This document provides guidance for responding to the required sections of the '**Application for Initial & *De Novo* IACUC Review**'. Excerpts of this guidance document were obtained from the publicly available journal article cited below:

Mohan, S., & Foley, P. L. (2019). Everything You Need to Know About Satisfying IACUC Protocol Requirements. *ILAR Journal*, *60*(1), 50-57. https://doi.org/10.1093/ilar/ilz010

For questions related to regulatory or ethical considerations of your proposed animal activity, please contact the IACUC at iacuc@une.edu for assistance.

A. Administrative Information

Key Considerations / Best Practices / Notes
▪ Per the federal regulations, only one individual can be named as the principal investigator of the project. The term 'Co-PI' is not regulatorily defined. A project may have several co-investigators, but only one principal investigator. ▪ The principal investigator has ultimate responsibility for the animal proposal, the use of animals on the protocol, oversight of the individuals involved with the project, and the implementation of the protocol in compliance with institutional rules and federal laws and regulations. ▪ UNE students are NOT permitted to serve in the role of principal investigator, but may be listed as a co-investigator in the IACUC application.

B. Lay Summary

Key Considerations	Best Practices / Notes
▪ **This section MUST be written in plain language that a non-scientist would understand.** Consider writing the lay summary as if your audience was a high school student or family member. Please see Appendix A for an example. ▪ Provide a brief (~300 words), high-level explanation of the proposed objectives or aims, and the knowledge you hope to gain by conducting the project. ▪ Identify the significance of the proposed project in advancing science or human or animal health (e.g., the potential benefits to human or animal health).	▪ The purpose of explaining the rationale of the project is to provide the IACUC with a simple and straightforward overview of the proposed project that can be easily understood by all members of the committee regardless of their scientific background. ▪ The lay summary also helps the committee understand the potential societal implications to balance the potential benefits of the project against any animal welfare concerns. ▪ Don't assume the reviewer has a background in your proposed research. ▪ Avoid the use of jargon and/or technical language. ▪ Be sure to spell out any acronyms at the time of first use. ▪ The summary should NOT be a reiteration of the aims of the grant application (however, you may use the grant application as a starting point)

C. Animal Requirements

Key Considerations / Best Practices / Notes

Use of Genetically Modified Animals

- When genetically modified animals are used in a project, describe any unanticipated phenotypic consequences (e.g., small size, aggressiveness, atypical behaviors) and any special care or monitoring required (e.g., feed placed on the housing floor, separating individuals, need for extra enrichment, increased observation of animals).
- Please reach out to the IBC Administrator to determine if your project requires review by the Institutional Biosafety Committee (IBC) prior to IACUC submission.

Animals Housed Outside of Central Facility > 12 hours

- Prospective IACUC approval (via the initial/de Novo application or a subsequent amendment application) is required to house animals in the field, a lab, or anywhere else outside of the central facility for more than 12 hours.

D. Rationale for Animal Use

Key Considerations	Best Practices / Notes
▪ Provide a rationale for why the use of animals is necessary to achieve the proposed aims of the project. ▪ Explain why non-animal models (e.g., in vitro, computational) cannot be used for the project.	▪ Animal use in scientific research should be carried out only after exhaustively searching options for nonanimal alternatives. ▪ The rationale for animal use should be scientifically based and relate directly back to the project objectives.

E. Species & Strain Justification

Key Considerations	Best Practices / Notes
▪ Provide a justification for the choice of species and specific animal strain(s) to be used in the proposed project. ▪ Explain why a species lower on the phylogenetic scale would not be equally (or more) appropriate.	▪ The justification should provide reviewers with assurance that the animal model has validity to address the specific hypotheses, research questions, or learning objectives of the project.

F. Justification of Animal Numbers

Key Considerations	Best Practices / Notes
▪ Provide a justification for the number of animals proposed for the project. See Appendix C for an example.	▪ A flow chart or table is often helpful when explaining animal numbers.

F. Justification of Animal Numbers

Key Considerations	Best Practices / Notes
▪ Describe the different experimental groups or subgroups necessary for the project (e.g., treatment group vs. control group), group sizes needed, time points, etc. ▪ If the project is being pursued for teaching or educational purposes, detail how animals will be used (e.g., shared among students or reused), and justify the number of animals based on the number of students, group size, and minimal use necessary to achieve the learning objectives of the project. ▪ Where applicable, there should be appropriate control groups, randomization, and a discussion of sex choice given the increased emphasis on performing studies using both male and female animals. ▪ The calculation of total animal numbers required for individual experimental procedures may be documented within Section G (Description of Experimental Design & Animal Procedures) to facilitate reviewer understanding of the study. See Appendix E for an example.	▪ This section of the IACUC application will receive particular scrutiny because of the ethical requirement to use as few animals as absolutely necessary. ▪ The IACUC will want to know how group sizes were determined, the statistical parameters used, and the power calculations used for achieving statistically valid results. ▪ Statistical methods such as a power analysis should be used when possible to determine appropriate group sizes (N) for the experiments and the total number of experimental groups stated. However, a power analysis is not appropriate for all projects and the regulations allow other methods such as published literature that uses the same or similar animal model or results from pilot studies. ▪ Justifying animal numbers based on how many experiments can be completed in a certain amount of time, rather than specific goals, is generally not adequate. ▪ Breeding colonies should be sized to provide the number of experimental animals needed without producing an abundance of extra animals that then must be needlessly euthanized.

G. Description of Experimental Design & Animal Procedures

Key Considerations / Best Practices / Notes

***General Instructions**:*

- **To the degree possible, this section should be written in plain language that a non-scientist would understand.** Don't assume the reviewer has a background in your proposed research. Avoid the use of jargon and/or technical language. Be sure to spell out any acronyms at the time of first use.
- Complete, concise descriptions of all procedures to be employed in the project are required in this section. *It is important for the IACUC to be able to discern the total "picture" of what will happen to animals from beginning to end to assess overall impact of the procedures on animal well-being.*
- Refer to Appendix F for examples of how to reference Behavior Core Service Book procedures in this section, whether you are using them as written (unmodified) or with modification.

G. Description of Experimental Design & Animal Procedures

Key Considerations / Best Practices / Notes

- A laboratory SOP approved by the UNE IACUC may be referenced to describe an animal procedure. Please specify the SOP # and the SOP title in your description of the procedure.
- The appropriate USDA pain category must be documented for each animal procedure described in this section (see Appendix B for USDA Pain & Distress Category table), except when an unmodified Behavior Core Service Book procedure is referenced.
- If sequential events are involved in the project, the sequence of events, time intervals, and other relevant details should be clearly described. Refer to Appendix D for guidance on designing an effective experimental timeline, including a simple example timeline formatted as a table. For an example of a narrative-style experimental timeline that includes animal numbers, see the '***Overall Experimental Design***' section within Appendix E.
- A flowchart may be an effective presentation of the planned procedure(s).
- It is acceptable to submit a stand-alone **Word** document to describe the experimental design and animal procedures in lieu of providing these details directly within Section G of the application.
- With regard to how much detail to include in this section, consider the "Goldilocks Effect": too little detail limits the ability of the IACUC to conduct a proper review; too much detail restrains the researcher's ability to make minor procedural changes during the course of the project and increases probability of an off-protocol or noncompliant event. *It is recommended that acceptable ranges be provided when applicable to allow for procedural flexibility in order to minimize potential protocol deviations.*
- **Include the following specific information outlined below (as applicable to your project).**

Animal Housing & Husbandry

***Note**: For field work or wildlife studies, do not provide these details in Section G of the application. Instead, provide them in '**Supplemental Form E: Field Work & Wildlife Studies**'.*

- Describe the living conditions of the animals and the routine animal husbandry activities that will be provided during the project.

  ***Note**: Any non-standard housing or husbandry should be detailed within Section K of the application.*

- Explain how the living conditions are appropriate for the species and contribute to their health and comfort.

Surgical Procedures

- If surgical or invasive procedures will be employed as part of the project, provide these details within '**Supplemental Form C: Animal Surgical Procedures**'.

Non-Surgical Procedures

- If substances will be administered to animals during the project, provide these details within '**Supplemental Form D: Use of Biological Materials, Chemicals, Drugs, Hazardous Agents, or Other Substances in Animal Studies**'.

G. Description of Experimental Design & Animal Procedures

Key Considerations / Best Practices / Notes

- **Animal Identification**: Specify the method(s) for identification *(e.g., ear tags, PIT tags, tattoos, collar, cage card, implant, etc.).*

  ***Note**: For field work or wildlife studies, do not provide these details in Section G of the application. Instead, provide them in '**Supplemental Form E: Field Work & Wildlife Studies**'.*

- **Blood Withdrawals**: Specify the volume, frequency, withdrawal site, and methodology.

  ***Note**: For field work or wildlife studies, do not provide these details in Section G of the application. Instead, provide them in '**Supplemental Form E: Field Work & Wildlife Studies**'.*

- **Animal Restraint**: Specify the method to be used *(e.g., restraint chairs, collars, vests, harness, slings, etc.)* and the duration of the restraint procedure. Describe how animals are restrained for routine procedures. Prolonged restraint must be scientifically justified with appropriate oversight to ensure it is minimally distressing. Describe any sedation, or animal acclimation procedures to the device used.

  ***Note**: For field work or wildlife studies, do not provide these details in Section G of the application. Instead, provide them in '**Supplemental Form E: Field Work & Wildlife Studies**'.*

- **Radiation**: Specify the dosage and schedule.

- **Food or Fluid Restriction**: If food, or fluid, or both food and fluid, will be restricted, describe the method for assessing the health and well-being of the animals.

- **Imaging Procedures**: Specify the methodology to be used *(e.g., anesthesia, monitoring of animals and/or depth of anesthesia during imaging, maintenance of body temperatures, etc.).* Relay the location of the equipment, transport to and from the location, any effect on animal well-being, and how equipment is sanitized between cohorts.

- **Behavioral Tests**: Specify the methodology, equipment used, monitoring, and acclimation process to be used. Any training given to animals prior to experiments, such as performing tasks for rewards, must be described along with any food or fluid regulation that is part of the motivation-reward process. *Positive reinforcement methods are preferred over negative reinforcement as motivators to perform and the latter requires clear justification.*

- **Tumor Production**: If tumor cell lines will be injected into animals to produce tumor growth, specify how tumor growth is measured, frequency of measuring, anticipated impact on health and well-being, endpoints for the study such as maximum size of tumor, ulceration, decreased body condition score or body weight, or other clinical impairments depending on tumor location.

Veterinary Care

- Specify if any painful or stressful outcomes are anticipated during the project. If yes, describe the plan for timely intervention, removal of animals from the project, or euthanasia. The details of the plan should also include promptly notifying the appropriate facility manager (fish or rodent) when timely intervention, removal of animals, or euthanasia is required during the project.

  ***Note**: Details about the euthanasia process should be described within Section J of the application.*

G. Description of Experimental Design & Animal Procedures

Key Considerations / Best Practices / Notes

- Any veterinary or clinical care provided after a non-surgical procedure (e.g., heated environment, extra bedding, etc.) must be described along with any anticipated clinical signs (e.g., behavioral changes, changes in vital signs, etc.) that would warrant administering the veterinary care.

  Note: *Details about veterinary or clinical care provided before/during/after a surgical procedure should be described in '**Supplemental Form C: Animal Surgical Procedures**'.*

- Specify a plan of action should an animal experience an unexpected injury or illness during the project (e.g., initiate treatment, call investigator prior to initiating treatment, euthanize).

Endpoint Criteria

- Define the humane vs. experimental endpoint criteria for the project.
- Humane endpoint criteria are established to determine when euthanasia should be performed. Humane endpoints are not necessarily the same as experimental endpoints, as experimental endpoints are planned based on experimental and scientific needs, whereas human endpoints recognize that some animals may need to be euthanized for ethical reasons prior to reaching their experimental endpoint.
- Some examples for indicators of euthanasia are tumor size, percentage of body weight loss, inability to eat or drink, behavioral abnormalities, signs of toxicity, or clinical signs of severe pain, infection, or respiratory distress.
- Anticipated clinical signs and endpoints must be described for any projects that are known to cause significant signs or that may be potentially lethal, such as administration of tumor cells, biologics, infectious agents, radiation, or toxic chemicals.
- Euthanasia is usually expected at the earliest stage when scientific objectives have been achieved.
- If death of the animal is used as the experimental endpoint, the study will be rigorously evaluated by the IACUC. Therefore, the requirement for using death as an experimental endpoint and unrelieved pain and distress must be scientifically justified.

H. Transportation of Animals

Key Considerations	Best Practices / Notes
▪ When animals are transported between rooms or buildings within UNE, describe the method, schedule, and route of transportation. ▪ Outlined the steps that will be taken to minimize stress and discomfort during and after the transportation of animals within UNE.	▪ Animals transported between buildings at UNE must be enclosed, covered at all times, and protected from the elements. Do not overload carts or stack cages/tanks. ▪ Consult with the appropriate facility manager (fish or rodent) if animals will be permanently moved from their original housing location to a different location within UNE.

H. Transportation of Animals

Key Considerations	Best Practices / Notes
	▪ Descriptions of the method, schedule, and route of transportation will help the IACUC assess whether there will be any exposure or health concerns (e.g., allergies) to non-research personnel (e.g., if a public elevator is used), if special containers/cages are required for transport, and whether special training is required for personnel transporting the animals.

I. Pain & Distress Classification & Consideration of Alternative Procedures

Key Considerations	Best Practices / Notes
▪ For projects involving USDA category D or E procedures, describe your consideration of alternatives and your determination that alternative procedures are not available or cannot be used. ***Note***: *Alternatives include methods that refine existing tests by minimizing animal distress, reduce the number of animals necessary for a project, or replace whole-animal use with in vitro or other tests.* ▪ For projects involving USDA category D or E procedures, list the databases that were searched to determine if alternatives exist to procedures that cause pain or distress in animals, and provide the data range of the search and keywords used in the search. ***Note***: *Consider using the '**Alternatives Literature Searching Worksheet**' available here for help in identifying the information needed to successfully develop a search strategy and run a multi-database literature search for alternatives.*	▪ For more information regarding pain and distress classifications, see Appendix B. ▪ Please note that the USDA pain categories specifically focus on the use of drugs to alleviate pain or distress in animals. For projects that induce chronic disease conditions (e.g., pain, tumor, or infectious disease), the appropriate pain category may not always be clear. The investigator should seek guidance from the IACUC. ▪ Category E procedures raise the bar for a harm-benefit analysis, and a detailed explanation of why appropriate analgesics or other drugs cannot be used in these procedures must be explained. ▪ The description of the database search should include information such as the database used for the search, date of search, the time period covered by the search (the past 5-10 years is recommended), and the search strategy used (e.g., keywords used in search). Click here to view recommended databases for literature searches. ▪ Provision of the keyword combinations and Boolean operators (e.g., simple words such as 'AND, OR, NOT, or AND NOT' used in conjunction to combine or exclude keywords in a search) allows the IACUC to determine whether the database searches are likely to be relevant and effective in searching for alternatives.

I. Pain & Distress Classification & Consideration of Alternative Procedures

Key Considerations	Best Practices / Notes
	▪ For procedures involving USDA category D or E procedures, the UNE attending veterinarian MUST be consulted prior to submitting an application for initial or *De Novo* review. Veterinary contact information is provided in the application.

J. Method of Euthanasia & Disposition of Animals

Key Considerations / Best Practices / Notes

Guidelines for Euthanasia of Rodents

- All activities related to the euthanasia of rodents deserve consideration equivalent to the euthanasia method itself, and may factor into the choice of method.
- Activities that contribute to distress in rodents include transport, handling (in animals not accustomed to it), disruption of compatible groups, and elimination of established scent marks. As eliminating all sources of distress may not be practical or possible, the selected method of euthanizing rodents should minimize these sources of potential distress.
- Methods of euthanasia likely to elicit distress vocalizations or pheromones that other animals in the room could hear or smell may be best performed in another location, if transportation distress can be minimized.
- Click here to view the acceptable AVMA methods of euthanasia for rodents.
- Death should be confirmed by physical examination, ensured by adjunctive physical method, or obviated by validation of euthanasia chambers or process. *When using CO_2 as the primary means of euthanasia, a secondary method of euthanasia should also be prescribed as a means to confirm animal death (e.g., use of cervical dislocation following CO_2 euthanasia or confirmed lack of respiration for a specified period of time).*
- For questions about carcass storage or disposal, please consult with the rodent facility manager.

Guidelines for Euthanasia of Fish

- Considerable evidence is accumulating that suggest it is appropriate to consider the possibility of pain perception in fish. The aim is to accomplish death rapidly with the minimum amount of pain and distress practicable.
- Because the environment associated with fish is different, and because knowledge about the evolutionary and societal status of lower vertebrate animals is limited, identifying and applying appropriate criteria for euthanasia can be difficult.
- The effectiveness of euthanasia methods described in the AVMA guidelines may vary by life stage, as well as by species. For example, some species may require a two-step euthanasia process depending on the recommended method of euthanasia.
- Click here to view the acceptable AVMA methods of euthanasia for fish.

J. Method of Euthanasia & Disposition of Animals

Key Considerations / Best Practices / Notes

- Additional care must be taken to ensure death following euthanasia in fish. Typically, a fish is considered to be dead 30 minutes after the last sign of gill movement and loss of eye-roll (the movement of the eye when the fish is rocked from side to side). In some species, a secondary step may be necessary to confirm death (e.g., decapitation, pithing, rapid chilling, etc.).
- For questions about carcass storage or disposal, please consult with the appropriate fish facility manager.

K. Special Concerns or Requirements

Key Considerations / Best Practices / Notes

Non-standard Housing or Husbandry

- Proposed deviations from standard husbandry practices should be described in detail and justified. Additionally, any anticipated effects from the alternative animal husbandry practices should be described. *This could include metabolic or other specialized housing, changes in light cycle, single housing, specialized diets, medicated water, food or fluid regulation, or a variety of other special project needs.*
- Social housing of species that normally live in social groups is considered the default by OLAW. Any requests for single housing will be closely scrutinized by the IACUC and must have specific justification.

Departures from the Guide

- If a departure from the recommendations of the *Guide* is required for the project, a scientific justification must be provided.

Appendix A

Original version of an example lay summary *NOT* written in plain language understandable to a non-scientist:

In human patients, breast cancer metastasis to bone induces severe pain. This pain is not well modulated by standard opiates. Cannabinoids have been used as homeopathic pain remedies for quite some time across many cultures but have not been well studied in controlled environments. This is no doubt partially due to legal concerns with cannabinoid use. In our studies we target cannabinoid receptor 2 specifically, which is predominantly expressed in immune cells and does not elicit psychotropic effects. Our preliminary data indicates that cannabinoid receptor 2 compounds may be effective in alleviating breast cancer induced bone pain. Here, we are proposing to evaluate the pain efficacy of our novel, experimental cannabinoid receptor 2 compounds by utilizing a direct to bone breast cancer metastasis model. This model incorporates murine breast cancer cells directly into the intramedullary space in one femur of Balb/C mice. Because the cells are sealed into the bone, complicating and life shortening factors such as extraneous metastasis to lung or brain can be avoided. This also allows us to study the mechanism of the cannabinoid receptor 2 compounds in the bone microenvironment. We consider the ability of cannabinoids to positively modify bone structural changes induced by tumor growth to be a fortunate effect- thus we are able to target three separate physiological effects of breast tumor growth with one single compound: the rate of primary tumor growth, pain alleviation, and positive modification of the bone microenvironment.

[Total word count: 238]

Modified version of an example lay summary written in plain language understandable to a non-scientist:

Breast cancer metastasis (the development of secondary malignant growths at a distance from the primary site of cancer) can cause severe pain in human patients. Physicians have struggled to help patients of the disease moderate the pain because standard opiate treatments (e.g., fentanyl, oxycodone, morphine, etc.) are less effective. Cannabinoids (a type of chemical in marijuana that causes drug-like effects all through the body, including the central nervous system and immune system) have been considered a homeopathic pain remedy (use of a natural substance as an alternative approach to medicine) for quite some time across many cultures, but studies in a controlled environment have been somewhat limited. This limited scope is likely connected to continuing legal concerns about cannabinoid usage for medicinal and recreational use.

Our study seeks to examine the potential health benefit of cannabinoids to bone cancer patients by replicating the disease using a mouse model. Murine (mouse) cancer cells will be introduced to the femurs (thigh bones) of healthy mice, creating a sealed microenvironment to study the effects of the disease that avoids the complications that occur when cancer spreads to other organs. We are chiefly interested in the cannabinoid receptor 2, which can be found inside immune cells and does not elicit the effects on the body typically associated with cannabinoids. Receptors are molecules inside or on the surface of a cell that bind to a specific substance and cause a specific response. Cannabinoid receptors are thought to impact factors such as mood, pain sensitivity, and immune function. We believe that cannabinoids possess an ability to positively affect the tissues and structures impacted by tumor growth. With that in mind, we would study the effect our cannabinoid compound has on tumor growth, pain alleviation, and positive effects on bone tissue.

[Total word count: 294]

Appendix B

USDA Pain & Distress Categories

Category B	Category C	Category D	Category E
Animals being bred or held for use in research but have not been subjected to any experimental procedures yet	Procedures involving no more than momentary or slight pain or distress to animals and no use of pain-relieving drugs, or procedures involving no pain or distress to animals	Procedures involving pain or distress to animals are appropriately relieved with anesthetics, analgesics, and/or tranquilizer drugs or other methods for relieving pain or distress	Procedures involving pain or distress or potential pain and distress to animals that are **NOT** relieved with anesthetics, analgesics and/or tranquilizer drugs or other methods for relieving pain or distress

Appendix C

Section F: Justification of Animal Numbers Example

In this example, the PI has provided a justification for the animal numbers per group. However, the exact total numbers of animals needed for individual proposed experiments is not detailed here (those totals have been presented in Section G to facilitate reviewer comprehension of the study – see Appendix E).

All animal numbers are determined based on extensive previously published data which uses the minimal number of rodents to achieve statistical significance. Further, whenever possible, power analysis and sample size estimation (using p=0.05 and power = 0.8 and above) are performed prior and during data collection. We will modify the numbers of animals needed based on preliminary statistical results as the project progresses.

The general guideline we used to determine animal numbers are listed below:

1) For behavioral studies, a minimum of 16 (8 males and 8 females) animals are needed. It should be noted that animals will be repeatedly assessed in these tests, so the total number of animals needed will not be affected by the time points selected.

2) For other post-euthanization studies: multiplex cytokine analysis needs about 12 mice per group (6 males and 6 females) and immunohistochemistry needs about 8 mice per group (4 males and 4 females). As mice need to be euthanized for sample collections, the total numbers of animals needed will be affected by the numbers of time points selected. Also, in each study, the nerve injury is compared to a sham surgery groups (e.g., two surgery groups are used for each treatment). However, only one group of mice is used at day 0 (before surgery) for each treatment. In general, the following time points will be used: days 0 (naïve mice), 3, 7, 14, 21, 28, 35, and 42 post-surgery (up to 8 time points total).

3) The numbers proposed above allow us to detect potential sex differences. However, when sex differences are suggested by statistical analysis, total numbers of mice may be increased in order to obtain sufficient data for both males and females to achieve statistical significance.

Exact numbers of mice needed for each experiment are calculated in Section G.

Appendix D

Tips for Creating an Effective Experimental Timeline

1. Consider using a tabular format. Tables are easier for IACUC reviewers to read than paragraphs.
2. Keep it clear and concise. Steer clear of jargon and complex technical language. Explaining the purpose behind each step in the timeline can help reviewers understand your project more easily.
3. Be chronological and specific. List events in the order they occur, from baseline to euthanasia. Include exact or estimated days (e.g., Day 1, Day 2-5, Week 2, etc.).
4. Account for repeated procedures. Note the frequency and duration of procedures like dosing or testing.
5. Include all key procedures. Cover everything that involves animal handling or manipulation:
    - Baseline testing
    - Surgeries or interventions
    - Drug administration
    - Behavioral or physiological assessments
    - Euthanasia and tissue collection
6. When applicable, define treatment groups. Clearly note when different groups receive different treatments. If complex, consider creating parallel timelines per group.
7. Clearly specify the planned euthanasia date or time.

Simple Experimental Timeline Example for a Mice Study

Day	Procedure/Event	Comments
1	Tail flick and hot plate test	Baseline testing
	Morphine injection @ 8:00 am	10 mg/kg, IP
	Tail flick conducted 30 minutes post-injection	Tolerance assessment
	Morphine injection @ 4:00 pm	10 mg/kg, IP
2	Morphine injection @ 8:00 am and 4:00 pm	10 mg/kg, IP
3	Morphine injection @ 8:00 am and 4:00 pm	10 mg/kg, IP
4	Morphine injection @ 8:00 am	10 mg/kg, IP
	Tail flick conducted 30 minutes post-injection	Tolerance assessment
	Naloxone injection 1 hour later	10 mg/kg, IP; naloxone will be administered to precipitate a withdrawal response
	Counts of jumping behavior for 20 minutes post-naloxone injection	Dependence assessment; measure of withdrawal symptom
	Weigh filter paper to quantify feces production	Dependence assessment; measure of withdrawal symptom
	CO2 asphyxiation followed by cardiac puncture (blood collection) and fresh brain collections	Euthanasia and tissue collection

Appendix E

Section G: Combined Narrative Example

In this example, the PI elected to combine the proposed animal numbers and experimental design together in Section G to facilitate reviewer comprehension of the study. The '***Overall Experimental Design***' section breaks down the total number of animals required and the combined pain level the animals will experience for each aim of the study. Pain levels for individual surgical and non-surgical procedures are documented elsewhere in this example.

Overall Experimental Design

Adult (8-10 weeks old) C57BL/6J (B6) mice will be used in three sets of experiments with one for each of the three specific aims. Equal numbers of males and females will be used in each experimental group. Animal numbers per group are determined based on the justifications provided in Section F. Mice will be randomly assigned to experimental groups within each experiment.

- **Aim 1** Characterize lumbar spinal cord cytokine responses via multiplex assay following sciatic nerve crush (SNC).
  - Experimental procedure **(Pain level = E)**: B6 mice arrival at animal facility -> at least one-week habituation -> subjected to either SNC or sham surgery, with one group of naive mice reserved for time 0 -> trans-cardiac perfusion followed by sample collection at selected times: days 0, 3, 7, 14, 21, 28, 35 and 42 post-surgery
  - Animals needed: 12 (6 males + 6 females) x 15 (groups: naïve + 2x7 time points) = 180 (60 males + 60 females)

- **Aim 2** Assess lumbar spinal cord CGRP expression via immunohistochemistry following SNC.
  - Experimental procedure **(Pain level = E)**: B6 mice arrival at animal facility -> at least one-week habituation -> subjected to either SNC or sham surgery, with one group of naive mice reserved for time 0 -> trans-cardiac perfusion followed by sample collection at selected times: days 0, 3, 7, 14, 21, 28, 35 and 42 post-surgery
  - Animals needed: 8 (4 males + 4 females) x 15 (groups: naïve + 2x7 time points) = 120 (60 males + 60 females)

- **Aim 3** Assess the effect of neuropeptide CGRP antagonist injection on cytokine responses and associated pain-like behavioral changes following SNC.

  *For behavioral tests (**Aim 3.1**)*:

  Experimental procedure **(Pain level = E)**:
  - B6 mice arrival at animal facility -> at least one-week habituation -> baseline behavioral testing (2-3 times before day 0) -> subjected to SNC surgery (day 0) -> daily intrathecal (i.t.) injection of CGRP antagonist from days 3-7 post- surgery, plus repeated behavioral testing at selected times: days 3, 7, 10 or 11, 14, 17or 18, 21, 28, 35 and 42 post-surgery -> euthanization following completion of the experiments
  - Animals needed: 16 (8 males + 8 females) x 2 (groups: CGRP antagonist + vehicle) = 32 (16 males + 16 females)

*For cytokine responses (**Aim 3.2**)*:

Experimental procedure (**Pain level = E**):

- B6 mice arrival at animal facility -> at least one-week habituation -> subjected to SNC surgery (day 0), with one group of naive mice reserved for time 0 -> daily intrathecal (i.t.) injection of CGRP antagonist from days 3-7 -> trans-cardiac perfusion followed by sample collection at selected times: days 0, 7, 14, 21, 28, 35 and 42
- Animals needed: 12 (6 males + 6 females) x 13 (groups: naïve + 2x6 time points) = 256 (128 males + 128 females)

Total numbers of mice needed for all 3 study aims: 180 + 120 + 32 +256 = **588 B6 mice** (294 males + 294 females)

Animal Housing & Husbandry

B6 mice (7-8 weeks old) will be obtained from JAX laboratory and housed within UNE Pickus animal facility under standard care by the facility staff. Mice will be housed 4/cage with free access to food and water. Animals will be habituated for at least one week before used in any experiments. They will be used when they are 8-10 weeks old.

Surgical Procedures

- **Sciatic nerve crush** (**Pain level E**) **–** please see details within the **Supplemental Form C: Animal Surgical Procedures.**

- **Intrathecal (i.t.) CGRP antagonist administration** (**Pain level D**) - CGRP antagonist, CGRP8-37 (Sigma-Aldrich) will be given intrathecally under inhalation anesthesia in a volume of 5 µl following the procedure described in (Hylden and Wilcox, 1980). Detailed procedure is described in **the Supplemental Form C: Animal Surgical Procedures**. Please also see **Supplemental Form D: Use of Biological Materials, Chemicals, Drugs, Hazardous Agents, or Other Substances in Animal Studies** for description of CGRP8-37.

Non-Surgical Procedures

- **Animal Identification** (**Pain level C**)**:** Adult mouse will be identified individually with an established numbering system using an ear puncher. Full or half holes will be punched on the edge of one or both ears of each mouse. Mouse will be restrained by hand during the procedure. It takes about 30-60 sec to number a mouse. The ear puncher is cleaned with 70% ethanol after each use. Occasionally, aggressive mice will be anaesthetized lightly with isoflurane prior to ear punch.

- **Behavioral Tests:**
  Mechanical sensitivity (**Pain level E**): The tactile sensitivity response is used and measured as the direct pressure stimulus required to elicit foot-withdrawal in non-restrained conditions. Within the same experiment, all tests are conducted consistently at the same time period of the day. Each mouse, under non-restrained conditions, is placed singly beneath an inverted ventilated plastic cover upon an elevated room-temperature aluminum mesh screen surface with 3-5 mm openings. Animals are previously acclimated to this environment and to the experimenter (about 30-60 min before testing). Each animal is subjected to stimulations from a series of von Frey filaments ranging from 0.008 g to 2 g (Stoelting, Wood Dale, IL) following the Up-Down paradigm [detailed in (Chaplan et al., 1994)]. During test, filament is pressed to the point of bending against the plantar surface of the ipsilateral hind paw. The 50% threshold force needed for paw withdrawal will be calculated and used to represent mechanical sensitivity. Animals are baseline tested before surgery (up to 3 separated times before surgery) and tested at selected time points post-surgery. The person performing the behavioral tests is blinded to the experimental groups.

Hargreaves test (**Pain level E**): Thermal sensitivities are assayed under non-restrained conditions similar to mechanical sensitivity. Each mouse is placed singly beneath an inverted plastic cover upon an elevated glass warmed to a constant 30°C or room temperature. A radiant heat source (24v halogen lamp focus through a convex lens to a 2x4 mm area) beneath the glass is focused on the ipsilateral plantar surface of the hind paw. The heat stimulus and an automatic timer are activated simultaneously by a hand-operated switch. Upon sensing discomfort, the mouse lifts its paw at which time the experimenter turns off the stimulus and stops the timer. Each latency score is an average of two-three trials separated by at least 10 min. The intensity of the light stimulus is set for a baseline latency of approximately 15-20 sec. In the absence of a response within a predetermined maximum latency (30 sec), the test is terminated to prevent tissue damage. The person performing the behavioral tests is blinded to the experimental groups.

Following the completion of all behavioral tests at the end of the experiment, animals will be euthanized as described in **Section J: Method of Euthanasia & Disposition of Animals.**

- **Intraperitoneal (i.p.) injection** (**Pain level C**): i.p. injection is performed with 27 or 25G needle attached to a 1cc syringe and drugs will be delivered through the peritoneal cavity of the mouse. Mouse will be restrained by hand during injection. Maximal 1000 µl of solution will be i.p. injected per mouse.

- **Trans-cardiac perfusion** (**Pain level C**): All mice are deeply anesthetized with avertin. Avertin solution is prepared by mixing 2.5gm 2,2,2 Tribromoethanol and 5 ml of 2-methyl-2-butanol (Tertiary amyl alcohol) and 200 ml of distilled water with a neutral pH (aliquoted and stored at -80°C; use within 2 weeks, when stored at 4°C in the dark). For anesthetizing mice prior to perfusion, each mouse is given avertin at ~250mg/kg via i.p. injection. And then the heart is exposed. A blunted 20G needle connected to perfusion buffer (Phosphate-buffered saline, PBS) is inserted into the left ventricle of the heart and the right atrium is cut open. Flow of the perfusion buffer is controlled by gravity. About 50-100 ml PBS will be needed for each mouse. The perfusion procedure will be performed over the sink. Following perfusion, selected tissue (may include spinal cord segments, brain, dorsal root ganglia (DRGs), paws, spleen, lymph nodes) are harvested. Lumbar spinal cord tissues will be used in this proposed project. Other tissues will be saved for future investigations to reduce animal usage.

Veterinary Care

Animals will be closely monitored throughout all experiments. Besides routine veterinary care, animals will be monitored for signs of distress, significant injury and illness (such as unexpected reduction of normal activities, excessive tissue injuries, signs of infections, etc.). Animals that suffer significantly will be euthanized following the description in **Section J: Method of Euthanasia & Disposition of Animals**.

Endpoint Criteria

Experimental endpoints are described in the ***Overall Experimental Design*** above. Additional humane endpoint is described in the ***Veterinary Care*** above.

Appendix F

How to Reference the Use of Unmodified Behavior Core Service Book Procedures

If your project involves the use of unmodified Behavior Core Service Manual procedures, you do not need to describe each procedure in Section G of the application or specify a USDA pain category. Refer to the example below for how to reference the use of unmodified Behavior Core Service Manual procedures within Section G.

Behavior Core Service Book (Version 1.0)

- Injections – Injections Without Anesthesia – Intraperitoneal (IP) – Section 2.1.1
- Measures – Reflexive Measures – Hot/Cold Plate – Section 4.1.6
- Measures – Reflexive Measures – Tail-withdrawal Test – Section 4.1.7
- Measures – Non-evoked Measures – Conditioned Place Preference (CPP) – Section 4.2.4
- End Points – Cardiac Puncture – Section 6.2

How to Reference the Use of a Modified Behavior Core Service Book Procedure

If your project involves the use of a modified Behavior Core Service Manual procedure, describe the modified procedure in Section G of the application and specify the USDA pain category. Refer to the example below.

Behavior Core Service Book (Version 1.0)
Models – Chemotherapy Induced Peripheral Neuropathy – Paclitaxel – Section 3.1.1 – USDA Pain Category E

This study will utilize mice and modify the approved Behavior Core Service Book procedure by altering the injection regimen. Mice in this study will be injected with 6 mg/kg, IP at a volume of 10 mL/kg, bodyweight once on day 0. A different group of experimental mice will be injected with 2 mg/kg, IP at a volume of 10 mL/kg, bodyweight on days 0, 2, 4, and 6. Refer to study groups and experimental timeline for additional study details.