Last Name of PI▶ [redacted]
Protocol No. Assigned by the IACUC▶ 9952-01P
Official Date of Approval ▶ [redacted]
Version 2 Approva
Version 3 Approval:
Version 4 Approval:

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▶ **Clement J. Zablocki VA Medical Center / 695**
3. Protocol Title▶ **Neuropharmacology of Pontine Control of Breathing Frequency**
4. Animal Species covered by this ACORP▶ **Dog**
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▶

      (2) If approved by the R&D Committee, give the date of approval▶

   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
      ▶

      (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
      ▶

      (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.

►

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 ► **Mu-opioid Effects on the Central Mechanisms that Control Breathing**
(2) IACUC approval number of other protocol ►**9952-01**
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 ► **Clement J. Zablocki VA Medical Center / 695**

7. Indicate the type(s) of animal use covered by this protocol (check all that apply):

►(X) Research
►( ) Teaching or Training
►( ) Testing
►( ) Breeding and colony management only; not for any specific research project
►( ) Holding protocol (as specified by local requirements; not required by VA, PHS, or USDA)
►( ) Other. Please specify►

Proposal Overview

B. **Description of Relevance and Harm/Benefit Analysis.** Using non-technical (lay) language that a senior high school student would understand, briefly describe how this research project is intended to improve the health of 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.

► Many pathologic conditions common to the Veteran population compromise respiratory function and include: diseases of the airways, lungs, and cardiovascular system, injuries to the head, brain, and cervical spinal cord, brain tumors, central and obstructive sleep apneas and hypertension. The Veteran population also has a frequent need for potent analgesics, be it for perioperative pain control or chronic pain from trauma, degenerative diseases or cancer. These drugs can cause serious respiratory depression leading to inadequate brain oxygen, loss of consciousness and even death.

Pharmacological strategies to restore respiratory function in these settings have been lacking due to a lack of detailed understanding of central respiratory control mechanisms and the effects of opioids and sedatives on it. The main purpose of this series of studies is to obtain detailed knowledge of the neurophysiology and neuropharmacology of specific functional groups of respiratory neurons that control breathing rate, which will serve as a prerequisite for designing specific medications and therapeutic strategies that allow adequate pain control without respiratory depression. All procedures will be carried out on animals rendered unconscious by adequately deep anesthesia and subsequent decerebration.

C. **Experimental Design.**

3. **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.

► Our previous studies have found that neurons in the brainstem region known as the pons are very sensitive

to low clinical opioid concentrations that are able to cause severe slowing of breathing rate and have the potential to cause respiratory arrest. Furthermore, we have discovered that neurons in a small sub region of the pons control breathing frequency and appear to be the *main channel* that mediates opioid-induced depression of breathing rate. Localized excitation of these neurons increases breathing rate, while localize depression of these neurons decrease breathing rate even to the point of apnea. Thus, any drugs that affect the activity of these neurons will have a major impact on breathing. The proposed series of studies are designed to precisely locate the subregion that controls breathing rate and to characterize the types of neurons in this subregion as well as their neurophysiological and neuropharmacologic properties. This information should suggest possible drug therapies to stimulate breathing that has been severely depressed by opioids and sedatives without compromising the beneficial effects of these potent pain relievers. From a functional point of view, these studies provide a better understanding of the mechanisms underlying the control of breath and the organization of the control neuronal network.

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

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

► All studies will use adult dogs, typically purpose-bred beagles, in acute experiments. They will be deeply anesthetized and subsequently decerebated so as to cause a complete loss of consciousness and sensation. Autonomic functions are well preserved in this model.

A specific group of animals is assigned to each of the 10 proposed protocols. These protocols are designed to address the following specific aims:
1: Precisely locate the parabrachial (PB) subregion in the dorsal pons that controls eupneic breathing frequency (f_B).
2: Identify the PB subregion neuron subtypes, determine if their axons project to the rhythmogenic preBötzinger Complex region and quantify their responses to pulmonary stretch receptor (PSR) inputs.
3: Determine how the discharge patterns of the various PB subregion neuron subtypes are generated and controlled by **A)** NMDA and non-NMDA receptor mediated glutamatergic endogenous excitation and **B)** by GABAergic and glycinergic endogenous inhibition.
4: Determine **A)** whether the $GABA_A$ receptors on neurons within this region are modulated by benzodiazapines (BZDs; e.g., midazolam), **B)** whether *systemically-administered* BZDs act on neurons within the PB subregion (antagonized by microinjected flumazenil), and **C)** whether microinjected BZDs modulate the effects of iv remifentanil-induced bradypnea.
5: Determine the role of $GABA_B$ receptors in the modulation of PB subregion neurons and inspiratory and expiratory phase durations (T_I and T_E) via microinjections of selective agonists and antagonists.
6: Identify preBC neuron subtypes that mediate increases in f_B evoked within the PB subregion by AMPA stimulation and highly localized electrical stimuli.
7: A) Characterize the modulation of the PSR reflex control of T_I and T_E mediated by the PB subregion, and **B)** and determine whether this modulation is due to PB subregion inputs to the solitary tract nucleus (NTS) that alter the PSR neurotransmission to the second order neurons.

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

► There are 2 protocols (1 & 4D, see Table below) for the microinjection studies without neuronal recording requiring a fewer dogs (~6 /protocol). In protocols of neuronal recordings w/wo microinjections (2, 3A, 3B, 4 A&B, 4C, 5 A&B, and 6) involving multiple subtypes of neurons, we

estimated numbers of neurons based on a 1-way ANOVA with a main factor of neuron subtype (7 levels). A sample size of 25 neurons/level would be required to detect 20% changes from control as significant using a within-group SD of 25% (based on data of ~100 premotor neurons) with α=0.05 and a power of 0.9. For protocols 2 & 6 without local microinjections, and due to the fixed distance (0.1 mm) between the 16 electrodes on the probe and based on our previous experience, we estimate 4-5 neurons/insertion and 2-3 complete protocols/dog (~10 neuron-protocols/dog). For protocols 3A, 3B, 4 A&B, 5 A&B, some subtypes will be combined to reduce the number of levels from 7 to 5, requiring ~125-neurons/protocol. Due to the microinjections of 2 drugs/neuron study, we estimate 1-2 complete protocols/dog with 4-5 neurons/probe insertion or ~6-neuron-protocols/dog. For protocol 4C that incorporates IV remi infusions and microinjections of midazolam in the PBSR, we will pool I- and E neuron subtypes and with NRM neurons this results in a factor level of 3 or a total 75 neuron-protocols. For protocol 7 A&B, only pump neurons will be studied and we estimate ~25 neurons would be required to detect significant changes in synaptic transmission. From the Table shown below, the estimated number of neurons for all 7 protocols is 950 obtained in 152 animals over a 48 month period. We have increased the number of animals by about 10% to account for experimental results affected by incomplete protocols due to the inability to maintain stable neuronal recordings during picoejections and/or technical difficulties, including poor signal-to-noise ratio, contamination by other unit activity, spontaneous dislodgment of the electrode, plugged ejection barrels, back flow between barrels, or ineffective drug action. If the success rate is higher, fewer animals will be used.

Table. Protocol Sequence for 4 years. Estimated number of neurons, dogs, and durations per protocol based on difficulty and experience. Power analysis information is given above. PBSR: parabrachial subregion.

#	Protocol	# Neurons	# dogs	Duration (mo.)	Year			
					1	2	3	4
1	Locate PBSR w/ microinj. AMPA	----	6	2	X			
2	Neurophysiology of PBSR neurons	175	13	5	X			
3A	PBSR neurons (5) w/ dual probe & AP5 & NBQX	125	23	5	X			
3 B	PBSR neurons w/ dual probe & BIC & STRY or PIC	125	23	5		X		
4 A&B	PBSR neurons w/ dual probe & local midazolam & flumazenil	125	23	5		X		
4 C	PBSR neurons w/ dual probe & IV remi & PBSR midazolam	75	12	6		X	X	
4 D	IV remi & midazolam w/ PBSR flumazenil microinjections	----	6	2			X	
5 A&B	PBSR neurons w/ dual probe & baclofen & SCH 50911	125	23	6			X	
6	preBC/BC neurons (7) / PBSR AMPA & electrical stimili	175	16	6				X
7 A&B	PSR/B-H reflex/PBSR modulation/pump NTS neurons	25	7	6				X
	TOTAL:	950	152	48				

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.)

▶ Anesthesia in mongrel dogs (8-15 kg) of either sex will be induced by mask with isoflurane and

maintained with the same agent (1-5% to effect) throughout the surgical preparation until completion of the decerebration procedure (described in section 4b, Methods of Procedure). The trachea will be intubated with a cuffed endotracheal tube and an adequate plane of surgical anesthesia will be ensured by lack of movement to skin incision, changes in blood pressure, salivation, isoflurane-induced decrease in blood pressure, and end-expired anesthetic concentrations at 1.5—2 times minimum alveolar concentration (MAC) for unresponsiveness associated with deep anesthesia. Mechanically ventilation with an air-O2 mixture will be used throughout the experiment to maintain hyperoxic isocapnia (FIO2 >0.6, end-tidal CO2 range 40–50 mmHg) monitored with an airway infrared gas analyzer. The left femoral artery and vein will be cannulated for measurement of blood pressure and infusion of maintenance fluids and test drugs (via 3-lumen catheter), respectively. Subsequently, a nondepolarizing muscle relaxant vecuronium (1mg/kg bolus, followed by 0.2 mg/kg/h IV infusion or by longer acting pancuronium bromide, 0.1 mg/kg/hr, if available) will be administered. Esophageal temperature will be measured and maintained within a target range of 37.5 - 38.5 °C with a servo-controlled heating pad. The animals will be positioned in a stereotaxic device. Bilateral dorsolateral neck dissections will be performed to isolate the vagus, and phrenic nerves for either recording (phrenic) or deafferentation and stimulation (vagus). Bilateral pneumothorax will be performed to minimize brainstem movement and phasic inputs from chest wall mechanoreceptors. Epsilon aminocaproic acid and dexamethasone 0.5 mg/kg IV will be given prophylactically every 6 hours to minimize potential brainstem edema related to surgical trauma. If marked brainstem swelling should occur, mannitol, an osmotic agent, will be administered IV at dose of 1.5 g/kg using a 10% solution. A carefully controlled midcollicular decerebration procedure will be carried out through bilateral parietal craniotomy windows. An occipital craniotomy will be performed and the dura mater will be cut along the midline and retracted to expose the medulla oblongata. A cerebellectomy and external sagittal and nuchal bone crest removal will be performed to completely expose the dorsal pontine surface. Respiratory neuronal activities will be recorded from the parabrachial region of the pons with a 16-electrode microprobe, and in other protocols with multibarrel micropipettes in conjunction with picoejection of neurotransmitter agonists and antagonists onto the neurons. These techniques will be used to examine the neurophysiology and neuropharmacology of neurons within a subregion of the pontine parabrachial region that appears to be essential for the control of breathing frequency and is a major location where opioids and sedatives act to depress breathing. Euthanasia for the decerebrate dogs at the conclusion of the proposed experiments will be accomplished with IV infusion of saturated KCl, in accordance with the current guidelines established by the American Veterinary Medical Association Panel on Euthanasia (AVMA Guidelines for the Euthanasia of Animals: 2013 Edition). Blood pressure is monitored to observe cardiac arrest.

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

► Alternative, non-animal methods cannot be used to meet the specific aims of this proposal. These *in vivo* experiments are designed to test hypotheses regarding the mechanism of action of neurotransmitters/modulators, μ-opioids and sedatives on specific types of respiratory neurons. Because of the highly complex and poorly understood nature of neural circuits within the mammalian CNS, an understanding of the control of breathing frequency and respiratory rhythm generation and the effects of drugs on critical portions of the network can only be obtained in intact, spontaneously active neural networks.

Our choice of dogs is based on our past 26 years of experience in 1) characterizing the integration of afferent inputs at expiratory and inspiratory premotor neurons, 2) identifying the associated neurotransmitter receptors for various afferent inputs to these neurons, 3) our detailed studies on the effects of volatile anesthetics on neurotransmission in respiratory premotor neurons, 4) studies on neurotransmitter systems and anesthetic effects in inspiratory hypoglossal motoneurons 5) studies characterizing the role of μ- and ∂-opioid receptors in respiratory bulbospinal premotor neurons, as well as data characterizing preBC neurons and their response to opioids, and 6) our recent studies of locating the pontine opioid-sensitive region that produces

bradypnea/apnea and oipoid-induced depression of the neurons in that region. The relatively large size of medullary respiratory and hypoglossal motoneurons in dogs (soma: 30-50 µm in diameter) allows stable recordings from a single neuron for hours, even in the presence of pressure picoejections and interventions, which may alter blood pressure, such as changes in anesthetic depth. It would be extremely difficult to use the picoejection technique in preparations with smaller and more densely spaced cells such as rodents where isolation of unit activity would be much harder and even small movements of the electrode tip lead to loss of the recording. In addition, the opioid-sensitive pontine region has not been identified/characterized in other species.

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]
Animal research experience ▶ [redacted] Laboratory and animal experience.
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
Training & Advising postdoc fellows & techs in all aspects of the acute in vivo preparation and data collection procedures	[redacted]

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

Name [redacted]
Animal research experience ▶ [redacted] Laboratory and animal experience.
Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this ACORP
Training postdoc fellows & techs in all aspects of the acute in vivo preparation	[redacted]

Name [redacted]
Animal research experience ▶ [redacted] Laboratory and animal experience.
Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this ACORP

Training postdoc fellows & techs in all aspects of the acute in vivo preparation	[redacted]

Name▶ [redacted]
Animal research experience ▶ [redacted] aboratory and animal experience.
Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this ACORP
Anesthetic induction, inturbation, ventilation, placement of femoral arterial and venous lines, dissection/isolation/preparation of phrenic and vagus nerves	[redacted]

Name [redacted]
Animal research experience ▶ [redacted] aboratory and animal experience.
Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this ACORP
Responsible for carrying out all aspects of the in vivo preparation and data collection procedures and protocols	[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]	Dogs	
		Dogs	
		Dogs	

[redacted]		[redacted] Dogs	
		-Dogs	

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"
► 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		
[redacted]	(X)	( )	( )	
	(X)	( )	( )	
	(X)	( )	( )	
	(X)			
	(X)			

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

► ( ) Yes. Describe them ►

► (X) No.

Animals Requested

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
dogs	**either**	**Adult; 8-16 kg**	[redacted]	**Conventionial**

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

USDA Category B

Procedures▶						
Species / 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**
dog	**42**	**50**	**37**	**23**		**152**

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**
	42	**50**	**37**	**23**		**152**

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

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

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

Procedure	Monitoring (indicate the method(s) to be used, and the frequency and duration of monitoring through post-procedure recovery)	Person(s) responsible for the monitoring	Method(s) by which pain or distress will be alleviated during or after the procedure (include the dose, route, and duration of effect of any agents to be administered)
See Appendix 5	**Continuous**	**PI or postdoc fellow**	**deep inhalational anesthesia until decerebration completed**

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

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

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

Veterinary Care and Husbandry

L. **Veterinary Support**.

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

Name [redacted]
Institutional affiliation ▶ [redacted]
email contact [redacted]

Name [redacted]
Institutional affiliation: [redacted]
email contact: [redacted]

Name [redacted]
Institutional affiliation: [redacted]
email contac [redacted]

4. Veterinary consultation during the planning of this protocol.

Name of the laboratory animal veterinarian consulted ▶ [redacted]

Date of the veterinary consultation (meeting date, or date of written comments provided by the veterinarian to the PI) ▶ [redacted]

M. **Husbandry.** As a reference for the animal husbandry staff, summarize here the husbandry requirements of the animals on this protocol. (Use Appendix 6 to justify the use of any special husbandry and to detail its effects on the animals. Use Appendix 9 to document any aspects of the husbandry that involve "departures" from the standards in the *Guide*. Consult the IACUC or the Attending Veterinarian for help in determining whether any "departures" are involved.)

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

a. Species	b. Type of housing*	c. Number of individuals per housing unit**	d. Is this housing consistent with the *Guide* and USDA regulations? (yes/no***)	e. Estimated maximum number of housing units needed at any one time
dog	stainless steel runs (4'x7') sanitized daily	2	yes	7

*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*.

4. 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
dog	Standard (See SOP)	
		[redacted]

*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.
▶

5. 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.

N. **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]		(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

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

▶ ( ) Some or all of the animals on this protocol WILL be used in the production and harvesting of antibodies. Check "Appendix 2" in Item Y, below, and complete and attach Appendix 2, "Antibody Production".

▶ (X) NO animals on this protocol will be used in the production and harvesting of antibodies.

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

▶ (X) This protocol INVOLVES administration of substances to the animals other than those used in routine husbandry and veterinary care. Check "Appendix 3" in Item Y, below, and complete and attach Appendix 3, "Biosafety".

▶ ( ) This protocol does NOT involve administration of any substances to the animals other than those used in routine husbandry and veterinary care.

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

Procedure	Surgical?		Bldg/Room Number	Requires transport through non-research areas?	
	Yes	No		Yes – describe method of discreet transport	No
Experiment	**(X)**	**( )**	[redacted]	**( )**	**(X)**
	( )	**( )**		**( )**	**( )**
	( )	**( )**		**( )**	**( )**
	( )	**( )**		**( )**	**( )**

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

Body Fluid, Tissue, or Device to be	Collected AFTER	Collected BEFORE Euthanasia

Collected	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")
Brainstem	**(X)**	( )	( )	( )
	( )	( )	( )	( )

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

▶ (X) Surgery WILL BE PERFORMED on some or all animals on this protocol. Check "Appendix 5" in Item Y, below, and complete and attach Appendix 5, "Surgery".

▶ ( ) NO animals on this protocol will undergo surgery.

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

▶ It is not anticipated that animals will develop signs of illness. If an animal develops clinical signs of illness, a VMU consulting veterinarian will be consulted for treatment options, including euthanasia as appropriate.

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

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

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

Check each method that may be used on this protocol	Method of Euthanasia	Species	AVMA Classification		
			Acceptable	Conditionally Acceptable	Unacceptable

( )	CO_2 from a compressed gas tank Duration of exposure after apparent clinical death▶ Method for verifying death▶ Secondary physical method▶		( )	( )	( )
( )	Anesthetic overdose Agent▶ Dose▶ Route of administration▶		( )	( )	( )
( )	Decapitation under anesthesia Agent▶ Dose▶ Route of administration▶		( )	( )	( )
( )	Exsanguination under anesthesia Agent▶ Dose▶ Route of administration▶		( )	( )	( )
(X)	Other (Describe) ▶ **Intravenous KCl 10 ml/kg** **Animals are decerebrate with pneumothorax**		(X)	( )	( )
( )	Other (Describe) ▶		( )	( )	( )

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

4. 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:
   ▶

5. 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]

6. 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.
   ► VMU staff will contact our staff. Carcass should be bagged and held for necropsy, with indication or note on the animal room as to when the animal was found dead or when euthanasia had to be performed.

   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.
   ► VMU staff will contact our staff via the contact information on file in the VMU office.

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

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
		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.)

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

3. Document the database searches conducted.
List each of the potentially painful or distressing procedures included in this protocol.
▶ PUBMED

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 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	8-26-14	2000-2014		opioids & resp. depression	(X)	(X)	(X)	(X)
PubMed	8-26-14	1970-2014		Respiratory neurons & opioids	(X)	(X)	(X)	(X)
PubMed	8-26-14	1970-2014		PreBotzinger & opioids	(X)	(X)	(X)	(X)
PubMed	8-26-14	1970-2014		pons&opioid&breathing	(X)	(X)	(X)	(X)

4. 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.

▶ Alternative methods cannot be utilized. The procedures require measurement of complex responses in a living, reflexly-responsive system or tissues obtained from such a system for which no *in vitro* system or computer model is sufficient. A comprehensive search and review of the available scientific literature via the PubMed database (see above) and national meetings of the American Society of Anesthesiologists and the Society for Neuroscience failed to identify possible alternative methods to the use of live animals in this project.

5. 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.

▶ A power analysis was performed to determine the required number of animals. Intermittent data analysis will be performed and if the success rate is better than expected, the number of planned experiments will be reduced. In addition, we have developed new technology that incorporates a 16-electrode probe in conjunction with a multibarrel pipette in order to record from and microeject drugs on *multiple* neurons during each protocol, thereby reducing the number of experimental runs.

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

▶ All surgical procedures have been performed in this experimental setup before. The current techniques are highly successful in maintaining homeo- and hemostasis, which are necessary for obtaining stable extracellular neuronal recordings over an 8-10 hour period.

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

▶ A careful search of the scientific literature, combined with annual attendance of National Scientific Meetings and many years working as an expert in the control of breathing indicate that these proposed studies do not unnecessarily duplicate work in the literature.

X. **Other Regulatory Considerations**.

3. **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 Phar-macy	Non-VA
Remifentanil	(X)	( )*	[redacted]	(X)	( )	(X)	( )
Midazolam	(X)	( )*		(X)	( )	(X)	( )
Phenylephrine	(X)	( )*		(X)	( )	(X)	( )

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

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 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► .

4. **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.

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

   ► ( ) Yes, some explosive agent(s) will be used on this protocol. Check "Appendix 3" and "Appendix 8" in Item Y, below, and complete and attach Appendix 8, "Use of Explosive Agent(s) within the Animal Facility or in Animals", as well as Appendix 3, "Biosafety".

   ► (X) No explosive agent(s) will be used as part of this protocol.

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

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

► ( ) 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			
M.1			
M.2	Veterinary Medical Unit SOPs		09/2010
U.4.a			
U.4.b			
V			

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

3. **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
[redacted]		[redacted]

b. **Certification by IACUC Officials.**

We certify that:

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

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

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

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

Name of Attending Veterinarian (VMO or VMC)	Signature	Date
[redacted]		
Name of IACUC Chair	Signature	Date
[redacted]		

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

5. **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)	Signature(s)	Date
[redacted]		08/27/2014
Name of Institutional Veterinarian	Signature	Date
[redacted]		
Name of IACUC Chair	Signature	Date
[redacted]		

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]		

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

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

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

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

7. **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]

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

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

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

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

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

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

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

Name of IACUC Chair	Signature	Date
NA	NA	NA
Name of Attending Veterinarian (VMO or VMC)	Signature	Date
NA	NA	NA
Name of the Chair of the Clinical Executive Board, or the Service Chief responsible for the Patient Care Area and Equipment	Signature	Date
NA	NA	NA
Name of ACOS for R&D	Signature	Date
NA	NA	NA
Name of Chief of Staff	Signature	Date
NA	NA	NA
Name of Director or CEO of the Facility (Hospital or Clinic)	Signature	Date
NA	NA	NA

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

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

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

I further certify that:

- Procedures involving explosive agent(s) will be performed within a properly operating, ventilated safety hood;

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

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

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

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

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

ACORP Appendix 3
Biosafety
Version 4

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

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

Material	Source	Nature of Material

(Identify the specific agent, device, strain, construct, isotope, etc.)	(Identify the vendor or colleague, or specify which animals on this protocol will serve as donors)	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
isoflurane	VMU	(X)	( )BSL_	( )	( )	( )	( )	( )
Lactated Ringer's (LR)	VMU	( )	( )BSL_	( )	( )	( )	(X)	( )
Dexamethasone (anti-inflammatory)	VMU	( )	( )BSL_	( )	( )	( )	(X)	( )
Epsilon-aminocaproic acid (antifibrinolytic)	VMU	( )	( )BSL_	( )	( )	( )	(X)	( )
Sodium Bicarbonate	VMU	( )	( )BSL_	( )	( )	( )	(X)	( )
lidocaine	VMU	(X)	( )BSL_	( )	( )	( )	( )	( )
epinephrine	VMU	(X)	( )BSL_	( )	( )	( )	( )	( )
Phenylephrine HCl	VMU	(X)	( )BSL_	( )	( )	( )	( )	( )
heparin	VMU	(X)	( )BSL_	( )	( )	( )	( )	( )
D,L-homocysteic acid (DLH)	Sigma	(X)	( )BSL_	( )	( )	( )	( )	( )
D-Ala2,N-Me-phe,gly5-ol]-enkephalin (DAMGO)	Sigma	(X)	( )BSL_	( )	( )	( )	( )	( )
naloxone	Sigma	(X)	( )BSL_	( )	( )	( )	( )	( )
Artificial cerebrospinal fluid (aCSF)	Sigma	(X)	( )BSL_	( )	( )	( )	(X)	( )
Pontamine sky blue	Sigma	(X)	( )BSL_	( )	( )	( )	( )	( )
paraformaldehyde	Sigma	(X)	( )BSL_	( )	( )	( )	( )	( )
vecuronium	VMU	(X)	( )BSL_	( )	( )	( )	( )	( )
cisatracurium	VMU	(X)	( )BSL_	( )	( )	( )	( )	( )
pancuronium	VMU	(X)	( )BSL_	( )	( )	( )	( )	( )

pancuronium	Sigma	(X)	( )BSL_	( )	( )	( )	( )	( )
KCl	VMU	( )	( )BSL_	( )	( )	( )	( )	(X)
Remifentanil	VMU	(X)	( )BSL_	( )	( )	( )	( )	( )
N-methyl-D-aspartate (NMDA)	Sigma	(X)	( )BSL_	( )	( )	( )	( )	( )
α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)	Sigma	(X)	( )BSL_	( )	( )	( )	( )	( )
2-amino-5-phosphonovaleriate (AP5)	Sigma	(X)	( )BSL_	( )	( )	( )	( )	( )
2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)-quinoxaline (NBQX)	Sigma	(X)	( )BSL_	( )	( )	( )	( )	( )
Bicuculline MCl	Sigma	(X)	( )BSL_	( )	( )	( )	( )	( )
Muscimol	Sigma	(X)	( )BSL_	( )	( )	( )	( )	( )
Glycine	Sigma	(X)	( )BSL_	( )	( )	( )	( )	( )
Strychnine	Sigma	(X)	( )BSL_	( )	( )	( )	( )	( )
Midazolam	VMU	(X)	( )BSL_	( )	( )	( )	( )	( )
Flumazenil	Sigma	(X)	( )BSL_	( )	( )	( )	( )	( )
Picrotoxin	Sigma	(X)	( )BSL_	( )	( )	( )	( )	( )
Baclofen	Sigma	(X)	( )BSL_	( )	( )	( )	( )	( )
SCH 50911 (GABA-B antag.)	Sigma	(X)	( )BSL_	( )	( )	( )	( )	( )
Mannitol	VMU	(X)	( )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)
Isoflurane	1-2 Vol%	Air/O2	ETT	Until decerebration complete	Maintenance anesthetic	C.2.c	Y
LR	1-20 ml/h	same	IV or IA	continuous	Carrier fluid, line flush	C.2.c	Y
Sodium Bicarbonate	12-25 mg/kg	LR	IV	Once, then as needed Induction & maintenance	Balance pH	C.2.c	Y
Dexamethasone (anti-inflammatory)	0.5 mg/kg	sterile H2O	IV	Every 6 hours	reduce brain swelling	C.2.c	Y
Epsilon-aminocaproic acid (antifibrinolytic)	125 mg/kg load and 15 mg/kg/h	sterile H2O	IV	continuous infusion	aid clotting	C.2.c	Y
Mannitol	1.5 g/kg	sterile H2O	IV	once	reduce brain swelling	C.2.c	Y
Lidocaine	10 mg/ml, 1-3 ml	LR	SQ	once	Local anesthesia	C.2.c	Y
epinephrine	2 mcg/ml, 1-20 ml/h	LR	IV	continuous	Blood pressure support	C.2.c	Y
Phenylephrine HCl	30µg/ml; 1-2 ml/min	LR	IV	continuous	Blood pressure support	C.2.c	Y
heparin	2.5 IU/ml	LR	IA	continuous	Line flush	C.2.c	Y
DLH	1 mM, 30nl	aCSF	Microinjection (µ-inject)	Seconds, ~20/animal	Glu Receptor agonist	C.2.a, b,c	Y
DAMGO	100µM, 100nl	aCSF	µ-inject	Seconds, 6-20/ animal	mu-opioid agonist	C.2.a, b,c	Y

naloxone	1mM, 100nl	aCSF	µ-inject	Seconds, 6-20 /animal	Opioid receptor antagonist	C.2.a, b,c	Y
naloxone	100µM, 1-3 ml	LR	IV	Bolus, 3x	Opioid receptor antagonist	C.2.a, b,c	Y
aCSF	See above	same	µ-inject	See above	Diluent	C.2.a, b,c	Y
Lumafluor Red Retrobeads	5% solution 200nl	dstl H2O	µ-inject	4x/animal	fluorescent dye electrode tip site marker	C.2.a, b,c	Y
paraformaldehyde	4%, 1-5 l	Phosphate buffered saline	Into carotid artery	once	Brainstem perfusion	C.2.a, b,c	Y
vecuronium	0.2 mg/kg PRN	LR	IV	PRN	paralysis	C.2.c	Y
cisatracurium	0.2 mg/kg	LR	IV	PRN	paralysis	C.2.c	Y
pancuronium	0.1 mg/kg	LR	IV	PRN	Paralysis	C.2.c	Y
KCl	Saturated, 2 ml/kg	NS	IV	once	Euthanasia	C.2.c	Y
Remifentanil	0.1–1.0 µg/kg/min	dstl H2O	IV	2-4x /animal	mu-opioid agonist	C.2.a, b,c	Y
N-methyl-D-aspartate (NMDA)	100-200 µM 100-200 nl	aCSF	µ-inject	10-20x /animal	NMDA –R agonist	C.2.a, b,c	Y
α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)	5-10 µM 20-100 nl	aCSF	µ-inject	20-50x /animal	AMPA-R agonist	C.2.a, b,c	Y
2-amino-5-phosphonovaleriate (AP5)	1-2 mM 100 nl	aCSF	µ-inject	10-20x /animal	NMDA-R antagonist	C.2.a, b,c	Y
2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)-quinoxaline (NBQX)	200-300 µM 100 nl	aCSF	µ-inject	10-20x /animal	AMPA-R antagonist	C.2.a, b,c	Y
Bicuculline MCl	100-200 µM 100 nl	aCSF	µ-inject	10-20x /animal	Competative GABAA-R antagonist	C.2.a, b,c	Y
Muscimol	10-20 µM 100 nl	aCSF	µ-inject	4-6x /animal	GABAA-R agonist	C.2.a, b,c	Y
Glycine	1-2 mM 100 nl	aCSF	µ-inject	4-6x /animal	Glycine-R agonist	C.2.a, b,c	Y
Strychnine	0.5-1 mM 100 nl	aCSF	µ-inject	4-6x /animal	Glycine-R antagonist	C.2.a, b,c	Y
Midazolam	10-100 µM 100 nl	aCSF	µ-inject	10-20x /animal	Benzodiaz-R agonist	C.2.a, b,c	Y

Midazolam	100-300 µg/kg	aCSF	IV	2-3x /animal	Benzodiaz-R agonist	C.2.a, b,c	
Flumazenil	10-100 µM 100 nl	aCSF	µ-inject	10-20x /animal	Benzodiaz-R antagonist	C.2.a, b,c	Y
Picrotoxin	1-5 mM 100 nl	aCSF	µ-inject	4-6x /animal	non-compet GABAA-R antagonist	C.2.a, b,c	Y
Baclofen	100 µM 100 nl	aCSF	µ-inject	10-20x /animal	GABAB-R agonist	C.2.a, b,c	Y
SCH 50911 (GABA-B antag.)	100 µM 100 nl	aCSF	µ-inject	20-50x /animal	GABAB-R antagonist	C.2.a, b,c	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.)

► Recent drug shortages have made it difficult to obtain sufficient muscle relaxants to ensure successful experiments. For this reason, non-pharmaceutical grade **pancuronium** may be reconstituted in normal saline from powder purchased from Sigma. Mixing of the substances will be performed in a flow hood under aseptic conditions. The substance will not be stored beyond the duration of the experiment, i.e., ~ 16h. Drug dosing will be equivalent to pharmaceutical grade pancuronium. As long as any other paralytic is available, use of non-pharmaceutical grade pancuronium will be avoided.

In compliance with the recent change in USDA requirements that all euthanasia drugs be pharmaceutical grade we will in the future purchase pharmaceutical grade KCl through the VMU. The highest available concentration is 40 meq/ml. We have not yet been able to establish which volume of this concentration is necessary to achieve euthanasia in our decerebrate animals. Should the required volume greatly exceed 20 ml, we will continue to use non-pharmaceutical grade, saturated KCl (Sigma) to ensure that euthanasia is achieved quickly and reliably.

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):

► All drugs will be given to the animal after induction of general anesthesia with isoflurane (1-5% to effect) and during maintenance of anesthesia with isoflurane (1-2 Vol%). Sufficient anesthetic depth will be judged from lack of withdrawal to pain, lack of salivation, and lack of blood pressure and heart rate increases with stimulation. General inhalational anesthesia will be terminated once decerebration is complete.

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	
isoflurane	( )	( )	( )	( )	( )	( )*	(X) sedation, only if inhaled in small, sealed compartment (mask, ETT)
lidocaine	( )	( )	( )	( )	( )	( )*	(X) none at the volume used SQ (<3ml), none with skin contact
epinephrine	( )	( )	( )	( )	( )	( )*	(X) blood pressure increase, arrhythmia, none with skin contact
Phenylephrine HCl	( )	( )	( )	( )	( )	( )*	(X) blood pressure increase, arrhythmia, none with skin contact
heparin	( )	( )	( )	( )	( )	( )*	(X) none at the volume used (<<1ml)
D,L-homocysteic acid (DLH)	( )	( )	( )	( )	( )	( )*	(X) none at the volume used (<<1ml)
D-Ala2,N-Me-phe,gly5-ol]-enkephalin (DAMGO)	( )	( )	( )	( )	( )	( )*	(X) none at the volume used (<<1ml)
naloxone	( )	( )	( )	( )	( )	( )*	(X) apnea when applied IV, none with skin contact
Pontamine sky blue	( )	( )	( )	( )	( )	( )*	(X) none at the volume used (<<1ml)
paraformaldehyde	( )	( )	( )	( )	( )	( )*	(X) irritating to skin and eyes
vecuronium	( )	( )	( )	( )	( )	( )*	(X) apnea when applied IV, none with skin contact
cisatracurium	( )	( )	( )	( )	( )	( )*	(X) apnea when applied IV, none with skin contact
pancuronium	( )	( )	( )	( )	( )	( )*	(X) apnea when applied IV, none with skin contact

KCl	( )	( )	( )	( )	( )	( )*	(X) apnea when applied IV, none with skin contact
Remifentanil	( )	( )	( )	( )	( )	( )*	(X) apnea when applied IV, none with skin contact
N-methyl-D-aspartate (NMDA)	( )	( )	( )	( )	( )	( )*	(X) none at the volume used (<10µl)
α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)	( )	( )	( )	( )	( )	( )*	(X) none at the volume used (<10µl)
2-amino-5-phosphonovaleriate (AP5)	( )	( )	( )	( )	( )	( )*	(X) none at the volume used (<10µl)
2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)-quinoxaline (NBQX)	( )	( )	( )	( )	( )	( )*	(X) none at the volume used (<10µl)
Bicuculline MCl	( )	( )	( )	( )	( )	( )*	(X) none at the volume used (<10µl)
Muscimol	( )	( )	( )	( )	( )	( )*	(X) none at the volume used (<10µl)
Glycine	( )	( )	( )	( )	( )	( )*	(X) none at the volume used (<10µl)
Strychnine	( )	( )	( )	( )	( )	( )*	(X) none at the volume used (<10µl)
Midazolam	( )	( )	( )	( )	( )	( )*	(X) apnea when applied IV, none with skin contact
Flumazenil	( )	( )	( )	( )	( )	( )*	(X) none at the volume used (<10µl)
Picrotoxin	( )	( )	( )	( )	( )	( )*	(X) none at the volume used (<10µl)
Baclofen	( )	( )	( )	( )	( )	( )*	(X) none at the volume used (<10µl)
SCH 50911 (GABA-B antag.)	( )	( )	( )	( )	( )	( )*	(X) none at the volume used (<10µl)
Mannitol	( )	( )	( )	( )	( )	( )*	(X) Diuresis, electrolyte imbalance with overdose

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

Name of agent ▶

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

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

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

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

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

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

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

Name of agent ▶

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

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

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

Name of Biological Agent	Screening for Infectious Agents

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

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

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

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

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

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

    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	(See detailed description at C.2.c)	(X)	( )	( )	( )*
2		( )	( )	( )	( )*
3		( )	( )	( )	( )*
4		( )	( )	( )	( )*

*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:
▶
b. Give the interval(s) between successive surgeries, and the rationale for choosing the interval(s):
▶

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.)

Dogs are manually but gently restrained, an anesthesia facemask is placed over the snout, then after the dog is anesthetized by isoflurane (1-5% to effect in 100% O2) using an anesthesia machine equipped with a waste gas scavenging via canister, an endotracheal tube is placed, and an adequate plane of surgical anesthesia is ensured by lack of palpebral and pedal reflexes, changes in blood pressure, salivation, isoflurane-induced decrease in blood pressure, and end-expired anesthetic concentrations at 1.5—2 times minimum alveolar concentration (MAC) for unresponsiveness associated with deep anesthesia.

The anesthetized dogs are mechanically ventilated with an air-O2 mixture and maintained in hyperoxic isocapnia (FIO2 >0.6, end-tidal CO2 range 40–50 mmHg) and surgically prepared to isolate the vagus, and phrenic nerves via bilateral neck incisions.

Vascular lines are placed in the femoral artery for continuous monitoring of blood pressure and heart rate and vein for administration of fluids and medications, such as corticosteroids and muscle relaxants and blood gas collection (if needed). After the arterial and venous cannulae have been placed and depth of anesthesia assessed via normal and stable values of blood pressure and heart rate, a nondepolarizing muscle relaxant vecuronium (1mg/kg bolus, followed by 0.2 mg/kg/h IV

infusion or by longer acting pharmaceutical grade pancuronium bromide, 0.1 mg/kg/hr, if available). If no pharmaceutical grade muscle relaxant is available due to manufacturing shortages, a non-pharmaceutical grade of pancuronium in powder form from Sigma, will be made fresh for each experiment.

The dogs will be positioned in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). A pneumothorax is performed. Animals will be decerebrated to prevent the confounding effects of background anesthesia on the interpretation of the neurotransmitter and opioid pharmacology. Complete separation of the brainstem from the diencephalon will be directly visualized as follows: A midline skin incision will be made from the spinous process of the axis to the nasion and the muscles and underlying connective tissue will be bilaterally dissected and reflected to expose the parietal bones. A high-speed drill with burr and rongeurs will be used to create 3.5 X 3.5-cm-wide bilateral parietal craniotomy windows. These windows are placed 0.5 cm lateral from the sagittal crest and 1.0 cm rostral from the nuchal crests to avoid disruption of the dorsal sagittal and transverse venous sinuses. After removal of the dura to expose the parietal and occipital lobes bilaterally, a bipolar coagulator and suction is used to remove small volumes of brain tissue sequentially until the caudal portions of the parietal and temporal lobes and the occipital lobe are removed bilaterally to expose the midbrain region. Blunt dissection will be use to expose the midline brain structures and the great cerebral vein will be clipped and then transected to avoid accidental tearing, which could lead to air embolism. The midcollicular line and the origins of the oculomotor nerves will be identified bilaterally to guide the brainstem transection in the desired plane with a spatula. The last few millimeters of the ventral brainstem are cut gently to avoid damage to the cavernous sinuses. The remaining brain tissue rostral to the transection will be gently removed by suction and cautery. Bleeding will be controlled by gentle bipolar coagulation, gauze packing and Surgicel (Ethicon Inc., Somerville, NJ). To prevent tissue drying, saline-soaked gauze will be placed on the transected brainstem. After the decerebration procedure is completed, isoflurane will be discontinued. In this type of model, most of the brainstem autonomic function is preserved, especially automatic control of breathing. Systemic blood pressure and fictive (neural equivalent of) breathing are continuously monitored and will be used to detected signs of responsiveness to paw pinch. After removal of the connective tissue from the dorsal atlas to the base of the scull, a drill and rongeurs will be used to remove the occipital bone bilaterally and the dura retracted to expose the medulla and floor of the 4th ventricle. Complete access of the dorsal surface of the brainstem will be obtained by cerebellectomy and external sagittal and nuchal bone crest removal.

Central respiratory neurons are recorded in the brainstem with micropipettes or microelectrodes and respiratory output is measured as changes in average phrenic nerve activity. Physiological changes in chemical respiratory drive are used to modify the breathing pattern. Neurotransmitters or transmitter antagonists are microejected through multibarrel glass pipettes at the site of the central neural recording. Experimental protocols to explore the neurotransmitter/modulator pharmacology of neurons in a subregion of the pontine parabrachial complex and their role in the control of breathing frequency will be performed in the decerebrate animals.

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

Name	Surgery #(s) (see Item 1)	Role in Surgery			
		Surgeon	Assistant	Manage Anesthesia	Other (describe)
[redacted]	all	(X)	( )	(X)	(X) supervise
	all	(X)	( )	(X)	( )
	all	(X)	( )	(X)	( )
	all	( )	(X)	(X)	( )
	all	(X)	( )	(X)	( )

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

Building	Room Number	Surgery #(s) (see Item 1)	Type of Space		
			Dedicated Surgical Facility	Other Dedicated Surgical Space	Other Space not Dedicated to Surgery
[redacted]	[redacted]	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

► **non-survival surgery, not requiring a sterile environment**

5. **Pre-operative protocol.**

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

Surgery #(s) (see Item 1)	Fast (Specify Duration)	Withhold Water (Specify Duration)	Place Intravenous Catheter(s) (Specify Site(s))	Other – Describe
1	(~12 hrs) --	( ) --	( ) --	( ) --
2	( ) --	( ) --	( ) --	( ) --
3	( ) --	( ) --	( ) --	( ) --
4	( ) --	( ) --	( ) --	( ) --

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)
Isoflurane	**All**	**1 to 5% to effect**	**Face mask for induction, and then after the endotracheal tube is placed, it is continuously delivered via closed circuit anesthesia machine and ventilator**	**Once**	**Induction & maintenance**

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.

Non-survival surgery. Hair in neck area; chest area and groin area will be clipped after anesthetic induction.

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

Isoflurane	( )*	**All**	**1-1.5 Vol%**	**inhalational**	**Continuous until decerebration**
Dexamethasone (anti-inflammatory)	( )*	**all**	**0.5 mg/kg**	**IV**	**Every 6 hours**
Epsilon-aminocaproic acid (antifibrinolytic)	( )*	**all**	**125 mg/kg load and 15 mg/kg/hour**	**IV**	**Continuous infusion**
Mannitol (osmotic agent)	( )*	**All**	**1.5 g/kg**	**IV**	**Once, if needed**
vecuronium	(X)*	**All**	**1mg/kg bolus, then 0.2 mg/kg/h**	**IV**	**Continuous infusion**
pancuronium	(X)*	**All**	**1mg/kg bolus, then 0.1 mg/kg/h**	**IV**	**Continuous infusion**
cisatracurium	(X)*	**all**	**1mg/kg bolus, then 0.2 mg/kg/h**	**IV**	**Continuous infusion**
Lactated Ringer Solution	( )*	**All**	**4-10 ml/kg/hour**	**IV**	**Continuous infusion**
Sodium Bicarbonate	( )*	**All**	**12-25 mg/kg**	**IV**	**Once, then as needed Induction & maintenance**

- 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.

► Delicate dissections of the phrenic nerves for recording neural activity and careful exposure of the highly vascular brainstem with maximal hemostasis via micro-cauterization require minimal movement, especially from spontaneous breathing. An adequate level of anesthesia (isoflurane: 2-5%) is assessed by lack of changes in continuously monitored arterial blood pressure and heart rate and lack of salivation and lacrimation during surgery leading up to decerebration, after which pain sensation and cognition are eliminated. An sign of discomfort will be used to signal that an increase in anesthetic is required.

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 warming pad will be used to maintain animal temperature between 36.5 and 37.5°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.

► Respiratory gas analysis including inspiratory and expiratory O2, CO2 and volatile agent will be continuously monitored with a Criticare gas analyzer. Alarm thresholds are set to maintain the parameters within normal limits. Blood pressure and respiratory rate are monitored continuously using a

PowerLab recording system. Increase in blood pressure or lacrimation or salivation before decerebration are interpreted as lightening of the anesthetic and will be treated with an increase in volatile agent. Changes in cardiac output are deduced from CO2 monitoring. Rectal temperature will be monitored continuously. Critical drops in blood pressure with preparation or drug application are immediately treated with increase in Lactated Ringer's until normalized. If required, phenylephrine HCl will be added to the IV fluid (~30 µg/ml final concentration) to aid the support of blood pressure.

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

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

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

   * 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.

   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					
2					
3					
4					

   *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)

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)

(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)

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.

(2) List the criteria for euthanasia related specifically to post-operative complications:
(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			( )	( )
2			( )	( )
3			( )	( )
4			( )	( )

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

Revision Signatures
Version 2 signatures

1. **Certification by Principal Investigator(s).**

To the best of my knowledge, I certify that the information provided in this Animal Component of Research Protocol (ACORP) Amendment is complete and accurate.

I further certify that:

- No personnel will perform any animal procedures until they have been approved by the IACUC. When new or additional personnel become involved in these studies, I will submit their qualifications, training, and experience to the IACUC and seek IACUC approval before they are involved in animal studies;
- I will ensure that all personnel are enrolled in an institutional Occupational Health and Safety Program prior to their contact with animals, or have declined in writing to participate, if allowed by local policy;

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

2. **Approval Signatures.** To the best of their abilities, the undersigned verify that the care and use of the animals described in this ACORP amendment has been evaluated 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,* VA Policy and local IACUC Policy, and find the use of animals described in this ACORP to be appropriate.

Name of Attending Veterinarian (VMO or VMC)	Signature	Date
[redacted]		
Name of IACUC Chair	Signature	Date
[redacted]		

Revision Signatures
Version 3 signatures

1. **Certification by Principal Investigator(s).**

To the best of my knowledge, I certify that the information provided in this Animal Component of Research Protocol (ACORP) Amendment is complete and accurate.

I further certify that:

- No personnel will perform any animal procedures until they have been approved by the IACUC. When new or additional personnel become involved in these studies, I will submit their qualifications, training, and experience to the IACUC and seek IACUC approval before they are involved in animal studies;
- I will ensure that all personnel are enrolled in an institutional Occupational Health and Safety Program prior to their contact with animals, or have declined in writing to participate, if allowed by local policy;

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

2. **Approval Signatures.** To the best of their abilities, the undersigned verify that the care and use of the animals described in this ACORP amendment has been evaluated 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*, VA Policy and local IACUC Policy, and find the use of animals described in this ACORP to be appropriate.

Name of Attending Veterinarian (VMO or VMC)	Signature	Date
[redacted]		
Name of IACUC Chair	**Signature**	**Date**
[redacted]		

Revision Signatures
Version 4 signatures

1. **Certification by Principal Investigator(s).**

To the best of my knowledge, I certify that the information provided in this Animal Component of Research Protocol (ACORP) Amendment is complete and accurate.

I further certify that:

- No personnel will perform any animal procedures until they have been approved by the IACUC. When new or additional personnel become involved in these studies, I will submit their qualifications, training, and experience to the IACUC and seek IACUC approval before they are involved in animal studies;
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Name of Principal Investigator(s)	Signature	Date
[redacted]	[redacted]	[redacted]

2. **Approval Signatures.** To the best of their abilities, the undersigned verify that the care and use of the animals described in this ACORP amendment has been evaluated 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,* VA Policy and local IACUC Policy, and find the use of animals described in this ACORP to be appropriate.

Name of Attending Veterinarian (VMO or VMC)	Signature	Date
[redacted]	[redacted]	[redacted]
Name of IACUC Chair		Date
[redacted]		[redacted]

Secondary Review

PI	[redacted]
STATION	**MILWAUKEE, WI #695**
FUNDING SOURCE	**DEPARTMENT OF VETERANS AFFAIRS**
APPLICATION TITLE	**NEUROPHARMACOLOGY OF PONTINE CONTROL OF BREATHING FREQUENCY**
SPECIES	**CANINE**
DATE OF REVIEW	**11/28/17**

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

REVIEWER FEEDBACK

General Comments: This ACORP uses a decereberate canine model to improve understanding of a specific sub-region of the pons that controls breathing rate; the goal is to develop new treatments that will relieve pain without depressing breathing. This research is particularly relevant in light of the opioid crisis in America. The investigator and nearly all the members of his research team have decades of experience and are highly skilled in performing the proposed procedures. The development of a new 16-electrode probe with a multi-barrel pipette that allows recording and microinjections of drugs on multiple neurons, which reduces the number of experimental runs, is noteworthy. The work involves complicated surgery and is highly technical to the point that the experimental plan cannot be readily grasped without considerable knowledge of the autonomic nervous system and spinal cord reflexes. Accordingly, we suggest that the experimental plan should be re-written in a manner that is more readily understood by lay persons so that the obvious value of this research to Veterans is better communicated. 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 response(s) is (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.

1. Although, item C.2.a outlines the specific aims, it could do a better job of communicating a clear understanding of the experimental plan, and the rationale for the agents used as well as the manipulations to be performed. As noted above, much of narrative is expressed in highly technical language. Two representative examples are: (1) Specific aim 2 states "Identify the PB subregion neuron subtypes, determine if their axons project to the rhythmogenic preBotzinger Complex region and quantify their response to pulmonary stretch receptor (PSR) inputs." and (2) "Respiratory neuronal activities will be recorded from the parabrachial region of the pons with a 16-electrode microprobe, and other protocols with multibarrel micropipettes in conjunction with

picojection of neurotransmitter agonists and antagonists onto the neurons." A simple and direct explanation of the term "decerebrate" and its ramifications is needed. The investigator does provide justification for use of the decerebrate model in Appendix 5– item 2 but this information needs to be clearly stated at the beginning of the ACORP. It would also seem worthwhile to indicate that (1) the autonomic nervous system works for the most part unconsciously to regulate body functions such that heart rate, respiratory rate, digestion, etc. and primarily controls the fight or flight response and (2) a decereberate animal will exhibit a flexor reflex (withdrawal reflex) in response to stimuli such as a toe pinch or pinprick even though the animal has no conscious awareness of the stimuli. The withdrawal reflex is mediated through the spinal cord not the higher centers of the brain. Please reconcile.

2. In item C.2.b, the investigator refers to multiple subtypes of neurons, seven levels of neuron subtypes, neurons/insertion, etc. and states "the estimated number of neurons for all 7 protocols is 950 obtained in 152 animals over a 48 month period. The explanation provided is not easy to follow. Please explain the seven levels of neuron subtypes and what is meant by neurons/probe insertion so it is more apparent how the animal numbers were derived.

3. In item C.2.a, the investigator indicates that purpose bred beagles will be used but item C.2.c lists mongrel dogs. The information provided in item D lists [redacted] as the vendor of adult dogs. Please clarify.

4. Concerns identified in the narrative of item C.2.c include:

   a) Mask induction with isoflurane can be challenging in dogs because of the likelihood of patient stress and the need for higher concentrations of isoflurane to achieve mask induction, which produces more cardiovascular and respiratory depression than comparable doses of intravenous pre-anesthetic agents (see: http://www.vasg.org/induction_protocols.htm). Please address.

   b) The investigator states "Mechanically ventilation with an air-O2 mixture will be used throughout the experiment to maintain hyperoxic isocapnia (FLO2>0.6, end-tidal CO2 range 40-50 mmHg)…." The paper shown at the link below indicates"There is growing evidence that the administration of oxygen in concentrations that produce hyperoxemia is associated cellular injury…" (http://anesthesiology.pubs.asahq.org/article.aspx?articleid=1932750). Please address this concern in item C.2 and Appendix 5**.**

   c) As noted above, the dogs will be mechanically ventilated because of the use of paralytic agents and also states that a bilateral pneumothorax will be performed to minimize brainstem movement and phasic inputs from chest wall mechanoreceptors. Please describe how and where the bilateral pneumothorax is created (e.g. bilateral incisions of the chest wall, bilateral incision of the diaphragm, etc.). Please address this concern in item C.2 and Appendix 5**.**

d) The narrative of item C.2.c describes the overall procedures but does not address why the extensive list of experimental agents in Appendix 3 was chosen or how and when they will be used.

5. The justification provided in items D and W should more specifically address why alternative or non-animal models cannot be used. It is quite obvious that such alternatives are not possible to use, but it should be addressed. The narratives also do not discuss whether other animal models such as zebrafish, rabbits, cats, pigs or monkeys were considered and why they were found to be unacceptable. Please address.

6. In item J, the response was "See Appendix 5," please list the procedures that will actually be performed (i.e. non-survival surgery, use of paralytics, decerebration, etc.)

7. In regard to item T, since healthy dogs are obtained from [redacted], are the dogs allowed to acclimate before surgery? Please describe the clinical signs that would be indicators of illness and would necessitate exclusion from the study.

8. Phenylephrine is listed in item X but is not a controlled substance, please delete.

9. An extensive list of potentially toxic agents was noted in item 4 of Appendix 3 but item 9 (potential for pain or distress) was left blank. Please reconcile.

10. Several concerns were identified in Appendix 5:
    a) In Item 2:
        i) The investigator states "Vascular lines are placed in the femoral artery for continuous monitoring of blood pressure and heart rate and vein for administration of fluids and medications, such as corticosteroids and muscle relaxants and blood gas collection (if needed). " Please specify all agents (clinical or experimental) administered to the dog and include dosage and route of administration. If blood samples will be collected, please indicate the amount and frequency of collection.

        ii) Three paralytic agents are listed in the ACORP (i.e. cisatracurium, vecuronium and pancuronium), but it is unclear when cisatracurium will be used as opposed to vecuronium and pancuronium. Please address.

        iii) The investigator uses the term "fictive" (neural equivalent of) breathing and indicates that along with blood pressure, it will be continuously monitored to detect signs of responsiveness to paw pinch. Please elaborate on the meaning

of fictive breathing and also clarify that paralytic agents are discontinued after decerebration in order to observe the withdrawal reflex to paw pinch.

b) In item 6.a, the investigator states that "An adequate level of anesthesia (isoflurane: 2-5%) is assessed by lack of changes in continuously monitored arterial blood pressure and heart rate and lack of salivation and lacrimation during surgery leading up to decerebration, after which pain sensation and cognition are eliminated. "An sign of discomfort will be used to signal that an increase in anesthetic is required." It appears the investigator meant to say "Any" sign of discomfort will be used to signal that an increase in anesthetic is required." Please correct and consider revising this paragraph so it is clear that the level of anesthesia is adjusted in response to pain before decerebration.

c) The *Guide*, page 118 states the following "In nonsurvival surgery, an animal is euthanized before recovery from anesthesia. It may not be necessary to follow all the techniques outlined in this section if nonsurvival surgery is performed but, at a minimum, the surgical site should be clipped, the surgeon should wear gloves, and the instruments and surrounding area should be clean (Slattum et al. 1991). For nonsurvival procedures of extended duration, attention to aseptic technique may be more important in order to ensure stability of the model and a successful outcome." Given that the non-survival procedures to be performed will last 8-10 hours (see item W), it is important to explain the measures used to be in compliance with *Guide* recommendations, please clarify with a notation to item 7.

Appendix - Additional Suggestions for Improvement

Comment 1: <u>Part B.</u> This section could be difficult for lay readers to understand. Try something like this:

Many Veterans have problems with respiration, and as many as 20% suffer from sleep apnea where they stop breathing for short periods while sleeping https://sleepfoundation.org/sleep-news/more-veterans-suffer-sleep-apnea . These breathing problems can be caused by things like battlefield injuries to the brain or spinal cord; or by lung diseases, brain tumors, or other medical problems. Many veterans also need to take strong pain control drugs (including opiates such as oxycontin) for pain right after having surgery, while some veterans need to take these drugs for constant pain from things like cancer or battlefield injuries. Unfortunately opiate pain killers slow down breathing, and in someone who already has trouble breathing this can cause serious problems including fainting or even death.

This project will study certain neurons in the brain that control respiration and that are very sensitive to opiate drugs. These neurons are in an area called the parabrachial nucleus that is located underneath the brain at the top of the spinal cord. We think how opiate drugs cause people to stop breathing and faint or die is that they turn these respiration neurons off. We will be testing these neurons to better understand how they are turned on and off by different kinds of drugs. Our ultimate goal is to develop drugs (or

possibly combinations of drugs) that will control pain without turning these cells off and causing breathing to slow down or stop.

Comment 2: <u>Part D:</u> The justification for using dogs may be somewhat difficult for the lay reader to follow. Try something like this:

These experiments require us to be able to record from individual neurons for hours at a time while testing various drugs and during changes in parameters such as blood pressure. Unfortunately, the parabrachial neurons of small animals are small and closely packed, which make it much more difficult to insert an electrode into an individual neuron. Furthermore, the electrode tip can easily move out of a small neuron at crucial times with the result that important information is not recorded.

Our only practical options for this work are large animals such as dogs. The relatively large size of the parabrachial neurons in dogs allows stable recordings from a single neuron for hours even while blood pressure is changing. We have 26 years of experience successfully studying various neurons that control respiration in dogs. Switching to another large species such as pigs would require us to largely start over, and we would have to run a lot of pig experiments to reach the point where we already are with dogs before we could even begin this particular study. This process would use many more pigs to get to that point than the number of dogs required for these experiments.

Comment 3, <u>part H:</u>

Please specify in the table that these are mongrel dogs.

Comment 4 <u>Part W3 table:</u>

The table is a good search for "lack of unnecessary duplication", however those searches do not really cover the other three columns in the table. A search on the ALTBIB or similar website covers the alternatives for you, as in the example below. See https://toxnet.nlm.nih.gov/altbib.html . Also, this should be item W1, not W3.

1. Document the database searches conducted.
   List each of the potentially painful or distressing procedures included in this protocol.

   ► ( ) **N/A**
   ► (X) **Painful or distressing procedures:**

   ► **Recording from the parabrachial nucleus**

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)
ALTBIB (Search PubMed using ALTBIB animal alternatives search strategy)	**9/28/ 2017**	**200-2017**	**Recording from the parabrachial nucleus**	**parabrachial nucleus, electrophysiology**	X	X	X	
PubMed	**8-26-2014**	**2000-2014**	**N/A**	**Opioids and respiratory depression**				X
PubMed	**8-26-2014**	**2000-2014**	**N/A**	**Respiratory neurons and opioids**				X
PubMed	**8-26-2014**	**2000-2014**	**N/A**	**PreBotzinger and opioids**				X
PubMed	**8-26-2014**	**2000-2014**	**N/A**	**Pons, opioid, breathing**				X

Comment 5: <u>part W4 (replacement)</u>

Please include a discussion of why you can't use smaller mammals instead of dogs such as rabbits or rats, and why you can use non-mammalian species. Also, this should have been item W2, not W4.

Comment 6 <u>part W6 (refinement).</u>

Please add a sentence about how pain and distress for the animals is eliminated. Try something like this: "Dogs are anesthetized and then decerebrated, which ensures they will experience no pain or distress. There is no way to further reduce pain and distress in this work." Also, this should have been item W4, not W6.

Comment 7: <u>Appendix 3, table 1:</u>

General comment – it is unclear why so many relatively benign substances (including artificial CSF and glycine) are listed as toxic substances.

Literature search Milwaukee [redacted]

1) How is this research relevant to Veterans health?

Many Veterans have problems with respiration, and as many as 20% suffer from sleep apnea where they stop breathing for short periods while sleeping (https://sleepfoundation.org/sleep-news/more-veterans-suffer-sleep-apnea *accessed 3-11-18*). These breathing problems can be caused by things like battlefield injuries to the brain or spinal cord; or by lung diseases, brain tumors, or other medical problems. Many veterans also need to take strong pain control drugs (including opiates such as oxycontin) for pain right after having surgery, while some veterans need to take these drugs for constant pain from things like cancer or battlefield injuries. Unfortunately opiate pain killers slow down breathing, and in someone who already has trouble breathing this can cause serious problems including fainting or even death.

2) Is this work <u>unnecessarily</u> 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	3-11-18	All available	opioids and respiratory depression and parabrachial	1

A PubMed search for the keywords opioids and respiratory depression and parabrachial brought up only one paper, which is an earlier paper from this same group. The current project will build upon that earlier work.

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	3/11/18	All available years	opioids and respiratory depression and parabrachial	0

An ALTBIB search for “alternatives to using animals” using the keywords opioids and respiratory depression yielded no papers at all. No computer models or in vitro models were found for this kind of work.

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	3/11/18	2000-present	opioids and respiratory depression and parabrachial	1

An ALTBIB search for all citations brought up only the one paper from this group that was noted above in section 2. That paper uses rabbits.

The current study requires recording from individual neurons for hours at a time while testing various drugs and during changes in parameters such as blood pressure. Unfortunately, the parabrachial neurons of small animals (such as mice, rats, or rabbits) are small and closely packed, which make it much more difficult to insert an electrode into an individual neuron. Furthermore, the electrode tip can easily move out of a small neuron at crucial times with the result that important information is not recorded.

Our only practical options for this work are large animals such as dogs. The relatively large size of the parabrachial neurons in dogs allows stable recordings from a single neuron for hours even while blood pressure is changing. This group has 26 years of experience successfully studying various neurons that control respiration in dogs. Switching to another large species such as pigs would require them to largely start over, and they would have to run a lot of pig experiments to reach the point where they already are with dogs before they could even begin this particular study. This process would use many more pigs to get to that point than the number of dogs required for these experiments.

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

The experiments all involve deeply anesthetizing the dogs and removing the cerebral cortex so there is a complete loss of consciousness and sensation. At the end of the study the animal is euthanized while still anesthetized. The animals will experience no pain or distress.