable period (approximately 1-2 months) of the implanted nerve cuff electrodes. These electrodes are known to have a limited duration of working life, due to unavoidable scar formation around the implants which ruins the recording and stimulating capability of the electrodes. To further optimize the experimental design, we will perform the acute (carbachol paradigm) study on the terminal day of a given chronically instrumented cat, instead of using a new cat.

   We calculated the minimal number of animals we need in order to get enough neurons recorded from – see section C2b for details.

4. Refinement. Describe the refinements that have been incorporated into this work and explain why no further refinements are feasible.

   ▶ We will adopt the following steps for refinement of our studies.

   Proper pre- and post-operation procedures are strictly followed in order to lessen or eliminate pain or distress, and infection. Veterinarian care will be provided by the [redacted] VMU staffs. Best appropriate use of analgesics, anesthetics, and tranquilizers will be adopted according to recommendations of the veterinary medical officer.

   Softened food will be given to the cats over the first few days post-surgery, since they may have some discomfort with swallowing at that time.

   Animals will be played with extensively to make them comfortable with the researchers and the laboratory before we start doing any recordings. After each recording session, animals will be given Vital Essentials Cat freeze-dried chicken breast treats, and played with some more to give them exercise and pleasure.

   Animals will be gradually accustomed to the restraint needed to record their sleep. It is essential that they be very comfortable and relaxed so they can sleep naturally.

   Humane endpoints will be used so animals are euthanized if they show signs of weight loss (10%), lack of grooming, cerebellar signs of motor deficits, or abnormal sleep-wake cycles. By feeding them Vital Essentials Cat freeze-dried chicken breast treats after each recording session, we will be able to see if they are having any problems chewing or swallowing (since these are controlled by the hypoglossal nucleus). We also will play with them with a laser pointer after each session which lets us see if there are any neurological deficits (problems with visual tracking, or motor problems affecting coordination in activities such as chasing and pouncing).

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

► Literature searches indicate that none of the proposed studies have been conducted previously and the data that we seek to obtain are new and unique. There are only a few groups working in this area, and no other group anywhere has been able to do chronic intracellular recordings. Many of the papers we brought up come from our group, and we are building on that earlier work.

X. **Other Regulatory Considerations.**

1. **Controlled drugs.**

► ( ) **N/A (Go to Question 2).**
► ( ) Complete the table below for each drug that is used in animals on this protocol and that is classified as a controlled substance by the DEA. See ACORP Instructions, for explanations about the information requested.

Controlled substances	Storage		Personnel Authorized to Access	Location for Use		Procurement	
	Double-locked	Not Double-locked*		VA Property	Not on VA Property	VA Phar-macy	Non-VA
buprenorphine	x	( )*	TBD	x		x	
ketamine	x	( )*		x		x	
pentobarbital	x	( )*		x		x	

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

► (x) **N/A**
► ( ) **Justification:**

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

► ( ) **N/A**
► (x) Some controlled substances will used on VA property, and all of these will be obtained through the local VA pharmacy.
► ( ) Some controlled substances will not be obtained through the local VA pharmacy, but none of these will be used on VA property. See the ACORP Instructions, for further information.
► ( ) Other. Explain►

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

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

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

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

► ( ) Yes, some explosive agent(s) will be used on this protocol. Check "Appendix 3" and "Appendix 8" in Item Y, below, and complete and attach Appendix 8, "Use of Explosive Agent(s) within the Animal Facility or in Animals", as well as Appendix 3, "Biosafety".
► (x) No explosive agent(s) will be used as part of this protocol.

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

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

► (x) Appendix 1, "Additional Local Information"
► ( ) Appendix 2, "Antibody Production"
► (x) Appendix 3, "Biosafety"
► ( ) Appendix 4, "Ante-mortem Specimen Collection"
► (x) Appendix 5, "Surgery"
► (x) Appendix 6, "Special Husbandry and Procedures"
► ( ) Appendix 7, "Use of Patient Care Equipment or Areas for Animal Studies"
► ( ) Appendix 8, "Use of Explosive Agent(s) within the VMU or in Animals"
► ( ) Appendix 9, "Departures from "Must" and "Should" Standards in the *Guide*"
► ( ) Appendix 10, "Overnight housing"

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

(NOTE TO REVIEWERS: This section will have to be developed over time. Some of the information will be pre-populated).

Item	SOP		Approval Date
	Title	ID	
C.2.c	This needs to be pre-populated		
M.1			
M.2			
U.4.a			
U.4.b			
V			

Z. **Certifications.** Signatures are required here for any ACORP that is to be submitted to VA Central Office in support of an application for VA funding. Include the typed names and dated signatures as shown below for the Main Body of the ACORP and for each of the Appendices that apply to this protocol. Do NOT include signatures for, or attach, any appendices that do NOT apply.

1. **Main Body of the ACORP.**

   a. **Certification by Principal Investigator(s):**

   I certify that, to the best of my knowledge, the information provided in this ACORP is complete and accurate, and the work will be performed as described here and approved by the IACUC. I understand that IACUC approval must be renewed at least annually, and that the IACUC must perform a complete *de novo* review of the protocol at least every three years, if work is to continue without interruption. I understand further that I am responsible for providing the information required by the IACUC for these annual and triennial reviews, allowing sufficient time for the IACUC to perform the reviews before the renewal dates, and that I may be required to complete a newer version of the ACORP that requests additional information, at the time of each triennial review.

   I understand that further IACUC approval must be secured before any of the following may be implemented:

   - Use of additional animal species, numbers of animals, or numbers of procedures performed on individual animals;
   - Changing any procedure in any way that has the potential to increase the pain/distress category to which the animals should be assigned, or that might otherwise be considered a significant change from the approved protocol;
   - Performing any additional procedures not already described in this ACORP;
   - Use of any of these animals on other protocols, or by other investigators.

   I further certify that:

   - No personnel will perform any animal procedures on this protocol until the IACUC has confirmed that they are adequately trained and qualified, enrolled in an acceptable Occupational Health and Safety Program, and meet all other criteria required by the IACUC. When new or additional personnel are to work with the animals on this protocol, I will provide this information to the IACUC for confirmation before they begin work;
   - I will provide my after-hours contact information to the animal care staff for use in case of emergency.

Principal Investigator	PI Signature	Date
[redacted], PhD	[redacted]	3/13/18

   b. **Certification by IACUC Officials.**

We certify that:

- We, with the IACUC, have evaluated the care and use of animals described on this ACORP, in accordance with the provisions of the USDA Animal Welfare Act Regulations and Standards, PHS Policy, the *Guide for the Care and Use of Laboratory Animals*, and VA Policy;
- The IACUC has determined that the care and use of animals described in this ACORP is appropriate, and has therefore approved the protocol;
- The full text of any minority opinions is documented here as indicated below:

▶ ( ) No minority opinions were submitted by any IACUC participant for inclusion.

▶ ( ) Minority opinions submitted by IACUC participants are copied here
▶

▶ ( ) Minority opinions submitted by IACUC participants are attached on separate pages labeled "IACUC Minority Opinion" (indicate the number of pages▶)

Name of Attending Veterinarian (VMO or VMC)	Signature	Date
[redacted], MA, DVM	[redacted]	5/25/18
Name of IACUC Chair		Date
for [redacted], MD		5/25/18

2. **Appendix 2. Antibody Production.** No signatures required.

3. **Appendix 3. Biosafety.**

   a. **Certification by PI(s) and IACUC Officials:**

   We certify that:

   - Before any animal experiments involving hazardous agents (identified in Item 10.a of Appendix 3) are performed, SOPs designed to protect all research and animal facility staff as well as non-study animals will be developed and approved by the appropriate VA or affiliated university safety committee and by the IACUC;

   - All personnel who might be exposed to the hazardous agents (identified in Item 10.a of Appendix 3) will be informed of possible risks and will be properly trained ahead of time to follow the SOPs to minimize the risks of exposure.

Principal Investigator	Signature	Date

[redacted], PhD	[redacted]	3/13/18
Name of Institutional Veterinarian	Signature	Date
[redacted], MA, DVM	[redacted]	5/25/18
Name of IACUC Chair		Date
for [redacted], MD	[redacted]	5/25/18

b. **Certification by Biosafety Official.** I certify that:

- Each agent to be administered to animals on this protocol has been properly identified in Item 1 of Appendix 3 as to whether it is "toxic", "infectious", "biological", or "contains recombinant nucleic acid";
- The use of each of the agents thus identified as "toxic", "infectious", or "biological", or "contains recombinant nucleic acid" is further documented as required in Items 4, 5, 6, and/or 8, as applicable, and in Item 10.a of Appendix 3;
- The use of each of these agents has been approved by the appropriate committee(s) or official(s), as shown in Item 10.a of Appendix 3.

Name of the Biosafety Officer, or of the Chair of the Research Safety or Biosafety Committee	Signature	Date
[redacted], PhD	[redacted]	5/21/18

c. **Certification by Radiation Safety Official.** I certify that:

- Each agent to be administered to animals on this protocol has been properly identified in Item 1 of Appendix 3 as to whether it is "radioactive";
- The use of each radioactive agent is further documented as required in Items 7 and 10.a of Appendix 3;
- The use of each radioactive agent has been approved by the appropriate committee(s), as shown in Item 10.a of Appendix 3.

Name of the Radiation Safety Officer, or of the Chair of the Radiation Safety or Isotope Committee	Signature	Date

4. **Appendix 4. Ante-mortem Specimen Collection.** No signatures required.

5. **Appendix 5. Surgery. Certification by the PI(s).** I certify that:

   - To the best of my knowledge, the information provided in Appendix 5 of this ACORP is complete and accurate;
   - The surgical procedures will be performed and the post-operative care (including administration of post-operative analgesics) will be provided as described;
   - The spaces where any survival surgical procedures will be performed (listed in Item 4 of Appendix 5) are suitable for sterile/aseptic surgery;
   - The names and contact information for research personnel to notify or consult in case of emergencies will be provided to the VMU supervisor and veterinary staff;
   - Post-operative medical records will be maintained and readily available for the veterinary staff and the IACUC to refer to, and will include the following:

     - Identification of each animal such that care for individual animals can be documented.
     - Daily postoperative medical records for each animal, that include documentation of daily evaluation of overall health and descriptions of any complications noted, treatments provided, and removal of devices such as sutures, staples, or wound clips;
     - Documentation of the administration of all medications and treatments given to the animals, including those given to reduce pain or stress.
     - Daily records covering at least the period defined as "post-operative" by local policy.
     - The signature or initials of the person making each entry.

Principal Investigator	PI Signature	Date
[redacted], PhD	[redacted]	3/13/18

6. **Appendix 6. Special Husbandry and Procedures.** No signatures required.

7. **Appendix 7. Use of Patient Care Equipment or Areas for Animal Studies.**

a. **Certification by the Principal Investigator(s).** I certify that, to the best of my knowledge, the information provided in Appendix 7 of this ACORP is complete and accurate, and the use of patient care equipment or areas for these animal studies will be as described.

Principal Investigator	PI Signature	Date

b. **Certification by the officials responsible for the use of any human patient care equipment in animal procedural areas.** Each of the following must sign to indicate that they have granted approval for the human patient care equipment to be moved to the VMU or other animal procedural area to be used on animals and then returned to the human patient care area, as described in Appendix 7. Leave this section blank, if not applicable.

Name of IACUC Chair	Signature	Date
Name of the Manager of the Human Patient Care Equipment	Signature	Date

c. **Certification by the officials responsible for the use of the equipment in human patient care areas for these animal studies.** Each of the following must sign to indicate that they have granted approval for animals to be transported into human patient care areas for study or treatment, as described in Appendix 7. Leave this section blank, if not applicable.

Name of IACUC Chair	Signature	Date
Name of Attending Veterinarian (VMO or VMC)	Signature	Date
Name of the Chair of the Clinical Executive Board, or the Service Chief responsible for the Patient Care Area and Equipment	Signature	Date

Name of ACOS for R&D	Signature	Date
Name of Chief of Staff	Signature	Date
Name of Director or CEO of the Facility (Hospital or Clinic)	Signature	Date

8. **Appendix 8. Use of Explosive Agent(s) within the Animal Facility or in Animals.**

   a. **Certification by the Principal Investigator(s).**

   I certify that, to the best of my knowledge, the information provided in Appendix 8 of this Animal Component of Research Protocol (ACORP) is complete and accurate, and the use of explosive agents in these animal studies will be as described.

   I further certify that:

   - Procedures involving explosive agent(s) will be performed within a properly operating, ventilated safety hood;
   - All electrical equipment operating when explosive agent(s) are in use will be positioned and powered outside of the hood;
   - Once the seal is broken on any containers of explosive agents, they will be kept in a safety hood throughout use, stored in an explosion-proof refrigerator or other approved storage area, and discarded properly once completely emptied;
   - Proper procedures will be used for safe and appropriate disposal of items (including animal carcasses) that may contain residual traces of the explosive agent(s).

Principal Investigator	PI Signature	Date

b. **Certification by the officials responsible for overseeing the use of explosive agent(s) in this protocol.** Each of the following must sign to verify that they or the committee they represent have granted approval.

Name of IACUC Chair	Signature	Date
Name of Attending Veterinarian (VMO or VMC)	Signature	Date
Name of Safety/Biosafety Officer for the Facility	Signature	Date
Name of ACOS for R&D	Signature	Date
Name of VISN Regional Safety Officer	Signature	Date

9. **Appendix 9. Departures from "Must" and "Should" Standards in the *Guide*.** No signatures required.

10. **Appendix 10. Certification by Principal Investigator is on the Appendix.**

Last Name of PI▶
Protocol No. Assigned by the IACUC▶
Official Date of Approval▶

ACORP Appendix 1
ADDITIONAL LOCAL INFORMATION
VERSION 4 V2 6/17/2015
(Required for all protocols)

(See ACORP App. 1 Instructions, for more detailed explanations of the information requested.)

Species covered by this Appendix: **Cat**

This protocol involves the following (check all that apply):

☐ Breeding ☐ Tumor Formation x Hazardous agents used in animals
x Multiple survival surgery ☐ Food and/or Fluid Restriction
☐ Antibody/Ascites Formation ☐ Hazard to VMU Personnel
x Prolonged Restraint (> than 15 minutes) ☐ Tumor formation

a.VA project # [redacted]	b. Protocol # [redacted]	c. 3 year expiration date **May 27, 2017**
d. PI name: [redacted] **PhD**		e. PI phone: [redacted]
f. PI e-mail: [redacted]		g. Species:**cat**
h. Protocol title: **Resolution Of The Mechanisms Responsible For Atonia During REM Sleep**		
i. Contact name 1: [redacted] **PhD**		j. e-mail: [redacted]
k.Contact name 2: [redacted]		l. e-mail: [redacted]
m. Lab phone:	n. Alternate phone: [redacted]	o. **EMERGENCY PHONE # (Cell preferred)** 1-[redacted]
p. Animals taken to lab? x Yes ☐ No If yes, Bldg [redacted] **and** [redacted] and Room(s) [redacted] **and** [redacted]		
q. Animals taken to lab and then returned to vivarium (*VMU return room only*) x Yes ☐ No If yes, provide a scientific justification here: **We conduct recording 3 days per week over 2-4 months period**		
r. Animals housed in the lab for 12 or more consecutive hours? ☐ Yes x No If yes, Bldg: and Room(s): and fill out part B below.		
s. Is wire-floored caging required? ☐ Yes x No		
t. Do animals need to be exempted from the environmental enrichment program? ☐ Yes x No If yes, provide a scientific justification here:		
u. Maximum allowable body weight loss (10% unless scientifically justified):**10%**		
v. Hazards used in animals (check all that apply): ☐ None x Toxic ☐ Infectious ☐ Biological ☐ Radioactive ☐ Other (list):		
w. Will VMU personnel be exposed to any of these hazards? (This includes animals housed in labs since VMU staff check them, wash the cages, etc.) ☐ Yes x No If yes, list which hazards:		
x. Body fluid, tissue and/or device collection? ☐ None ☐ Live x Dead ☐ Both		

y. Surgery? ☐ None ☐ Minor x Major ☐ Both ☐ Non-survival Multiple survival surgeries? x Yes ☐ No If there are multiple survival surgeries, list surgery types: **head implant; hypoglossal nerve cuff and genioglossal EMG implant**
z. Anesthetics/analgesics used (excluding euthanasia)? x Yes ☐ No If yes, list: **isoflurane, buprenorphine, ketamine, carprofen, lidocaine, alpha-chloralose**
aa. Euthanasia methods (must include anesthetic plus physical method unless scientifically justified): **Pentobarbital iv**
bb. All controlled substances used: **buprenorphine, ketamine, Pentobarbital**
cc. List any other drugs from Appendix 5 (surgery appendix): **xylazine, baytril, atropine sulphate, dexamethasone, betadine, rubbing alcohol, saline.**

Delegation of Authority: Complete this section for every employee in this study, starting with the PI, specifying which procedures each is allowed to perform. All should be listed in the ACORP main body. Everyone listed must also have current employment status (VA or WOC) and be up-to-date with all required training and medical clearances.
Please note: There must always be at least one person responsible for task codes A, D, and H.

Species : **cat**

Last name, first name, degree(s):	Task codes (use the list below):
[redacted], PhD	**A,B,C,D,G,H,I,J**
[redacted], PhD	**A,B,C,D,G,H,I,J**
[redacted], PhD	**B,I,J**
[redacted], PhD	**I,J**

Task codes

A = Routine daily care of animal B = Performs survival surgery C = Performs non-survival surgery D = Evaluates endpoint criteria E = Collects samples with anesthesia F = Collects samples without anesthesia G = Collects or works with samples postmortem H = Euthanizes animal subjects	I = Performs in vivo procedures other than sample collection or surgery, such as behavioral studies J = Other work with animals, please specify: K = PI with no animal contact. The PI must be listed in section E of the ACORP Main Body. If the PI does have animal contact, list them with the appropriate task codes. L = Non-PI with no animal contact. This person does not need to be listed in section E of the ACORP.

Last Name of PI▶
Protocol No. Assigned by the IACUC▶
Official Date of Approval▶

ACORP APPENDIX 3
BIOSAFETY
VERSION 4 V2 6-17-2015

See ACORP App. 3 Instructions, for more detailed explanations of the information requested.

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

Material Identify the specific materials including radioisotopes, chemicals, drugs (standard clinical agents as well as test agents), controlled substances, infectious agents, biomaterials, prosthetic devices, minipumps, special diets, and cells, tissues, or body fluids. *(Do not list drinking water and the standard food from the VMU)*	**Source** (Identify the vendor or colleague, or specify which animals on this protocol will serve as donors)	**Nature of Material**							
		Toxic Agent (such as mutagens, carcinogens, teratogens, neurotoxins, Select Agents, ect. - Item 4)	Infectious Agent (Item 5) – Enter the CDC Biosafety Level (BSL 1, 2, or 2*)	Biological Agent (Item 6)	Radioactive Agent (Item 7)	Contains Recombinant Nucleic Acid (Item 8)	Routine Pre- or Post-Procedural Drug	Euthanasia agent	Other
formaldehyde	Fisher Scientific	x	BSL-						x
strychnine	Sigma-Aldrich	x	BSL-						x
methysergide	Tocris Bioscience	x	BSL-						x
prazocin	Tocris Bioscience	x	BSL-						x
isoflurane	VMU pharmacy		BSL-				x		
lidocaine	VMU pharmacy		BSL-				X		
xylazine	VMU pharmacy						X		
buprenorphine	VMU pharmacy						X		
carprofen	VMU pharmacy						X		
baytril	VMU pharmacy						X		
atropine sulphate	VMU pharmacy						X		
dexamethasone	VMU pharmacy						X		

ketamine	VMU pharmacy						X		
saline	VMU pharmacy						X		
carbachol	Sigma-Aldrich	x							
alpha-chloralose	Sigma-Aldrich	x							
Pentobarbital	VMU pharmacy	x					X	X	
Ophthalmic ointment	VMU pharmacy						X		
betadine	VMU pharmacy	x					X		
Rubbing alcohol	VMU pharmacy						x		

Only BSL 1, 2 or 2* work is permitted at VA-GLA. No BSL 3 or 4 work.

2. **Summary of How Materials will be Administered.** Complete the table below for each of the materials shown in the table in Item 1 above. For each item, note if it is USP grade, FDA approved, a fixative, or a special diet.

Material* (Identify the specific agent, device, strain, construct, isotope, etc.)	**Dose** (e.g., mg/kg, CFU, PFU, number of cells, mCi) and **Volume** (ml)	**Diluent* or Vehicle***	**Route** of admin	**Frequency or duration** of admin	**Reason** for Administration and **Expected Effects**	Location of **Further Details** in this ACORP (specify "Main Body" or "App #", and identify the Item)	Administration Under **Anesthesia, sedation, or tranquilization** (Y/N)
formaldehyde*	10%, 2000ml	saline	intracardiac	Once, 30 minutes	Fixative, brain will be fixed for histology	ACORP main body, item C2	yes
strychnine*	50mM, $0.2x10^{-3}$ml	saline	iontophoresis, pressure ejection, reverse dialysis	Multiple (2-3) trials, 3-10 minutes	Glycine receptor antagonist; motoneuron excitability increases	ACORP main body, item C2	No for chronic cat, yes for acute cat
methysergide*	1mM, $0.2x10^{-3}$ ml	saline	iontophoresis, pressure ejection, reverse dialysis	Multiple (2-3) trials, 3-10 minutes	Serotonin mixed receptor antagonist; motoneuron excitability decreases	ACORP main body, item C2	No for chronic cat, yes for acute cat

prazocin*	0.2mM, $0.2x10^{-3}$ ml	saline	iontophoresis, pressure ejection, reverse dialysis	Multiple (2-3) trials, 3-10 minutes	Norepinephrine receptor antagonist; motoneuron excitability decreases	ACORP main body, item C2	No for chronic cat, yes for acute cat
isoflurane	2-4%	N/A	inhalation	Once,3-4 hrs	General anesthesia for survival and non-survival surgeries	ACORP appendix 5	yes
lidocaine	2%	N/A	local application	Once, 1 minute	Inhibits gag reflex during endotracheal intubation	ACORP appendix 5	yes
xylazine	2mg/kg, 0.1ml/kg	N/A	i.m.	Once, 1 minute	Tranquilizer, aids survival surgeries	ACORP appendix 5	yes
buprenorphine	0.02 mg/kg, 0.07 ml/kg	N/A	s.c	Once, 1 minute	Analgesic, eliminates postsurgical pain	ACORP appendix 5	yes
carprofen	5 mg/kg, 0.2 ml	N/A	s.c.	Once, 1 minute	Pre-emptive and post-operative analgesia to eliminate pain	ACORP appendix 5	yes
baytril	5mg/kg, 0.1ml/kg	N/A	s.c.	Once, 1 minute	Antibiotic, prevents postsurgical infection	ACORP appendix 5	yes
atropine sulphate	0.04 mg/kg, 0.07 ml/kg	N/A	i.m.	Once, 1 minute	Antimuscarinic agent, reduce mucous secretion of trachea	ACORP appendix 5	yes
dexamethasone	0.5mg/kg, 0.25ml/kg	N/A	i.m.	Once, 1 minute	Anti-edematic agent, prevents brain edema	ACORP appendix 5	yes
ketamine	8-12 mg/kg, 0.08-0.12 ml/kg	N/A	i.m.	Once, 1 minute	Short-acting anesthetic, aids endotracheal intubation	ACORP appendix 5	yes

saline	4ml/kg, 12ml	N/A	s.c.	Once, 1 minute	For fluid balance, prevents dehydration	ACORP appendix 5	yes
carbachol*	4mg/ml, $0.2x10^{-3}$ ml	saline	pressure ejection	Once, 1 minute	To induce REM sleep, for simulating naturally occurring REM sleep	ACORP main body, item C2	yes
alpha-chloralose*	120mg/kg loading, 2ml; 60mg/kg, 1ml; maintenance	saline	i.v.	Multiple (2-3) times in acute cats,1 minute	For acute cat studies, this is compatible with carbachol's action to induce REM sleep	ACORP main body, item C2	yes
pentobarbital	100mg/kg, 1ml	N/A	i.v.	Once, 1 minute	For euthanasia	ACORP main body, item C2	yes
ophthalmic ointment	N/A	N/A	topical	Once, 1 minute	For ocular lubricant	ACORP appendix 5	yes
betadine	10%, 5ml	N/A	topical	3 times, 1 minute	Used as an antiseptic prior to skin incision	ACORP appendix 5	yes
rubbing alcohol	70%, 5ml	N/A	topical	3 times, 1 minute	Used as an antiseptic	ACORP appendix 5	yes

*Each material, diluent, or vehicle that is listed as FDA approved or is labeled "USP" is pharmaceutical grade. Check on-line for formulations that are FDA approved for administration to humans http://www.accessdata.fda.gov/scripts/cder/ob/default.cfm or animals http://www.accessdata.fda.gov/scripts/animaldrugsatfda/

Designate with a * each material and each diluent or vehicle to be used that is not pharmaceutical grade. For each of these, fill out tables 2a and 2b below to explain why the use of a non-pharmaceutical grade formulation is necessary, and to describe how it will be ensured that the material is suitable for use. (See ACORP App. 3 Instructions, for specifics about the level of detail required.)

Table 2a

List all items from table 2 that are not USP grade, FDA approved, a fixative, or a special diet	Why the use of a non-pharmaceutical grade formulation necessary? *Please put an X in the appropriate column, and add rows as needed.*			
	No FDA approved version exists	The FDA approved injectable forms are too dilute or have the wrong diluents for this study*	The FDA approved versions are only in pills or other forms that aren't suitable for this study	Other (please explain)

strychnine	x			
methysergide			x	
prazocin	x			
carbachol			x	
alpha-chloralose	x			

**Note: Injectables that are too concentrated can usually be diluted with saline.*

Table 2b

List all items from table 2 that are not USP grade, FDA approved, a fixative, or a special diet	How it will be ensured that the material is suitable for use? *Please put YES, N/A, or an explanation in each column, and add rows as needed.*					
	Purity/grade/pyro-genicity	Sterility	Osmolality	Stability	Formulation and pharmacokinetics	pH
	The certificate of analysis from the manufacturer will be examined to ensure the material is suitable.	If the drug does not come as a sterile solution, it will be sterile filtered before use.	Sterile USP grade isotonic diluents will be used, such as USP grade normal saline.	The supplier's guidelines on storage and stability will be followed.	The literature has been consulted to determine the appropriate formulation and that the pharmacokinetics are suitable	The pH of the solution will be tested (with pH paper or a meter) before injection
strychnine	yes	yes	yes	yes	yes	yes
methysergide	yes	yes	yes	yes	yes	yes
prazocin	yes	yes	yes	yes	yes	yes
carbachol	yes	yes	yes	yes	yes	yes
alpha-chloralose	yes	yes	yes	yes	yes	yes

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

   a. For each material with "Y" entered in the last column of the table in Item 2 above, describe the anesthesia, sedation, or tranquilization to be used, identifying the anesthetic, sedative, or chemical tranquilizer, and detailing the dose, volume, and route of administration (Make sure that these agents are also included in Item 1 of this appendix, as materials to be administered):
   ► For acute (non-survival) cat studies: all surgical procedures will be performed under Isoflurane (2-4%, via inhalation). A second injectable anesthetic (alpha-chloralose, loading dose, 120 mg/kg, i.v., 2 ml; maintenance dose, 60 mg/kg, i.v., 1 ml) will then be used for data acquisition in conjunction with carbachol microinjection into the nucleus pontis oralis to induce REM sleep. At the end of the experimental procedures, the animals are euthanized using Pentobarbital (100 mg/kg, i.v.). The animal will be perfused with fixatives and the brain will be removed for histology.
   It is known that isoflurane inhibits the muscarinic cation current (Dryn et al., Eur J Pharmacol. 2018, 820:39-44) and therefore disrupts the carbachol effects on neurons. In contrast, alpha-choralose has been proven to be ideal for the elicitation of pharmacological induction (via carbachol injection to the pontine reticular formation) of REM sleep-like state in cats (e.g., Yamuy et al., Exp Neurol. 2010, 221(2):335-45; Fung et al., Brain Res. 2000, 885(2):262-72; Kohlmeier et al., Neuroscience 1998, 86(2):557-69).

b. For each material with "N" entered in the last column of the table in Item 2 above, explain why no anesthesia, sedation, or tranquilization is necessary, or can be provided, and describe any alternate methods of restraint that will be used.
► For chronic (survival) cat studies: Neurons (including hypoglossal motoneurons) do not possess any pain receptors and the applied chemicals will not produce any pain sensation to the animal subjects. The various drugs will be applied by: (1) iontophoresis as ions, (2) pressure ejection in minute volumes not exceeding 0.2 microliter for each trial, or (3) reverse dialysis as molecules diffusing across the dialysis membrane to adjacent hypoglossal motoneurons. Therefore, no anesthesia, sedation, or tranquilization are administered for the chronic cat studies. In addition, cats will be well adapted to the head-restraint device and the body restraining bag which will not cause any pain or distress as judged by (1) the calm behavior of the animal during recording sessions as well as (2) normal phase transitions throughout the sleep-waking cycles of the cat during experiments. Therefore, no further restraint will be necessary for the experiment. No anesthesia, sedation, or tranquilization can be used because it will interfere with the natural sleep we are studying.

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

Name of Toxic Agent	a. Mutagen	b. Carcinogen	c. Teratogen	d. Select Agent?			e. Other – specify toxic properties (neurotoxin, etc.)
				Not a Select Agent	Select Agent Used in Sub-threshold Quantities	Select Agent that Requires Registration/Approval	
formaldehyde	x	x	x	x		( )*	( )►
strychnine				x		( )*	(x)► fatal if swallow
methysergide	x		x	x		( )*	(x)► acute oral toxicity
prazocin				x		( )*	(x)► harmful if swallowed
carbachol				x		( )*	(x)► acute oral toxicity, fatal if swallow
alpha-chloralose		x		x		( )*	(x)► acute oral toxicity, harmful if swallow

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

Name of agent ►

Registered with CDC or USDA ►
Registration Number ►
Registration Date ►
Expiration Date of Registration ►

Name of official who granted approval on behalf of VACO▶
Date of approval▶

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

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

*Complete the following for each agent for which the ABSL Number given is less than the BSL Number shown (copy the lines below for each agent):

Name of agent ▶
Justification for applying ABSL measures that are less protective than those recommended ▶

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

Name of agent ▶

Registered with CDC or USDA ▶
Registration Number ▶
Registration Date ▶
Expiration Date of Registration ▶

Name of official who granted approval on behalf of VACO▶
Date of approval▶

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

Name of Biological Agent	Screening for Infectious Agents

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

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

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

Name of Agent that Contains Recombinant Nucleic Acid	Subject to the *NIH Guidelines for Research Involving Recombinant DNA Molecules*	Exempt

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

Neurons (including hypoglossal motoneurons) do not possess any pain receptors and the applied chemicals will not produce any pain sensations or distress to the animal subjects.
To adapt the animal to sit calmly (with head restrained to the recording apparatus) during the intracellular recording sessions, the initial adaptation period is always brief and the animal will be given a treat (Vital Essentials Cat freeze-dried chicken) and playtime following each adaptation. Training over time will ameliorate the stress response to prolonged restrain.

Name of Agent	Nature of Potential Pain/Distress	Measures to Alleviate Pain/Distress

10. **Protection of Animal Facility Staff from Hazardous Materials.** Complete Items 10.a and 10.b, below, for each of the agents listed in the table in Item 1, above, as "toxic", "infectious", "biological", "radioactive", or "contains recombinant nucleic acid" (detailed in Items 4 – 8). This item specifically addresses members of the animal facility staff; protection of the research staff from each of these agents must be addressed in Item G of the main body of the ACORP. See ACORP App.3 Instructions, for details.

    a. Complete the table below.

Name of Hazardous Agent	Approving Committee or Official	Institution (VA or affiliate)	Names of Animal Facility Staff Members at Risk (for VA-GLA put "to be determined")
formaldehyde	SRS	VA	to be determined
strychnine	SRS	VA	to be determined
methysergide	SRS	VA	to be determined
prazocin	SRS	VA	to be determined
carbachol	SRS	VA	to be determined
alpha-chloralose	SRS	VA	to be determined

    b. Detail how the individuals listed in the table above (Item 10.a.) have been (or will be) informed of the possible risks of exposure, and have been (or will be) trained to avoid exposure to these agents.
    ▶The following Agent Management Plan is provided for the VMU staff and others who come into contact with these animals.

AGENT MANAGEMENT PLAN
11. Names and strains (if applicable) of agent(s), chemical(s) or toxin(s) covered in this plan: **All these drugs (listed above in table 10a) need similar PPE, etc. so we are including them in one agent management plan.**
12. ☐ Yes **x** No: Is baseline screening of serum required for VMU animal care workers? If you are unsure if this is necessary, please contact the RBSO via email at [redacted]@va.gov
13. Discuss appropriate PPE to be worn by PI, PI's staff, and VMU staff: **Wear safety goggles for eye protection and face mask to protect inhaling chemicals in fine particle/dust and vapor form. For handling the chemicals, wear nitrile rubber gloves for skin protection. Laboratory coats are essential for whole-body protection against accidental contact with chemicals in all forms. VMU staff will not be directly exposed to any of these chemicals, since the chemicals will be microinjected into the cat's brains in minute amounts.**

14. Identify any risks associated with bedding, and precautions/handling measures to be observed by VMU staff. This includes cages/bedding returned to the VMU from work conducted in the laboratory.
There will be no risk to VMU staffs as they will not be in direct contact such as weighing, dissolution and administration of the chemicals. VMU staff will not be directly exposed to any of these chemicals, since they will be microinjected into the cat's brains in minute amounts. Most of the chemicals administered are membrane receptor antagonists and they will be converted by neurons and glia to metabolites which then will be further catabolized by the liver and renal systems to form non-hazardous final products. When alpha-chloralose is used in acute cat studies, the animal will be euthanized at the end of the experiment and the carcasses will be double-bagged in biohazard bags before disposing in the proper biohazard storage room in VMU.

15. Indicate any particular instructions/precautions regarding potential hazards for project and/or VMU staff for handling animal food and water.
The animals will receive normal food and water, no precautions needed.

16. Discuss any special medical treatment guidelines that the project and/or VMU staff need to be aware of in case of animal medical emergency. For instance, are there specific compounds or circumstances that cannot be used or should be avoided?
All drugs are acceptable.

17. Discuss specific health risks relative to the agent/chemical/toxin addressed in this Plan for persons who come in contact with animals/returned carcasses covered under this PSP, to include routes/mechanisms of transmission. Describe initial signs and symptoms of exposure.
With the proper personal PPE (see item 13 above), there should not be any chemical-induced health risks for people in contact with animals/returned carcasses.

In case of direct contamination of a person with the chemicals, the general route of transmission of contaminants is by skin absorption (for solid and liquid forms), swallowing, or by inhalation to the lungs (for dust forms). Once inside the body, chemicals will be transmitted to various organs via systemic (blood circulatory) system.

Common initial symptoms of chemical contaminations are skin irritation, eye damage/irritation, respiratory sensitization (coughing).

18. Discuss precautions/protocols that should be taken in case of escape of a live animal to which the agent/chemical/toxin addressed in this Plan has been administered.
Under our protocol, chemicals for experiemental studies will only be administered: (1) when the chronic cat is painlessly head-restrained, or (2) when the cat is under anesthesia (for acute study) and the head of the animal is mechanically fixed to a stereotaxic head-holder (using the mouth clamp and ear bars). Therefore, there will not be any possibility for a live animal with the administered chemicals to escape. To further prevent escape, we will keep the door of the laboratory locked at all times when the animal is in it. All researchers involved in the research protocol will be trained by taking the species-specific (cat) course (CITI program).

If an animal should get loose, no special precautions are needed beyond those noted above. Please capture the animal and return it to it's VMU housing.

19. **Check all cage card requirements:**

☐ Carcinogens	☐ Infectious Agents	x Neurotoxins	x Mutagens/Teratogens
☐ Biologic Toxins	☐ Human Cells and/or Cell Lines	☐ Other (specify):	

11. **Signatures.** Provide the applicable signatures on the signature pages (Item Z.3) of the main body of this ACORP.

Last Name of PI▶
Protocol No. Assigned by the IACUC▶
Official Date of Approval▶

ACORP Appendix 5

SURGERY
VERSION 4 V2 6-17-2015

See ACORP App. 5 Instructions, for more detailed explanations of the information requested.

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

Surgery		Terminal	Survival		
#	Description (specify the species, if ACORP covers more than one)		Minor	Major	One of Multiple*
1	Head electrodes implant (cat)				(x)*
2	Hypoglossal nerve cuff and genioglossal EMG implants (cat)				(x)*
3	Non-survival surgery for carbachol study (cat)	x			( )*
4	Thoracotomy for perfusion (cat)	x			( )*

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

a. Provide a complete scientific justification for performing the multiple survival surgeries on an individual animal:
► Whereas the cortical EEG, EOG, LGN and EMG recording electrodes last for months, the hypoglossal nerve cuff electrode is known to have a limited working life, due to scar tissue forming adjacent to the cuff electrodes over a period of 1-2 months. It takes 10-14 days for the cat to recover from the initial head implant surgery. After this recovery period, the cat will be adapted incrementally (from 15 min initially to 4 hrs by the end of 1 month), 3 times per week, to the recording environment. Once adapted, it will exhibit multiple episodes of normal phase switching between sleep-waking cycles. When this condition is reached, a second surgery to implant the hypoglossal nerve cuff and genioglossal EMG electrodes will follow. By matching the functional lifespan of the implanted cuff electrode with the behavior of the cat during adaptation as well as by polysomnography, we will be able to optimize the yield of data acquisition that depends on the viability of the cuff electrode such that it will enable us to identify electrophysiologically (based upon antidromic activation technique) individual hypoglossal motoneurons.

b. Give the interval(s) between successive surgeries, and the rationale for choosing the interval(s):
► An optimal interval between the 2 survival surgeries will be approximately 2-3 months apart. We need the cat to be completely recovered from the head implant surgery first, and it also becomes well adapted to the recording lab environment (as judged by normal cycling through sleep and waking states). To achieve both of these goals, it will take at least 2-3 months if not longer.

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

Surgery 1 ► **For chronic (survival) cat studies implanting head and neck electrodes**: cats are prepared for chronic recordings according to procedures that are described, in detail, in Morales and Chase,1981 (Brain Res 225:279-95), Soja et al.,1991 (J Neurosci 9:2804-11), Fung and Chase 2015 (Sleep 38:139-45). Specifically, cats will be pre-medicated by administering a short-acting anesthetic (ketamine, 8-12 mg/kg, i.m., 0.08-0.12 ml/kg), a tranquilizer (xylazine, 2 mg/kg, i.m., 0.1 ml/kg), analgesics (buprenorphine, 0.02 mg/Kg, s.c., 0.07 ml/kg; carprofen, 5 mg/kg, 0.2 ml, s.c.), an antibiotic (baytril, 5 mg/kg, s.c,, 0.1 ml/kg), a mucous suppressant (atropine sulphate, 0.04 mg/kg, i.m.,0.07 ml/kg), and an anti-edematic agent (dexamethasone, 0.5 mg/kg, i.m., 0.25 ml/kg). Once sedated, the glottis and the pharynx will be swabbed with lidocaine (2%) to inhibit the gag reflex during endotracheal intubation. The cephalic vein on the forelimb will be catheterized for i.v. infusion of sterile saline or injections of supplemental ketamine (when needed) during the surgery. Sterile ophthalmic ointment will be applied over both eyes before shaving the hair over the surgical areas (scalp and neck). With the animal placed in a David Kopf stereotaxic head holder, the scalp and neck will be scrubbed with 3 alternating applications of chlorhexidine (2%) and alcohol (70%). Surgical procedures will then be performed under the anesthetic (isoflurane, 2-4%, via inhalation). Surgical drapes will be placed to cover up the areas other than the scalp and neck regions. A mid-sagittal skin incision will be made on the scalp (from orbital to occipital bone rostrocaudally). With the skin retracted, small holes will be drilled to allow the placement of four cortical screw electrodes (bilaterally in the parietal bones; for EEG recording), and one ground screw electrode. The right frontal sinus will be exposed (for an area of 8 mm diameter) by rongeur to permit access to implant 2 screw electrodes for recordings of EOG. Two flexible wire electrodes will be implanted into the nuchal musculature for recording EMG activity. An access hole (2 mm in diameter) will be drilled at occipital bone for passage of glass microelectrodes for intracellular recordings from individual hypoglossal motoeneurons. This hole will be sealed and protected by a stainless steel recording chamber with a stainless steel screw closure (cap) which can be screwed on and off of a "finish" on the top of the chamber. The recording chamber will be bonded to the cranium using sterile acrylic cement. All electrical leads from individual electrodes will be soldered to a Winchester-connector (head plug). Two nylon tubes which are instrumented to fit with the stereotaxic head restraining device will be aligned adjacent to the Winchester-connector. The connector and head restraining tubes will be bonded to the calvarium with sterile acrylic cement. The skin on the neck and around the base of the head implant will be sutured using Ethilon monofilament nylon. Post-operational care includes daily injections of Baytril (to prevent infection), Buprenorphine (12 hrs apart) and Carprofen (every 24 hrs) as analgesics for 3 days. The sutures will be removed in 7 -10 days after surgery.

Surgery 2 ► **For chronic (survival) cat studies implanting the hypoglossal nerve cuff electrode**: After a 2-week period, the animals are placed in a recording apparatus in order to adapt them to the experimental conditions. The adaptation period lasts 1-2 months, and is completed when animals exhibit multiple spontaneous cycles of sleep and wakefulness during individual recording sessions (4 hrs per day, 3 days per week). A second surgery will be performed to implant a hypoglossal nerve cuff electrode and 2 flexible EMG wire electrodes to the tongue (genioglossal) muscle. Standard preparation procedures for aseptic surgery as described above will be followed. Under isoflurane anesthesia, the skin of the chin will be incised (3 cm) for implanting a cuff electrode (for stimulation and recording purposes) to the hypoglossal nerve and a paired flexible EMG electrode wires implanted to the tongue (genioglossal) muscle. The flexible leads of the electrodes will be tunneled subcutaneously behind the ear and will be externalized dorsally adjacent to the acrylic head implant. The leads will be cemented to the head implant. Skin wounds will be sutured using Ethilon monofilament nylon. Routine post-operational care will be followed and the sutures will be removed in 7 -10 days after surgery.

Surgery 3 ► **For acute (non-survival) cat studies**: cats will be first anesthetized with Isoflurane (2-4%). Surgical anesthesia is induced and maintained with isoflurane initially by inhalation via a mask and subsequently via an endotracheal tube. After the animal is sedated, the hair on the ears will be

removed with clippers for the purpose of attaching a micro-sensor to a SurgiVet pulse oximeter. Hair on the scalp and submandibular-neck regions will be removed with clippers. These areas will be scrubbed with 3 alternating applications of chlorhexidine (2%) and alcohol (70%). The skin of the ventral head and neck region will be incised and the underlying muscles will be retracted to expose the trachea and the external jugular vein. An endotracheal tube will be inserted into the trachea. Following tracheotomy, the tracheal tube will be connected to the ventilation system for continual administration of isoflurane. The external jugular vein will be catheterized. A cuff electrode will be implanted to the right hypoglossal nerve proximal to the bifurcation to medial and lateral branches of the hypoglossal nerve. Skin wound will be closed with wound clips. A midline incision will be made in the skin over the calvarium; the skin and muscles will be retracted. A small hole (approximately 2mm) will be made in the bone with a dental drill over the occipital bone in order to provide access for glass microelectrodes for recording intracellularly from hypoglossal motoneurons. Another access hole will be drilled over the parietal bone for stereotaxic injection of carbachol, via a Hamilton microsyringe, to the pontine reticular formation (to induce REM sleep). Upon completion of these surgical procedures, the anesthetic agent will be switched to alpha-chloralose (120 mg/kg loading dose; 60 mg/kg maintenance dose, i.v.). During the entire experiment, the vital signs of the animal, which include oxygen saturation levels, cardiac and respiratory activity (monitored with a SurgiVet pulse oximeter) and rectal temperature will be continuously monitored. At the end of the experimental procedures, the animals are euthanized using Pentobarbital (100 mg/kg, i.v.). A thoracotomy (see below) will be performed to expose the heart for intracardial perfusion with fixative. The brain will be removed post-mortem for histology.

Surgery 4 ►► **Thoracotomy for perfusion (cat)**: Under deep anesthesia using pentobarbital (100 mg/kg, i.v.), the animal will be placed on its back and the four limbs tethered to a perfusion table. Hair over the thorax will be shaved with a hair clipper. The level of anesthesia will be monitored by the corneal reflex and toe-pinch withdrawal reflex. Once the breathing and heartbeat stop, a deep bilateral incision to the thorax will be made with a scalpel. The rib cage will then be retracted forward towards the head direction. The pericardial membrane will be cut to expose the heart. Following a cut to the right atrium (to allow the outflow of blood and perfusate), the left ventricle will be cut to enable the insertion of a perfusion guide tube to the ascending aorta. Once in position, the guide tube will then be secured using a Babcock clamp and the perfusion can begin. Blood will be rinse off the animal's body by perfusing with 0.9% saline. This is followed by perfusing with a fixative of 10% formaldehyde. The brain will be removed for histology confirmation of electrode placements. The carcass will be double bagged and taken to freezer room in VMU and the perfusate will be drained into a biohazard-labeled container for proper disposal.

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

Name	Surgery #(s) (see Item 1)	Role in Surgery			
		Surgeon	Assistant	Manage Anesthesia	Other (describe)

[redacted]	1,2,3,4	x			
[redacted]	1,2,3,4		x	x	

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

Building	Room Number	Surgery #(s) (see Item 1)	Type of Space		
			Dedicated Surgical Facility	Other Dedicated Surgical Space	Other Space not Dedicated to Surgery
[redacted]	VMU Surgical room	1	x	( )*	( )*
[redacted]	VMU Surgical room	2	x	( )*	( )*
[redacted]	[redacted]	3	x	( )*	( )*
[redacted]	VMU Room[redacted]	4	x	( )*	( )*

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

5. **Pre-operative protocol.**

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

Surgery #(s) (see Item 1)	Fast (Specify Duration)	Withhold Water (Specify Duration)	Place Intravenous Catheter(s) (Specify Site(s))	Other – Describe
1	(x) -- 12 hrs	( ) --	(x) -- cephalic vein	( ) --
2	(x) -- 12 hrs	( ) --	(x) -- cephalic vein	( ) --
3	( ) --	( ) --	(x) -- jugular vein	( ) --

4	( )--	( )--	(x) --cephalic vein	( )--

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

Agent	Surgery #(s) (see Item 1)	Dose (mg/kg) & volume (ml)	Route of administration	Frequency of administration (e.g., times/day)	Pre-operative period of treatment (e.g., immediate, or # of days)
Xylazine	1, 2	2mg/kg, 0.1ml/kg	i.m.	once	immediate
Buprenorphine	1, 2	0.02mg/kg, 0.07ml/kg	s.c.	2 times/day	3 days
Baytril	1, 2	5mg/kg, 0.1ml/kg	s.c.	1 time/day	3 days
Atropine sulphate	1, 2	0.04mg/kg, 0.07ml/kg	i.m.	once	immediate
Dexamethasone	1, 2	0.5mg/kg, 0.25ml/kg	i.m.	once	immediate
Ketamine	1, 2	8-12mg/kg, 0.08-0.12 ml/kg	i.m.	once	immediate
Carprofen	1, 2	5 mg/kg, 0.2 ml	s.c.	1 time/day	3 days

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

Surgery 1 ▶ **Head implant surgery**: The animals will be pre-medicated as described above. Once sedated, the glottis and the pharynx will be swabbed with lidocaine (2%) to facilitate the endotracheal intubation. Sterile ophthalmic ointment will be applied over both eyes before shaving the hair. Hair will be removed on the scalp and the neck with clippers. With the animal placed in a David Kopf stereotaxic head holder, the scalp overlying the parietal bones and the contiguous region of the neck will be scrubbed with 3 alternating applications of betadine and alcohol, followed by a final betadine solution spray. Surgical drapes will be placed to cover up the areas other than the zone of operation (scalp and neck regions).

Surgery 2 ▶ **Hypoglossal nerve cuff and tongue (genioglossal) EMG electrodes implant surgery**: Standard preparation procedures for premedication as described above will be followed. Once sedated, sterile ophthalmic ointment will be applied over both eyes before shaving the hair. Hair will be removed under the chin with clippers. With the animal placed in a supine position, the skin from the mandible to the throat will be scrubbed with 3 alternating applications of betadine and alcohol, followed by a final betadine solution spray. Surgical drapes will be placed to cover up the areas other than the zone of operation.

Surgery 3 ▶ **Acute (non-survival) surgery**: This requires no pre-medication procedures. General anesthesia is induced and maintained with isoflurane (2-4%) by inhalation via a mask initially and

subsequently via an endotracheal tube. After the animal is sedated, the hair on the scalp, the ears, and submandibular-neck regions will be shaved with hair clippers. These areas will be scrubbed with 3 alternating applications of chlorhexidine (2%) and alcohol (70%). The skin of the ventral head and neck region will be incised and the underlying muscles will be retracted to expose the trachea and the external jugular vein. An endotracheal tube will be inserted into the trachea. Following tracheotomy, the tracheal tube will be connected to the ventilation system for continual administration of isoflurane. The external jugular vein will be catheterized. A cuff electrode will be implanted to the right hypoglossal nerve proximal to the bifurcation to medial and lateral branches of the hypoglossal nerve. Skin wound will be closed with wound clips. A midline incision will be made in the skin over the calvarium. A small hole (approximately 2 mm) will be made in the bone with a dental drill over the occipital bone in order to provide access for glass microelectrodes for recording intracellularly from hypoglossal motoneurons. Another access hole will be drilled over the parietal bone for stereotaxic injection of carbachol, via a Hamilton microsyringe, to the pontine reticular formation (to induce REM sleep). Upon completion of these surgical procedures, isofluane will be discontinued and the anesthetic will be maintained using alpha-chloralose (initial dose 120 mg/kg, i.v.; supplemental dose 60 mg/kg, i.v.). The vital signs of the animal (oxygen saturation level and heart rate) will be monitored using a SurgiVet oximeter and rectal temperature will be monitored continuously throughout the experiment.

Surgery 4 ▶ **Thoracotomy (terminal) surgery**: The animal will be euthanized using Pentobarbital (100 mg/kg, i.v.) prior to perfusion.

6. **Intra-operative management.**

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

NOTE: If saline is being administered, it must be warmed to body temperature first.

Agent	Paralytic*	Surgery #(s) (see Item 1)	Dose (mg/kg) & volume (ml)	Route of administration	Frequency of dosing
Sterile saline (0.9%)	( )*	1,2,3	4 ml/kg, 12 ml	i.v.	Slow drips during surgery
Pentobarbital	( )*	4	100 mg/kg, 1 ml	i.v.	Once
	( )*				

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

b. **Intra-operative physical support.** For each surgery, describe any physical support that will be provided for the animals during surgery (e.g., warming, cushioning, etc.).
▶ A water-circulating heating pad will be used to maintain the core temperature during surgery.

c. **Intra-operative monitoring.** Describe the methods that will be used to monitor and respond to changes in the state of anesthesia and the general well-being of the animal during surgery.
► The depth of anesthesia of the animal will be monitored using the SurgiVet Pulse-oximeter. Readings of the vital signs (rectal temperature, pulse rate, breathing frequency) will be recorded on a surgical log sheet at 5-minute intervals.

7. **Survival surgery considerations.** For each survival surgical procedure indicated in Item 1 and described in Item 2, complete Items 7.a. – 7.g.

a. Complete the table below for each survival surgery listed in Item 1, above.

Surgery # (see Item 1)	Survival Period	Measures for Maintaining Sterility							
		Sterile Instruments	Surgical Cap	Sterile Gloves	Surgical Scrub	Sterile Drapes	Sterile Gown	Face Mask	Other*
1	2-4 months	x	x	x	x	x	x	x	(x)*
2	2-4 months	x	x	x	x	x	x	x	(x)*
3									( )*
4									( )*

* Describe any "other" measures to be taken to maintain sterility during surgery.
► All cortical screw, EMG wire and hypoglossal nerve cuff electrodes with leads that are implanted to the animal will be autoclaved prior to implantation surgery.

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

Surgery 1 ► Heating pad and blanket will be used to keep the animal warm.

Surgery 2 ► Heating pad and blanket will be used to keep the animal warm.

Surgery 3 ►

Surgery 4 ►

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

Surgery # (see Item 1)	Agent*	Dose (mg/kg) & Volume (ml)	Route of Administration	Frequency of Dosing (e.g., times/day)	Period of treatment (e.g. days)
1	Buprenorphine	0.02mg/kg, 0.07ml/kg	s.c.	2 times/day	3 days

1	carprofen	5 mg/kg, 0.2 ml	s.c.	1 time/day	3 days
2	Buprenorphine	0.02mg/kg, 0.07ml/kg	s.c.	2 times/day	3 days
2	carprofen	5 mg/kg, 0.2 ml	s.c.	1 time/day	3 days
3	none				
4	none				

*For each surgery for which NO post-operative analgesic will be provided, enter "none" in the "Agent" column, and explain here why this is justified:
► For surgery #3, it is an acute, terminal procedure and therefore no post-operative analgesic is necessary.

For surgery #4, the perfusion step is also an acute, terminal procedure and therefore no post-operative analgesic is necessary.

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

Surgery # (see Item 1)	Medication	Dose (mg/kg) & Volume (ml)	Route of Administration	Frequency of dosing (e.g. times/day)	Period of treatment (e.g. days)
1	Baytril	5mg/kg, 0.1ml/kg	s.c.	1 time/day	3 days
2	Baytril	5mg/kg, 0.1ml/kg	s.c.	1 time/day	3 days
3					
4					

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

(1) Immediate post-operative monitoring

Surgery # (see Item 1)	Frequency of Monitoring	Duration at this Frequency	Name(s) of Responsible Individual(s)
1	Continuous monitoring immediately after surgery until animal is sternal and conscious	From cessation of Isoflurane anesthesia to the point the animal is conscious and able to assume a sternal position instead of lying on its sides, vital signs will be documented every 15 minutes.	[redacted]

2	Continuous monitoring immediately after surgery until animal is sternal and conscious	From cessation of Isoflurane anesthesia to the point the animal is conscious and able to assume a sternal position instead of lying on its sides, vital signs will be documented every 15 minutes.	[redacted]
3			
4			

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

Surgery # (see Item 1)	Frequency of Monitoring	Duration at this Frequency	Name(s) of Responsible Individual(s)
1	Twice daily for initial 3 days postsurgery	20-30 minutes	[redacted]
2	Twice daily for initial 3 days postsurgery	20-30 minutes	[redacted]
3			
4			

f. Post-operative consequences and complications.

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

Surgery 1 ▶ Skin wounds will be swollen and sensitive. Buprenorphine and carprofen will be administered to lessen or eliminate postsurgical pain and/or distress. Baytril will be administered to prevent infection of the animal. Softened food will be fed to the animal over the first few days postsurgery.

Surgery 2 ▶ Same as listed in Surgery 1 above.

Surgery 3 ▶

Surgery 4 ▶

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

Surgery 1 ▶ Continual depression, loss of appetite, significant weight loss (over 10%), aggression, neurological signs (abnormal gait, cerebellar signs of motor deficit (e.g., ataxia and dysmetria),

lethargy), abnormal phase-switching of sleep-wake cycles, failure to groom, illness refractory to veterinary intervention, wound dehiscence, and dislodgement of head implant.

Surgery 2 ▶ Same as listed in Surgery 1 above.

Surgery 3 ▶

Surgery 4 ▶

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

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

Surgery # (see Item 1)	Location of Records	Name(s) of Individual(s) Responsible for Maintaining Written Records	Research Personnel	Veterinary Staff
1	[redacted] Bldg [redacted] Room [redacted] and [redacted] Bldg [redacted] Room [redacted]	[redacted]	x	
2	[redacted] Bldg [redacted] Room [redacted] and [redacted] Bldg [redacted] Room [redacted]	[redacted]	x	
3	[redacted] Bldg [redacted] Room [redacted] and [redacted] Bldg [redacted] Room [redacted]	[redacted]	x	
4	[redacted] Bldg [redacted] Room [redacted] and [redacted] Bldg [redacted] Room [redacted]	[redacted]	x	

8. **Certification.** The PI must sign the certification statement in Item Z.5 of the main body of the ACORP.

Last Name of PI ▶
Protocol No. Assigned by the IACUC ▶
Official Date of Approval ▶

ACORP APPENDIX 6

SPECIAL HUSBANDRY AND PROCEDURES
VERSION 4 v2 6-17-2015

See ACORP App. 6 Instructions, for more detailed explanations of the information requested.

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

Special Procedure		Features							
Number	Brief Description	Special Husbandry	Restraint	Noxious Stimuli	Exercise	Behavioral Work	Irradiation	Imaging	Other**
1	Head and body restraint for chronic cat studies		x						
2	Electrophysiology study in sleeping cats		x						x
3	Electrophysiology study in sleeping cats under hypoxic condition		x						x
4	Acute electrophysiological recording								x

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

**Describe any "Other" features that are involved.
▶ We will perform intracellular recording from individual hypoglossal motoneurons in order to determine the effects of neurotransmitter antagonists on cellular excitability changes during naturally-occurring and carbachol-induced REM sleep states under normoxic and hypoxic conditions.

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

Special Procedure 1 ▶ Following a full recovery (10-14 days) from the head implant surgery, each cat will be placed within a body restraining bag. The restraining device on the head implant will then be connected to the head pins of the David Kopf chronic cat stereotaxic head holder. The cat will be accompanied by the research staff during each period of the adaptation to the stereotaxic equipment in the lab. The cat will be restrained for an incremental, adaptation period from 15 min to 2 hrs at the initial phase until up to 4 hrs per day (for 3 days per week) when full adaptation is achieved. The latter phase will be indicated by behavioral (sitting still with minimal body movements) and polysomnographic signs of normal cycling of sleep and wakefulness stages (approximately one REM sleep episode per 45 minutes during each 4- hrs recording session) with the head and body restrained.

Special Procedure 2 ► When the chronically instrumented animal (i.e., with head and nerve cuff implants) becomes fully adapted to head and body restraints over a period of 2-3 months, recording sessions (4 hrs per day, 3 days per week) will begin. We will record intracellularly from hypoglossal motoneurons throughout the sleep-waking cycles. This recording procedure will be performed in conjunction with monitoring the states of consciousness (waking, NREM sleep, REM sleep) based on standard polysomnographic (EEG, EOG, PGO, and EMG) criteria of the restrained cat. A glass microelectrdoe will be advanced stereotaxically, using a David Kopf Hydraulic Microdrive, to the hypoglossal nucleus. Search stimulus will be provided by stimulating the hypoglossal nerve at low intensities (0.2 to 5V, 0.2 msec duration) while monitoring the evoked antidromic field potential. Upon successful impalement of an electrophysiologocal identified hypoglossal motoneuron, baseline excitability indices (e.g., membrane potential, threshold of discharging action potentials) will be recorded first, followed by changes produced by test chemicals applied e.g., via iontophoresis. Similar control-postdrug paradigm will be used to record the cell's excitability changes during different states of waking, NREM and REM sleep under normoxic condition.

Special Procedure 3 ► All surgical, adaptation, recording and drug administration procedures are the same as described in Special Procedure 2 above. The only change is the experimental (hypoxia) condition under which pre- and post-drug data acquisition will be carried out. Specifically, a breathing mask designed for cats will be positioned in close proximity to the animal's nose and mouth. The animal will be subjected to breathing under a hypoxic condition by replacing the normal air supply (21% oxygen in air; oxygen saturation in blood or SpO2 = 100%) with one that contains a lower oxygen level (10% oxygen in air; oxygen saturation in blood or SpO2 = 75%). The SpO2 levels will be continuously monitored using the SurgiVet Pulse oximeter with the sensor attached to the ear of the animal. The hypoxic condition will last for approximately 10-15 minutes during REM sleep and normal air will be re-introduced to the animal once the animal awakens. This hypoxic program will only be activated during the REM sleep stage of the cat which last for a maximal duration of 15 minutes.

Special Procedure 4 ► For acute electrophysiological recording, all surgical procedures will be performed under isoflurane anesthesia. Briefly, the animal will be tracheotomised for pump-ventilation and the external jugular vein cannulated for drug injection. A cuff electrode will be implanted to the hypoglossal nerve for stimulation in order to identify electrophysiologically the hypoglossal motoneurons. Wound clips will be used to close the skin of the incised ventral neck area. The animal will be placed onto a stereotaxic instrument using ear bars, orbital and mouth clamps. A skin incision will be made to expose the dorsal calvarium. Small (5mm diameter) holes will be drilled in the calvarium for stereotaxic placements of (1) glass microelectrode to record intracellularly from the hypoglossal motoneurons, (2) a Hamilton micro-syringe needle aiming stereotaxically at the nucleus pontis oralis (for injection of carbachol to induce REM sleep). Once these surgical procedures are finished, the isoflurane will be stopped and replaced by an injectable anesthetic (alpha-chloralose) which is proven to be compatible with the carbachol-induced REM sleep study. Test drugs will be applied by e.g., iontophoresis, micropressure injection, or reverse microdialysis to the hypoglossal nucleus while intracellular data will be collected pre- and post drug at baseline (pre-carbachol) and carbachol-REM sleep states (post-carbachol).

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

Special Procedure 1 ► The head restraint is necessary for successful intracellular recording of hypoglossal motoneurons of the behaving cat throughout sleep and waking cycles. Accurate placement of the recording electrodes into the hypoglossal motoneurons can only be achieved by placing the head (and the brain) in a stereotaxic head-holder (similar to human patients undergoing any stereotactic neurosurgery). Our research team is one of a handful in the world that has the unique experience

and success in conducting intracellular studies in chronic cats. Our experience is that once the cat is adapted to the head restraint over a period of approximately 2-3 months, the animal subject shows no sign of distress during each experiment that lasts for 4 hrs. To reward the animal after each restraint conditioning session, we will play with the cat (e.g., using a laser pointer light to simulate novel moving objects around the cat), feed it with freeze-dried chicken breast as a treat, and watch it's gait and jumping movements in the laboratory. This play-time for the cat will take half an hr before we transport the cat in a pet carrying box covered with drape back to the vivarium.

Special Procedure 2 ▶ The electrophysiological (intracellular recording) study is the only direct measurement of a given motoneuron's level of activity at any given state of consciousness. The REM sleep-dependent reduction in the motoneuron's activity is important in explaining why the upper airway narrows and the airway resistance increases during REM sleep – these events could result in obstructive sleep apnea in pathological conditions. By using appropriate pharmacological (receptor) blockers while measuring the changes in cellular indices of excitability, we can determine the receptor mechanisms that are involved in REM sleep muscle atonia. The translational significance of our study will be to facilitate future therapeutic development of drugs that might relieve apnea-induced health risks.

Special Procedure 3 ▶ The experimental (hypoxic) condition is designed to mimic the human sleep apnea condition that occurs intermittently during REM sleep. Data collected will be important for the elucidation of a different set of neurotransmitters are involved in regulating the activity of hypoglossal motoneurons under hypoxic REM sleep as compared to other neurotransmitters that are specific for controlling the muscle atonia phenomenon during normal REM sleep.

Special Procedure 4 ▶ Because natural REM sleep lasts on average for 15 minutes in cats, the extended period (1 hr) of pharmacologically induced REM sleep (by carbachol) enables us to increase the yield of intracellular data such that we can compare the drug effects over repeated trials on multiple cells between the baseline (similar to non-REM sleep) and REM sleep states.

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

Procedure Number (see Item 1)	Responsible Individual(s)	
	Carrying Out Procedure	Monitoring the Animals
1	[redacted]	[redacted]
2	[redacted]	[redacted]
3	[redacted]	[redacted]
4	[redacted]	[redacted]

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

Procedure Number (see Item 1)	Expected Potential Pain and/or Distress			
	No	Yes		
		Description	To Be Relieved	Not to Be Relieved
1		Animal will show discomfort before adaptation is achieved but it will adjust to the head and body restraints and will sleep normally in the restrained condition	( )[a]	(x)[b]
2	x		( )[a]	( )[b]
3	x		( )[a]	( )[b]
4		Acute surgical procedures will induce pain	(x)[a]	( )[b]

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

Procedure Number (see Item 1)	Agent	Dose (mg/kg) & vol (ml)	Route of admin	Freq of admin (times/day)	Duration of admin (days post-procedure)
4	isoflurane	2-4 %	inhalation	Throughout the surgery	N/A
4	Alpha-chloralose	120 mg/kg loading, 60 mg/kg maintenance, 4 ml	i.v.	2-3 times	N/A
4	Pentobarbital for euthanasia	100 mg/kg, 1 ml	i.v.	1 time	N/A

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

Special Procedure 1 ► The cat will be restrained for an incremental, adaptation period from 15 min to 2 hrs at the initial phase until up to 4 hrs per day (3 days per week) when full adaptation is achieved. The entire adaptation phase will take 2-3 months for individual cats. We will accompany the cat during each adaptation period to enable the animal to overcome its anxiety of the laboratory environment and also it will become comfortable to be around with the researcher in the room. In case the animal urinates inside the body bag, we will dry and clean up its body and abort the adaptation process for the day. Upon full adaptation, the cat will show normal behavior of relaxed sitting when awake. It will show normal, multiple transitions throughout the sleep-waking cycle (approximately one REM sleep episode per 45 minutes during each 4 hrs recording session) with the head and body restrained. After each recording session, we will let the cat roam free within the laboratory room (with door closed and locked). The animal will have a 20-30 minutes period of play-time with pet toys, cotton balls, etc. We will use a laser pointer to simulate novel moving objects so the cat likes to pounce on or attempts to catch. We will reward the cat with freeze-dried chicken breast as a treat and water before returning it to its home cage.

Special Procedure 2 ► Same as in Special Procedure 1 above.

Special Procedure 3 ► Same as in Special Procedure 1 above.

Special Procedure 4 ►N/A

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

Special Procedure 1 ► In case of administering tranquilizer or anti-anxiety drugs to alleviate the head and body restraint, these drugs are usually long-acting (over days). The injected drugs will then interact with the baseline recordings of the motoneuron's activity at all states throughout the sleep-waking cycles. In order to avoid this prolonged drug effects on our intended drug-free cat study we adopt instead the restraint conditioning technique which has been proven to work with minimal or no obvious sign of distress from the head and body restrained cat. The conditioning procedure is gradual, with initially 15 min to 2 hrs up to 4 hr maximal daily (3 days per week) when fully adapted (over a period of 2-3 months). During the initial stage of adaptation process, we will minimize the distress of the cat by relieving the cat from the head restraint immediately when the cat shows persistent vocalization and excessive body movements inside the restraining bag. Usually the cat is easier to adjust to be placed inside a body bag (without the head-restraint) and shows no sign of distress. The head restraint will then be re-tried the next experiment day of the week until the animal is adjusted to the physical restraint of the head and the body.

It is important to note that the head-restrained preparation is a well-established and widely accepted model used by electrophysiologists, anatomists, behavioral scientists, physiologists, etc. in the field of neuroscience research (Vanini et al., J Neurosci. 2011 31:2649-56; Ttaepavarapruk et al., J Neurophysiol. 2008, 100: 598 – 608; Levine and Jacobs, J Neurosci. 1992, 12: 4037-4044; May et al., J Neurosci Methods. 1991, 40: 155-169; also see our publications below). It is also critical for us to utilize this preparation to obtain a stable and long-term intracellular recording during sleep and waking state in unanethetized, chronic cats and achieve our specific aims that are described in the grant. we have extensive experiences with this animal preparation (Drs. [redacted] and [redacted] have performed these types of experiment with survival, chronic animal preparations for more than 30 years and more than 20 years, respectively). we have published over 45 studies using this preparation (selected peer-reviewed publication: Fung and Chase, sleep. 2015, 38:139-46; Fung and Chase, J Sleep Res. 2014, 23:469-74; Xi and Chase, Sleep. 2010, 33:1236-43; Xi and Chase, Neuroscience. 2006,140:335-42; Xi et al., J Neurosci. 2004, 24:10670-8; Xi et al., J Neurophysiol. 2002, 87:2880-8; Xi et al., Brain Res. 2001, 901:259-64; Chase et al., J Neurosci. 1989, 9:743-51; Chase and Morales, Science. 1983, 221:1195-8; Chase et al., Exp Neurol. 1984, 84:364-73; Morales and Chase, Exp Neurol. 1982, 78:471-6; Chase et al., Exp Neurol. 1981, 71:226-33; Morales and Chase, Exp Neurol. 1978, 62:821-7; Nakamura et al., Science. 1978, 199:204-7).

Special Procedure 2 ► Same as in Special Procedure 1 above.

Special Procedure 3 ► Same as in Special Procedure 1 above.

Special Procedure 4 ►N/A

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

Procedure Number (see Item 1)	Monitoring Methods	Endpoint Criteria
1	Continual monitoring for signs of distress (persistent vocalization, excessive body movements) and polysomnography (EEG desynchrony and maximal EMG levels, failure to fall asleep within the first 2 hrs being restrained).	continual depression, loss of appetite, significant weight loss (over 10%), aggression, neurological signs (abnormal gait, cerebellar signs of motor deficit (e.g., ataxia and dysmetria), lethargy, abnormal phase-switching of sleep-wake cycles, failure to groom, illness refractory to veterinary intervention, wound dehiscence, and dislodgement of head implant.
2	Same as above	Same as above
3	Same as above	Same as above
4	Throughout the acute study under isoflurane and alpha-chloralose anesthesia, we will monitor the depth of anesthesia based on vital signs of heart rate, respiration rate and core temperature using the SurgiVet Pulsimeter technique. Toe-pinch withdrawal reflex will also be tested to affirm the adequacy of anesthesia.	If unforeseen deterioration signs of cardiopulmonary function occurs (as judged by vital signs) or failure of carbachol to induced REM sleep occur, the acute study will be terminated and the animal euthanized

5. **Animal restraint.** Complete the table below for each special procedure in which animals are put under restraint for more than 15 minutes.

Prolonged Restraint (defined by the IACUC as over 15 minutes)						
Method of restraint	**Species**	**Approved Duration of Restraint**	**Method of acclimatization**	**Monitoring**	**Criteria for removing animals that do not adapt or acclimate**	**Provision of veterinary care for animals with adverse clinical consequences**
Head and body restraint (Head restrained to the David Kopf stereotaxic head-holder; body placed	cat	1 to 4 hrs	We adopt an incremental, adaptation that lasts minimally (15 min-2 hrs) at the beginning several days until up to 4 hrs per day (3 days per week) when full adaptation	Continual monitoring by direct observation and polysomnography	Presence of signs of distress (persistent vocalization, excessive body movements) and behavioral agitation (EEG desynchrony	TBD by VMO

inside a body restraining bag)			is achieved. A reward strategy of play-time and cat treat at end of each adaptation day will enable the animal to become relaxed while being restrained and accompanied by the researcher within the laboratory environment.		and maximal EMG levels, failure to fall asleep within the first 2 hrs of being restrained).	