Research Strategy

SIGNIFICANCE

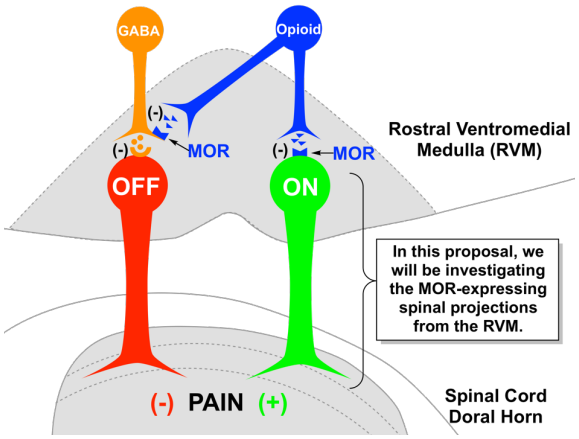

Figure 2: Model by which MOR signaling in the RVM inhibits ON-cells and activates OFF-cells. Dissecting the underlying circuitry using pharmacological approaches is complicated because MOR signaling modulates the activity of several distinct cell types. In this proposal, I will use the MOR-cre mouse to test a specific aspect of this model by investigating the anatomy, circuitry and function of MOR+ spinal projections from the RVM to the dorsal horn. Adapted from *Wall and Melzack's Textbook of Pain*[59].

The **premise** of this proposal is that **mu-opioid receptor (MOR)** signaling modulates the activity of several distinct cell types in the **rostral ventromedial medulla (RVM)**, including ON-cells, which facilitate pain, and OFF-cells, which inhibit pain. This supported by a wealth of *in vitro* and *in vivo* pharmacological studies[5–9,12–14]. Morphine, an MOR agonist, is believed to suppress pain partially through direct inhibition of ON-cells[5–7] and indirect activation of OFF-cells, through MOR-mediated inhibition of GABAergic interneurons presynaptic to OFF-cells[8,9] **(Figure 2).** Although there is strong support for this model, there are also a number of observations that hint at further complexity. Moreover, since multiple neuronal subtypes within the RVM express MOR, current studies have been unable to tease apart the underlying circuitry by which MOR+ spinal projections facilitate pain.

Until recently, we lacked the tools to selectively visualize and manipulate the activity of MOR+ RVM spinal projections. With the recent development of a MOR-cre allele, we are now positioned to address new questions about the descending circuitry which facilitates pain. My proposal seeks to address these gaps in knowledge by using the recently developed Oprm1^{cre} mouse in combination with anatomy, electrophysiology, and behavior to dissect the descending circuitry involving MOR-expressing spinal projections at a new level of detail. Based on the model described above, **I hypothesize that MOR-expressing RVM neurons that project to the spinal cord will innervate the dorsal horn (anatomy, Aim 1), inhibit post-synaptic interneurons in the dorsal horn (electrophysiology, Aim 2), and facilitate pain (behavior, Aim 3).** The proposed experiments will shed light on key unanswered questions regarding the organization of the RVM, in particular the specific circuitry through which MOR+ spinal projections facilitate pain.

The **significance** of this proposal lies in the unfortunate reality that opioid medications remain the mainstay of treatment for chronic pain, despite a collection of central nervous system (CNS) side effects including addiction of epidemic proportion across the United States[15]. There is a demand for novel therapeutics which hijack the endogenous opioid system without producing harmful side effects. In particular, long-term opioid use produces a paradoxical hyperalgesia, which may be mediated by the RVM[11]. Thus, a greater understanding of MOR+ RVM neurons have an immediate impact on our understanding of opioid-induced hyperalgesia and chronic pain.

Furthermore, the proposed experiments will provide me with invaluable training in many **innovative** approaches, including modern genetic techniques, slice electrophysiology, and pain behavioral paradigms. Mentorship from my sponsor, Dr. Ross, my co-sponsor, Dr. Koerber, and training in the Pittsburgh Center for Pain Research will all prepare me to succeed in a research track in a competitive anesthesiology residency and will contribute to my goal to become a physician-scientist specializing in pain medicine.

BACKGROUND AND RATIONALE

Descending modulation of pain. Nociception is encoded by pseudounipolar primary afferents specialized to respond to noxious mechanical, thermal, or chemical stimuli[3]. Nociceptors are influenced heavily by interneurons in the dorsal horn and modulation from a descending circuit in which the RVM plays a critical role. While non-selective electrical stimulation of the RVM produces analgesia[16,17], further studies indicate that modulation of pain from the RVM is bidirectional with facilitation as well as inhibition. Electrophysiological studies by Fields, Heinricher and others have created a framework from which to consider descending modulation. Their model comprises three functional classes of neurons: 1) ON-cells, which facilitate nociceptive responses; 2) OFF-cells, which inhibit nociceptive responses; and 3) NEUTRAL-cells, which have no effect on nociceptive responses[1,7,17,18]. Although this framework is an important conceptual advance, many details remain unclear. Moreover, it is likely that this model does not fully capitulate the complexity of the cell types that are involved.

MOR is an inhibitory GPCR which inhibits neuronal activity[19] and is expressed on both spinal projections and interneurons within the RVM[20,21]. We have known for decades that both systemic and local administration of morphine and other mu-opioid agonists exert differential effects on the RVM, where they inhibit the activity of

ON-cells while increasing the activity of OFF-cells[5–7]. This is due to direct inhibition of ON-cells which are believed to express MOR, and disinhibition of OFF-cells[5,9,13] **(Figure 2).** Current anatomical data shows that between 20-50% of RVM spinal projections express MOR[21]; however this proportion increases in persistent inflammatory pain states[10], strengthening the evidence that these neurons are pain facilitatory. Interestingly, ablation of MOR+ RVM neurons does not affect RVM and PAG-induced analgesia[13], further suggesting that MOR+ RVM neurons are pro-nociceptive rather than analgesic. Together, these data strengthen the assertion that MOR+ RVM neurons produce a pro-nociceptive circuit underlying persistent pain and opioid-induced hyperalgesia.

Which RVM spinal projections could express MOR? Numerous studies have sought to categorize RVM spinal projections by neurochemical phenotype. Most RVM spinal projections are GABAergic[14,22,23] or serotonergic[9,20], and subsets of both these populations respond to MOR agonism. There is some controversy regarding how GABAergic neurons fit into the ON-cell/OFF-cell framework. In one model, GABAergic projections synapse on primary afferents to *inhibit* mechanical pain[23]. In a contrasting model, GABAergic projections synapse on inhibitory interneurons to *facilitate* mechanical pain via disinhibition[14]. Electrophysiological studies with post-hoc immunostaining has revealed GAD67 (a marker for GABAergic neurons) is expressed on both ON- and NEUTRAL-cells[24,25]. Furthermore, RVM spinal projections can also be distinguished by connectivity, as most RVM spinal projections synapse on interneurons in multiple dorsal horn laminae[26–29], while some synapse on primary afferents[23]. Thus, GABAergic neurons are too broad of a population to categorize into ON-cells or OFF-cells, and MOR+ RVM spinal projections likely consist of multiple neurochemical and anatomical phenotypes.

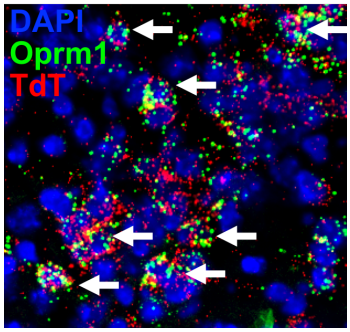

Figure 3: FISH performed by Eileen Nguyen of the Ross Lab validating the Oprm1-cre mouse.

No study has comprehensively investigated the neurochemical phenotype of MOR+ RVM spinal projections, identified the interneurons which receive input from this subpopulation, or observed the effect of selectively stimulating MOR+ spinal projections on acute and chronic pain behaviors. To study these neurons, we have acquired the Oprm1^{cre} mouse developed by Richard Palmiter **(Letter of Support)**. Our lab has performed FISH experiments to validate this Cre allele **(Figure 3).** I am therefore poised to use tracing techniques, the Oprm1^{cre} mouse, FISH, and IHC to investigate the anatomy of the MOR+ RVM spinal projections **(Aim 1)**. I will then use slice electrophysiology and optogenetics to define interneuron post-synaptic currents from MOR+ RVM neurons and phenotype interneurons based on firing pattern, pharmacological responsivity, and cell morphology **(Aim 2)**. Finally, I will use chemogenetics and behavior to determine whether MOR+ spinal projections facilitate noxious mechanical, thermal, and chemical pain responses, as well as persistent pain **(Aim 3)**. Together, the proposed experiments will characterize in a new way a critically important pathway for pain facilitation, thereby informing our understanding of the neural circuits mediating chronic pain.

APPROACH

Aim 1: Determine the spinal targets and neurochemical phenotypes of MOR+ RVM neurons.

Rationale: The RVM sends both GABAergic[22,25,30–32] and serotonergic[9] spinal projections from the RVM; a subset of these projections respond to MOR agonism[9,12]. Subsequent data has shown that MOR is expressed on interneurons and spinal projections from the RVM, identified by FISH[20] and IHC[21]. Preliminary FISH data from our lab has found MOR-expressing neurons in the RVM which express Vgat (GABAergic), VGlut2 (glutamatergic), and Tph (serotonergic), indicating there exist MOR+ RVM neurons of multiple neurotransmitter phenotypes **(Figure 4)**. However, the neurochemical phenotypes of all MOR+ spinal projections are unknown, and it is unknown in which laminae MOR+ RVM neurons terminate. Using FISH for the markers Vgat, VGlut2, Tph, and Oprm1 and tracing with Fluoro-Gold, the neurochemical phenotypes of MOR+ RVM spinal projections can be determined. (Experiment 1A). Through anterograde viral tracing, MOR+ RVM terminals can be visualized in the dorsal horn (Experiment 1B). **I hypothesize that the RVM sends primarily GABAergic MOR+ neurons to laminae I and II.**

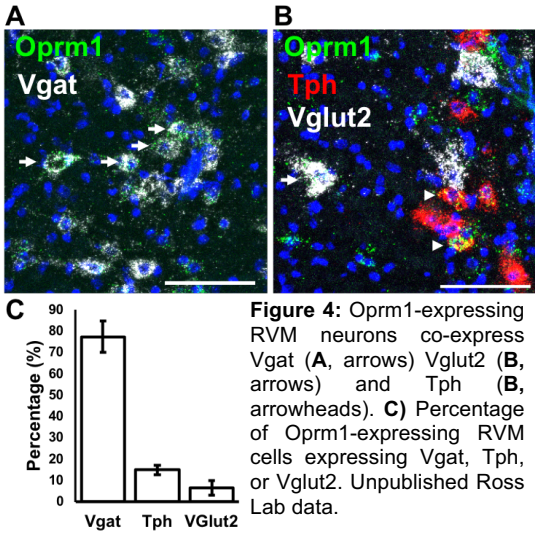

Figure 4: Oprm1-expressing RVM neurons co-express Vgat (**A**, arrows) Vglut2 (**B,** arrows) and Tph (**B,** arrowheads). **C)** Percentage of Oprm1-expressing RVM cells expressing Vgat, Tph, or Vglut2. Unpublished Ross Lab data.

Experimental Design: The neurochemical phenotype of MOR+ RVM spinal projections will be determined through injections of the retrograde tracer Fluoro-Gold into the spinal cord of WT mice to trace spinal projections

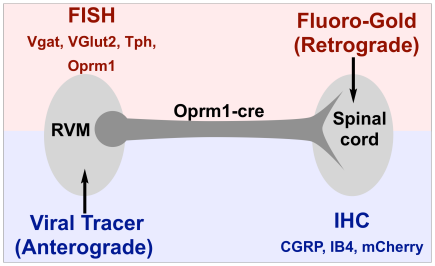

Figure 5: In experiment 1A **(Red)**, MOR+ spinal projections will be retrogradely traced from the spinal cord to the RVM; FISH will then be performed in RVM sections. In experiment 1B **(Blue)**, MOR+ RVM spinal projections will be anterogradely traced from the RVM; IHC will be performed on spinal cord sections.

back to the RVM. Co-staining of traced MOR+ neurons for Vgat, VGlut2, Tph, and Oprm1 will then be achieved via FISH on RVM sections. Secondly, identification of spinal cord laminae receiving input from MOR+ RVM neurons will be achieved through injections of a Cre-dependent anterograde AAV encoding a fluorescent probe into the RVM of Oprm1^{cre} mice, followed by IHC of the spinal cord **(Figure 5)**. Experiment 1A: WT mice will be anesthetized to receive spinal injections of Fluoro-Gold (2% w/v in water) into the dorsal horn. Five weeks after injections, mice will be decapitated. brainstems will be prepared fresh-frozen and sectioned in a cryostat. FISH will be performed for Vgat, VGlut2, Tph, and Oprm1 under the guidance of the RNAScope kit protocol by ACD Technologies. Experiment 1B: Oprm1^{cre} mice will be anesthetized to receive stereotaxic injections of AAV2-Ef1-DIO-mCherry into the RVM via the following coordinates: -6.0 mm bregma, -5.5 mm ventral, and midline. Five weeks after injections, mice will be transcardially perfused with 4% paraformaldehyde (PFA); spinal cords will be collected and post-fixed. IHC will be performed on spinal cord slices to amplify the mCherry signal and co-stain with calcitonin gene-related peptide (CGRP) and (IB4), two primary afferent markers which aid in visualizing dorsal horn laminae[33].

Scientific Rigor and Statistical Analysis: To ensure appropriate representation of soma sizes in the RVM, I will only quantify neurons with nuclei that are clearly visible. Based on previous immunostaining work from our lab[34], 60x confocal images of the RVM will be taken from each mouse, which will be repeated in 6 animals for each experiment. I will quantify the percentage of RVM neurons which co-express each marker, and will quantify the percentage of each subtype that co-expresses MOR. All steps for these experiments will also be performed on wild-type mice as negative controls. To account for potential sex differences, pilot analyses will be performed to determine whether there are differences in the distribution of MOR or other markers. If differences exist, sex will be considered a biological variable in future studies.

Anticipated Results: For Experiment 1A, I expect to visualize co-localized FG/Oprm1/Vgat in the RVM, consistent with current literature identifying mu opioid-sensitive GABAergic spinal projections, which are presumed to express MOR. The identification of glutamatergic, MOR+ RVM spinal projections would be an unprecedented finding which would prompt further investigation into the role of glutamate signaling in the RVM. For Experiment 1B, I expect to identify mCherry-labeled axons in laminae I and II of the dorsal horn, consistent with previous studies identifying the projection targets of the RVM[26–29]. The results of **Aim 1** will identify for the first time the dorsal horn targets of MOR+ RVM neurons with a higher level of granularity the RVM spinal projections which express MOR, as well as identify the supraspinal targets of MOR+ RVM inputs.

Potential Pitfalls and Alternative Approaches: Our lab has experience with FISH (RNAScope) and viral techniques. In addition, I am familiar with immunohistochemistry and stereotaxic injections. I will consult Eileen Nguyen, a graduate student in the Ross Lab with experience performing RNAscope and stereotaxic injections in the RVM, for technical assistance **(Letter of Support).** Therefore, I do not anticipate technical challenges in completing this aim. However, it is possible that I will identify a significant population of glutamatergic MOR+ spinal projections, which would be inconsistent with my hypothesis. It is possible that, unlike GABAergic ON-cells which are pro-nociceptive, glutamatergic ON-cells may mediate analgesia. Therefore, even if my hypothesis is wrong, this study will provide novel insights into the subpopulations of RVM spinal projections expressing MOR and will still inspire the further studies detailed in **Aim 2 and Aim 3.** It also is possible that we will identify MOR+ RVM spinal projections in deeper laminae; to investigate this further, we will adjust the experiments detailed in **Aim 2A** and **2B** to include interneurons residing in identified laminae.

Contribution and Training Opportunity: The proposed experiments will comprehensively phenotype all of the spinal projections from the RVM which express MOR and identify the dorsal horn targets of MOR+ RVM neurons. The completion of **Aim 1** will provide me with training in applying mouse genetics, viral labeling, and FISH to trace the anatomy of spinal circuits and I will refine my skills in stereotaxic surgery and confocal microscopy.

Aim 2A: Identify the MOR+ RVM inputs received by interneurons in lamina I and II of the dorsal horn.

Rationale: Spinal projections from the RVM modulate nocifensive reflexes, and synapse on interneurons in the superficial dorsal horn[26–29]. If MOR+ RVM spinal projections are pain facilitating, it would follow that these neurons would inhibit interneurons which normally gate pain. Inhibitory enkephalinergic interneurons are one subpopulation of interneurons which have been shown to receive GABAergic input[14] from the RVM. However, no study has taken a comprehensive approach to record the type of RVM input received from an unbiased

sample of lamina I and II interneurons. Identifying the input received by MOR+ RVM spinal projections is essential for uncovering the circuitry through which these neurons facilitate pain. **I hypothesize that MOR+ RVM spinal projections inhibit interneurons in dorsal horn lamina I and II.**

Experimental Design 2A: Selective activation of MOR+ RVM neurons and recording of responsive interneurons will be achieved through activation of a virally-inserted channelrhodopsin (ChR2) and whole-cell patch-clamp electrophysiology in slice **(Figure 6).** Briefly, Oprm1^{cre} mice will be anesthetized to receive injections of a Cre-dependent AAV-FLEX-rev-ChR2-tdTomato into the RVM. Three weeks after injections, mice will be anesthetized and decapitated. The spinal cord will be rapidly isolated and cut into 400 μm sagittal sections with a vibratome. Neurons within lamina I and II will be visualized and whole-cell patch-clamp recordings will be made in a K-gluconate internal solution. Baseline membrane potential will be measured in a patched cell in current clamp mode. Light-evoked excitatory and inhibitory post-synaptic currents (EPSCs and IPSCs) will be detected at -70 mV and -40 mV holding potentials, respectively. For cells exhibiting a light-evoked current, glutamate and GABA receptor antagonists (CNQX and bicuculline) will be used to confirm the receptor subtype(s) underlying the evoked current.

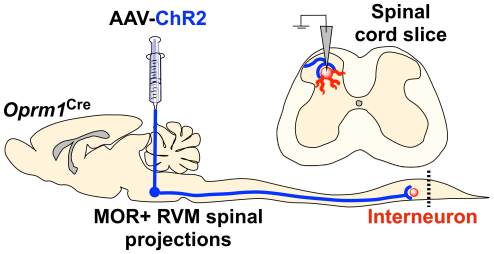

Figure 6: Schematic of **Aim 2A**. Using the Oprm1^{cre} mouse and a virus containing ChR2, I will optogenetically activate the MOR+ spinal projections from the RVM and record the inputs received by spinal interneurons.

Aim 2B: Explore the electrophysiological, neurochemical and morphological phenotype of dorsal horn interneurons receiving input from MOR+ RVM projections.

Rationale: Both inhibitory and excitatory interneurons reside in the superficial dorsal horn. Work from many groups has contributed to an emerging scheme by which dorsal horn interneurons can be divided into numerous subtypes based on firing pattern[35–37], response to cell-type specific agonists[38,39], genetic markers[40–43], and morphology[35,37,39,44]. Little is known about the interneurons which receive input from the RVM, including from MOR+ projections. Thus, the goal of this aim is to apply the emerging classification scheme for dorsal horn interneurons to the discovery of diverse interneuron subtypes which receive MOR+ RVM inputs.

Experimental Design 2B: Tissue will be prepared similarly to Experimental Design 2A. Once an interneuron responsive to light-evoked MOR+ RVM stimulation is patched, experiments will be conducted to phenotype the interneuron by the classification scheme detailed in **Figure 7**. The recording electrode will be filled with neurobiotin for post-hoc morphological analysis**.** Action potentials will be recorded in current clamp mode to measure the firing pattern of the interneuron. The pharmacological responsivity will then be evaluated by administration of somatostatin (SST), gastrin-releasing peptide (GRP), and substance P (SP) separated by wash-out periods, all in the presence of tetrodotoxin (TTX) and excitatory and inhibitory synaptic receptor blockers; the cell's responses to these agonists will be recorded. After recordings, slices will be post-fixed in 4% PFA, cut into 60 μm sections, and incubated in avidin-rhodamine to stain the neurobiotin-labeled cells.

Scientific Rigor and Statistical Analysis: I will sample cells randomly to maintain an unbiased approach. To consistently identify dorsal horn lamina I and II, I will define lamina II as a translucent band across the dorsal horn ventral to lamina I, within 100 μm from the II/III border. For **Aim 2A,** a power analysis was performed based on previous experiments performing optogenetic stimulation and slice electrophysiology[45]. With an expected effect size of 0.9 and a significance level of 0.05, a total of 12 interneurons will be recorded from 4 mice to achieve a power of 0.8. Paired t-tests will be used for statistical comparisons between the amplitude of IPSCs and EPSCs before and after light stimulation, as well as before and after bicuculline and CNQX administration respectively; $p<0.05$ will be taken as significant. I expect the MOR+ RVM inputs to be monosynaptic, which is distinguished from polysynaptic inputs by a short latency and minimal latency variability between recordings (known as jitter). However, if I identify a significant proportion of polysynaptic inputs to interneurons, monosynaptic versus polysynaptic input will be considered a variable. For **Aim 2B,** a power analysis based on a chi-squared comparison of the eight categories of interneurons determined that I will need to characterize ~60 neurons that receive direct input from MOR-cre RVM neurons, from 20 mice. Morphology of neurobiotin-labelled cells will be reconstructed with confocal microscopy using 1.0 μm z-stacks; morphology will be quantified through quantifying axonal arbors and the length of the dendritic dimensions in the rostrocaudal and dorsoventral axes.

Anticipated Results: For **Aim 2A,** identification of monosynaptic light-evoked IPSCs but not EPSCs in patched interneurons would confirm my hypothesis that MOR+ RVM spinal projections inhibit interneurons. This would contribute to our current model of MOR+ RVM neurons, which may facilitate pain by inhibiting inhibitory

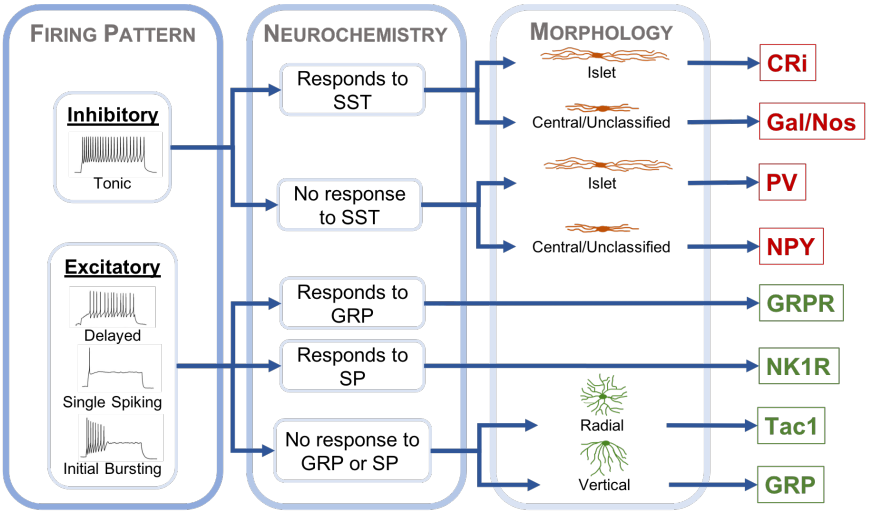

Figure 7: Interneurons exhibiting a light-evoked current will be classified by this scheme*. Inhibitory neurons subtypes: SST, somatostatin; GRP, gastrin-releasing peptide; SP, substance P; CRi, calretinin inhibitory; Gal/Nos, galanin/nitric oxide synthase; PV, parvalbumin; NPY, neuropeptide Y. Excitatory neuron subtypes: GRPR, gastrin-releasing peptide receptor; NK1R, neurokinin-1 receptor; Tac1, tachykinin precursor 1; GRP, gastrin-releasing peptide. * I acknowledge that this classification scheme is based on incomplete and emerging data and does not fully capture the complexity of dorsal horn interneuron subtypes.

interneurons which normally gate pain. For **Aim 2B,** if MOR+ RVM neurons inhibit inhibitory interneurons, as predicted, I would expect the recorded cells to display a tonic firing pattern and to fall into one of five categories of inhibitory interneurons based on their response to SST and central or islet morphology (**Figure 7**, red cells). Alternatively, the unexpected finding that the neurons that receive IPSCs from MOR+ RVM show a delayed, single spiking, or initial bursting firing pattern and respond to either GRP or SP, and/or show a radial or vertical morphology would suggest that MOR-cre RVM neurons provide inhibitory input onto one of the subtypes of excitatory interneurons (**Figure 7**, green cells), which would be expected to decrease nociception. In either case, these results would represent the first comprehensive data set identifying and characterizing all of the interneurons in the superficial dorsal horn receiving input from spinally projecting MOR+ RVM cells.

Potential Pitfalls and Alternative Approaches: Our lab has expertise with whole-cell patch clamping and confocal microscopy[46,47]. I will consult Dr. Kelly Smith, a postdoctoral fellow in the Ross Lab with expertise in electrophysiology[48–51], for training in the required techniques **(Letter of Support)**. I will also receive direct mentorship from my co-sponsor, Dr. H. Richard Koerber, a renowned expert in electrophysiology. Therefore, I do not anticipate technical challenges in completing this aim. For **Aim 2A,** however, it is possible that I will identify excitatory interneurons which receive EPSCs. If so, this would represent a new mechanism through which MOR+ RVM neurons could facilitate afferent signaling. For **Aim 2B,** it is possible I may discover cell types which do not fit into the classification scheme detailed in **Figure 7.** Should this occur, we will develop a post-hoc immunohistochemistry protocol to label the specific marker which defines the cell population.

Contribution and Training Opportunity: These experiments would establish, for the first time, a comprehensive dataset identifying the subtypes of interneurons responsive to spinally projecting MOR+ RVM cells and will quantify the type of input received by these interneurons. Completion of **Aim 2** would provide me with invaluable training in whole-cell patch-clamp electrophysiology and confocal reconstruction of neuron morphology.

Aim 3: Elucidate the functional role of MOR+ RVM spinal projections in behavioral assays of pain.

Rationale: Numerous studies have demonstrated that systemic and local injection of MOR agonists inhibit the firing rate of RVM neurons identified as ON-cells by in vivo recordings[5,6,8,9,12,13], thereby attenuating nocifensive reflexes. These opioid-sensitive neurons, which include local interneurons and spinal projections, play an unclear role in pain behaviors. No study has investigated the pain facilitating effects of MOR-expressing neurons through selective stimulation. With the Oprm1^{cre} mouse, a chemogenetic approach serves to fill this critical gap in knowledge. Designer receptors exclusively activated by designer drugs (DREADDs) are modified GPCRs which are only activated by the exogenous ligand clozapine-N-oxide (CNO), and can be inserted into cre-expressing cells with AAVs. The benefits of this approach include the ability to selectively activate the receptor with injections of CNO, and that injection of CNO will activate the DREADD receptor for hours, allowing us to conduct an array of behavioral tests. MOR+ RVM spinal projections can therefore be activated for the first time to determine their effects on acute and chronic pain models. **I hypothesize that MOR+ RVM spinal projections facilitate mechanical, chemical, and thermal hyperalgesia, and exacerbate chronic neuropathic pain.**

Experimental Design: MOR+ RVM spinal projections will be excited or inhibited to determine the effect of these neurons on pain behaviors. This will be accomplished through expression of an excitatory DREADD (hM3D(Gq)) or inhibitory DREADD (hM4D(Gi)), respectively, via injection of a retrograde AAV into the lumbar spinal cord or Oprm1^{cre} mice. A guide cannula will be placed into the RVM to allow for local injections of CNO into the RVM, such that only MOR+ RVM neurons retrogradely traced from the spinal cord will be targeted. Following CNO

injection, acute and chronic behavioral responses will be measured **(Figure 8)**. Details: Oprm1^{cre} mice will be anesthetized and receive injections of a Cre-dependent AAV-retro containing the excitatory DREADD into the lumbar spinal cord, so that only descending spinal projections express the DREADD **(Figure 8)**. In parallel, a guide cannula will be stereotaxically imbedded in the RVM, at -6.0 mm bregma, -4.5 mm ventral (with the needle extending 1 mm past the guide), and midline. Six weeks after injections, mice will be habituated to each testing apparatus for 60 minutes on two consecutive days before undergoing behavioral experiments. Mice will receive injections by cannula of either vehicle (0.5% DMSO in saline) or CNO 30 minutes before behavioral experiments. Spontaneous nocifensive behaviors: The total time spent engaging in licking and biting will be quantified over a 30-minute period before and after receiving CNO. Thermal pain assay: Mice will be placed in individual Plexiglass boxes atop a Hargreaves' apparatus. Paw withdrawal latency to a hot stimulus will be measured over three 20-second trials. Mechanical pain assay: 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 (10 μg/10 μl) 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. Persistent neuropathic pain state: A spared nerve injury (SNI) model[52,53] will be achieved with surgeries ligating the tibial and common peroneal nerve, sparing the sural nerve. Seven days after surgery, spontaneous nocifensive behaviors will be measured before and after CNO administration, followed by hyperalgesia measures with Hargreaves and Von Frey filaments.

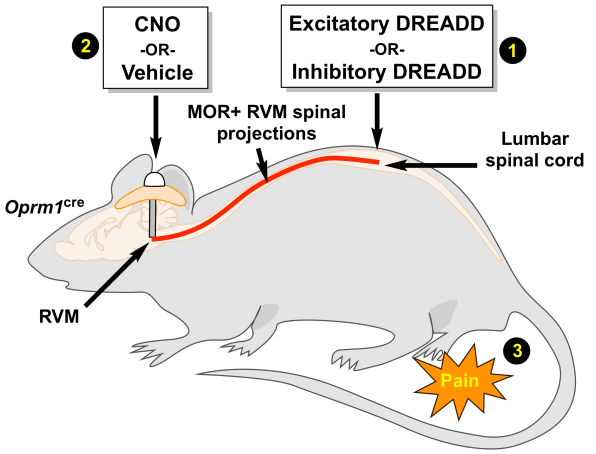

Figure 8: Schematic of **Aim 3. 1)** I will chemogenetically target MOR+ RVM spinal projections by injecting a retrograde AAV containing the gene for a DREADD receptor into the lumbar spinal cord of Oprm1^{cre} mice. **2)** I will activate the DREADD through injections of CNO into the RVM. **3)** After CNO injection, I will measure behavioral responses to noxious mechanical, chemical, and thermal stimuli on the hindpaws.

Scientific Rigor and Statistical Analysis: To confirm successful retrograde infection and cannula placement, I will section and image each mouse RVM blindly. If the cannula is placed outside the RVM or there is no expression of AAV reporter, I will exclude that mouse from the statistical analysis. A power analysis was performed based on previous studies using chemogenetic manipulation of pain behaviors[54]. With an expected effect size of 0.6 and a significance level of 0.05, ~9 mice will be required per group to achieve a power of 0.8. Experiments will be conducted blindly to treatment group. Data will be analyzed via two-way ANOVA with Bonferroni post hoc test for repeated measures, and $p<0.05$ will be taken as significant. To account for sex differences, pilot analyses will be performed to determine whether there are differences in behavioral responses to MOR+ RVM cell stimulation. If differences exist, sex will be considered a biological variable in subsequent studies.

Anticipated Results: A finding of CNO-induced mechanical, chemical, or thermal hypersensitivity in mice which received hM3D(Gq) DREADD would show for the first time that selective activation of MOR+ spinal projections from the RVM facilitate pain behaviors in mice, and would provide strong evidence that MOR+ cells are ON-cells. Inhibition of MOR+ cells in the RVM via the hM4D(Gi) DREADD are expected to have little effect in acute pain (where facilitation is not recruited), but is expected to reduce pain hypersensitivity in the spared nerve injury. If so, this would suggest that the inhibition of ON-cells may have therapeutic benefit for persistent pain.

Potential Pitfalls and Alternative Approaches: The Ross Lab has extensive experience with pain behavioral experiments[55]. I will consult Dr. Tayler Sheahan, a postdoctoral fellow in the Ross Lab with expertise in stereotaxic surgeries and pain behavioral experiments[56–58], for training and guidance **(Letter of Support)**. Therefore, I do not anticipate technical challenges in completing this aim. However, should technical challenges arise from using chemogenetics, we will try optogenetics and modify our experimental conditions to allow for continuous attachment of the mouse to the fiber optic cable. There is also a strong body of evidence supporting the hypothesis that MOR+ ON-cells facilitate pain. However, MOR+ spinal projections from the RVM may inhibit pain behaviors or have no effect. These neurons could mediate other forms of somatosensation, such as itch. We will investigate further by performing behavioral assays of itch on the mice described above.

Contribution and Training Opportunity: These experiments would elucidate the role of MOR+ RVM spinal projections on acute and chronic pain behaviors. Completion of **Aim 3** would provide me with excellent training in stereotaxic injections and pain behavioral paradigms.