

## Vertebrate Animals

### A. Description of Procedures

All procedures have been approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC) review board.

Lumbar spinal cord injections: Adult mice will be deeply anesthetized with ketamine/xylazine (0.1 ml/20g). The lumbar spinal cord will be exposed by performing a laminectomy over the segment between L3 and L5. The dura will be cut, and a pulled glass micropipette will be advanced into the dorsal horn of the spinal cord at a 45-degree angle to the rostrocaudal axis. Three 50 nL injections of viral vector will be made bilaterally and the incision site will be closed. Mice will be allowed to recover from anesthesia in a warmed box before returning to holding cages and will be given three weeks to recover from surgeries and for viral transfection to occur.

Stereotaxic RVM injections: Adult mice will be deeply anesthetized with ketamine/xylazine (0.1 ml/20g). Animals will be positioned in a stereotaxic device. A midline sagittal incision will be made in the skin to expose the calvarium and 30% hydrogen peroxide will be applied to visualize suture lines. Pulled glass micropipettes will be used to inject 200 nL of viral vectors into the RVM according to the following coordinates: 6.0 mm posterior from bregma, 0.0 mm lateral from midline, and 5.5 mm ventral to the skull surface. The micropipette will be kept in the injection site for 10 minutes after infusion is complete and will then be retracted slowly over 15 minutes. Mice will be allowed to recover from anesthesia in a warmed box before returning to holding cages and will be given three weeks to recover from surgeries and for viral transfection to occur.

Stereotaxic cannula placement surgeries: Adult mice will be deeply anesthetized with ketamine/xylazine (0.1 ml/20g). Animals will be positioned in a stereotaxic device. A midline sagittal incision will be made in the skin to expose the calvarium and 30% hydrogen peroxide will be applied to visualize suture lines. A 30-gauge guide cannula will stereotaxically implanted into the RVM according to the following coordinates: 6.0 mm posterior from bregma, 0.0 mm lateral from midline, and 4.5 mm ventral to the skull surface, with the injector aimed 1.0 mm below the guide cannula. The cannula will then be secured to the skull with dental cement. Cannula placements will be histologically confirmed postmortem with an injection of Chicago sky blue ink; animals with injection sites that were not within the RVM will be excluded from the analyses.

FISH: Adult mice will be overdosed with urethane (2 g/kg). Once mice are unresponsive to a noxious tail pinch, animals will be decapitated. The brainstem and spinal cord will subsequently be removed and immediately frozen on dry ice.

Immunohistochemistry: Adult mice will be overdosed with urethane (2 g/kg). Once mice are unresponsive to a noxious tail pinch, animals will be transcardially perfused with 4% paraformaldehyde in phosphate-buffered saline (4% PFA-PBS). Spinal cords will be removed and post-fixed in 4% PFA-PBS for 4 hours, and cryoprotected in 30% sucrose-PBS at 4C overnight.

Electrophysiology: Adult mice will be overdosed with urethane (2 g/kg). Once mice are unresponsive to a noxious tail pinch, animals will be decapitated. The brainstem and spinal cord will be rapidly removed and placed in an ice-cold, oxygenated sucrose-based artificial cerebrospinal fluid (in mM; 234 sucrose, 2.5 KCl, 0.5 CaCl<sub>2</sub>, 10 MgSO<sub>4</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, and 11 glucose).

Pain Behavioral assays: Mice will be acclimated to the testing room prior to testing. Mice will then receive CNO or vehicle injections into the RVM via cannula and will undergo these behavioral tests back-to-back. To assess spontaneous nocifensive behaviors, the total time spent engaging in licking and biting will be quantified over a 30-minute period. To assess thermal pain, mice will be placed in individual Plexiglass boxes atop a Plexiglass platform held at 30°C. A radiant source will be applied to the surface beneath the hindpaw and latency to paw withdrawal will be recorded three times, with a maximum 20 seconds of exposure over each trial. To assess mechanical pain: Mice will be placed in individual Plexiglass boxes atop a mesh platform. Von Frey filaments will be applied to the hindpaw, and mechanical pain threshold will be quantified via the up-down method. Chemical pain assay: Mice will receive capsaicin injection (10ug/10ul) into the footpad; total time spent engaging in nocifensive behaviors will be quantified over a 30-minute period. Capsaicin-induced hypersensitivity: After capsaicin injections, mice will undergo Von Frey testing to measure hyperalgesia.

Spared Nerve Injury (SNI) procedure<sup>52,53</sup>: Mice will be deeply anesthetized with ketamine/xylazine (0.1 ml/20g). A small incision will be made on the lateral thigh. Gentle blunt dissection through the biceps femoris will reveal the sciatic nerve. The common peroneal and tibial nerves will be ligated distal to the trifurcation of the sciatic nerve, leaving behind the spared sural nerve. Sham surgeries will be performed on control mice, where the nerve

will be visualized but no branches ligated. Mice will be allowed to recover from anesthesia in a warmed box before returning to holding cages and will be given one week for the spared nerve injury phenotype to develop. 7 days after surgery, mice will undergo behavioral assays as detailed below.

Persistent neuropathic pain behavioral assay: Seven days after the SNI procedure described above, spontaneous nocifensive behaviors will be measured before and after CNO administration, followed by thermal and mechanical pain assays described above.

## **B. Justification of Species**

The mouse nervous system adequately replicates the essential components of human somatosensation, particularly nociception. Furthermore, powerful molecular genetic tools have been developed for use in mice that permit clear visualization of distinct neuronal populations; optical and pharmacogenetic control of neural circuits; and generation of knock-in mouse models that make possible an enormous repertoire of genetic manipulations and electrophysiological experiments. The mouse model system also exhibits the strong advantage of a rapid generational turnaround, given that mice reach sexual maturity at eight weeks and produce sizeable pup litters. In order to specifically target and activate/inhibit neurons of the MOR-expressing phenotype, I will use the Oprm1<sup>cre</sup> knock-in mouse, which has been acquired by the Ross Lab. Wild-type (WT) mice are used as controls in some experiments.

Selection of species, strain, sex, and age of animals: For all experiments, adult mice (*Mus musculus*) that are of 2-3 months of age of both sexes will be used. If no differences are observed between males and females, results will be pooled. If any differences are observed in preliminary experiments, sex will be used as a variable for all subsequent experiments. Mice are maintained on a C57Bl/6 background.

Justification for the number of animals used:

**Aim 1:** Based on previous immunostaining work from our lab<sup>34</sup>, 60x confocal images of the RVM will be taken from each mouse, which will be repeated in 6 WT mice for FISH experiments (Experiment 1A) and 6 Oprm1<sup>cre</sup> mice for IHC experiments (Experiment 1B). Additionally, I will use 2 WT mice, which don't express cre recombinase, as negative controls for Experiment 1B, for a total of 8 WT mice and 6 Oprm1<sup>cre</sup> mice needed.

**Aim 2A:** A power analysis was performed based on previous experiments performing optogenetic stimulation and slice electrophysiology<sup>45</sup>. Assuming a paired t-test with an expected effect size of 0.9, a significance level of 0.05, and a desired power of 0.8, I will need to record from 12 interneurons. I will record from 3 interneurons per mouse, so I will need 4 Oprm1<sup>cre</sup> mice. Additionally, I will need 20 WT mice for training and practice.

**Aim 2B:** A power analysis was performed based on previous studies categorizing dorsal horn interneurons<sup>39</sup>. Assuming a chi-squared analysis for 8 groups, a significance level of 0.05, and a desired power of 0.8, I will need to record from 60 interneurons. I will record from 3 interneurons per mouse, so I will need approximately 20 Oprm1<sup>cre</sup> mice.

**Aim 3:** A power analysis was performed based on previous work from our lab using chemogenetics to manipulate pain behaviors<sup>55</sup>. Assuming a two-way ANOVA with an expected effect size of 0.6, a significance level of 0.05, and a desired power of 0.8, 9 Oprm1<sup>cre</sup> mice will be used per group for a total of 36 Oprm1<sup>cre</sup> mice.

**Total mice used:**

Oprm1<sup>cre</sup> mice: 6 (Aim 1) + 4 (Aim 2A) + 20 (Aim 2B) + 36 (Aim 3) = **66 Oprm1<sup>cre</sup> mice**

WT mice: 8 (Aim 1) + 20 (Aim 2A) = **28 WT mice**

## **C. Minimization of pain and distress**

Description of veterinary care. The University of Pittsburgh animal care facility is a fully accredited facility. Mice are currently housed in a barrier facility within the AAALAC accredited Division of Laboratory Animal Resources (DLAR) in the Biomedical Sciences Tower-3, which is adjacent to and connected with the building in which our laboratory is located. Adult male and female mice will be used in these studies. All mice used in these studies will be bred within the DLAR facilities. Animals are maintained in accordance with the applicable portions of the Animal Welfare Act and the NRC Guide for the Care and Use of Laboratory Animals. Animal research at the University of Pittsburgh is overseen by the DLAR. The expert veterinary staff of DLAR ensure the implementation of a humane animal care and use program by providing a range of services to the university's biomedical research community. All animals are housed in specific pathogen free (SPF) environments and are monitored

daily by the DLAR staff for infection or disease. If animal care issues arise, these issues will be immediately treated and resolved through interaction between laboratory personnel and the DLAR staff.

Minimizing pain and distress during procedures: Overall, the outstanding veterinary care and research team, in accordance with the IACUC, have implemented the proper protocols to ensure that animals experience minimal discomfort. Surgical procedures: Following all surgical procedures, direct and continuous observation will be used to determine if any mice manifest overt signs of including, but not limited to: hunched posture, weight loss, spiked coat, and signs of irritation or infection at the surgical site. Mice exhibiting signs of distress will be euthanized. Otherwise, mice will be euthanized upon completion of experiments. To minimize pain and discomfort, ketoprofen (nonsteroidal anti-inflammatory, 5mg/kg) and buprenorphine (opioid, 0.1 mg/kg) will be given before and following the procedure (2 days). A broad-spectrum antibiotic (enrofloxacin, 10 mg/kg) will be administered prior to and following the procedure to minimize the risk of infection. Acute pain behavioral assays: It is anticipated that the mice will experience some mild, brief discomfort. Spared Nerve Injury (SNI): The hallmark of the SNI model is its ability to produce a robust chronic neuropathic pain model. In order to minimize long-term suffering from this procedure, we will perform behavioral experiments on these animals one week after the surgery and euthanize after behavioral experiments are complete.

Methods of euthanasia. Adult mouse euthanasia will be performed under deep anesthesia. For electrophysiology and immunohistochemistry, animals will be transcardially perfused followed by decapitation. For in vivo experiments, mice will receive urethane euthanasia solution (IP 200 mg/kg) followed by cervical dislocation. These methods are consistent with the recommendations of the 1993 Report of the American Veterinary Medical Association Panel on Euthanasia and will only be performed by trained personnel. The proposed euthanasia procedures have already been approved by our IACUC.