

DEPARTMENT OF
VETERANS AFFAIRS

Memorandum

Date:

From: [REDACTED] Coordinator, IACUC

Subj: **Approval Notice**

ACORP #: 14-007-DG-17-002

Titled: High Frequency Spinal Cord Stimulation to Restore Cough

To:

Your new ACORP was reviewed by the Louis Stokes Cleveland DVA Medical Center Institutional Animal Care and Use Committee and was approved by designated committee review on [REDACTED]

A copy of this form will be filed with your ACORP in the Research Office. If you have any questions, please contact [REDACTED] IACUC Coordinator at [REDACTED] or via email at [REDACTED]

[REDACTED]
IACUC Coordinator

The Louis Stokes Cleveland DVA Medical Center Institutional Animal Care and Use Committee is accredited by the American Association for Accreditation of Laboratory Animal Care International (AAALAC) and operates under the Public Health Service (PHS) Assurance number A3928-01.

Protocol #: 14-007-DG-17-002

Protocol Title: High Frequency spinal Cord Stimulation to Restore Cough

Principal Investigator [REDACTED]

Investigator Assurances

I agree to abide by the policies of the Louis Stokes Cleveland DVA Medical Center Institutional Animal Care and Use Committee (IACUC) and all applicable federal regulations.

I will adhere to the protocol as described and as modified.

I will submit any modifications of the protocol to the IACUC for review and approval before initiating them.

I will notify the IACUC of changes in the location of the animal research.

I will assist the IACUC in verifying compliance with the regulations.

I will notify the IACUC of any unexpected results that affect the welfare of the animals. I will report any unanticipated pain or distress, morbidity, or mortality to the attending veterinarian and the IACUC.

I understand and agree that emergency veterinary care, including euthanasia, will be administered to animals exhibiting unbearable pain distress or illness. Prior to any emergency treatment, the veterinary staff will make every effort to contact my representative or me.

I declare that all experiments involving live animals will be performed under my supervision or that of another qualified scientist. All other personnel involved in animal use in this project have been or will be trained in proper procedures relevant to this protocol, including but not limited to animal handling, administration of anesthetics and analgesics, aseptic technique, postoperative monitoring, and euthanasia. I will notify the IACUC when new employees are hired and will certify when their training to perform the relevant experimental procedures on live animals is complete.

I declare that the information provided in this protocol is accurate. If this project is to be funded, I certify that this protocol accurately describes all procedures in which I intend to involve laboratory animal subjects.

I declare that the studies described here do not unnecessarily duplicate previous

[REDACTED]

[REDACTED]

Date

ANIMAL COMPONENT OF RESEARCH PROTOCOL (ACORP)
Main Body
VERSION 4

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]
2. VA Station Name (City) and 3-Digit Station Number ▶ Cleveland, 541-Cleveland
3. Protocol Title ▶ High Frequency Spinal Cord Stimulation to Restore Cough
4. Animal Species covered by this ACORP ▶ Dogs
5. Funding Source(s). Check each source that applies:
 - ▶ (X) Department of Veterans Affairs.
 - ▶ () 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, identify the project:
 - (1) Title of project ▶ N/A
 - (2) If approved by the R&D Committee, give the date of approval ▶ [REDACTED]
 - 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
 - ▶ ACORP#: 14-007-DG-14-004

- (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

Animal Species	Previously approved ACORP#: 14-007-DG-14-004				New ACORP
	Total number of animals approved	Number of animals used to date	Number of animals to be used under current ACORP	Number of animals remained	Total number of animals requested
Dogs	80	24	5	51	51

- (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.

► Under previous animal protocol (ACORP#: 14-007-DG-14-004) we accomplished:

Experiment #1a: Determination of optimal stimulus paradigm and electrode location during monopolar HF-SCS (spinal cord intact).

Experiment #1c: Evaluation of expiratory muscle activation via bipolar HF-SCS.

Experiment #1d: Evaluation of non-expiratory muscle contraction during HF-SCS.

and partially:

Experiment #2c: Localization of spinal cord pathways mediating activation of the expiratory muscles during HF-SCS.

Timetable:

		Previously approved ACORP#: 14-007-DG- 14-004	New ACORP
Objective 1			
	Exp. 1a		
	Exp. 1b		
	Exp. 1c		
	Exp. 1d		
Objective 2			
	Exp. 2a		
	Exp. 2b		
	Exp. 2c	Partially done	
	Exp. 2d		
Objective 3			
	Exp. 3a		
	Exp. 3b		

- c. List any other relevant previously approved animal use protocols (copy the lines below as needed for each protocol listed).

- (1) Title of other protocol ► Electrical stimulation of the respiratory muscles in a pig model
IACUC approval number of other protocol ► 2012-0127
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 ► IACUC-CWRU
- (2) Title of other protocol ► Electrical stimulation of the respiratory muscles in dogs
IACUC approval number of other protocol ► 2013-0028
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 ► IACUC-CWRU
- (3) Title of other protocol ► Electrical stimulation of the respiratory muscles
IACUC approval number of other protocol ► 2010-0041
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 ► IACUC-CWRU
- (4) Title of other protocol ► Electrical stimulation of the respiratory muscles
IACUC approval number of other protocol ► 2007-0064
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 ► IACUC-CWRU
- (5) Title of other protocol ► Electrical stimulation of the respiratory muscles
IACUC approval number of other protocol ► 2008-0106
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 ► IACUC-CWRU
- (6) Title of other protocol ► Expiratory muscle activation to produce cough
IACUC approval number of other protocol ► 2004-0040
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 ► IACUC-CWRU
- (7) Title of other protocol ► Evaluation of lower thoracic spinal cord stimulation on expiratory muscle function
IACUC approval number of other protocol ► 960068
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 ► IACUC-CWRU
- (8) Title of other protocol ► Evaluation of lower thoracic spinal cord stimulation on expiratory muscle function
IACUC approval number of other protocol ► 950080
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 ► IACUC-CWRU
- (9) Title of other protocol ► High Frequency Spinal Cord Stimulation to Restore Cough
IACUC approval number of other protocol ► VA Protocol #: 14-007-DG-14-004
- (10) Title of other protocol ► Electrical stimulation of the respiratory muscles in dogs
IACUC approval number of other protocol ► 2016-0038

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 ▶ IACUC-CWRU

1. 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 people and/or other animals, or otherwise to serve the good of society, and explain how these benefits outweigh the pain or distress that may be caused in the animals that are to be used for this protocol.

- ▶ Patients with spinal cord injury (SCI) suffer from a high incidence of recurrent respiratory tract infections including bronchitis and pneumonia due to their inability to cough and clear secretions. Moreover, diseases of the respiratory system are the leading cause of death in this patient population. These individuals lack an effective cough mechanism since their injury results in paralysis of virtually all of the muscles of expiration. Since the spinal cord below the level of injury is intact in most patients, the neuromuscular system innervating the expiratory muscles is functional. It is possible, therefore, to electrically activate these muscles to restore an effective cough. In recent preliminary animal studies, however, we have demonstrated that large positive airway pressures, comparable to those achieved with higher stimulus amplitudes, can also be achieved with very low stimulus amplitudes (1-2mA) and high stimulus frequencies ($\geq 300\text{Hz}$). This method, therefore, could theoretically be applied in all study populations who would benefit from restoration of an effective cough, including stroke and amyotrophic lateral sclerosis (ALS). There is no precise threshold level of positive pressure generation above which an adequate cough is assured. However, based upon numerous previous clinical studies, positive airway pressures of $60\text{cmH}_2\text{O}$ and peak flow rates of 270L/min are considered effective in clearing secretions.¹⁻³ Moreover, in our previous clinical trial evaluating SCS to restore cough, our subjects also found that pressures in this range were usually effective in clearing secretions⁴⁻⁸.

The major thrust of the proposed studies, therefore, is to resolve important basic science issues and further develop this technique in an animal model in advance of clinical trials.

This is a nonsurvival dog study which limits pain/distress to the experimental animals.

References:

1. Birnkrant DJ, Panitch HB, Benditt JO, Boitano LJ, Carter ER, Cwik VA, Finder JD, Iannaccone ST, Jacobson LE, Kohn GL, Motoyama EK, Moxley RT, Schroth MK, Sharma GD, Sussman MD. American College of Chest Physicians consensus statement on the respiratory and related management of patients with Duchenne muscular dystrophy undergoing anesthesia or sedation. *Chest*. 132:1977-1986, 2007. PMID: 18079231.
2. Senent C, Golmard JL, Salachas F, Chiner E, Morelot-Panzini C, Meninger V, Lamouroux C, Similowski T, Gonzalez-Bermejo J. A comparison of assisted cough techniques in stable patients with severe respiratory

insufficiency due to amyotrophic lateral sclerosis. Amyotroph Lateral Scler 12:26-32, 2011. PMID: 21091398.

3. Bach JR, Ishikawa Y, Kim H. Prevention of pulmonary morbidity for patients with Duchenne muscular dystrophy. Chest 112:1024-1028, 1997. PMID: 9377912.
4. DiMarco AF, Geertman RT, Tabbaa K, Polito RR, Kowalski KE. Economic consequences of an implanted neuroprosthesis in spinal cord injured subjects for restoration of an effective cough. Topics in Spinal Cord Injury Rehabilitation (in Press).
5. DiMarco AF, Kowalski KE, Geertman RT, Hromyak DR. Lower thoracic spinal cord stimulation to restore cough in patients with spinal cord injury: results of a National Institutes of Health-sponsored clinical trial. Part I: methodology and effectiveness of expiratory muscle activation. Arch Phys Med Rehabil 2009;90:717-725.
6. DiMarco AF, Kowalski KE, Geertman RT, Hromyak DR, Frost FS, Creasey GH, Nemunaitis GA. Lower thoracic spinal cord stimulation to restore cough in patients with spinal cord injury: results of a National Institutes of Health-sponsored clinical trial. Part II: clinical outcomes. Arch Phys Med Rehabil 2009;90:726-732.
7. DiMarco AF, Kowalski KE, Geertman RT, Hromyak DR. Spinal cord stimulation: a new method to produce cough in patients with spinal cord injury. Am J Respir Crit Care Med 2006;173:1386-1389.
8. DiMarco AF, Kowalski KE, Hromyak DR, Geertman RT. Long-term follow-up of spinal cord stimulation to restore cough in subjects with spinal cord injury. J Spinal Cord Med 2014;37:380-388.

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.

► Cough is an airway defensive reflex consisting of an inspiratory phase followed by a forced expiratory effort initially against a closed glottis, followed by active glottal opening and rapid expiratory flow. Lack of an effective cough may result in the frequent aspiration of airway secretions and foreign material, which could lead to serious recurrent respiratory tract infections. Many patients with spinal cord injury have paralysis of their expiratory muscles and, consequently, lack an effective cough. Cough effectiveness can be assessed by measurements of maximum airway pressure generation and maximum expiratory airflow rates. We expect that high frequency spinal cord stimulation (HF-SCS) is capable of activating the expiratory muscles to generate an effective cough. This technique has the potential to reduce the morbidity and mortality rates in certain patient populations that have an inadequate cough mechanism. To resolve important basic science issues and further develop this technique in advance of clinical trials, animal studies must be completed to accomplish the following objectives: I) determine the optimal stimulus parameters and electrode location that produce optimal activation of the expiratory muscles with the consequent development of large expiratory airway pressures and high peak expiratory airflow rates (the maximum flow of air during forced expiration) characteristic of an effective cough with minimal side effects; Maximal expiratory airway pressure is a well established measurement that is used to assess expiratory muscle strength, II) evaluate the mechanism by which the expiratory muscles are activated during HF-SCS, and III) evaluate the electric field distribution during HF-SCS and determine alternative electrode designs.

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

List of Abbreviations, Acronyms, and Symbols:**Abbreviations**

CO₂ – carbon dioxide
EMG – electromyogram
ENG – electroneurogram
FEM – finite element model
HF-SCS – high frequency spinal cord stimulation
ID – internal diameters
IV – Intravenous
Laminectomy – surgical procedure that removes a portion of the vertebral bone called the lamina
PB – pentobarbital
PCO₂ – partial pressure of carbon dioxide in the blood
Paraplegia – results when an injury to the spinal cord is below the first thoracic spinal nerve
PEF – Peak expiratory flow, also called peak expiratory flow rate (PEFR); maximum speed of expiration
PFF – peak firing frequencies
PO₂ – partial pressure of oxygen; reflects the amount of oxygen gas dissolved in the blood
Post mortem – Latin for "after death"
Quadriplegia (tetraplegia) refers to a spinal cord injury within the cervical sections of C1-C8.
SC – spinal cord
SCI – spinal cord injury
SCS – spinal cord stimulation
SE – standard error
SMU – single motor unit
T – thoracic spinal level
VC – ventral column of spinal cord

Symbols

Hz – Hertz; defined as one cycle per second
µm – micrometer
mA – milliamps
mm – millimeter
mg – milligram
min – minute
ms – millisecond
mL – milliliter
mmHg – millimeter of mercury
s – second

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

► If successful, this data will provide critically important information for future clinical trials of these techniques in a broad patient population with neuromuscular diseases. We plan to address these issues by performing several experiments which are organized within the three major objectives.

Objective I) determine the optimal stimulus paradigm and electrode location that results in optimal activation of the expiratory muscles with the consequent development of large positive airway pressures and high peak expiratory airflow rates characteristic of an effective cough,

Experiments accomplished under VA Protocol #: 14-007-DG-14-004

- #1a: Determination of optimal stimulus paradigm and electrode location during monopolar HF-SCS (spinal cord intact)
- #1c: Evaluation of expiratory muscle activation via bipolar HF-SCS
- #1d: Evaluation of non-expiratory muscle contraction during HF-SCS

Specific experiments remaining to address objective I:

- #1b: Assessment of global expiratory muscle force generation during monopolar HF-SCS (animals spinalized at different levels to mimic different levels of spinal cord injury)

Objective II) evaluate the mechanisms by which the expiratory muscles are activated during HF-SCS.

Specific Experiments to address Objective II:

- #2a and #2b: Identification of the pattern of expiratory muscle activation via electromyographic (EMG) recordings
- #2c: Localization of spinal cord pathways mediating activation of the expiratory muscles during HF-SCS
- #2d: Characterization of expiratory muscle activation via single motor unit (SMU) recordings

Objective III) Evaluate the electric field distribution during HF-SCS with the goal of evaluating alternative electrode designs.

Specific Experiments to address the objective II:

- #3a: Assessment of the distribution of the electric field during HF-SCS.

Studies will be performed on purpose-bred mongrel dogs. Prior to surgery, animals will undergo an overnight fast. All animals will be anesthetized initially with IV pentobarbital (PB), 25mg/kg. Supplemental anesthesia (3-5mg/kg PB) will be administered as required. Animals will be tracheostomized and intubated with a cuffed endotracheal tube (10mm ID) and placed on a mechanical ventilator. We have found that tracheostomy access allows for a more secure airway and more accurate measurements of airway pressure, peak expiratory airflow and volume, as there are often leaks around an oral endotracheal tube with this preparation, compared to direct tracheal intubation. Furthermore, these experiments are non-survival. Catheters will be placed in the femoral vein and artery for administration of intravenous fluids and medications and to monitor arterial blood pressure, respectively. A homeothermic blanket will be used to maintain body temperature at $38 \pm 0.5^\circ\text{C}$. End-tidal pCO_2 will be monitored with a rapidly responding CO_2 analyzer at the tracheal opening. Tracheal airway, gastric and esophageal pressures will be measured with a differential pressure transducer during airway occlusion; airflow, volume and muscle EMGs will be monitored and stored on a computer utilizing a data acquisition and analysis system (Spike 2 software).

Laminectomies will be performed at the lower thoracic spinal cord levels. Stimulating leads with 8 platinum-iridium disc electrodes (4mm) will be inserted epidurally and positioned on the dorsal or ventral surface of the spinal cord and advanced to the $\text{T}_5\text{-T}_8$ and to $\text{T}_9\text{-T}_{11}$ spinal cord regions to activate the expiratory muscles via spinal cord stimulation. Stimulation will be applied in intact animals, as well as varying models of spinal cord injury. While the animal is still able to breathe spontaneously (prior to high spinal cord injury that would abolish spontaneous inspiratory activity) measurements of spontaneous airway pressure, volume and airflow may be measured. Electrical stimulation will be applied with a square wave pulse stimulator (Model S88, Grass Technologies, US) over a wide range of stimulus parameters (0-15mA; 0-1kHz, 0-0.5ms pulse width). Electromyographic electrodes (bipolar stainless steel wires) will be placed into several respiratory and non-respiratory muscles to assess the level of their activation. Animal will be removed from the ventilator for brief

periods while electrical stimulation is applied. This will allow for the accurate measurement of airflow and airway pressures.

Optimal electrode location and stimulus parameters for maximum respiratory output while minimizing lower extremity movement will be determined. Studies will be performed to assess the pattern of expiratory muscle recruitment during HF-SCS applied at different spinal cord levels. Several designs of multi-contact stimulating electrodes will be positioned over the surface of the thoracic spinal cord. Recording electromyographic electrodes will be placed at several locations in the abdominal and internal intercostal muscles.

After completion of all experiments, animals will be euthanized with euthanasia solution without ever recovering from anesthesia (Euthasol 100 mg/kg given IV).

See below and ACORP App. 5 for more details.

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

	# of dog requested
Objective 1	
Exp. 1a	
Exp. 1b	10
Exp. 1c	
Exp. 1d	
Objective 2	
Exp. 2a	10
Exp. 2b	
Exp. 2c	1
Exp. 2d	10
Objective 3	
Exp. 3a and Exp. 3b	10
Total Number Requested	41
	Accomplished under the previously approved ACORP#: 14-007-DG-14-004

► The information to be acquired is directly applicable to human health and disease. To obtain statistically valid results, it is necessary to study 8-10 animals in each group of experiments for a maximum total of 41 animals (10 animals to meet Objective I, 21 animals to meet Objective II and 10 animals to meet Objective III).

Sample Size Calculations. Assuming a satisfactory analysis, 8 dogs will be evaluated in each group of experiments. This sample size is necessary to test the primary efficacy outcome hypotheses as estimated using the methods of Lenth.¹ Based on our existing data, a high percentage of dog can be expected to demonstrate satisfactory results. However, for small samples, a single dog could have a big impact on the size needed. Using an alpha of 0.05, a power of 90%, and assuming a normal distribution, the required sample size is 8. Based upon an assumed success rate of 80%, we are requesting 10 animals in each group of experiments. The number of animals requested is approximately a 20% increase over the numbers anticipated to be actually required for statistical conclusions. Additional animals are requested and will be used to

defray the anticipated non-success rate (muscle fatigue, difficulty to obtain data etc.). It must be pointed out that a 20% non-success rate in these complex experiments is moderate. The sample size of 8 successful dogs is actually required for statistical conclusions. Additional two animals are requested to cover the anticipated non-success rate. So, we are requesting minimum 8 and maximum 10 dogs for each experiment.

Reference

1. Lenth R V (2006-9). Java Applets for Power and Sample Size [Computer software]. Retrieved January 23, 2012 from <http://www.stat.uiowa.edu/~rlenth/Power>.

There is no alternative to animal models to evaluate this technique in a controlled fashion. We recognize the importance to collect the most data possible with each animal, not only for ethical reasons, but also due to the high cost of the animal and anesthesia. For that reason, we often combine experimental aims in a single animal. However, we have found that repeated stimulation of the expiratory muscles results in muscle fatigue and unreliable data points. For that reason, we are limited in the number of times that stimulation can be applied in a single animal during this short period of the experiment. Additionally, some of the experimental designs require different models of spinal cord injury (i.e. spinal cord sections of varying degrees and at various levels along the spinal cord), which cannot necessarily be achieved with one single animal. Whenever

possible then, experiments will be combined in a single animal (see Objective III; Experiments #3a and #3b). In all cases, the numbers of animals requested are those which are required to provide statistically conclusive results.

Adult mongrel dogs, weighing 15-25 kg of either sex, will be used, as they have a similar size spinal cord compared to humans allowing us to accurately evaluate the effects of stimulating electrodes differing in size and shape. Therefore, the efficacy of HF-SCS, the pattern of muscle recruitment and mechanism of HF-SCS should be applicable to humans. Additionally, extensive studies of thoracoabdominal mechanics and respiratory muscle activity have been performed on the dog model but not on pigs, sheep or other more socially acceptable animals. Furthermore, the respiratory neurons and spinal cord pathways in the dog model are comparable to those found in the human. We have many years of experience using the dog model, which has proved very useful for translational research with regards to the evaluation of electrical stimulation techniques to restore muscle function.

- 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.)

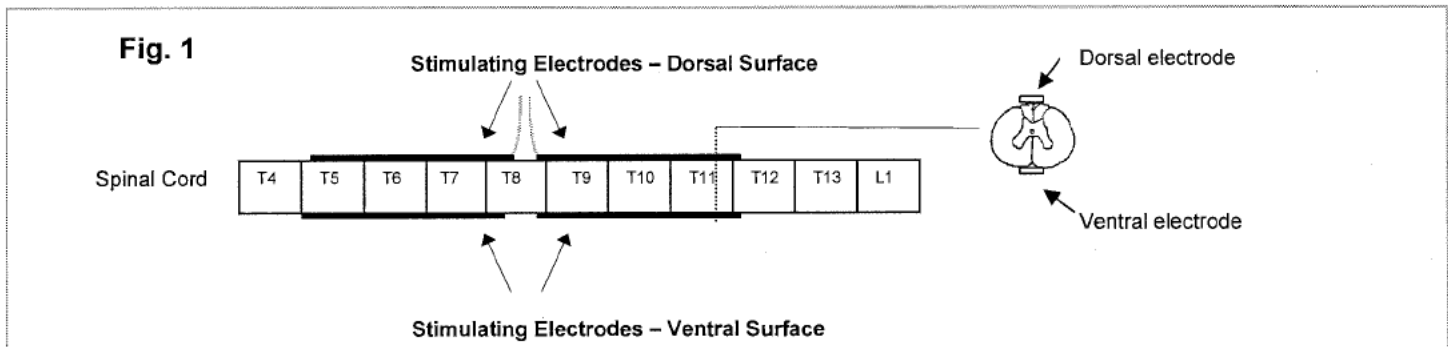
All experimental animals will undergo multiple surgical procedures including: tracheostomy, femoral cannulation, laminectomies and exposure of respiratory and non-respiratory muscles for EMG recordings. See ACORP App. 5 for more details.

► **OBJECTIVE I: Determine the optimal stimulus paradigm and electrode location that result in optimal activation of the expiratory muscles with the consequent development of large positive airway pressures and high peak expiratory airflow rates characteristic of an effective cough.**

While our preliminary studies suggest that monopolar HF-SCS applied on the dorsal epidural surface at the T₉ level results in the production of large positive airway pressures and peak airflow rates, it is possible that alternate electrode locations will result in similar output parameters but with even lower current requirements. It is also possible that similar output parameters can be achieved over one or more spinal levels. The results of the proposed studies will provide useful information concerning the specificity with which the stimulating electrode must be positioned and determination of the optimal stimulus paradigm.

Animal Preparation. Animals will be anesthetized initially with IV pentobarbital (PB), 25mg/kg. Supplemental anesthesia (3-5mg/kg PB) will be administered as required. Level of anesthesia will be monitored by pupillary responses to light, response to noxious stimuli, and corneal reflex. All animals will undergo a cervical tracheostomy and intubation with a cuffed endotracheal tube (10mm ID). Animals will be mechanically ventilated with a [REDACTED] pump ventilator ([REDACTED]). End-tidal CO₂ will be continuously monitored with a rapidly responding CO₂ analyzer ([REDACTED]). A catheter will be placed in the femoral artery to monitor blood pressure ([REDACTED]). Temperature will be maintained at 38° ± 0.5°C with a homeothermic blanket ([REDACTED]).

For HF-SCS studies, a laminectomy will be performed at the T₈ level; two strip electrodes, each with 8 disc contacts, will be inserted onto the dorsal surface of the spinal cord and advanced one to span the T₅-T₈ levels, and the second to span the T₉-T₁₁ spinal levels. Subsequently, the leads will be inserted onto the ventral surface and advanced to the same levels (Fig.1). Animals will be euthanized with a euthanasia solution at the conclusion of these studies.



Experiment # 1b: Assessment of global expiratory muscle force generation during monopolar HF-SCS (animals spinalized at different levels to mimic different levels of spinal cord injury). Initial studies will be performed at the optimal site(s) determined in Experiment #1a. Since this technique may ultimately be applied in patients with spinal cord injury, we plan to evaluate the influence of upper spinal and supraspinal centers on expiratory pressure generation during HF-SCS. We hypothesize that HF-SCS may have variable effects on pressure generation in patients with SCI depending upon the level of spinal cord injury.

Protocol. HF-SCS will be performed following sequential section of the spinal cord: at the C₁ (high tetraplegia with injury above the phrenic pool of motoneurons), C₈ (low tetraplegia with injury below the phrenic pool of motoneurons) and T₆ (paraplegia) spinal levels. Spinal section will be performed with watchmaker forceps. Complete section will be verified by lifting the hook across the area of transaction. After spinal cord section, studies will commence following stabilization of hemodynamic variables. In our experience, this typically occurs within 30-40min.

Experiment #1b will be performed in 8-10 animals.

OBJECTIVE II: Evaluation of the mechanism by which the expiratory muscles are activated during HF-SCS.

It is beyond the scope of this grant proposal to completely delineate all of the mechanisms by which HF-SCS results in activation of the expiratory muscles. However, we have outlined a number of experiments which relate to the practical application of this technique in humans.

Initially, the specific expiratory muscles activated during HF-SCS will be identified by assessment of the multi-unit EMG recordings of the internal intercostal, external and internal obliques and transversus abdominis muscles. Expiratory muscle activation will be further characterized by single motor unit (SMU) recordings. Studies will then be performed to determine the location of spinal cord pathways by which the motoneuron pools are activated by serial section of the spinal cord and spinal roots (Experiment #2a and #2b).

Animal Preparation. Initial animal preparations will be the same as that described in Objective I. Stimulating electrode(s) will be positioned at the optimal site for peak expiratory airflow and airway pressure generation, based upon the results of Objective I.

SPECIFIC EXPERIMENTS

Experiment #2a and #2b: Identification of the pattern of expiratory muscle activation via EMG recordings. In these experiments, the pattern of activation of the expiratory muscles during SCS will be

examined by analyses of their respective EMGs. EMG signals from different portions of the internal intercostal, external and internal obliques and transversus abdominis muscles will be recorded during LF-SCS and HF-SCS (see Fig. 8 in Preliminary Studies section). These studies will allow us to identify which expiratory muscles are activated during HF-SCS, determine their threshold for activation and latencies, and qualitatively compare their activation patterns. The results of this study will also allow us to roughly quantitate the relative participation of these muscles during HF-SCS. Moreover, we previously demonstrated that electrical stimulation with 15mA (50Hz) results in direct activation of motor roots within two to three segments of the stimulating electrode and more distal roots via spinal cord pathways. The purpose of these studies will be to assess the mechanism by which the spinal roots are activated during HF-SCS.

Protocol. Laminectomies will be performed at the T₈ level and between T₁₂ and L₂ spinal levels on one side of the animal to expose the spinal nerves. Recording electrodes will be positioned in the superior, upper, middle and lower portions of the external and internal obliques, transversus abdominis, and internal intercostal muscles. Electrodes will be placed following small skin incisions over the muscles of interest. The pattern of EMG activation of the various expiratory muscles will be recorded under several conditions. Supramaximal stimulus paradigms will be applied initially. Subsequently, a wide range of stimulus amplitudes (at optimal pulse width) at supramaximal frequencies will be applied. The threshold for activation and EMG latency of each of the expiratory muscles will be determined from this data. Subsequently, the laminectomy incision will be extended from the T₇ to the L₂ spinal level and spinal roots between T₇ and L₂ will be severed sequentially; all parameters will be reassessed following section of the roots at each level in one group of animals in the cephalad to caudal direction (Experiment #2a) and, in a second group of animals, in caudal to cephalad direction (Experiment #2b).

Experiments #2a and #2b will be performed in 16-20 animals.

Experiment #2c: Localization of spinal cord pathways mediating activation of the expiratory muscles during HF-SCS.

In this group of animals, the influence of spinal cord pathways mediating activity to more rostral and caudal spinal segments will be assessed by sequential spinal cord section (two to three segments from the stimulation electrode) at the T₆ and the T₁₁-T₁₂ spinal levels.

Protocol. With the animal in the prone posture, the relationship between stimulus intensity and stimulus frequency and pressure generation during HF-SCS will be determined. A stimulating electrode will be positioned on the dorsal epidural surface of the spinal cord at the optimal electrode site as determined in Objective #1 following a laminectomy incision extending between T₉ through T₁₂. Airway and gastric pressures will be monitored over a range of stimulus amplitudes (0-2mA) under control conditions (animal intact). Subsequently, the spinal cord at the T₁₁-T₁₂ level will be sequentially sectioned with watchmakers forceps. Repeat pressure measurements will be made, in separate trials, following sequential section of the a) dorsal columns, b) lateral funiculi, and c) ventral funiculi. For histological verification, section of the lateral funiculi will be performed 2-3mm below the dorsal column section; total section will be performed 2-3mm below the lateral funiculi section. Subsequently, sequential section at the T₆ spinal level will be performed. Spinal cord section will be confirmed histologically, post mortem. Blood pressure and heart rate will be carefully monitored following the sectioning procedures.

Assuming that activation of spinal cord pathways located within the dorsal columns play an important role in the activation of more caudal ventral roots (as our preliminary results suggest), the results of this experiment will help further characterize and identify these tracts. The dorsal columns contain cutaneous, joint and muscle primary afferent fibers. The largest proportion of fibers in the dorsal columns transmits cutaneous information. These afferents, as well as joint afferents, make connections with motoneurons, but with bi- or trisynaptic linkage. Group Ia afferents from muscle spindles, however, make monosynaptic connections with motoneurons. The dorsal columns also contain ascending fibers from nerve cells in the spinal gray matter

(postsynaptic dorsal column pathway). This system, however, has not been described in the dog or in humans. The differences in synaptic connections among the different afferent fibers present in the dorsal columns will be used to identify the specific pathways stimulated during thoracic HF-SCS.

Experiment #2c will be performed in 8-10 animals.

Experiment #2d: Characterization of expiratory muscle activation via SMU recordings. Based upon our preliminary results with HF-SCS, the applied stimulus frequencies necessary to achieve optimal activation of the expiratory muscles are far beyond the physiologic range of peripheral nerve stimulation, i.e. 400-600Hz or higher. It is necessary, therefore, to better characterize the output of the motoneuron pools by determining the firing frequencies of individual motoneurons. This experiment will also allow us to test our hypothesis that pre-motoneuron stimulation via pathway activation allows for processing of the stimulus, resulting in motoneuron firing frequencies in the physiologic range.

Protocol. With animals in the supine posture, recording electrodes will be positioned in each of the expiratory muscles as described in Experiment #2a and #2b. Electrodes will be placed at specific sites within each muscle where SMUs can be discriminated with a high signal to noise ratio. In each muscle, an average of 50-80 SMU will be identified and analyzed. Based upon previous studies, this number provides a satisfactory sample size of the firing frequencies of a given expiratory muscle. Since 1-3 SMUs can be identified at each recording site, recordings from multiple sites from each muscle will be obtained. The presence of pre- and post- expiratory activity in each muscle will also be determined. Airway pressure generation will be recorded during SMU recordings. To assess the stability of SMU firing during prolonged expiratory muscle stimulation, the effects of HF-SCS provided over a 1-2min period will also be evaluated. For these studies, SMUs of the internal intercostal, external and internal obliques and transversus abdominis muscles will be evaluated. In each animal, the pattern of EMG activity will be determined during HF-SCS before and following C₁ spinal section.

Experiment #2d will be performed in 8-10 animals.

OBJECTIVE III: Evaluation of the electric field distribution during HF-SCS with the goal of evaluating alternative electrode designs.

To further our goal of maximizing pathway activation of the expiratory motoneuron pools while minimizing stimulus amplitude, the distribution of the electric field during HF-SCS and thresholds for pathway activation will be measured. This data will be fit to a computer model of the spinal cord to predict electrode designs resulting in optimal expiratory muscle activation. Since currently employed electrodes require a laminectomy for implantation, another major goal of these studies is the design of stimulating electrodes that can be implanted much less invasively, i.e. through a large bore needle. Electrodes will then be tested in acute animal studies. It is anticipated that the results of these experiments will lead to improved electrode designs resulting in optimal expiratory muscle activation and that can be implanted non-invasively.

Animal Preparation. Initial animal preparations will be the same as that described in Objective I.

SPECIFIC EXPERIMENTS

Experiment #3a: Assessment of the distribution of the electric field (E) during HF-SCS. In this experiment, the surface electrical fields generated during HF-SCS will be measured. This data will be used to determine the thresholds for pathway activation of the expiratory motoneuron pools and threshold for activation of the ventral roots. This data will also be used to validate predictions from computer modeling developed in experiment #3b (see below).

Protocol. With the animal in the prone posture (studies will be performed before and after C₁ section), the multiple contact electrode will be positioned in the optimal location for expiratory muscle activation (as determined in Objective I) via a laminectomy incision with as minimal surgery as possible since the surgery itself is likely to alter the electric field. Each set of contacts will be activated (with the stimulus paradigm determined in Objective I) to determine the optimal location for stimulation. The tripolar configuration is used to minimize the stimulation artifact. This method has been highly successful for recording nerve signals during stimulation.

Electroneurogram (ENG) electrodes will be placed on the ventral roots (T₉, T₁₀ and T₁₁ levels) to detect ventral root activation. Expiratory motoneuron pool activation will also be monitored with ENG electrodes on the T₁₀ root of the internal intercostal nerve (mid-axillary region).

During HF-SCS, signal recordings from the internal intercostal nerve and ventral root may occur consequent to pathway activation of the intercostal motoneuron pools and/or from direct ventral root stimulation. Therefore, the threshold for direct activation of the ventral roots by the applied electrical field will be re-assessed following section of the 7th thru 13th ventral roots at their exit point from the spinal cord. Since the point of activation of the ventral roots via direct stimulation is 1-2cm distal to the spinal cord, direct ventral root stimulation will be maintained while potential pathway stimulation will be eliminated by this procedure.

The electrode will be positioned to allow recording of the surface electrical fields both longitudinal (E_l) and transverse (E_t). The amplitude of the field is determined by dividing the measured electrical potential between two adjacent recording contacts by the distance between them (X = 0.5cm).

Experiment #3b: Assessment of alternative electrode placement and design. Computer models have traditionally been very useful to facilitate the design of complex systems. When animal experiments are required, modeling systems can decrease the number of necessary experiments by providing computer assisted electrode designs. We propose to generate a model of the spinal cord and surrounding tissue to design the best configuration of an electrode capable of stimulating the expiratory motoneuron pools via spinal cord pathways without activating the ventral roots directly. There will be special emphasis on electrode designs that can be placed with minimally invasive surgery. This experiment consists of 3 components: a) Field calculations; b) Axon calculation and c) Design optimization.

a) Electric field calculations. The first step is to model the volume conductor in which the current is flowing. The structure consists of neural tissue, high conductivity CSF and low conductivity bone. The volume conductor is, therefore, highly non-homogeneous and current distribution is difficult to predict accurately using standard techniques. In order to calculate the electric field distribution, the finite element method (FEM) will be applied. The FEM divides the volume conductor to be simulated into smaller volumes with a resolution high enough to describe accurately its geometric and electrical properties. The Poisson equations that govern the sources and the electric fields are then solved using commercially available software (ANSOFT). The model will be validated by stimulating each tripole and comparing the resulting electric field to that obtained in the *in vivo* experiments.

b) Axonal modeling. The second step in this process is to simulate the axons in the spinal cord. A motoneuron axon will be situated with the myelin sheath and the nodes of Ranvier. A cell body with few dendrites will be placed in the gray matter of the spinal cord and the axons will be located transversely and into the ventral root. Another set of axons will be placed longitudinally into the ventro-lateral portion of the cord. Each axon will be myelinated and with [REDACTED] Electrical stimulation will be applied to determine if these axons can be excited. The model will be modified (position and diameters of the

axons within the cord) until the threshold current for activating the ventral roots and spinal cord pathways for expiratory motoneuron activation are similar to those obtained in vivo.

c) Design optimization. With the model validated, it will be possible to place stimulating electrodes on the surface of the cord and modify their design. The activating threshold for each axon will then be estimated and the map of the axons that are activated can be obtained. The configuration of the stimulating electrodes capable of activating the target axons without resulting in direct activation of the ventral roots will be determined using computer simulation. Since our preliminary data suggest that the area of excitation is within the ventrolateral portion of the cord, our analysis will initially focus on electric fields generated on this area. The model will be employed to predict ideal electrode size and shape with emphasis on configurations that can be placed with minimally invasive techniques.

The ratio of the activation threshold of the target spinal cord axons to the ventral root axons will be measured and compared to those obtained in the model. The effectiveness of the two best (lowest threshold ratio) electrodes will then be tested in acute animal studies. The model will be updated and the simulations run again until a high threshold ratio can be obtained.

Experiments #3a and #3b will be performed in 8-10 animals.

Timetable

Task	Previously approved ACORP# 14-007-DG-14-004					New ACORP				
	2015	2016	2017	2018	2019	2015	2016	2017	2018	2019
Objective 1. Determine the optimal stimulus paradigm and electrode location										
Objective 2. Evaluate the mechanism by which the expiratory muscles are activated during HF-SCS										
Objective 3. Evaluate the electric field distribution during HF-SCS										

To determine the optimal stimulus paradigm and electrode location resulting in optimal activation of the expiratory muscles to produce large positive airway pressures and high peak expiratory airflow rates characteristic of an effective cough (Objective I) will be evaluated in year 2017. This will be followed by an assessment of the mechanism of HF-SCS (Objective II). Electric field measurements will then be utilized in the determination of optimal electrode designs (Objective III) in year 2019.

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

► **Animal Selection.** We have employed a dog model in most of our previous work involving upper¹⁻⁵ and lower thoracic spinal cord stimulation^{6-9,12} and physiological evaluation of respiratory muscle function and, therefore, have considerable experience with this model. There is also extensive neurophysiologic and anatomic information available in this species. The size of the spinal cord and distance between roots are relatively similar in humans and dogs.^{10,11} The dog's larger size compared to smaller species makes it easier to

assess optimal electrode location (Objective I), the pathways by which the motor nerves innervating the expiratory muscles are stimulated (Objective II), and construct a model of current distribution (Objective III).

All animal care and experimental procedures will be performed according to the guidelines set forth in "National Institutes of Health Publication Guide for the Care and Use of Laboratory Animals."

References:

1. DiMarco AF, Altose MD, Cropp A, Durand D. Activation of the inspiratory intercostal muscles by electrical stimulation of the spinal cord. *Am Rev Respir Dis* 136:1385-1390, 1987.
2. DiMarco AF, Supinski GS, Budzinska K. Inspiratory muscle interaction in the generation of changes in airway pressure. *J Appl Physiol* 66:2573-2579, 1989.
3. DiMarco AF, Romaniuk JR, Supinski GS. Parasternal and external intercostal muscle shortening during eupneic breathing. *J Appl Physiol* 69:2222-2226, 1990.
4. DiMarco AF, Romaniuk JR, Supinski GS. Action of the intercostal muscles on the rib cage. *Resp Physiol* 82:295-306, 1990.
5. DiMarco AF, Romaniuk JR, Supinski GS. Mechanical action of the interosseous intercostal muscles as a function of lung volume. *Am Rev Respir Dis* 142:1041-1046, 1990.
6. DiMarco AF, Romaniuk JR, Kowalski KE, Supinski G. Mechanical contribution of individual expiratory muscles to pressure generation during spinal cord stimulation. *J Appl Physiol* 87:1433-1439, 1999.
7. DiMarco AF, Romaniuk JR, Kowalski KE, Supinski G. Pattern of expiratory muscle activation during lower thoracic spinal cord stimulation. *J Appl Physiol* 86:1881-1889, 1999.
8. DiMarco AF, Romaniuk JR, Kowalski KE, Supinski G. Efficacy of combined intercostal and expiratory muscle pacing to maintain artificial ventilation. *Am J Respir Crit Care Med* 156:122-126, 1997.
9. DiMarco AF, Kovvuri S, Romaniuk J, Romaniuk JR, Supinski GS. Effect of synchronous intercostal muscle and diaphragm contraction on inspired volume production. *Am Rev Resp Dis* 143:A566, 1991.
10. Miller ME, Chrtistensen GC, Evand E. *Anatomy of the dog*. W.B. Saunders; Philadelphia, 1964
11. Struijk JJ, Holsheimer J, Boom HB. Excitation of dorsal root fibers in spinal cord stimulation. *IEEE Trans Biomed Eng* 40:632-639, 1993.
12. Kowalski KE, Romaniuk JR, Brose S, Richmond MA, Kowalski T, DiMarco AF. High Frequency Spinal Cord Stimulation – New Method to Restore Cough. *Respir Physiol Neurobiol* 232:54-56, 2016. PMID: 27395446.

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]

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
Surgeries (Tracheostomy, cannulation, laminectomy and muscle exposure)	[REDACTED]
Anesthesia	
Euthanasia	

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

Name ▶ [REDACTED]

Animal research experience ▶ [REDACTED]

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this ACORP
Surgery (Tracheostomy, cannulation, laminectomy and muscle exposure)	[REDACTED]
Anesthesia and Euthanasia	

Name ▶ [REDACTED]

Animal research experience ▶ [REDACTED]

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this ACORP
Surgery (Tracheostomy, cannulation, laminectomy and muscle exposure)	[REDACTED]
Anesthesia and Euthanasia	[REDACTED]

3. VMU animal care and veterinary support staff personnel (copy the lines below for each individual)

Name ▶

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

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

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

Name of Individual	Working with the VA IACUC	ORD web-based species specific course (Identify the species)	Any other training required locally (Identify the training)
[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]
[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]
[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]

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

G. **Occupational Health and Safety.**

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

Name	Enrollment in OHSP		Declined optional services	Current on Interactions with OHSP? (yes/no)
	VA program	Equivalent Alternate Program – identify the program		

				()	
				()	
				()	

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

► (X) Yes. Describe them ► ARC procedures, Pre exposure rabies immunization, Knowledge of procedures to follow in the event of a penetrating bite wound from a dog.

► () No.

Animals Requested

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

Description (include the species 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
Mongrel dogs	Either	>9 months		Purpose Bred

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

J.

USDA Category B

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

USDA Category C

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

USDA Category D

Procedures ►						
Species / Experimental Group / Procedure(s)	Year 1	Year 2	Year 3	Year 4	Year 5	Category D TOTAL
Dogs/ laminectomy and stimulation	16	15	10			41

USDA Category E

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

TOTALS over all Categories

Species / Experimental Group / Procedure(s)	Year 1	Year 2	Year 3	Year 4	Year 5	GRAND TOTAL
Dogs	16	15	10			41

K. **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)

Laminectomy, intact spinal cord	Nociception reflexes such as toe pinch, corneal reflex and jaw tone as well as sudden changes in heart, blood pressure and/or respiratory rate	All personnel (Non-survival experiments)	Animals will be anesthetized initially with IV pentobarbital (PB), 25mg/kg. Supplemental anesthesia (3-5mg/kg PB) will be administered as required. (Non-survival experiments)
Laminectomy, transected spinal cord	Nociception reflexes such as toe pinch, corneal reflex and jaw tone, respiratory rate, sudden changes in heart rate and blood pressure (elevation in any vital parameter of 10%-20% indicates the need for supplemental anesthesia).	All personnel (Non-survival experiments)	Same as above

L. **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

M. **Veterinary Support.**

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

Name ►

Institutional affiliation ►

email contact ►

2. Veterinary consultation during the planning of this protocol.

Name of the laboratory animal veterinarian consulted ►

Date of the veterinary consultation (meeting date, or date of written comments provided by the veterinarian to the PI) ►

N. **Husbandry.** As a reference for the animal husbandry staff, summarize here the husbandry requirements of the animals on this protocol. (Use Appendix 6 to justify the use of any special husbandry and to detail its

effects on the animals. Use Appendix 9 to document any aspects of the husbandry that involve “departures” from the standards in the *Guide*. Consult the IACUC or the Attending Veterinarian for help in determining whether any “departures” are involved.)

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

a. Species	b. Type of housing*	c. Number of individuals per housing unit**	d. Is this housing consistent with the <i>Guide</i> and USDA regulations? (yes/no***)	e. Estimated maximum number of housing units needed at any one time
Dogs	Standard	animals will be group housed in compatible pairs when appropriate	Yes	4-5

*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:

►

** 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)

►

***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*.):

a. Species	b. Description of Enrichment*	c. Frequency
Dogs	Standard see sop	Standard

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

►

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.



► () 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.



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

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

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

Building	Room number	Inside of VMU?	
		Yes	No
[REDACTED]	[REDACTED]	(X)	()
		()	()
		()	()

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

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

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

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

Special Features

P. **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.

Q. **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.

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

Procedure	Surgical?		Bldg/Room Number	Requires transport through non-research areas?	
	Yes	No		Yes – describe method of discreet transport	No
Non-survival surgery	(X)	()		()	(x)
	()	()		()	()
	()	()		()	()
	()	()		()	()

S. **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")

None	(X)	(X)	(X)	(X)
	()	()	()	()
	()	()	()	()

T. **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.

U. **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.)

▶ N/A (non-survival surgery) If any animal develops a serious illness or injury that is determined to be untreatable, per the attending veterinarian, the animal will be euthanized.

V. **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 ▶ Euthasol Dose ▶ 100mg/kg Route of administration ▶ Intravenously	Dogs	(x)	()	()
()	Decapitation under anesthesia Agent ▶ Dose ▶ Route of administration ▶		()	()	()
()	Exsanguination under anesthesia Agent ▶ Dose ▶ Route of administration ▶		()	()	()
()	Other (Describe) ▶		()	()	()
()	Other (Describe) ▶		()	()	()

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:

▶ Animal death will be recognized and confirmed by a combination of criteria, including lack of pulse and respiration, pale mucous membranes, absence of all reflexes, including corneal reflex and response to firm toe pinch.

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:

▶

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.

[REDACTED]

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.
- ▶ N/A (non-survival surgery)
- b. Describe how the PI's staff should be contacted.
- ▶ () Please contact a member of the PI's staff immediately. (Copy the lines below for each individual who may be contacted)
- Name ▶
- Contact Information ▶
- ▶ (X) 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.
- ▶

- W. **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.

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
N/A		Items:	()**
		Items:	()**
		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.)

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

1. Document the database searches conducted.

List each of the potentially painful or distressing procedures included in this protocol.

- Direct contact with colleagues: there is not suitable alternative to the methods that we employ in these procedures. The current state-of-art for anesthetic administration is with Pentobarbital in these types of acute dogs studies.

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

Name of the database	Date of search	Period of years	Potentially painful or	Key words and/or search strategy used	Indicate which mandate each search addressed
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		covered by the search	distressing procedures 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	2/22/17	10 years	Non-survival surgery	Spinal cord stimulation, laminectomy, tracheostomy, spinal sectioning, respiration, cough, respiratory muscle electromyography, animal model, nonsurvival surgery	(X)	(X)	(X)	(x)
Journals (on line)	2/22/17	10 years	Non-survival surgery	Spinal cord stimulation, laminectomy, tracheostomy, spinal sectioning, respiration, cough, respiratory muscle electromyography, animal model, nonsurvival surgery	(X)	(X)	(X)	(x)
OhioLink	2/22/17	10 years	Non-survival surgery	Spinal cord stimulation, laminectomy, tracheostomy, spinal sectioning respiration, cough, respiratory muscle electromyography, animal model, nonsurvival surgery	(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.

► An important consideration concerning animal studies, is their potential applicability in clinical trials. In this regard, we have had considerable success with several previous translational studies in the dog species (see references below). For example, we have performed intra-muscular diaphragm pacing using electrical stimulation in dogs to restore ventilation and replicated these results in human clinical trials. This technique is now being applied world-wide to restore breathing in those ventilator dependent tetraplegics with intact bilateral phrenic. We have also developed combined upper thoracic ventral root stimulation and unilateral diaphragm pacing in dogs and then demonstrated that full-time ventilatory support could be maintained with this technique in ventilator dependent tetraplegics with only a single intact phrenic nerve. Moreover, we have performed extensive studies to restore cough via epidural spinal cord stimulation and have successfully replicated these studies in clinical trials. This method holds promise to become the new standard of care for

spinal cord injury patients. However, necessary refinements of these techniques are badly needed. We have used a dog model in all of our studies since dogs have a similar size spinal cord compared to humans allowing us to accurately evaluate the effects of stimulating electrodes differing in size and shape. Additionally, extensive studies of thoracoabdominal mechanics and respiratory muscle activity have been performed on the dog model. Furthermore, the respiratory neurons and spinal cord pathways in the dog model are comparable to those found in the human. We have many years of experience using the dog model, which has proved very useful for translational research with regards to the evaluation of electrical stimulation techniques to restore muscle function.

Stated differently, the same electrode to be used in humans cannot be evaluated in smaller animal species due to size considerations. Smaller electrodes must be used in lower animal species, the use of which results in different electric field distributions. Obviously, the ventral roots are much closer together in smaller animals. This data obtained from lower animal species therefore may provide misleading results, not applicable to humans.

There is little information concerning the effects of electrical current applied on the epidural surface of the spinal cord in terms of the specific respiratory muscles activated. Moreover, there is no suitable computer simulation to reproduce the innumerable variables involved in the complex interaction of respiratory muscles during stimulation. With the basic science data accumulated from our studies, however, it may be possible to develop study models in the future. The goal of this objective 2 and 3 is to address some of the mechanisms of HF-SCS. This knowledge can be used in the future to predict optimal stimulus waveform patterns of stimulation, electrode placement and electrode design. Since this knowledge can be difficult to gain experimentally, it is conceivable to develop a computer model to investigate the direct effects of HF-SCS on the SC and proximate motor roots. The model infrastructure might consist of a finite element model of a SCS lead implanted in the epidural space along with multi-compartment cable models of axons within the white matter of the SC which can be used to define the stimulation waveforms, geometries and configurations that maximize outcomes. The results of this analysis may limit the future need for animal studies.

References (Clinical Translation):

DiMarco AF, Onders RP, Kowalski KE, Miller ME, Ferek S, Mortimer JT, 2002. Phrenic nerve pacing in a tetraplegic patient via intramuscular diaphragm electrodes. *Am J Respir Crit Care Med* 166:1604-1606. PMID: 12471076.

DiMarco AF, Onders RP, Ignagni A, Kowalski KE, Mortimer JT, 2005a. Phrenic nerve pacing via intramuscular diaphragm electrodes in tetraplegic subjects. *Chest* 127:671-678. PMID: 15706014.

DiMarco AF, Takaoka Y, Kowalski KE, 2005b. Combined intercostal and diaphragm pacing to provide artificial ventilation in patients with tetraplegia. *Arch Phys Med Rehabil* 86:1200-1207. PMID: 15954060.

DiMarco AF, Onders RP, Ignagni A, Kowalski KE, 2006a. Inspiratory muscle pacing in spinal cord injury: case report and clinical commentary. *J Spinal Cord Med* 29:95-108. PMID: 16739553.

DiMarco AF, Takaoka Y, Kowalski KE. Combined intercostal and diaphragm pacing to provide artificial ventilation in patients with tetraplegia. *Arch Phys Med Rehabil* 86:1200-1207, 2005. PMID: 15954060, doi: 10.1016/j.apmr.2004.11.027, URL: [http://www.archives-pmr.org/article/S0003-9993\(05\)00077-8/pdf](http://www.archives-pmr.org/article/S0003-9993(05)00077-8/pdf).

DiMarco AF, Kowalski KE, Geertman RT, Hromyak DR. Spinal cord stimulation: a new method to produce cough in patients with spinal cord injury. *Am J Respir Crit Care Med* 2006;173:1386-1389.

DiMarco AF, Kowalski KE, Geertman RT, Hromyak DR. Lower thoracic spinal cord stimulation to restore cough in patients with spinal cord injury: results of a National Institutes of Health-sponsored clinical trial. Part I: methodology and effectiveness of expiratory muscle activation. Arch Phys Med Rehabil 2009;90:717-725.

DiMarco AF, Kowalski KE, Geertman RT, Hromyak DR, Frost FS, Creasey GH, Nemunaitis GA. Lower thoracic spinal cord stimulation to restore cough in patients with spinal cord injury: results of a National Institutes of Health-sponsored clinical trial. Part II: clinical outcomes. Arch Phys Med Rehabil 2009;90:726-732.

DiMarco AF, Kowalski KE, Hromyak DR, Geertman RT. Long-term follow-up of spinal cord stimulation to restore cough in subjects with spinal cord injury. J Spinal Cord Med 2014;37:380-388.

DiMarco AF, Geertman RT, Tabbaa K, Polito RR, Kowalski KE. Economic consequences of an implanted neuroprosthesis in spinal cord injured subjects for restoration of an effective cough. Topics in Spinal Cord Injury Rehabilitation (in Press).

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.

▶ We propose to perform studies in 60 dogs. We propose to use 8-10 dogs in each of the experimental designs (1 experiment to satisfy Objective 1, 4 total experiments to meet Objective 2 and 2 total experiments to meet Objective 3). We recognize the importance to collect the most data possible with each animal, not only for ethical reasons but also due to the high cost of the animal and anesthesia. For that reason, we often combine experimental aims in a single animal. However, we have found that repeated stimulation of the expiratory muscles results in muscle fatigue and unreliable data points. For that reason, we are limited in the number of times that stimulation can be applied in a single animal during this short period of the experiment. Additionally, some of the experimental designs require different models of spinal cord injury (i.e. spinal cord sections of varying degrees and at various levels along the spinal cord), which cannot necessarily be achieved with one single animal. Whenever possible, experiments will be combined in a single animal.

While we initiated this work under the previous IACUC (), to complete the work and answer new questions raised. Our previous work has led to the development of inspiratory muscle pacing systems that have been successful in providing ventilator-dependent spinal cord injured patients freedom from mechanical ventilation. Our work has also led to the development of a system to provide expiratory muscle stimulation to generate an effective cough, which has resulted in a significant reduction in respiratory tract infections and improvements in life quality. However, these first generation systems are somewhat crude and require further refinement. The major thrust of our current investigation is to test a novel method of expiratory muscle activation, i.e. high frequency spinal cord stimulation (HF-SCS) to electrically activate the expiratory muscles, in an animal model. If successful, this method will be applied in clinical trials and may significantly facilitate the management of respiratory secretions, reduce the incidence of respiratory tract infections and ultimately reduce the morbidity and mortality in these patient populations. Prior to initiating new procedures in clinical trials, however, these methods need to be rigorously tested in animals to meet FDA requirements to obtain IDEs.

Our previous work has been published in a number of scientific journals. Our most recent publications include:

Kowalski KE, Romaniuk JR, DiMarco AF. Changes in expiratory muscle function following spinal cord section. J Appl Physiol 102:1422-1428, 2007. PMID: 17158247.

DiMarco AF, Romaniuk JR, Kowalski KE. Effects of diaphragm activation on airway pressure generation during lower thoracic spinal cord stimulation. Respir Physiol Neurobiol 159: 102-107, 2007. PMID: 17681870.

DiMarco AF, Kowalski KE. Effects of chronic electrical stimulation on paralyzed expiratory muscles. *J Appl Physiol* 104: 1634-1640, 2008. PMID: 18403449.

DiMarco AF, Kowalski KE. High frequency spinal cord stimulation of inspiratory muscles in dogs: a new method of inspiratory muscle pacing. *J Appl Physiol* 107: 662-669, 2009. PMID: 19520839.

DiMarco AF, Kowalski KE, Geertman RT, Hromyak DR. Lower thoracic spinal cord stimulation to restore cough in patients with spinal cord injury: results of a National Institutes of Health-sponsored clinical trial. Part I: methodology and effectiveness of expiratory muscle activation. *Arch Phys Med Rehabil* 90: 717-725, 2009. PMID: 19406289.

DiMarco AF, Kowalski KE, Geertman RT, Hromyak DR, Frost FS, Creasey GH, Nemunaitis GA. Lower thoracic spinal cord stimulation to restore cough in patients with spinal cord injury: results of a National Institutes of Health-sponsored clinical trial. Part II: clinical outcomes. *Arch Phys Med Rehabil* 90: 726-732, 2009. PMID: 19406290.

DiMarco AF, Kowalski KE. Intercostal muscle pacing with high frequency spinal cord stimulation in dogs. *Respir Physiol Neurobiol* 171:218-224, 2010. PMID: 20338266.

DiMarco AF, Kowalski KE. Distribution of electrical activation to the external intercostal muscles during high frequency spinal cord stimulation in dogs. *J Physiol* 589:1383-1395, 2011. PMID: 21242258.

Kowalski KE, DiMarco AF. Comparison of wire and disc leads to activate the expiratory muscles in dogs. *J Spinal Cord Med* 34:600-608, 2011. PMID: 22330116.

Kowalski KE, DiMarco AF. Comparison of wire and disc leads to activate the expiratory muscles in dogs. *J Spinal Cord Med* 34:600-608, 2011. PMID: 22330116.

DiMarco AF, Kowalski KE. Spinal cord pathways mediating phrenic activation during high frequency spinal cord stimulation. *Respir Physiol Neurobiol* 186:1-6, 2013. PMID: 23261850.

Kowalski KE, Hsieh YH, Dick TE, DiMarco AF. Diaphragm activation via high frequency spinal cord stimulation in a rodent model of spinal cord injury. *Exp Neurol* 247:689-693, 2013. PMID: 23499833.

DiMarco AF, Kowalski KE. Activation of inspiratory muscles via spinal cord stimulation. *Respir Physiol Neurobiol* 189:438-449, 2013. PMID: 23751522.

DiMarco AF, Kowalski KE, Hromyak DR, Geertman RT. Long-term follow-up of spinal cord stimulation to restore cough in subjects with spinal cord injury. *J Spinal Cord Med* 37:380-388, 2014. PMCID: PMC4116721.

DiMarco AF, Kowalski KE. Electrical Activation to the Parasternal Intercostal Muscles during High Frequency Spinal Cord Stimulation in Dogs. *J Appl Physiol* 118:148-155, 2015. PMCID: PMC4297776.

Kowalski KE, Kowalski T, DiMarco AF. Safety Assessment of Epidural Wire Electrodes for Cough Production in a Chronic Pig Model of Spinal Cord Injury. *J Neurosci Methods* 268:98-105, 2016. PMCID: PMC4903884.

Kowalski KE, Romaniuk JR, Brose S, Richmond MA, Kowalski T, DiMarco AF. High Frequency Spinal Cord Stimulation – New Method to Restore Cough. *Respir Physiol Neurobiol* 232:54-56, 2016. PMID: 27395446.

DiMarco AF, Geertman RT, Tabbaa K, Polito RR, Kowalski KE. Economic consequences of an implanted neuroprosthesis in spinal cord injured subjects for restoration of an effective cough. *Topics in Spinal Cord Injury Rehabilitation* (in Press).

DiMarco AF, Geertman RT, Tabbaa K, Polito RR, Kowalski KE. Minimally Invasive Method to Activate the Expiratory Muscles to Restore Cough. *Journal of Spinal Cord Medicine* (Submitted) 2017.

The techniques to be employed under this protocol are similar to those explored in previous studies; therefore we would expect that the magnitude of response and differences will be similar. After reviewing our previous data, however, we believe that a sample size of 8-10 animals per experimental design would be sufficient. For a listing of experimental procedures and the estimated number of animals required for each procedure, please refer to section #2 of the protocol.

Repeated-measure ANOVA and post hoc Newman-Keuls Tests have been used in the past to determine statistical significance and will be used for these studies as well.

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

► Our laboratory is one of the leading laboratories in the world in this field. All researchers who study this problem cooperate and discuss their results at major scientific meetings. From that source, literature searches and human applications of our systems, we are convinced that we are using the most appropriate techniques to achieve our research goal. Based upon a comprehensive literature review, there is no suitable alternative that can be utilized to address the research questions that need to be answered prior to human application.

The primary aim of our study design is the measurement of muscle activity and effects of muscle contraction secondary to electrical stimulation. Many anesthetics and sedatives, including inhalational agents, result in muscle relaxation and impair the responses to electrical stimulation. We have attempted to use a sedative, such as Xylazine, and even after administration of the reversal agent, Yohimbine, the responses to electrical stimulation were markedly reduced. We have tried a variety of anesthetic agents, including inhalational Halothane and Isoflurane, and found that muscle activation is markedly suboptimal. Of note, the expiratory muscles are particularly sensitive to these agents. We have found that pentobarbital and alpha chloralose provide the least muscle relaxation and therefore are our primary and secondary anesthetic agents respectively. Moreover, non-survival surgery under general anesthesia will limit pain/distress.

Animals will be anesthetized using 1 of 2 methods:

1. Pentobarbital. An initial dose of 30mg/kg will be given intravenously. This will be supplemented by additional doses (3-5ml/kg) as required to maintain sufficient anesthesia.
2. Alpha chloralose with Pentobarbital. Animals will receive pentobarbital (25-30mg/kg) given intravenously. After surgical preparation, anesthesia will be maintained with alpha chloralose IV (100 mg/kg) as required to maintain sufficient anesthesia.

Anesthetic level will be monitored by corneal, snout and lip pinch reflexes and changes in respiratory rate, heart rate and blood pressure.

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

► Based upon the literature review there is not suitable alternative to the methods that we employ in these procedures.

Y. Other Regulatory Considerations.

1. Controlled drugs.

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

Controlled substances	Storage		Personnel Authorized to Access	Location for Use		Procurement	
	Double-locked	Not Double-locked*		VA Property	Not on VA Property	VA Pharmacy	Non-VA
Pentobarbital	(x)	()*	All personnel	(x)	()	(x)	()
Euthasol	(x)	()*	All personnel	(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.



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

▶ (x) Some controlled substances will be 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.

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

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

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

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

Item	SOP		Approval Date
	Title	ID	
C.2.c	N/A		
M.1	Animal Care and Use Program Standard Operating Procedures	Sec. 5.1	2/2014
M.2	Animal Care and Use Program Standard Operating Procedures	Sec. 5.2	2/2014
U.4.a	N/A		
U.4.b	N/A		
V	N/A		

AA. 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.

Name(s) of Principal Investigator(s)	Signature	Date

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
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Name of IACUC Chair	Signature	Date
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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.

Name(s) of Principal Investigator(s)		Date
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Name of Institutional Veterinarian	Signature	Date
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Name of IACUC Chair	Signature	Date
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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]	[REDACTED]	[REDACTED]

4. 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.

Name(s) of Principal Investigator(s)	Signature(s)	Date
[REDACTED]	[REDACTED]	[REDACTED]

ACORP APPENDIX 3
BIOSAFETY
VERSION 4

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

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

Material (Identify the specific agent, device, strain, construct, isotope, etc.)	Source (Identify the vendor or colleague, or specify which animals on this protocol will serve as donors)	Nature of Material						
		Toxic Agent (Item 4)	Infectious Agent (Item 5) -- Enter the CDC Biosafety Level (BSL 1, 2, 3, or 4)	Biological Agent (Item 6)	Radioactive Agent (Item 7)	Contains Recombinant Nucleic Acid (Item 8)	Routine Pre - or Post-Procedural Drug	Euthanasia agent
Pentobarbital sodium-anesthesia	VA pharmacy	()	() BSL_	()	()	()	(x)	()
Euthasol (pentobarbital sodium + phenytoin sodium)	VA pharmacy	()	() BSL_	()	()	()	()	(x)
0.9% Sodium Chloride	VA pharmacy	()	() BSL_	()	()	()	(x)	()

<u>Atropine sulfate</u>	VA pharmacy	()	()BSL_	()	()	()	(x)	()
Epinephrine	VA pharmacy	()	()BSL_	()	()	()	(x)	()
Alpha chloralose	Sigma	()	()BSL_	()	()	()	(x)	()

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

Material* (Identify the specific agent, device, strain, construct, isotope, etc.)	Dose (e.g., mg/kg, CFU, PFU, number of cells, mCi) and Volume (ml)	Diluent* or Vehicle*	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)
Pentobarbital	25-30mg/kg	0.9% Sodium chloride	IV	Initial	Initial Anesthesia	App 5	Y
							Y
Euthasol's use	100mg/kg	0.9% Sodium chloride	IV	1	Euthanasia	Item U	Y
0.9% sodium chloride	5-10mL/kg/h	N/A	IV	Continuous administration	Hydration during surgery	App 5	Y
Atropine	0.02-0.04 mg/kg	N/A	SC, IM or IV	1	Reduce respiratory tract secretions, prevent bradycardia	App 5	Y
Epinephrine	0.1mg/kg	N/A	IV, IM	1	Emergency cardiac stimulation (increasing blood pressure, stimulating the heart muscle, accelerating the heart rate, and increasing cardiac output).	App5	Y
*Alpha chloralose	100mg/kg	0.9% Sodium chloride	IV	Continuous administration	Anesthesia (post- surgical preparation)	App 5	Y

*Each material, diluent, or vehicle that is listed as FDA approved or is labeled "USP" is pharmaceutical grade. Check on-line for formulations that are FDA approved for administration to humans (<http://www.fda.gov/Drugs/InformationOnDrugs/ucm129662.htm>) or animals (<http://www.fda.gov/AnimalVeterinary/Products/ApprovedAnimalDrugProducts/UCM042847>). Designate with a * each material and each diluent or vehicle to be used that is not pharmaceutical grade. For each of these, explain here why the use of a non-pharmaceutical grade formulation is necessary, and describe how it will be ensured that the material is suitable for use. (See ACORP App. 3 Instructions, for specifics about the level of detail required.) We plan to perform all experiments under pentobarbital anesthesia alone.

However, if may be necessary to assess the effects chloralose anesthesia on expiratory output in some animals. Since its initial description in 1893, alpha-chloralose has undergone extensive pharmacologic evaluation. It has been characterized as a compound possessing potent central nervous system activity and has been employed widely as an animal anesthetic in the laboratory setting. Surgical preparation in the present studies will be performed and completed under pentobarbital anesthesia alone. Alpha chloralose will be given after the entire surgical preparation is completed. We have found that pentobarbital and alpha-chloralose provide the least muscle relaxation and therefore lessen the impact on measured outcomes. Moreover, this non-pharmaceutical grade compounds is not available from a veterinary or medical supplier. It is generally administered IV as an initial loading dose of 100mg/kg of body weight. Drug preparation: Alpha-chloralose (10g) will be added to a 100ml of 0.9%NaCl at a temperature of 52 to 57°C. Prepared solutions will be passed through a syringe filter (0.22 µm or finer) at the time of preparation and usage. Alpha-chloralose will be administered as a continuous infusion at the estimated rate (30mg/kg/h) throughout the experiment. Supplements with the same alpha-chloralose solution will be provided after re-warming and stirring for a short time before using it, so that the concentration will be the same.

►

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):

► An initial dose of 25mg/kg will be given intravenously. This will be supplemented by additional doses (3-5ml/kg) as required to maintain sufficient anesthesia. Anesthetic level will be monitored by corneal, snout and lip pinch reflexes and heart rate and blood pressure. The animals will be under continuous hemodynamic and cardiorespiratory monitoring. IV fluids will be administered throughout the surgical procedures. Ventilation will be supported by a respirator.

- 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.

►

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
				Not a Select Agent	Select Agent Used in Sub-threshold Quantities	Select Agent that Requires Registration/Approval	
N/A	()	()	()	()	()	()*	() ▶

*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
N/A		(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
N/A	

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
N/A		

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
N/A	()	()
	()	()

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

Instructions, for details).

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

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
N/A			

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



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

ACORP Appendix 5
SURGERY
VERSION 4

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	Spinal Cord Stimulation (SCS) a) Tracheostomy (mid cervical region) b) Femoral cannulation c) Laminectomies d) Exposure of respiratory and non-respiratory muscles for EMG recording	(x)	()	()	()*

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

- a. Provide a complete scientific justification for performing the multiple survival surgeries on an individual animal:
 ► N/A
- b. Give the interval(s) between successive surgeries, and the rationale for choosing the interval(s):
 ► N/A

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

Animals will be anesthetized with pentobarbital sodium (*or in some experiments if necessary to study the influence on anesthesia on the observed effects: alpha-chloralose will be used as an anesthetic agent. However, the alpha chloralose will be given after the entire surgical preparation is completed*). An initial dose of PB (25 mg/kg) will be given intravenously; additional doses of 3-5 mg/kg will be provided, as needed.

Animals will be tracheostomized and intubated with a cuffed endotracheal tube (10 mm ID). A catheter will be placed in the femoral vein to administer fluids and supplemental anesthesia. A second catheter will be placed in the femoral artery for continuous monitoring of blood pressure and heart rate (Waveline Pro Multi-Function Monitor, DRE Inc., Louisville, KY). Body temperature will be maintained with a heating blanket (Harvard Apparatus, Cambridge, MA) at $38 \pm 0.5^\circ\text{C}$. Airway pressure generation during SCS will be measured at functional residual capacity (FRC) following airway occlusion with a pressure transducer (Validyne, MP45, Northridge, CA) connected to the airway opening.

Surgical procedures:

(a) ► Tracheostomy (mid cervical region) in non-survival experiments

Animals will be tracheostomized and intubated with a cuffed endotracheal tube (10mm ID) and placed on a mechanical ventilator. We have found that tracheostomy access allows for a more secure airway and more accurate measurements of airway pressure, peak expiratory airflow and volume, as there are often leaks around an oral endotracheal tube with this preparation, compared to direct tracheal intubation. Furthermore, these experiments are non-survival. Tracheostomy is a surgical procedure, which involves creating an opening into the trachea, to allow placement of an endotracheal tube. A tracheostomy provides a more secure airway and more accurate measurements of airway pressure, peak expiratory airflow and volume. In contrast, there are often leaks around an oral endotracheal tube, compared to direct tracheal intubation. Tracheostomy will be performed with the animal in the supine posture with the neck hyperextended. The cricothyroid cartilage will be palpated and a 1-2" incision will be made over the cricothyroid membrane (immediately caudal to cricothyroid cartilage) in the ventral midline. The sternohyoideus muscles will be separated longitudinally, and the tracheal cartilage will be identified. The membrane between the second and third tracheal ring will be incised and an endotracheal tube (10mm ID) will be placed within the trachea. Tracheostomized and intubated animals will be placed on a mechanical ventilator.

(b) ► Femoral cannulation

Catheters will be placed in the femoral vein and artery for administration of intravenous fluids and medications and to monitor arterial blood pressure, respectively. Both femoral vessels will be approached by making a longitudinal incision in the inguinal area over the medial aspect of the hind leg. Blunt dissection will be used to separate the connective tissue until the femoral artery and vein are exposed. The artery and vein will be separated. Using a micro-dissecting scissors, a small incision will be made in the vein (and later in the artery) approximately ¼ thru and at a 45 degree angle. The catheter (previously flushed with saline) will be inserted and tightened around the vein and artery, respectively. The arterial line will be attached to the blood pressure transducer; the vein will be connected to a constant infusion line. The venous line will be used to administer fluids and supplemental anesthesia.

(c) ► Laminectomies

Laminectomies will be performed at the cervical and thoracic spinal cord levels. Stimulating leads with 8 platinum-iridium disc electrodes (4mm) will be inserted epidurally and positioned on the dorsal or ventral surface of the spinal cord and advanced to the T₅-T₈ and to T₉-T₁₁ spinal cord regions to activate the expiratory muscles via spinal cord stimulation. Stimulation will be applied in intact animals, as well as varying models of spinal cord injury (following sequential section of the spinal cord: at the C₁ (high tetraplegia with injury above the phrenic pool of motoneurons), C₈ (low tetraplegia with injury below the phrenic pool of motoneurons) and T₆ (paraplegia) spinal levels). Spinal section will be performed with watchmaker forceps. Complete section will be verified by lifting the hook across the area of transaction.

(d) ► Exposure of respiratory and non-respiratory muscles for EMG recordings

Electromyographic electrodes (bipolar stainless steel wires) will be placed into several respiratory and non-respiratory muscles to assess the level of their activation. Studies will be performed to assess the pattern of respiratory and non-respiratory muscle recruitment during HF-SCS applied at different spinal cord levels. Because of the large size of the respiratory muscles and the fact that they are innervated by multiple spinal nerve roots, multiple electrodes will be placed in each of these muscles to assess the regional activation of the various portions of these muscles. Electromyographic electrodes will be implanted into the external oblique muscles in the midaxillary line at four levels: 1) superior (within 1–2 cm of muscle origin), 2) upper (3 cm above

the costal margin), 3) middle (just below the costal margin), and 4) lower (just above the iliac crest); in the transversus abdominis muscles in the anterior axillary line and in the rectus abdominis muscles just lateral to the midline at three levels corresponding to the upper, middle, and lower portion of the external oblique; in the upper and lower portions of the internal oblique; in the internal intercostal muscles (between 1th and 12th interspaces); in the external intercostal muscles (between 1th and 12th interspaces); in the parasternal intercostal muscles (between 1th and 6th interspaces) and in the diaphragm (costal portion). Activation of non-respiratory muscle during stimulation will be assessed by evaluating EMG activity from the hind limb (middle portion of: quadriceps, hamstrings, gastrocnemius, tibialis).

Length under anesthesia - Expected duration of each procedure will be between 5 and 8 hours.

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)
	1,2,3,4	(x)	()	(x)	(x) He will supervise and coordinate all aspects of the experiments.
	1,2,3,4	(x)	(x)	(x)	()
	1,2,3,4	(x)	(x)	(x)	()

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

Building	Room Number	Surgery # (s) (see Item 1)	Type of Space		
			Dedicated Surgical Facility	Other Dedicated Surgical Space	Other Space not Dedicated to Surgery
		All	(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
all	(x) overnight ~15 hrs	()	(x) –	() --

- 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)
Pentobarbital	all	25-30 mg/kg	intravenously	Initially and general anesthesia	Immediate (non-survival surgery)
Atropine (for emergency and excessive respiratory secretions)	all	0.02-0.04mg/kg	SC, IM or IV	As needed usually once	Immediate
Epinephrine - emergency	all	0.1mg/kg	IV, IM	As needed	Immediate

- 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.

Since the animal will not regain consciousness and will be euthanized while under anesthesia, all aseptic techniques normally performed in survival surgery are not necessary for these non-survival experiments. However, as a minimum for these non-survival experiments, the following procedures will be followed: surgeon will wear gloves, instruments will be cleaned using an autoclave prior to each experiment and finally, the work area will be cleaned. The animal, prior to surgery, will be prepared by having both hair removed and pre-surgical scrubbing (using betadine surgical scrub) of the area of incision (see below).

Surgery 1 ► Tracheostomy (mid cervical region) – neck will be shaved, wiped clean and pre-surgically scrubbed

Surgery 2 ► Femoral cannulation - inner leg region will be shaved, wiped clean and pre-surgically scrubbed

Surgery 3 ► Laminectomies – back will be shaved, wiped clean and pre-surgically scrubbed

Surgery 4 ▶ Exposure of respiratory and non-respiratory muscles for EMG recording – abdomen/chest/legs will be shaved and wiped clean

6. Intra-operative management.

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

Agent	Paralytic*	Surgery #(s) (see Item 1)	Dose (mg/kg) & volume (ml)	Route of administration	Frequency of dosing
Pentobarbital	()*	all	3-5 mg/kg	intravenously	as needed
Fluids	()*	all	5-10mL/kg/hr	IV	continuous
Alpha chloralose	()*	all	100mg/kg	IV	continuous

* 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 homeothermic blanket will be used to maintain body temperature at $38 \pm 0.5^{\circ}\text{C}$.

- 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.

▶ Anesthetic level will be monitored by corneal, snout and lip pinch reflexes, changes in blood pressure, heart rate and also ECG and end-tidal CO_2 to monitor the general well-being of the animal during and after surgery.

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	Survival Period	Measures for Maintaining Sterility
---------	-----------------	------------------------------------

# (see Item 1)		Sterile Instruments	Surgical Cap	Sterile Gloves	Surgical Scrub	Sterile Drapes	Sterile Gown	Face Mask	Other*
	N/A	()	()	()	()	()	()	()	()*

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



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

Surgery 1 ► N/A

- 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	N/A				

*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:



- 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)
	N/A				

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

(1) Immediate post-operative monitoring

Surgery # (see Item 1)	Frequency of Monitoring	Duration at this Frequency	Name(s) of Responsible Individual(s)
N/A			

(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)
N/A			

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 ► N/A

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

Surgery 1 ► N/A

- (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.)

►

- 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	N/A		()	()

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

Secondary Review

PI	STATION	FUNDING SOURCE	APPLICATION TITLE
	Cleveland, OH - 541	Department of Veterans Affairs	High Frequency Spinal Cord Stimulation to Restore Cough

ACTION NEEDED BY IACUC

The IACUC must review the concerns listed below and decide what response is needed. This action must be documented in the IACUC minutes and the changes required by the IACUC must be incorporated into the ACORP(s) and the revised ACORP(s) must be forwarded to the CVMO for archiving.

In case of questions about this review, please contact [REDACTED] Assistant Chief Veterinary Medical Officer at [REDACTED]

REVIEWER FEEDBACK

ACORP Item number(s)	Comments/Concerns
ACORP (dog)	This ACORP uses a nonsurvival canine model of spinal cord injury to investigate the potential of high frequency spinal cord stimulation (HF-SCS) to restore the ability to effectively cough. The investigator is commended for providing an extensive number of supporting literature citations, a detailed justification for the use of dogs as opposed to other models (see items C.2.b, D, and W), a clear rationale for the use of pentobarbital and alpha-chloralose over other anesthetics, and an explanation of the relevancy and applicability of this work to clinical trials. A few aspects of protocol should be clarified. An appendix to this review provides additional information for the IACUC's consideration. The specific numbered comments provided below must be reviewed by the IACUC, to determine what responses are needed. These actions must be documented in the IACUC minutes, and the changes required by the IACUC must be incorporated into the ACORP and the revised ACORP provided to the CVMO for archiving.
Items C.2.b, J, and W	Items C.2.b and J account for 41 dogs but item W indicates the proposed experiments will be performed in 60 dogs, please explain.
Item U	Presumably, the dogs are received and acclimated for several days prior to performing the nonsurvival surgery; during this period, the dogs could become ill or develop some other health issue. Please indicate who will perform daily health assessments, list general indicators of illness/disease, and conditions that would warrant veterinary treatment or euthanasia, if necessary.
Appendix 5	The investigator states that laminectomies will be performed but does not address the approach, the skin incision, or how the portions of the vertebra are removed to access the spinal cord; providing this information would improve understanding of the protocol.

(cont.)

Appendix – Additional Suggestions for Improvement

Comment 1: Part B. This section is well written, and would benefit from a brief discussion of the relevance to Veterans' health. Try something like this between the third and fourth sentence:

These kinds of problems are so common in the Veteran population that the VA has instituted 24 spinal cord injuries and disorders (SCI/D) centers around the country, and has a webpage discussing healthy breathing for these patients (see http://www.veteranshealthlibrary.org/142,41161_VA accessed 1/11/2018).

Comment 2: Part D: The justification for the species choice is good, but could be made clearer and easier for the lay reader to understand.

Try something like this:

The electrodes we are using are platinum-iridium disc electrodes that are 4 mm in diameter, similar to what will eventually be used in clinical trials. These electrodes are an appropriate size for use in humans and dogs, but are far too large to use in small animals such as rats, mice, or rabbits (see <http://www.entomoljournal.com/archives/2017/vol5issue6/PartAI/5-6-202-439.pdf>). Furthermore, since the size of the spinal cord and distance between roots are relatively similar in humans and dogs^{10, 11} we will be able to more accurately determine optimal electrode location (Objective I), the pathways by which the motor nerves innervating the expiratory muscles are stimulated (Objective II), and construct a model of current distribution (Objective III).

While there is a lot of information available on dog respiration mechanics and muscle functioning, not much has been done on other large animals such as pigs or sheep. Switching to other large animals would require us to start over to a large degree, running a lot of experiments to reach the point where we already are with dogs before we could even perform this particular study. This process would use many more animals to get to that point than the 41 dogs required for this study. We have employed a dog model in most of our previous work involving upper¹⁻⁵ and lower thoracic spinal cord stimulation^{6-9, 12} and physiological evaluation of respiratory muscle function and therefore have considerable experience with this model.

Comment 4, section W1 table (literature search). Note: this section is normally section W – something got changed in the numbering that made it section X.

This literature search would be strengthened by doing the following:

Change how the search terms are run. Running all the potentially painful or distressing procedures together in a single search means only a paper that includes all of those search terms would be found. Run separate searches instead, since there may be papers that address individual procedures.

In the example below, the first row is a search on PubMed for unnecessary duplication, focusing on this particular study.

The second row is an ALTBIB search specifically for papers where alternatives to using animals for this kind of research is the main topic. This was done using the ALTBIB website run by NIH

(cont.)

<https://toxnet.nlm.nih.gov/altbib.html> (This website works with Google Chrome, but not with Internet Explorer)

The rest are for potentially painful or distressing procedures, using the ALTBIB search for citations from 2000 to present. Each of these searches brings up less than 30 papers. The term “anesthesia” is included in these searches since as noted in the ACORP section W4 many anesthetics can result in muscle relaxation and impair the responses to electrical stimulation.

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	1/22/18	1966-2018	N/A	electrode, spinal cord, expiration	()	()	()	(X)
ALTBIB Citations with Animal Use Alternatives as the main topic	1/22/18	1966-2018	N/A	electrode, spinal cord, expiration	(X)	()	()	()
PubMed using ALTBIB animal alternatives search strategy	1/22/18	2000-2018	Tracheostomy under anesthesia	tracheostomy, anesthesia	(X)	(X)	(X)	()
PubMed using ALTBIB animal alternatives search strategy	1/22/18	2000-2018	Femoral cannulation under anesthesia	femoral cannulation, anesthesia	(X)	(X)	(X)	()
PubMed using ALTBIB animal alternatives search strategy	1/22/18	2000-2018	Laminectomy under anesthesia	Laminectomy, anesthesia	(X)	(X)	(X)	()
PubMed using ALTBIB animal alternatives search strategy	1/22/18	2000-2018	Exposure of respiratory and non-respiratory muscles for EMG recordings	anesthesia AND muscles AND EMG AND (respiratory OR non-respiratory)	(X)	(X)	(X)	()

(cont.)

Comment 5, section W2: (Replacement)

This section would be strengthened by including the following information:

Please explain why this research cannot be done with computer models or *in vitro* methods (it may be obvious to any biomedical scientist, but this still has to be included). You can say something like “We ran a search specifically looking for animal use alternatives for this kind of research, and there were no in vitro, computer, or other non-animal models available.”

Comment 6, section W3: (Reduction) A line can be added to the end of this part directing the reader to the detailed statistical analysis in section C2b. Try something like: “See section C2b for details.”

Comment 7, section W5 (lack of unnecessary duplication):

The answer provided in this ACORP would be strengthened by providing some more detail.

Try something like this:

Our PubMed literature search for “electrode, spinal cord, expiration” yielded 11 papers, of which 8 were on the roles of various parts of the brainstem in respiration, while our focus is on directly stimulating the thoracic spinal cord to stimulate respiration. Of the remaining three papers, one was a review article, one studied upper cervical neurons, and only one was relevant to our study. That paper was a clinical trial (discuss how your work is not an unnecessary duplication of this earlier paper. In particular, since it was a clinical trial why do you still need to do this kind of work in dogs?).

(cont.)

1) How is this research relevant to Veterans health?

This study is refining methods to restore the ability to cough when certain sections of the spinal cord have been damaged. This group has already shown they can stimulate the cough reflex through spinal cord stimulation, and the goal of this study is to determine the optimal parameters, electrode location, etc., so they can then move into clinical trials.

These kinds of problems are so common in the Veteran population that the VA has instituted 24 spinal cord injuries and disorders (SCI/D) centers around the country, and has a webpage discussing healthy breathing for these patients (see http://www.veteranshealthlibrary.org/142.41161_VA accessed 3/10/2018).

2) Is this work unnecessarily duplicating work already documented in the literature?

Name of the database	Date of search	Period of years covered by the search	Key words and/or search strategy used	How many papers were found?
PubMed	3/10/18	All available years	electrode, spinal cord, expiration	11

A PubMed literature search for “electrode, spinal cord, expiration” yielded 11 papers, of which 8 were on the roles of various parts of the brainstem in respiration, while the focus of this study is on directly stimulating the thoracic spinal cord to stimulate respiration. Of the remaining three papers, one was a review article, one studied upper cervical neurons, and only one was relevant to this study. That paper was a clinical trial that used very high levels of stimulation (50 – 450 mA 200) [Butler, 2011] while this study will be using a stimulation intensity of only 1-2 mA along with minimally invasive surgical techniques. The lower stimulation intensity would have the advantages of far fewer problems with tissue injury and electrode damage and a much longer battery life. If successful, it will then move into clinical trials.

3) Could this work be done in computer models or in vitro (tissue culture)?

Name of the database	Date of search	Period of years covered by the search	Key words and/or search strategy used	How many papers were found?
ALTBIB Citations with <u>Animal Use Alternatives</u> as the main topic	3/10/18	All available years	spinal cord stimulation, expiration	0
PubMed	3/10/18	All available years	spinal cord stimulation, expiration, computer model	0
PubMed	3/10/18	All available years	spinal cord stimulation, expiration, in vitro	3

An ALTBI search for alternatives to animal use brought up no papers.

No computer models at all were found for this research.

There were three papers on in vitro work:

One paper looked at the medulla in the peri-natal period, while this protocol is working with the thoracic spinal cord in the adult which is very different and not comparable.

One was actually a drug study in rats and not in vitro at all.

The third paper studied locomotion and respiration, which is unrelated to the coughing reflex that this protocol is studying.

4) Could it be done in non-mammals or in other mammals?

Name of the database	Date of search	Period of years covered by the search	Key words and/or search strategy used	How many papers were found?
ALTBIB animal alternatives search strategy - all citations	3/10/18	2000-present	spinal cord stimulation, cough	0

PubMed	3/10/18	All available years	coughing AND spinal cord AND (bird OR reptile OR amphibian)	0
PubMed	3/10/18	All available years	coughing AND spinal cord stimulation AND (rat OR mouse OR guinea pig OR rabbit OR pig)	3

Fish and invertebrates do not have lungs and so cannot be used as a model for coughing.

An ALTBIB search for relevant papers looking at alternatives for studying spinal cord stimulation and cough brought up no papers.

A PubMed search for non-mammalian alternatives (bird OR reptile OR amphibian) for studying spinal cord stimulation and cough also brought up no papers.

A PubMed search for other mammalian models (rat OR mouse OR guinea pig OR rabbit OR pig) brought up only 3 papers. Two were papers looking at chemical stimulation, which is not relevant to the work we are doing. The third was an earlier paper from this group using mini-pigs [Kowalski 2016]. Unfortunately, the mini-pig spinal cord has only half the diameter of the human spinal cord. Since the size of the spinal cord and distance between roots are relatively similar in humans and dogs this protocol will be able to more accurately determine optimal electrode location, the pathways by which the motor nerves innervating the expiratory muscles are stimulated, and construct a model of current distribution. This information is essential for designing a proper clinical trial and the dog is the best model for obtaining this information.

5) **Are the methods used the best available (least painful or distressing to the dogs)?**

All dogs will be fully anesthetized throughout the experiment, and then immediately euthanized without regaining consciousness once the experiment is complete. Euthanasia will be performed with the same method used in regular veterinary clinics. They will experience no pain or distress from the experiment.

Animals will be anesthetized using 1 of 2 methods:

1. Pentobarbital. An initial dose of 30mg/kg will be given intravenously. This will be supplemented by additional doses (3-5ml/kg) as required to maintain sufficient anesthesia.

2. Alpha chloralose with Pentobarbital. Animals will receive pentobarbital (25-30mg/kg) given intravenously. After surgical preparation, anesthesia will be maintained with alpha chloralose IV (100 mg/kg) as required to maintain sufficient anesthesia.

Anesthetic level will be monitored by corneal, snout and lip pinch reflexes and changes in respiratory rate, heart rate and blood pressure.

The primary aim of this study design is the measurement of muscle activity and effects of muscle contraction secondary to electrical stimulation. Many anesthetics and sedatives, including inhalational agents, result in muscle relaxation and impair the responses to electrical stimulation. This group has attempted to use a sedative, such as Xylazine, and even after administration of the reversal agent, Yohimbine, the responses to electrical stimulation were markedly reduced. They have tried a variety of anesthetic agents, including inhalational Halothane and Isoflurane, and found that muscle activation is markedly suboptimal. Of note, the expiratory muscles are particularly sensitive to these agents. They have found that pentobarbital and alpha chloralose provide the least muscle relaxation and therefore are used as the primary and secondary anesthetic agents respectively.