Table of Contents

OMB Number: 4040-0001
Expiration Date: 10/31/2019

APPLICATION FOR FEDERAL ASSISTANCE

SF 424 (R&R)

3. DATE RECEIVED BY STATE	State Application Identifier

1. TYPE OF SUBMISSION

[ ] Pre-application [X] Application [ ] Changed/Corrected Application

2. DATE SUBMITTED | **Applicant Identifier**

4. a. Federal Identifier

b. Agency Routing Identifier

c. Previous Grants.gov Tracking ID

5. APPLICANT INFORMATION **Organizational DUNS:** 004514360

Legal Name: University of Pittsburgh
Department: Office of Research Division:
Street 1: 123 University Place
Street 2: B21 University Club
City: Pittsburgh County/Parish: Allegheny
State: PA: Pennsylvania Province:
Country: USA: UNITED STATES ZIP / Postal Code: 15213-2303

Person to be contacted on matters involving this application
Prefix: First Name: Jennifer Middle Name: E.
Last Name: Woodward Suffix:
Position/Title: Vice Chancellor for Research Operations
Street 1: 123 University Place
Street 2: B21 University Club
City: Pittsburgh County/Parish: Allegheny
State: PA: Pennsylvania Province:
Country: USA: UNITED STATES ZIP / Postal Code: 15213-2303
Phone Number: 412-624-7400 Fax Number:
Email: offres@pitt.edu

6. EMPLOYER IDENTIFICATION *(EIN) or (TIN)*: 125-0965591A6

7. TYPE OF APPLICANT: X: Other (specify)

Other (Specify): Private, non-profit, state-related, educ inst

Small Business Organization Type [ ] Women Owned [ ] Socially and Economically Disadvantaged

8. TYPE OF APPLICATION:

[X] New [ ] Resubmission

[ ] Renewal [ ] Continuation [ ] Revision

If Revision, mark appropriate box(es).

[ ] A. Increase Award [ ] B. Decrease Award [ ] C. Increase Duration [ ] D. Decrease Duration

[ ] E. Other (specify):

Is this application being submitted to other agencies? Yes [ ] No [X] What other Agencies?

9. NAME OF FEDERAL AGENCY:

National Institutes of Health

10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER:

TITLE:

11. DESCRIPTIVE TITLE OF APPLICANT'S PROJECT:

Descending facilitation of pain by mu-opioid receptor-expressing neurons in the rostral ventromedial medulla

12. PROPOSED PROJECT:

Start Date	Ending Date
04/01/2020	03/31/2023

13. CONGRESSIONAL DISTRICT OF APPLICANT

PA-018

SF 424 (R&R) APPLICATION FOR FEDERAL ASSISTANCE

14. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION

Prefix: First Name: Ruby Middle Name: Andrea
Last Name: Holland Suffix:
Position/Title: MD/PhD Student
Organization Name: University of Pittsburgh
Department: Neurobiology Division: Medicine
Street 1: M250 Scaife Hall
Street 2: 3550 Terrace Street
City: Pittsburgh County/Parish: Allegheny
State: PA: Pennsylvania Province:
Country: USA: UNITED STATES ZIP / Postal Code: 15261
Phone Number: 412-648-2324 Fax Number:
Email: rah143@pitt.edu

15. ESTIMATED PROJECT FUNDING

a. Total Federal Funds Requested	$284,829.00
b. Total Non-Federal Funds	$0.00
c. Total Federal & Non-Federal Funds	$284,829.00
d. Estimated Program Income	$0.00

16. IS APPLICATION SUBJECT TO REVIEW BY STATE EXECUTIVE ORDER 12372 PROCESS?

a. YES [ ] THIS PREAPPLICATION/APPLICATION WAS MADE AVAILABLE TO THE STATE EXECUTIVE ORDER 12372 PROCESS FOR REVIEW ON:

DATE:

b. NO [X] PROGRAM IS NOT COVERED BY E.O. 12372; OR

[ ] PROGRAM HAS NOT BEEN SELECTED BY STATE FOR REVIEW

17. By signing this application, I certify (1) to the statements contained in the list of certifications* and (2) that the statements herein are true, complete and accurate to the best of my knowledge. I also provide the required assurances * and agree to comply with any resulting terms if I accept an award. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. (U.S. Code, Title 18, Section 1001)

[X] I agree

** The list of certifications and assurances, or an internet site where you may obtain this list, is contained in the announcement or agency specific instructions.*

18. SFLLL or other Explanatory Documentation

19. Authorized Representative

Prefix: First Name: Jennifer Middle Name: E.
Last Name: Woodward Suffix:
Position/Title: Vice Chancellor for Research Operations
Organization: University of Pittsburgh
Department: Office of Research Division:
Street 1: 123 University Place
Street 2: B21 University Club
City: Pittsburgh County/Parish: Allegheny
State: PA: Pennsylvania Province:
Country: USA: UNITED STATES ZIP / Postal Code: 15213-2303
Phone Number: 412-624-7400 Fax Number:
Email: offres@pitt.edu

Signature of Authorized Representative — Completed on submission to Grants.gov

Date Signed — Completed on submission to Grants.gov

20. Pre-application	
21. Cover Letter Attachment	Cover Letter

University of Pittsburgh
School of Medicine
Department of Neurobiology

E1440 Biomedical Science Tower
200 Lothrop Street
Pittsburgh, PA 15213
Fax: (412) 648-1441
Email: rah143@pitt.edu

August 2, 2019

National Institutes of Health
Center for Scientific Review
6701 Rockledge Drive MSC 7768
Bethesda MD 20892-7768

Re: Submission of F31 Application: **"Descending facilitation of pain by mu-opioid receptor-expressing neurons in the rostral ventromedial medulla"**

Dear Sir or Madam:

Based on the scientific content of this F31 application (FOA: **PA-19-195**), I respectfully request that this F31 application be assigned the following awarding institute:

Institutes/Centers: National Institute of Neurological Disorders and Stroke – **NINDS**

As requested, I have provided below the list of referees that I have asked to submit reference letters on my behalf:

1. **Bart De Jonghe, PhD**
   Associate Professor and Assistant Director of Nutrition Science Programs, Department of Biobehavioral Health Sciences, University of Pennsylvania School of Nursing
2. **Matthew Hayes, PhD**
   Associate Professor of Nutritional Neuroscience, Department of Psychiatry, Perelman School of Medicine at the University of Pennsylvania
3. **Richard Steinman, MD, PhD**
   Director, Medical Scientist Training Program
   University of Pittsburgh and Carnegie Mellon University MSTP

Sponsor/Dissertation Advisor: Sarah E. Ross, PhD
Associate Professor, Department of Neurobiology, University of Pittsburgh

Co-Sponsor: H. Richard Koerber, PhD
Professor, Department of Neurobiology, University of Pittsburgh

Thank you for your consideration.

Sincerely,

Ruby A. Holland
MD/PhD Candidate
Department of Neurobiology
University of Pittsburgh School of Medicine
rah143@pitt.edu

OMB Number: 4040-0010
Expiration Date: 10/31/2019

Project/Performance Site Location(s)

Project/Performance Site Primary Location

[ ] I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name: University of Pittsburgh
DUNS Number: 004514360
Street 1: 123 University Place
Street 2: B21 University Club
City: Pittsburgh
State: PA: Pennsylvania
Province:
Country: USA: UNITED STATES
ZIP / Postal Code: 15213-2303

County: Allegheny

Project/Performance Site Congressional District: PA-018

Additional Location(s):

RESEARCH & RELATED Other Project Information

OMB Number: 4040-0001
Expiration Date: 10/31/2019

1. * Are Human Subjects Involved? [ ] Yes [X] No

1.a If YES to Human Subjects

Is the Project Exempt from Federal regulations? [ ] Yes [ ] No

If yes, check appropriate exemption number. [ ] 1 [ ] 2 [ ] 3 [ ] 4 [ ] 5 [ ] 6 [ ] 7 [ ] 8

If no, is the IRB review Pending? [ ] Yes [ ] No

IRB Approval Date:

Human Subject Assurance Number:

2. * Are Vertebrate Animals Used? [X] Yes [ ] No

2.a. If YES to Vertebrate Animals

Is the IACUC review Pending? [X] Yes [ ] No

IACUC Approval Date:

Animal Welfare Assurance Number D16-00118

3. * Is proprietary/privileged information included in the application? [ ] Yes [X] No

4.a. * Does this Project Have an Actual or Potential Impact – positive or negative - on the environment? [ ] Yes [X] No

4.b. If yes, please explain:

4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed? [ ] Yes [ ] No

4.d. If yes, please explain:

5. Is the research performance site designated, or eligible to designated, as a historic place? [ ] Yes [X] No

5.a. If yes, please explain:

6. * Does this project involve activities outside the United States or partnerships with international collaborators? [ ] Yes [X] No

6.a. If yes, identify countries:

6.b. Optional Explanation:

7. Project Summary/Abstract	Project Summary
8. Project Narrative	Project Narrative
9. Bibliography & References Cited	Bibliography
10. Facilities & Other Resources	Facilities
11. Equipment	Equipment
12. Other Attachments	

Project Summary/Abstract

Pain is a debilitating and prevalent condition which severely impacts quality of life. Unfortunately, our current treatment options are limited by adverse effects, and the pathways modulating pain signaling are not fully understood. The rostral ventromedial medulla (RVM) is a brainstem site which plays a critical role in pain modulation, primarily by sending descending projections to the spinal cord. While non-specific electrical stimulation of the RVM produces analgesia, RVM neurons can either facilitate or inhibit pain. RVM spinal projections can be characterized as ON-cells, OFF-cells, or NEUTRAL-cells based on their response to, and effect on, nocifensive reflexes, but many details remain unclear. Morphine acts at the mu-opioid receptor (MOR) to inhibit neuronal activity and produces analgesia in part by activating OFF-cells and inhibiting ON-cells. Thus, it follows that ON-cells express MOR. Numerous studies support the assertion that ON-cell circuitry is implicated in chronic pain and opioid-induced hyperalgesia. However, until recently we lacked the genetic tools necessary to dissect the circuitry of MOR-expressing neurons, including ON-cells, in the RVM. With the recent development of the *Oprm1-Cre* knock-in mouse, we now have the genetic tool necessary to study these circuits in better detail. The goal of this proposal is to therefore test the hypothesis that MOR+ RVM neurons facilitate pain by inhibiting interneurons in the superficial dorsal horn. I will test this hypothesis using a combination of genetic, molecular, electrophysiological, and behavioral approaches. **Aim 1** will investigate which cell types in the RVM express MOR through viral tracing, fluorescent in situ hybridization (FISH), and immunohistochemistry (IHC). **Aim 2A** will identify the MOR+ RVM inputs received by interneurons in the dorsal horn through optogenetics and slice electrophysiology. **Aim 2B** will explore the electrophysiological, neurochemical, and morphological phenotype of dorsal horn interneurons receiving input from MOR+ RVM neurons using a combined electrophysiology and anatomical approach. **Aim 3** will test the hypothesis that MOR+ RVM neurons facilitate mechanical, chemical, and thermal pain through the use of chemogenetics and behavioral assays of acute and chronic neuropathic pain. The work detailed in this proposal is critically important because an enhanced understanding of the circuitry underlying the RVM can pave the way for the development of novel pain therapeutics and will advance the field of neuroscience.

Furthermore, this proposal is heavily inspired by my clinical interest in anesthesiology, where I plan to work as a pain specialist and investigate pain signaling mechanisms to minimize patient suffering and improve quality of life. The professional, technical, and intellectual skills which will be developed over the course of this fellowship will position me for success as a physician-scientist in academic anesthesiology.

Project Narrative

Chronic pain is a debilitating condition which severely impacts quality of life for millions of people worldwide. Ascending pain signals undergo descending modulation by mu-opioid receptor (MOR)-positive neurons in the rostral ventromedial medulla (RVM) through circuits which are not completely understood. In this proposal, I will elucidate the specific circuitry through which MOR+ RVM spinal projection neurons facilitate pain using a combination of genetic, electrophysiological, and behavioral approaches.

Bibliography

1. Fields, H. L. & Heinricher, M. M. Anatomy and physiology of a nociceptive modulatory system. *Philos Trans R Soc Lond, B, Biol Sci* **308,** 361–374 (1985).
2. Chen, Q. & Heinricher, M. M. Descending control mechanisms and chronic pain. *Curr Rheumatol Rep* **21,** 13 (2019).
3. Koch, S. C., Acton, D. & Goulding, M. Spinal circuits for touch, pain, and itch. *Annu Rev Physiol* **80,** 189–217 (2018).
4. Heinricher, M. M., Tavares, I., Leith, J. L. & Lumb, B. M. Descending control of nociception: Specificity, recruitment and plasticity. *Brain Res Rev* **60,** 214–225 (2009).
5. Heinricher, M. M., Morgan, M. M. & Fields, H. L. Direct and indirect actions of morphine on medullary neurons that modulate nociception. *Neuroscience* **48,** 533–543 (1992).
6. Heinricher, M. M., Morgan, M. M., Tortorici, V. & Fields, H. L. Disinhibition of off-cells and antinociception produced by an opioid action within the rostral ventromedial medulla. *Neuroscience* **63,** 279–288 (1994).
7. Basbaum, A. I., Clanton, C. H. & Fields, H. L. Opiate and stimulus-produced analgesia: functional anatomy of a medullospinal pathway. *Proc Natl Acad Sci U S A* **73,** 4685–4688 (1976).
8. Barbaro, N. M., Heinricher, M. M. & Fields, H. L. Putative pain modulating neurons in the rostral ventral medulla: reflex-related activity predicts effects of morphine. *Brain Res* **366,** 203–210 (1986).
9. Marinelli, S., Vaughan, C. W., Schnell, S. A., Wessendorf, M. W. & Christie, M. J. Rostral ventromedial medulla neurons that project to the spinal cord express multiple opioid receptor phenotypes. *J Neurosci* **22,** 10847–10855 (2002).
10. Zhang, L. & Hammond, D. L. Cellular basis for opioid potentiation in the rostral ventromedial medulla of rats with persistent inflammatory nociception. *Pain* **149,** 107–116 (2010).
11. Li, Z. *et al.* CaMKIIα may modulate fentanyl-induced hyperalgesia via a CeLC-PAG-RVM-spinal cord descending facilitative pain pathway in rats. *PLoS ONE* **12,** e0177412 (2017).
12. Pedersen, N. P., Vaughan, C. W. & Christie, M. J. Opioid receptor modulation of GABAergic and serotonergic spinally projecting neurons of the rostral ventromedial medulla in mice. *J Neurophysiol* **106,** 731–740 (2011).
13. Harasawa, I., Johansen, J. P., Fields, H. L., Porreca, F. & Meng, I. D. Alterations in the rostral ventromedial medulla after the selective ablation of μ-opioid receptor expressing neurons. *Pain* **157,** 166–173 (2016).
14. François, A. *et al.* A Brainstem-Spinal Cord Inhibitory Circuit for Mechanical Pain Modulation by GABA and Enkephalins. *Neuron* **93,** 822–839.e6 (2017).
15. Benyamin, R. *et al.* Opioid complications and side effects. *Pain Physician* **11,** S105-20 (2008).
16. Mayer, D. J., Wolfle, T. L., Akil, H., Carder, B. & Liebeskind, J. C. Analgesia from electrical stimulation in the brainstem of the rat. *Science* **174,** 1351–1354 (1971).
17. Zorman, G., Hentall, I. D., Adams, J. E. & Fields, H. L. Naloxone-reversible analgesia produced by microstimulation in the rat medulla. *Brain Res* **219,** 137–148 (1981).
18. Heinricher, M. M., Barbaro, N. M. & Fields, H. L. Putative nociceptive modulating neurons in the rostral ventromedial medulla of the rat: firing of on- and off-cells is related to nociceptive responsiveness. *Somatosens Mot Res* **6,** 427–439 (1989).
19. Gris, P. *et al.* A novel alternatively spliced isoform of the mu-opioid receptor: functional antagonism. *Mol Pain* **6,** 33 (2010).
20. Wang, H. & Wessendorf, M. W. Mu- and delta-opioid receptor mRNAs are expressed in spinally projecting serotonergic and nonserotonergic neurons of the rostral ventromedial medulla. *J Comp Neurol* **404,** 183–196 (1999).
21. Pinto, M., Sousa, M., Lima, D. & Tavares, I. Participation of mu-opioid, GABA(B), and NK1 receptors of major pain control medullary areas in pathways targeting the rat spinal cord: implications for descending modulation of nociceptive transmission. *J Comp Neurol* **510,** 175–187 (2008).

22. Antal, M., Petkó, M., Polgár, E., Heizmann, C. W. & Storm-Mathisen, J. Direct evidence of an extensive GABAergic innervation of the spinal dorsal horn by fibres descending from the rostral ventromedial medulla. *Neuroscience* **73,** 509–518 (1996).
23. Zhang, Y. *et al.* Identifying local and descending inputs for primary sensory neurons. *J Clin Invest* **125,** 3782–3794 (2015).
24. Morgan, M. M., Whittier, K. L., Hegarty, D. M. & Aicher, S. A. Periaqueductal gray neurons project to spinally projecting GABAergic neurons in the rostral ventromedial medulla. *Pain* **140,** 376–386 (2008).
25. Winkler, C. W. *et al.* Kappa opioid receptor (KOR) and GAD67 immunoreactivity are found in OFF and NEUTRAL cells in the rostral ventromedial medulla. *J Neurophysiol* **96,** 3465–3473 (2006).
26. Holstege, G. & Kuypers, H. G. J. M. in **57,** 145–175 (Elsevier, 1982).
27. Martin, G. F., Vertes, R. P. & Waltzer, R. Spinal projections of the gigantocellular reticular formation in the rat. Evidence for projections from different areas to laminae I and II and lamina IX. *Exp Brain Res* **58,** 154–162 (1985).
28. Basbaum, A. I., Clanton, C. H. & Fields, H. L. Three bulbospinal pathways from the rostral medulla of the cat: an autoradiographic study of pain modulating systems. *J Comp Neurol* **178,** 209–224 (1978).
29. Fields, H. L., Malick, A. & Burstein, R. Dorsal horn projection targets of ON and OFF cells in the rostral ventromedial medulla. *J Neurophysiol* **74,** 1742–1759 (1995).
30. Heinricher, M. M. & Tortorici, V. Interference with GABA transmission in the rostral ventromedial medulla: disinhibition of off-cells as a central mechanism in nociceptive modulation. *Neuroscience* **63,** 533–546 (1994).
31. Kato, G. *et al.* Direct GABAergic and glycinergic inhibition of the substantia gelatinosa from the rostral ventromedial medulla revealed by in vivo patch-clamp analysis in rats. *J Neurosci* **26,** 1787–1794 (2006).
32. Aicher, S. A., Hermes, S. M., Whittier, K. L. & Hegarty, D. M. Descending projections from the rostral ventromedial medulla (RVM) to trigeminal and spinal dorsal horns are morphologically and neurochemically distinct. *J Chem Neuroanat* **43,** 103–111 (2012).
33. Snider, W. D. & McMahon, S. B. Tackling pain at the source: new ideas about nociceptors. *Neuron* **20,** 629–632 (1998).
34. Kardon, A. P. *et al.* Dynorphin acts as a neuromodulator to inhibit itch in the dorsal horn of the spinal cord. *Neuron* **82,** 573–586 (2014).
35. Gatto, G., Smith, K. M., Ross, S. E. & Goulding, M. Neuronal diversity in the somatosensory system: bridging the gap between cell type and function. *Curr Opin Neurobiol* **56,** 167–174 (2019).
36. Grudt, T. J. & Perl, E. R. Correlations between neuronal morphology and electrophysiological features in the rodent superficial dorsal horn. *J Physiol (Lond)* **540,** 189–207 (2002).
37. Yasaka, T., Tiong, S. Y. X., Hughes, D. I., Riddell, J. S. & Todd, A. J. Populations of inhibitory and excitatory interneurons in lamina II of the adult rat spinal dorsal horn revealed by a combined electrophysiological and anatomical approach. *Pain* **151,** 475–488 (2010).
38. Gutierrez-Mecinas, M., Furuta, T., Watanabe, M. & Todd, A. J. A quantitative study of neurochemically defined excitatory interneuron populations in laminae I-III of the mouse spinal cord. *Mol Pain* **12,** (2016).
39. Boyle, K. A. *et al.* A quantitative study of neurochemically defined populations of inhibitory interneurons in the superficial dorsal horn of the mouse spinal cord. *Neuroscience* **363,** 120–133 (2017).
40. Sathyamurthy, A. *et al.* Massively Parallel Single Nucleus Transcriptional Profiling Defines Spinal Cord Neurons and Their Activity during Behavior. *Cell Rep* **22,** 2216–2225 (2018).
41. Wildner, H. *et al.* Genome-wide expression analysis of Ptf1a- and Ascl1-deficient mice reveals new markers for distinct dorsal horn interneuron populations contributing to nociceptive reflex plasticity. *J Neurosci* **33,** 7299–7307 (2013).
42. Bröhl, D. *et al.* A transcriptional network coordinately determines transmitter and peptidergic fate in the dorsal spinal cord. *Dev Biol* **322,** 381–393 (2008).
43. Huang, M. *et al.* Ptf1a, Lbx1 and Pax2 coordinate glycinergic and peptidergic transmitter phenotypes in dorsal spinal inhibitory neurons. *Dev Biol* **322,** 394–405 (2008).

44. Todd, A. J. Identifying functional populations among the interneurons in laminae I-III of the spinal dorsal horn. *Mol Pain* **13,** 1744806917693003 (2017).
45. Chen, Q. *et al.* Optogenetic Evidence for a Direct Circuit Linking Nociceptive Transmission through the Parabrachial Complex with Pain-Modulating Neurons of the Rostral Ventromedial Medulla (RVM). *Eneuro* **4,** (2017).
46. Hachisuka, J. *et al.* Semi-intact ex vivo approach to investigate spinal somatosensory circuits. *elife* **5,** (2016).
47. Hachisuka, J. *et al.* Wind-up in lamina I spinoparabrachial neurons: a role for reverberatory circuits. *Pain* **159,** 1484–1493 (2018).
48. Smith, K. M. *et al.* Distinct forms of synaptic inhibition and neuromodulation regulate calretinin-positive neuron excitability in the spinal cord dorsal horn. *Neuroscience* **326,** 10–21 (2016).
49. Smith, K. M. *et al.* Functional heterogeneity of calretinin-expressing neurons in the mouse superficial dorsal horn: implications for spinal pain processing. *J Physiol (Lond)* **593,** 4319–4339 (2015).
50. Smith, K. M., Madden, J. F., Callister, R. J., Hughes, D. I. & Graham, B. A. The search for novel analgesics: re-examining spinal cord circuits with new tools. *Front. Pharmacol.* **5,** 22 (2014).
51. Tae, H.-S. *et al.* Gabapentin Modulates HCN4 Channel Voltage-Dependence. *Front. Pharmacol.* **8,** 554 (2017).
52. Decosterd, I. & Woolf, C. J. Spared nerve injury: an animal model of persistent peripheral neuropathic pain. *Pain* **87,** 149–158 (2000).
53. Cichon, J., Sun, L. & Yang, G. Spared nerve injury model of neuropathic pain in mice. *Bio Protoc* **8,** (2018).
54. Alhadeff, A. L. *et al.* A neural circuit for the suppression of pain by a competing need state. *Cell* **173,** 140–152.e15 (2018).
55. Saloman, J. L. *et al.* Gi-DREADD Expression in Peripheral Nerves Produces Ligand-Dependent Analgesia, as well as Ligand-Independent Functional Changes in Sensory Neurons. *J Neurosci* **36,** 10769–10781 (2016).
56. Sheahan, T. D., Copits, B. A., Golden, J. P. & Gereau, R. W. Voluntary Exercise Training: Analysis of Mice in Uninjured, Inflammatory, and Nerve-Injured Pain States. *PLoS ONE* **10,** e0133191 (2015).
57. Sheahan, T. D. *et al.* Metabotropic glutamate receptor 2/3 (mglur2/3) activation suppresses TRPV1 sensitization in mouse, but not human, sensory neurons. *Eneuro* **5,** (2018).
58. Sheahan, T. D. *et al.* Inflammation and nerve injury minimally affect mouse voluntary behaviors proposed as indicators of pain. *Neurobiol. Pain* **2,** 1–12 (2017).
59. McMahon, S. B., Koltzenburg, M., Tracey, I. & Turk, D. *Wall and Melzack'sTextbook of Pain*. (Elsevier, 2006).

Facilities and Other Resources

Laboratory: Dr. Ross's lab consists of approximately 1000 square feet of recently renovated space in the Department of Neurobiology on the 14th floor of the Thomas E. Starzl Biomedical Science Tower (BST) building at the University of Pittsburgh main campus. It includes desks and wet benches for six people as well as a perfusion room with a backdraft table and a confocal microscope room equipped with a Nikon A1R for immunohistochemistry. I will have exclusive access to a patch-clamp that is set up in the Ross Laboratory for slice electrophysiology recordings. The laboratory also offers ample bench space for solution preparation, a chemical fume hood complete with reagents, a small workshop for electronics and small part fabrication, and a station for micropipette fabrication with a Sutter 2000 microelectrode puller.

Animals: Mice used for the proposed experiments will be housed in the University of Pittsburgh Division of Laboratory Animal Resources (DLAR) vivarium facilities. These facilities have state of the art caging systems and rooms for quarantine and for breeding specialized populations. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care and professionally staffed by a manager and animal care technicians who provide basic animal care. The Ross Lab keeps ~130 cages of mice and has a full-time technician who maintains the animal colonies and performs genotyping for the lab.

Computer: Dr. Ross's lab has ten computers. Three are linked to equipment, and the rest are at dedicated workstations available to lab personnel. All computers are networked behind the University of Pittsburgh firewall and are supported by dedicated IT staff in the Department of Neurobiology.

Office: I have dedicated bench space within the lab that promotes productive interactions with lab members. I also have a desk on the 14th floor of BST right down the hall from our lab space, seated with postdoctoral fellows from collaborating laboratories. My sponsor's and co-sponsor's (Drs. Ross and Koerber) offices are both a few doors down from my desk.

Other: My training brings together the expertise of **Dr. Sarah Ross (sponsor)** and **Dr. H. Richard Koerber (co-sponsor** in a collaborative project which will provide excellent training. Dr. Ross, my official mentor within the graduate program, has extensive expertise with modern genetic techniques, as well as seven years of experience with electrophysiology and optogenetics. Dr. Koerber, who will serve as my co-mentor, is a nationally-renowned expert in electrophysiology. I am also a member of the Pittsburgh Center for Pain Research, which consists of basic science researchers and clinical faculty. Most members of the Pain Center are physically located on the 14th floor of the Biomedical Science Tower, which facilitates collaboration. This proximity to other research groups fosters a collaborative environment among trainees, featuring weekly seminars and journal clubs attended by multiple groups.

As a member of the Center for Neuroscience at the University of Pittsburgh (CNUP) graduate program, I have the unique advantage of working closely with two renowned universities, University of Pittsburgh and Carnegie Mellon University, which are located less than half a mile apart. Being a member of the CNUP allows me to interact frequently with graduate students, postdoctoral fellows, and investigators from both universities studying diverse aspects of neuroscience. We also have an annual retreat weekend in the fall, which exposes me to a breadth of neuroscience research through speakers and posters, and provides me with the opportunity to present my research and gain valuable feedback from experts across the two institutions.

Finally, as a member of the Department of Neurobiology, I have access to shared freezers, water purification, autoclaves, a glass washer, a cold room, a warm room, cell culture facilities, a sterilization apparatus, dry ice, liquid nitrogen, ultracentrifuges, numerous -20°C and -80°C freezers, electronic hardware, software, the University of Pittsburgh Machine Shop, the Center for Biologic Imaging, and The University of Pittsburgh Brain Institute. I will also present my research annually to members of the Department of Neurobiology to gain feedback and share ideas.

Equipment

General: The Ross Lab is well-equipped with multiple freezers (-20°C and -80°C), three centrifuges, two epifluorescent microscopes (an Olympus BX53 upright and a Nikon TS100 inverted), a Coolsnap camera, a Leica CM1950 cryostat, a Leica 1200 vibratome, a dissection hood, two Leica stereo dissecting microscopes (one for fresh and one for fixed tissue) a Leica EC3 camera (to watch and record dissections), and a backdraft table (for animal perfusions).

Stereotaxic and Spared Nerve Injury Surgery: Located in the vivarium is a surgical setup with stereotaxis (KOPF) for surgeries such as craniotomy and viral injection. Ear and bite bars are in place on this apparatus, as is a complete homeothermic blanket system. A stereoscope (Leica Microsystems M80, 6x) allows for magnification and visualization of surgical site. Surgical tools (Fine Science Tools), a dental drill for craniotomy (Foredam), surgical sterilization equipment (Dent-Eq glass bead sterilizer), and a modified microliter syringe-pump (Hamilton 700 series) for injections are located onsite.

Microscopes: The Ross Laboratory has access to a Nikon A1R resonance confocal microscope and Neurolucida system for reconstructing cell morphology. Dr. Zachary Wills, a PI within the Department of Neurobiology, provides extensive training and assistance as well as maintenance for this equipment. We are very fortunate to have access to advice from Simon Watkins, who runs the Center for Biologic Imaging (CBI) at the University of Pittsburgh. The CBI currently employs four faculty members and 20 staff, including multiple post-doctoral fellows, students, and technicians. The CBI facility is connected to the same building as Dr. Ross's laboratory and has fully equipped microscopy suites, computer labs, as well as wet and dry bench space for light and electron microscopic preparations. There are over 19 confocal microscopes of different types, as well as multiple systems dedicated to super-resolution imaging. These are available to students after completion of training.

Behavior: For the acute and chronic pain assays, the Ross Laboratory has four Sony Handycam HDRXR260V high definition video cameras and a Panasonic BMP-BD85 Blu-ray player connected to a 27" monitor. These will be used to record and analyze pain behaviors. Behavioral experiments will be performed within the Rodent Behavioral Analysis Core (RBAC) within the university's DLAR vivarium. The RBAC gives access to open field boxes for recording spontaneous and capsaicin-induced pain behaviors, as well as a Hargreaves apparatus and Von Frey microfilaments.

Slice Electrophysiology and Optogenetic Stimulation: The Ross Lab owns two set-ups for *in vitro* brain slice experiments. Each setup includes an air table, upright microscopes (Olympus), a CED power1401-3 interface with associated Signal 5 software, an Axoclamp 2B microelectrode clamp, AC and DC amplifiers, Sutter and Scientifica micromanipulators, temperature control units, an XM10-IR Olympus CCD camera, a Lumencor SOLA LED light source, and a microelectrode puller (Sutter P2000). For the duration of the proposed award, I will have exclusive access to one of the electrophysiology setups. Additionally, the Ross Laboratory has facilities, equipment, and materials for preparing solutions, cutting spinal cord slices, and fabricating electrodes.

OMB Number: 4040-0001
Expiration Date: 10/31/2018

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE – Project Director/Principal Investigator					
Prefix:		First Name:	Ruby	Middle Name:	Andrea
Last Name:	Holland			Suffix:	
Position/Title:	MD/PhD Student	Department:	Neurobiology		
Organization Name:	University of Pittsburgh		Division:	Medicine	
Street 1:	M250 Scaife Hall				
Street 2:	3550 Terrace Street				
City:	Pittsburgh	County/Parish:	Allegheny		
State:	PA: Pennsylvania		Province:		
Country:	USA: UNITED STATES		Zip / Postal Code:	15261	
Phone Number:	412-648-2324	Fax Number:			
E-Mail:	rah143@pitt.edu				
Credential, e.g., agency login		HOLLANDR			
Project Role:	PD/PI	Other Project Role Category:			
Degree Type:	BA				
Degree Year:	2016				
Attach Biographical Sketch		Holland Biosketch			
Attach Current & Pending Support					

PROFILE - Senior/Key Person					
Prefix:		First Name:	H	Middle Name:	Richard
Last Name:	Koerber			Suffix:	
Position/Title:	Professor	Department:	Neurobiology		
Organization Name:	University of Pittsburgh		Division:	Medicine	
Street 1:	W1447 Biomedical Science Tower				
Street 2:	200 Lothrop Street				
City:	Pittsburgh	County/Parish:	Allegheny		
State:	PA: Pennsylvania		Province:		
Country:	USA: UNITED STATES		Zip / Postal Code:	15213	
Phone Number:	412-648-9518	Fax Number:			
E-Mail:	rkoerber@pitt.edu				
Credential, e.g., agency login		koerber			
Project Role:	Other (Specify)	Other Project Role Category:			Co-Sponsor
Degree Type:	PHD				
Degree Year:	1981				
Attach Biographical Sketch		Koerber Biosketch			
Attach Current & Pending Support					

OMB Number: 4040-0001
Expiration Date: 10/31/2018

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Senior/Key Person					
Prefix:		First Name:	Sarah	Middle Name:	Elizabeth
Last Name:	Ross			Suffix:	
Position/Title:	Associate Professor	Department:	Neurobiology		
Organization Name:	University of Pittsburgh		Division:	Medicine	
Street 1:	W1456 Biomedical Science Tower				
Street 2:	200 Lothrop Street				
City:	Pittsburgh	County/Parish:	Allegheny		
State:	PA: Pennsylvania		Province:		
Country:	USA: UNITED STATES		Zip / Postal Code:	15213	
Phone Number:	412-624-9178	Fax Number:			
E-Mail:	saross@pitt.edu				
Credential, e.g., agency login		ross_sa			
Project Role:	Other (Specify)	Other Project Role Category:		Sponsor	
Degree Type:	PHD				
Degree Year:	2001				
Attach Biographical Sketch		Ross Biosketch			
Attach Current & Pending Support					

ADDITIONAL SENIOR/KEY PERSON PROFILE(S)

Additional Biographical Sketch(es)

Additional Current and Pending Support(s)

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. DO NOT EXCEED FIVE PAGES.

NAME: HOLLAND, RUBY

eRA COMMONS USER NAME (credential, e.g., agency login): HOLLANDR

POSITION TITLE: MD/PhD Student

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE (if applicable)	END DATE MM/YYYY	FIELD OF STUDY
University of Pennsylvania, Philadelphia, PA	BA	05/2016	Chemistry
University of Pittsburgh School of Medicine -- Carnegie Mellon University, Pittsburgh, PA	PHD	05/2023	Neurobiology
University of Pittsburgh School of Medicine, Pittsburgh, PA	MD	05/2025	Medicine

A. Personal Statement

I am currently an MD/PhD candidate at the University of Pittsburgh School of Medicine and Carnegie Mellon University MSTP. My career goal is to become a physician-scientist in academic anesthesiology, applying my insights from long-term pain management in the clinic to my research interests in neurophysiological mechanisms of pain signaling. Throughout my training, I have been motivated by the impact of basic science research on patient quality of life. This began as early as my freshman year of college, when I began working as a research assistant in a nutritional neuroscience lab with Dr. Bart De Jonghe. Through my work studying the neural pathways implicated in chemotherapy-induced anorexia and malaise, I learned a variety of technical skills such as working with laboratory rats and mice, immunohistochemistry, PCR, and other tissue processing techniques. Dr. De Jonghe encouraged me to take on independent projects, contribute to manuscripts from early on in my training, and communicate my work with poster presentations. While this work was solely preclinical, the potential applications of this research on medicine became apparent to me while fundraising for the American Cancer Society and volunteering in a cancer unit at the Hospital of the University of Pennsylvania, I met patients whose severe nausea dominated their lives inside and outside of the hospital. After graduating from the University of Pennsylvania, I continued to work with Dr. De Jonghe, taking on a full-time technician position which entailed more responsibility and freedom to design experiments. These experiences solidified my passion for symptom management and quality of life, and how my research could benefit these endeavors through informing the development of targeted antiemetics.

I became interested in pain research during a collaboration with Drs. Amber Alhadeff and Nicholas Betley investigating the integration of competing hunger and pain signals in the lateral parabrachial nucleus. In this set of projects, I conducted pain experiments utilizing novel behavioral paradigms, chemogenetics, and tissue processing techniques. This research experience inspired me to apply to the University of Pittsburgh MSTP with the vision of conducting my graduate work at the Center for Pain Research. This core of faculty consists of many exceptional MSTP mentors who are experts in pain research, including Dr. Sarah Ross and Dr. Michael Gold, both of whom I have worked with on central and peripheral mechanisms of pain. During my first two years of medical school I took active steps to pursue my interest in anesthesiology by taking on a leadership role in the Anesthesiology Interest Group, enrolling in a peripheral nerve block elective course, and shadowing multiple anesthesiologists. In my OB/GYN clerkship course, I also actively sought out opportunities to care for patients with pelvic pain conditions. Through these experiences I have become acquainted with an expansive community of anesthesiologists and neuroscientists who share my passion for pain management and improving quality of life. I believe that pursuing an M.D./Ph.D at the University of Pittsburgh, alongside award-winning investigators and passionate clinicians will prepare me to take significant steps towards my goal of becoming an independent investigator in academic anesthesiology.

B. Positions and Honors

ACTIVITY/ OCCUPATION	START DATE	ENDING DATE	FIELD	INSTITUTION/ COMPANY	SUPERVISOR/ EMPLOYER
Undergraduate Research Assistant	09/2012	05/2016	Nutritional Neuroscience	University of Pennsylvania	Dr. Bart De Jonghe, PhD
Research Technician/Lab Manager	06/2016	05/2017	Nutritional Neuroscience	University of Pennsylvania	Dr. Bart De Jonghe, PhD
Teaching Assistant	07/2015	05/2016	Chemistry	University of Pennsylvania School of Nursing	Dr. Antonio Davila, PhD
PhD Student	07/2019	06/2023 (anticipated)	Neurobiology	University of Pittsburgh	Dr. Sarah Ross, PhD

Academic and Professional Honors

2012-2013 Dean's List, University of Pennsylvania
2015 Lt. Col. M. Richman Scholarship, University of Pennsylvania
2016 Graduated *cum laude*, University of Pennsylvania
2019 Best Workshop Award, University of Pittsburgh MSTP

Academic Activities

2014-2015 Pre-Medical Volunteer, Hospital of the University of Pennsylvania
2014-2015 Webmaster, Penn Colleges Against Cancer, American Cancer Society
2015-2016 Entertainment Committee Chair, Penn Colleges Against Cancer, American Cancer Society
2015-2016 President, Stitch for Kids, University of Pennsylvania
2018-2018 MSTP Second Look Committee, University of Pittsburgh
2018-2018 MSTP Welcoming Committee, University of Pittsburgh
2018-2018 MSTP Hosting Committee, University of Pittsburgh
2018-Present Coordinator, Anesthesiology Interest Group, University of Pittsburgh
2018-Present Coordinator, Knitt Med, University of Pittsburgh
2019-Present Wellness Committee Member, University of Pittsburgh

Research Support

2013 Penn Undergraduate Research Mentorship Grant, University of Pennsylvania
2013 Biology and Control of Nausea and Vomiting 2013 Conference Travel Award, Cyclic Vomiting Syndrome Association
2014 University Scholars Summer Stipend, University of Pennsylvania
2015 University Scholars Summer Stipend, University of Pennsylvania
2015 University Scholars Travel Grant, University of Pennsylvania
2017-2019 T32 Grant, Medical Scientist Training Program, University of Pittsburgh
2019-Present R01 Diversity Supplement, University of Pittsburgh

C. Contribution to Science

1. **Characterization of glutamate receptor signaling implicated in chemotherapy-induced anorexia and malaise**

Cisplatin chemotherapy is used commonly to treat a variety of cancers despite severe side effects such as nausea, vomiting, and anorexia that compromise quality of life and limit treatment adherence. The neural mechanisms mediating these side effects remain elusive. Because glutamate signaling in the hindbrain is implicated in inhibitory feeding pathways, the De Jonghe lab investigated glutamatergic circuits implicated in chemotherapy-induced malaise and anorexia in rodent models. I was an undergraduate student when I contributed to the discovery that cisplatin activates neurons within the nucleus tractus solitarius (NTS), lateral parabrachial nucleus (lPBN), central nucleus of the amygdala (CeA), and bed nucleus of the stria terminalis (BNST) in rats. Using PCR, we determined cisplatin induces increased AMPA and NMDA receptor subunit expression in all of these regions. We then demonstrated through local injections of glutamate receptor

antagonists and food intake measurements that CeA glutamate receptor signaling mediates cisplatin-induced anorexia and body weight loss. Together, these findings help to characterize the neural mechanisms mediating cisplatin-induced anorexia, advancing opportunities to develop better-tolerated chemotherapies and adjuvant therapies to prevent anorexia and concurrent nutritional deficiencies during cancer treatment. My contributions to this work included experimental design, behavioral experiments on rats and mice, stereotaxic surgeries, as well as tissue harvesting and processing. I was also extensively involved in data analysis and preparation of manuscripts.

a. **Holland RA**, Leonard JJ, Kensey NA, Hannikainen PA, De Jonghe BC (2014). Cisplatin induces neuronal activation and increases central AMPA and NMDA receptor subunit gene expression in mice. Physiol Behav. 2014 Sep;136:79-85. PubMed PMID: 24582677.

b. Alhadeff AL, **Holland RA**, Nelson A, Grill HJ, De Jonghe BC (2015). Glutamate Receptors in the Central Nucleus of the Amygdala Mediate Cisplatin-Induced Malaise and Energy Balance Dysregulation through Direct Hindbrain Projections. J Neurosci. 2015 Aug 5;35(31):11094-104. PubMed PMID: 26245970; PubMed Central PMCID: PMC4524978.

c. **Holland RA**, De Jonghe BC (2013). Cisplatin induces a dose-dependent increase in Fos expression in the mouse brain. Center for Undergraduate Research and Fellowships Open House, University of Pennsylvania. Poster Presentation. Philadelphia, PA.

d. **Holland RA**, Leonard JJ, Kensey K, Hannikainen, P, De Jonghe BC (2013). Anorectic doses of cisplatin induce neural activation and alterations in AMPA and NMDA receptor subunit gene expression in mice. Biology and Control of Nausea and Vomiting Conference. Poster Presentation. Pittsburgh, PA.

2. **Investigation of a hindbrain-forebrain circuit involved in cisplatin-induced anorexia and weight loss.**

Previous work from the De Jonghe lab identified three major brain regions activated by injection of cisplatin chemotherapy: the NTS, lPBN, CeA, and BNST. However, the connectivity between these brain regions and the role of these projections in anorexia and weight loss behaviors, however, was unknown. Therefore, we sought to determine the neuroanatomical connections between brain regions involved in chemotherapy-induced anorexia and weight loss and elucidate the role of these projections in rodent behaviors. Using the retrograde tracer Fluoro-Gold and the anterograde tracer Fluoro-Ruby, we found cisplatin-induced neuronal activation in projections from the NTS to the PBN, as well as projections from the PBN to the CeA. Utilizing chemogenetic approaches, we then discovered that chemogenetic inhibition of lPBN to CeA projections attenuated cisplatin-induced anorexia and body weight loss in rats. Together, these experiments build on previous work by the De Jonghe lab and identify a hindbrain to forebrain circuit which is required for cisplatin-induced anorexia and weight loss in rats. My specific contributions to this project include assisting with stereotaxic injections, conducting behavioral experiments, tissue harvesting, and immunohistochemistry.

a. Alhadeff AL, **Holland RA**, Zheng H, Rinaman L, Grill HJ, De Jonghe BC (2017). Excitatory Hindbrain-Forebrain Communication Is Required for Cisplatin-Induced Anorexia and Weight Loss. J Neurosci. 2017 Jan 11;37(2):362-370. PubMed PMID: 28077715; PubMed Central PMCID: PMC5242394.

b. De Jonghe BC, **Holland RA**, Olivos DR, Rupprecht LE, Kanoski SE, Hayes MR (2016). Hindbrain GLP-1 receptor mediation of cisplatin-induced anorexia and nausea. Physiol Behav. 2016 Jan 1;153:109-14. PubMed PMID: 26522737; PubMed Central PMCID: PMC4862654.

c. **Holland RA** (2015). Neural Circuits of Nausea: Amygdala glutamate signaling in chemotherapy-induced nausea. University Scholars Talk. Philadelphia, PA.

d. **Holland RA**, Zimmer DJ, Hayes MR, De Jonghe BC (2015). Hindbrain neuroinflammation mediates cisplatin-induced pica and anorexia in the rat. Biology and Control of Nausea and Vomiting Conference. Poster Presentation. Pittsburgh, PA.

3. **Discovery of a neural circuit for the suppression of pain during hunger.**

Hunger and pain are two competing signals that individuals must resolve to ensure survival. However, the neural processes that prioritize conflicting survival needs are poorly understood. While working as a research technician, I contributed to a collaborative project investigating the effect of hunger on pain responses in rodents. My primary contribution to this project involved conducting rodent experiments and injections, tissue collection and processing, and optimizing an immunohistochemistry protocol for co-staining AgRP and NPY in mouse brain sections. We discovered that hunger attenuates behavioral responses and affective properties of inflammatory

pain without altering acute nociceptive responses. This effect is centrally controlled, as activity in hunger-sensitive agouti-related protein (AgRP)-expressing neurons abrogates inflammatory pain. Systematic analysis of AgRP projection subpopulations revealed that the neural processing of hunger and inflammatory pain converge in the hindbrain parabrachial nucleus (PBN). Strikingly, activity in AgRP → PBN neurons blocked the behavioral response to inflammatory pain as effectively as hunger or analgesics. The anti-nociceptive effect of hunger is mediated by neuropeptide Y (NPY) signaling in the PBN. By investigating the intersection between hunger and pain, we have identified a neural circuit that mediates competing survival needs and uncovered NPY Y1 receptor signaling in the PBN as a target for pain suppression. My primary contribution to this project involved conducting rodent injections, tissue collection and processing, and optimizing an immunohistochemistry protocol for co-staining AgRP and NPY in mouse brain sections.

a. Alhadeff AL, Su Z, Hernandez E, Klima ML, Phillips SZ, **Holland RA**, Guo C, Hantman AW, De Jonghe BC, Betley JN. A Neural Circuit for the Suppression of Pain by a Competing Need State. Cell. 2018 Mar 22;173(1):140-152.e15. PubMed PMID: 29570993; PubMed Central PMCID: PMC5877408.

4. **Investigation of a rat model of chemotherapy-induced peripheral neuropathy and cognitive impairments**

Paclitaxel is a chemotherapy commonly paired with carboplatin in the treatment of a variety of solid tumor cancers. Under this regimen, patients report an array of debilitating peripheral neurological symptoms including chemotherapeutic-induced peripheral neuropathy (CIPN) often experienced as a numbness and tingling in the hands and feet which develops into debilitating pain. In my first lab rotation in the Pittsburgh Center for Pain Research, I worked in the Gold Lab to study the mechanisms underlying paclitaxel-induced peripheral neuropathy and cognitive impairments. We hypothesized that the cognitive impairments caused by chemotherapy were due, in part, to changes in the gut microbiome, based on prior studies linking microbiome dysregulation to cognitive function. I contributed to this project by administering a paclitaxel/carboplatin cocktail to male and female rats and testing thermal and mechanical pain responses periodically over two months. I then measured memory through open field tests and anxiety through open field tests. Rats were then euthanized, and fecal samples and peripheral nerves were collected. Sex was found to be an important biological variable in this study, as my experiments showed female mice experienced significant increases in mechanical and thermal hyperalgesia as well as anxiety while males did not. This finding was consistent with human data indicating women report more severe symptoms of paclitaxel chemotherapy, indicating that rats were a viable model for paclitaxel-induced cognitive impairments.

a. **Holland RA,** Farias JP, Loeza-Alcocer E, Hartung J, Gold MS (2017). The Gut Microbiome: A Therapeutic Target for Paclitaxel-Induced Peripheral Neuropathy and Cognitive Impairments? Medical Scientist Training Program Annual Retreat. Poster Presentation. University of Pittsburgh MSTP. Pittsburgh, PA.

5. **Characterization of primary afferent phenotypes implicated in sexual pleasure and pelvic pain signaling.**

In my second lab rotation in the Pittsburgh Center for Pain Research, I worked in the Ross Lab to investigate a new frontier in somatosensory signaling: sexual pleasure. I used immunohistochemistry, neuroanatomical tracing, and a viral/genetic approach to visualize and characterize genital corpuscles, a specialized form of afferent which had not yet been identified in the mouse. I visualized two broad categories of primary afferents: a significant population of unique, clustered terminals within the glans of the mouse penis and clitoris, resembling genital corpuscular receptors described in prior literature, as well as free nerve endings extending to the epidermis. The genital corpuscular receptors are VGlut2 positive, identifying a genetic target by which these cells can be optogenetically or chemogenetically targeted to elucidate their role in sexual reward pathways.

a. **Holland RA**, Ross SE (2018). Neuroanatomical Tracing and Immunohistochemical Phenotype of the Genital Corpuscular Receptors of the Mouse Penis. Medical Scientist Training Program Annual Retreat. Poster Presentation. University of Pittsburgh MSTP. Pittsburgh, PA.

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.

Follow this format for each person. DO NOT EXCEED FIVE PAGES.

NAME: Koerber, H Richard

eRA COMMONS USER NAME (agency login): koerber

POSITION TITLE: Professor

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)*

INSTITUTION AND LOCATION	DEGREE (if applicable)	Completion Date MM/YYYY	FIELD OF STUDY
Marietta College, Marietta, Ohio	BS	05/1975	Biology
West Virginia University, Morgantown, WV	MS	09/1977	Physiology and Biophysics
West Virginia University, Morgantown, WV	PHD	10/1981	Physiology and Biophysics

A. Personal Statement

I have over 40 years of experience in recording from spinal and sensory neurons. During the early years of my career I was involved with developing novel physiological approaches for the recording and stimulation of specific primary sensory neurons in cats. In addition, these techniques also allowed for the simultaneous recording of neuronal response to individual afferent stimulation inputs in the spinal cord. Subsequently, I developed a novel *ex vivo* spinal cord/DRG/skin preparation to allow similar studies to be carried out in mice. We are now using this preparation to investigate the function of specific spinal neural networks while employing optogenetics to stimulate specific populations of sensory neurons. Thus, I am highly qualified to provide expert assistance in the experiments in this proposal.

1. Brown AG, **Koerber HR**, Noble R. An intracellular study of spinocervical tract cell responses to natural stimuli and single hair afferent fibres in cats. **J Physiol**. 1987 Jan; 382:331-54. PMID: 3625552; PMCID: PMC1183027.
2. **Koerber HR**, Seymour AW, Mendell LM. Tuning of spinal networks to frequency components of spike trains in individual afferents. **J Neurosci**. 1991 Oct;11(10):3178-87. PMID: 1941079.
3. Kardon AP, Polgár E, Hachisuka J, Snyder LM, Cameron D, Savage S, Cai X, Karnup S, Fan CR, Hemenway GM, Bernard CS, Schwartz ES, Nagase H, Schwarzer C, Watanabe M, Furuta T, Kaneko T, **Koerber HR**, Todd AJ, Ross SE. Dynorphin acts as a neuromodulator to inhibit itch in the dorsal horn of the spinal cord. **Neuron**. 2014 May 7;82(3):573-86. PMID: 24726382; PMCID: PMC4022838.
4. Hachisuka J, Omori Y, Chaing MC, Gold MS, **Koerber HR**, Ross SE. Wind-up in lamina I spinoparabrachial neurons: a role for reverberatory circuits. **Pain**. 2018 Aug 159(8):1484-1493. PMID: 29578943 PMCID: PMC6053328.

B. Positions and Honors

Positions and Employment

1981 - 1982	Postdoctoral Fellow, University of Utah, School of Medicine, Salt Lake City, UT
1982 - 1984	Postdoctoral Fellow, University of Edinburgh, Edinburgh
1984 - 1986	Research Associate, SUNY at Stony Brook, Stony Brook, NY
1986 - 1989	Research Assistant Professor, SUNY at Stony Brook, Stony Brook, NY
1989 - 1993	Assistant Professor, Department of Neurobiology, Anatomy and Cell Science, University of Pittsburgh School of Medicine, Pittsburgh, PA
1993 - 1996	Assistant Professor, Department of Neurobiology, University of Pittsburgh School of Medicine, Pittsburgh, PA

1996 - 2007	Associate Professor, Department of Neurobiology, University of Pittsburgh School of Medicine, Pittsburgh, PA
2007 -	Professor, Department of Neurobiology, University of Pittsburgh, School of Medicine, Pittsburgh, PA

Other Experience and Professional Memberships

1977 -	Member, Society for Neuroscience
2005 -	Member, IASP
2007 -	Member, APS

C. Contributions to Science

1. Over the past 40+ years I have made several significant contributions to our understanding of the organization, function and plasticity in the somatosensory system. The first seminal contributions come from my work as an NIH (NRSA) funded postdoctoral fellow at the University of Edinburgh in the lab of Dr. Alan G. Brown. One of the historic difficulties in understanding the integration of sensory information in the spinal cord is the fact that cutaneous stimulation activates a wide range of sensory fibers. During the two years of this fellowship I developed a method for establishing dual recordings from in vivo cat preparations. First, I established an intracellular recording from an identified cutaneous sensory neuron located in the dorsal root ganglia. While maintaining this recording, a second was established from an identified spinocervical tract cell in the spinal dorsal horn. Using this approach, we were able to stimulate individual primary afferent fibers while recording the evoked responses in the identified spinal projection neuron. These studies resulted in a series of three manuscripts describing the functional properties of these specific spinal networks. I extended these findings as a Research Associate and later as an Assistant Professor at SUNY Stony Brook working with Dr. Lorne Mendell describing how spinal networks receiving input from different fiber types were tuned to respond to differently to frequency modulated inputs.

    a. Brown AG, Koerber HR, Noble R. An intracellular study of spinocervical tract cell responses to natural stimuli and single hair afferent fibres in cats. J Physiol. 1987 Jan;382:331-54. PubMed PMID: 3625552; PubMed Central PMCID: PMC1183027.
    b. Brown AG, Koerber HR, Noble R. Actions of trains and pairs of impulses from single primary afferent fibres on single spinocervical tract cells in cat. J Physiol. 1987 Jan;382:313-29. PubMed PMID: 3625551; PubMed Central PMCID: PMC1183026.
    c. Koerber HR, Mendell LM. Functional specialization of central projections from identified primary afferent fibers. J Neurophysiol. 1988 Nov;60(5):1597-614. PubMed PMID: 3199174.
    d. Koerber HR, Seymour AW, Mendell LM. Tuning of spinal networks to frequency components of spike trains in individual afferents. J Neurosci. 1991 Oct;11(10):3178-87. PubMed PMID: 1941079.

2. While at Stony Brook I began my first independently funded NIH research project examining the anatomical and functional plasticity in the spinal cord following peripheral nerve injury and regeneration. I continued this project in my next position as an Assistant Professor at the University of Pittsburgh. The rationale for these studies stemmed from the clinical observation that following nerve injury and regeneration, patients initially loose the ability to localize stimuli applied to the reinnervated skin. However, with time the ability to localize the stimulus is frequently restored. I hypothesized that the recovery in function was due to spinal plasticity. Over the course of these studies we made two major findings. First, we found that following regeneration some primary afferent fibers appeared to sprout into the superficial laminae of the dorsal horn apparently confirming the results of earlier studies by Clifford Woolf and colleagues. Second, we found that immediately following regeneration, dorsal horn cells had very large cutaneous receptive fields and with time these receptive fields became smaller and more concise. Finally, we determined that this process involved the establishment of new functional synapses in the spinal dorsal horn.

    a. Koerber HR, Mirnics K, Brown PB, Mendell LM. Central sprouting and functional plasticity of regenerated primary afferents. J Neurosci. 1994 Jun;14(6):3655-71. PubMed PMID: 8207480.

b. Koerber HR, Mirnics K, Mendell LM. Properties of regenerated primary afferents and their functional connections. J Neurophysiol. 1995 Feb;73(2):693-702. PubMed PMID: 7760128.

c. Koerber HR, Mirnics K. Plasticity of dorsal horn cell receptive fields after peripheral nerve regeneration. J Neurophysiol. 1996 Jun;75(6):2255-67. PubMed PMID: 8793739.

d. Koerber HR, Mirnics K, Lawson JJ. Synaptic plasticity in the adult spinal dorsal horn: the appearance of new functional connections following peripheral nerve regeneration. Exp Neurol. 2006 Aug;200(2):468-79. PubMed PMID: 16696973.

3. Subsequently, I was intrigued by two widely accepted findings. First was the suggestion that during development large diameter low threshold mechanoreceptors initially project into the superficial laminae of the dorsal horn and with time retract into their adult locations in deeper laminae. The second was the suggestion that following injury these large diameter fibers could sprout and reenter the superficial laminae. This sprouting was considered to be the possible anatomical substrate for the appearance of mechanical allodynia in chronic pain patients. In order to study the possible mechanisms involved in this extensive plasticity of the afferent fiber projections, we developed an ex vivo mouse somatosensory preparation consisting of spinal cord, DRGs, nerves and innervated skin. Using this preparation we were able to determine that large low threshold mechanoreceptive afferent fibers did not initially project into the superficial laminae of the dorsal horn nor did they sprout into these laminae following injury. In addition, we identified a group of fast conducting high threshold mechanoreceptive fibers that projected into these and deeper laminae that were probably the source of the prior findings suggesting sprouting. These studies demonstrated that the spinal plasticity resulting in mechanical allodynia did not require anatomical reorganization, but rather plasticity in existing spinal circuits.

   a. Woodbury CJ, Ritter AM, Koerber HR. Central anatomy of individual rapidly adapting low-threshold mechanoreceptors innervating the "hairy" skin of newborn mice: early maturation of hair follicle afferents. J Comp Neurol. 2001 Jul 30;436(3):304-23. PubMed PMID: 11438932.

   b. Koerber HR, Woodbury CJ. Comprehensive phenotyping of sensory neurons using an ex vivo somatosensory system. Physiol Behav. 2002 Dec;77(4-5):589-94. PubMed PMID: 12527004.

   c. Woodbury CJ, Koerber HR. Widespread projections from myelinated nociceptors throughout the substantia gelatinosa provide novel insights into neonatal hypersensitivity. J Neurosci. 2003 Jan 15;23(2):601-10. PubMed PMID: 12533620.

   d. Woodbury CJ, Kullmann FA, McIlwrath SL, Koerber HR. Identity of myelinated cutaneous sensory neurons projecting to nocireceptive laminae following nerve injury in adult mice. J Comp Neurol. 2008 May 20;508(3):500-9. PubMed PMID: 18335545; PubMed Central PMCID: PMC2664515.

4. The advent of novel mouse genetic techniques and our ex vivo mouse preparation has also allowed us to make a number of other important contributions to the field. While other recording methods allow for recording from primary afferents, our preparation allows for comprehensive phenotyping of sensory neurons. This approach, in collaboration with different groups producing novel transgenic and knock-out mouse lines, has allowed us to address many fundamental questions concerning the roles of specific receptor and channels in the development and function of cutaneous sensory neurons. The first of these studies addressed the roles of neurotrophic factors during development. Drs. Kathryn Albers and Brian Davis had developed transgenic mice that overexpressed different neurotrophic factors in the skin. We found that in addition to being required for survival of particular afferent types, these neurotrophic factors play profound roles in the functional properties of different subsets of afferent fibers including both nociceptive fibers as well as low threshold tactile afferents. These collaborations have lead to additional studies examining the roles of specific receptors and channels in primary afferent function. Examples of these studies are referenced below.

   a. Albers KM, Woodbury CJ, Ritter AM, Davis BM, Koerber HR. Glial cell-line-derived neurotrophic factor expression in skin alters the mechanical sensitivity of cutaneous nociceptors. J Neurosci. 2006 Mar 15;26(11):2981-90. PubMed PMID: 16540576.

   b. McIlwrath SL, Lawson JJ, Anderson CE, Albers KM, Koerber HR. Overexpression of neurotrophin-3 enhances the mechanical response properties of slowly adapting type 1 afferents and myelinated nociceptors. Eur J Neurosci. 2007 Oct;26(7):1801-12. PubMed PMID: 17897394.

c. Rau KK, McIlwrath SL, Wang H, Lawson JJ, Jankowski MP, Zylka MJ, Anderson DJ, Koerber HR. Mrgprd enhances excitability in specific populations of cutaneous murine polymodal nociceptors. J Neurosci. 2009 Jul 1;29(26):8612-9. PubMed PMID: 19571152; PubMed Central PMCID: PMC2756673.

d. Molliver DC, Rau KK, McIlwrath SL, Jankowski MP, Koerber HR. The ADP receptor P2Y1 is necessary for normal thermal sensitivity in cutaneous polymodal nociceptors. Mol Pain. 2011 Feb 10;7:13. PubMed PMID: 21310055; PubMed Central PMCID: PMC3049184.

5. Our studies of the effects peripheral nerve injury and regeneration demonstrated that many different types of nociceptive fibers reinnervating the skin were either sensitized or had undergone phenotypic change. In order to investigate potential mechanism for this injury induced plasticity we examined expression changes in the DRG as well as in the skin following injury and during regeneration. We found a number of changes in expression in both skin and DRG that were correlated with the changes in nociceptor function. In order to determine any causal relationships between these expression and functional changes we developed a novel method for in vivo siRNA knockdown of expression of specific gene products. The results from these studies have identified specific molecular mechanisms underlying the functional and phenotypic changes observed following peripheral injury.

   a. Jankowski MP, Lawson JJ, McIlwrath SL, Rau KK, Anderson CE, Albers KM, Koerber HR. Sensitization of cutaneous nociceptors after nerve transection and regeneration: possible role of target-derived neurotrophic factor signaling. J Neurosci. 2009 Feb 11;29(6):1636-47. PubMed PMID: 19211871; PubMed Central PMCID: PMC2768416.

   b. Jankowski MP, Rau KK, Soneji DJ, Anderson CE, Koerber HR. Enhanced artemin/GFRα3 levels regulate mechanically insensitive, heat-sensitive C-fiber recruitment after axotomy and regeneration. J Neurosci. 2010 Dec 1;30(48):16272-83. PubMed PMID: 21123573; PubMed Central PMCID: PMC3018779.

   c. Jankowski MP, Rau KK, Soneji DJ, Ekmann KM, Anderson CE, Molliver DC, Koerber HR. Purinergic receptor P2Y1 regulates polymodal C-fiber thermal thresholds and sensory neuron phenotypic switching during peripheral inflammation. Pain. 2012 Feb;153(2):410-9. PubMed PMID: 22137295; PubMed Central PMCID: PMC3264839.

   d. Jankowski MP, Soneji DJ, Ekmann KM, Anderson CE, Koerber HR. Dynamic changes in heat transducing channel TRPV1 expression regulate mechanically insensitive, heat sensitive C-fiber recruitment after axotomy and regeneration. J Neurosci. 2012 Dec 5;32(49):17869-73. PubMed PMID: 23223305; PubMed Central PMCID: PMC3533441.

Complete List of Published Work available at: http://www.ncbi.nlm.nih.gov/pubmed/?term=koerber+hr

D. Research Support

Ongoing Research Support

R01 AR069951-01 Koerber, H Richard (MPI) 04/01/16-03/31/21
Characterization of Epithelial-Neural Communication
This study will determine how ChR2-mediated activation of skin keratinocytes impacts activation of different functional classes of cutaneous sensory afferents under normal and inflamed conditions.
Role: **MPI**

R01 NS096705 Koerber, H Richard (PD/PI) 09/01/16-08/31/21
Molecular Genetic Dissection of the Spinal Microcircuits of Wind-up
Chronic pain is a serious health concern effecting millions of American annually. In this project, we are using use innovative genetic tools together with a novel somatosensory preparation to dissect the spinal circuitry involved in processing nociceptive information. These studies will provide new knowledge about how specific spinal networks process peripheral sensory information under normal and inflamed conditions. This knowledge should provide targets for new pharmaceutical therapies for the treatment of chronic pain.
Role: **PD/PI**

Completed Research Support (past 7 years)

R01 NS023725-28 Koerber, H Richard (PI) 09/01/89-06/30/19

Peripheral Nerve Regeneration and Sensory Neuron Plasticity

The goal of this project is to understand the molecular mechanisms underlying nociceptor sensitization following peripheral nerve injury and regeneration.

Role: **PI**

R01NS050758 Davis, Brian M (PI) 01/15/05-02/28/13

Characterization and Plasticity of Visceral Nociceptors

The goal of this project was to determine the distribution and comprehensive phenotype of visceral nociceptors and to determine the effects of inflammation of these properties.

Role: **Co-Investigator**

R01NS059003 Albers, Kathy M (PI) 12/01/08-11/30/12

Sox11 and Functional Recovery of Sensory Neurons

The goal of these experiments was to determine the role of the transcription factor Sox11 in sensory neuron survival and response to injury. In addition, we also identified factors downstream of Sox11 and their potential roles in survival and regeneration.

Role: **Co-Investigator**

R01NS031826 Davis, Brian M (PI) 02/01/07-01/31/12

Role of Growth Factors in Persistent Pain

The goal of this project was to further determine the cellular mechanisms that mediate the persistent alterations in pain observed following injury and inflammation, by studying both the peripheral nerve and the spinal cord mechanisms initiated by persistent nociception.

Role: **Co-Investigator**

R01 NS052848 Koerber, H Richard (PI) 04/17/06-03/31/11

Primary and Secondary Nociceptors in Persistent Pain

In this project we used our ex vivo spinal cord/skin/nerve preparation and a combination of behavioral, anatomical (both LM and EM) and electrophysiological techniques to determine the time course of post-injury changes in cutaneous sensory neurons and superficial dorsal horn cells in both wild type and transgenic mice following the induction of persistent pain states.

Role: **PI**

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.

Follow this format for each person. DO NOT EXCEED FIVE PAGES.

NAME: Ross, Sarah

eRA COMMONS USER NAME: ross_sa

POSITION TITLE: Associate Professor, Department of Neurobiology

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE (if applicable)	Completion Date MM/YYYY	FIELD OF STUDY
University of Western Ontario, London, Canada	BS (Hons)	1991 - 1995	Physiology
University of Michigan, Ann Arbor, MI	PhD	1995 - 2001	Physiology (Ormond MacDougald)
Harvard Medical School, Boston, MA	Post-doc	2002 - 2011	Neurobiology (Michael Greenberg)

A. Personal Statement

I am an Associate Professor at the University of Pittsburgh Medical School in the Department of Neurobiology and the Pittsburgh Center for Pain Research. My lab is focused on dissecting the neural circuits of pain and itch using a combination of molecular approaches, electrophysiology and behavior. I am very committed to the mentorship of Ruby Holland, and I believe that my lab will be an outstanding environment for her PhD training as an MSTP student, thereby preparing her for a successful career as a physician scientist in the field of pain. In support of this idea, I have a growing track record of successful trainees, funded grants, and strong publications.

A new direction for my lab is the dissection of descending circuits from the RVM. For the last year, we have been successfully targeting this region of the brain with viruses to manipulate neurons therein, and thus I have no concerns about the feasability of Ruby's project. Moreover, we have very recently (in the last few months) acquired and validated the MOR-cre allele (from Richard Palmiter), which will allow us to dissect the role of MOR-expressing RVM neurons in a new way. I am very excited about Ruby's proposal — to focus on a specific type of MOR-expressing RVM neurons that project to the spinal cord. These are putative ON-cells which are proposed to facilitate pain. Now, Ruby will be able to visualize these cells and manipulate their activity. Since no one has ever selectively activated ON-cells in the context of pain behaviors, Ruby's experiments have the potential to provide important new insight into the function and circuitry of descending modulation.

B. Positions and Honors

Positions and Employment

2011 – 2017 Assistant Professor, Department of Neurobiology and Pittsburgh Center for Pain Research, University of Pittsburgh, Pittsburgh, PA
2012 – Secondary Appointment, Department of Anesthesiology
2016 – Secondary Appointment, Department of Clinical and Translational Science
2018 – Associate Professor, Department of Neurobiology and Pittsburgh Center for Pain Research

Other Experience and Professional Memberships

2011 – Member, American Pain Society
2011 – Member, International Federation for the Study of Itch
2013 Co-organizer, 7th World Congress on Itch
2014 Ad hoc reviewer, Wellcome Trust
2015 – Member, International Association for the Study of Pain
2015 – Member, Society for Neuroscience
2015 Ad hoc reviewer, ETS Zurich Research Grants
2015 – Member, Rita Allen Review Committee

2016	Add hoc reviewer, NIH (NIDCR)
2016 – 2017	Member, Interagency Pain Research Coordinating Committee (IPRCC)
2016 –	Editorial board member, *Pain*
2016 –	Editorial board member, *Itch*
2018 –	Co-chair, basic science SIG (American Pain Society)
2019 –	Co-chair, itch SIG (International Association for the Study of Pain)
2019 –	Co-organizer, Keystone Conference (Somatosensation: From Detection to Perception)

Honors

1996	Henry Caulkins Scholarship, University of Michigan
1997	Henry Caulkins Scholarship, University of Michigan
1997	Susan Lipschutz Award for Women Graduate Students, University of Michigan
1997	NSERC Predoc Award, The Natural Sciences and Engineering Research Council of Canada
1997	Outstanding Graduate Teaching Award, University of Michigan
1998	Henry Caulkins Scholarship, University of Michigan
2000	Henry Caulkins Scholarship, University of Michigan
2000	Rackham Predoctoral Training Award, University of Michigan
2002	Distinguished Dissertation Award, University of Michigan
2002	CIHR post-doctoral award (declined), Canadian Institute of Health Sciences
2002	Jane Coffin Childs Fellowship, Jane Coffin Childs Foundation
2007	Career Development Fellowship, Harvard Medical School
2007	William Randolph Hearst Award, Harvard Medical School
2012	Rita Allen Foundation Pain Scholar, Rita Allen Foundation
2012	Whitehall Award, Whitehall Foundation
2017	Mallinckrodt Scholar Finalist (1 of 4)

C. Contribution to Science

Complete List of Published Work in Google Scholar (42 publications; h-index 24; >8000 citations): https://scholar.google.com/citations?user=ILpDSFYAAAAJ&hl=en

1. **Functional circuitry of the dorsal horn** My research program is aimed at understanding the neural basis of somatosensory integration. In particular, we use molecular genetic, electrophysiological and behavioral experiments to analyze neural circuits in the spinal dorsal horn. Recently, we provided key evidence showing that B5-I neurons are a population of spinal inhibitory interneurons that function to inhibit itch. We found that B5-I neurons release the kappa opioid dynorphin, and our experiments revealed that kappa opioids modulate itch tone bidirectionally at the level of the spinal cord (*Neuron 82: 573*). We also investigated which afferents respond to dynorphin by characterizing the sensory neurons that express the kappa opioid receptor *(Neuron, 99: 1274).*

   Since mice lacking B5-I neurons mice develop neuropathic itch, these mice provided us with an opportunity to test novel therapeutic treatments for this condition. Working in collaboration with Allan Basbaum, we found that neuropathic itch can be treated by restoring inhibition via spinal cord transplantation of inhibitory interneurons. These findings illustrate the utility of a cell-based therapy to ameliorate severe neuropathic itch (*JCI 124: 3612*). We have also collaborated with Qiufu Ma and Martyn Goulding to study the role of dynorphin-expressing in mechanical sensation. These studies revealed that dynorphin-expressing inhibitory interneurons are involved in the gating of mechanical pain (*Cell 159: 1417*).

   a. Kardon AP, Polgár E, Hachisuka J, Snyder LM, Cameron D, Savage S, Cai X, Karnup S, Fan CR, Hemenway GM, Bernard CS, Schwartz ES, Nagase H, Schwarzer C, Watanabe M, Furuta T, Kaneko T, Koerber HR, Todd AJ*, **Ross SE***. (2014) Dynorphin acts as a neuromodulator to inhibit itch in the dorsal horn of the spinal cord. *Neuron*. 82(3):573-86. PMC4022838

   b. Snyder, L. M., M. C. Chiang, E. Loeza-Alcocer, Y. Omori, J. Hachisuka, T. D. Sheahan, J. R. Gale, P. C. Adelman, E. I. Sypek, S. A. Fulton, R. L. Friedman, M. C. Wright, M. G. Duque, Y. S. Lee, Z. Hu, H. Huang, X. Cai, K. A. Meerschaert, V. Nagarajan, T. Hirai, G. Scherrer, D. H. Kaplan, F. Porreca, B. M. Davis, M. S. Gold, H. R. Koerber and S. E. Ross (2018). "Kappa Opioid Receptor Distribution and Function in Primary Afferents." *Neuron* **99**(6): 1274-1288 PMID30236284

c. Braz JM, Juarez-Salinas D, **Ross SE**, Basbaum AI. (2014) Transplant restoration of spinal cord inhibitory controls ameliorates neuropathic itch. *J Clin Invest*. 124(8):3612-6. PMC4109547

d. Duan B, Cheng L, Bourane S, Britz O, Padilla C, Garcia-Campmany L, Krashes M, Knowlton W, Velasquez T, Ren X, **Ross SE**, Lowell BB, Wang Y, Goulding M, Ma Q. (2014) Identification of spinal circuits transmitting and gating mechanical pain. *Cell*.159(6):1417-32 PMC4258511

2. **Developed novel approaches and created novel genetic tools to study spinal circuitry** We spent several years developing a novel preparation that enables us to ask questions about spinal circuitry that were previously elusive. In our new preparation, the lumbar spinal cord, saphenous nerve, and hindpaw skin are dissected in continuum, allowing us to record from spinal projection neurons while we provide natural stimulation to the skin and optogenetic manipulation of spinal interneurons (*eLIFE*, e22866).

   In parallel to these efforts, we have generated a series of novel genetic tools that allow us to dissect spinal circuits by giving us access to specific cell types. In particular, we generated three new knockin alleles: a *Bhlhb5-flpo* knockin mouse, so that we could use intersectional genetic strategies to target B5-I neurons (*Dev. Biol. 414:149*), a *KOR-cre* knockin mouse, in order to visualize and manipulate the cells that express KOR (*Genesis, 54:29*), and a *NK1R-creER* mouse, to give us a genetic handle on spinal projection neurons (*Genesis 54:593*). These alleles have now been described and made freely available to others.

   a. Hachisuka J, Baumbauer, KM, Omori Y, Snyder LM, Koerber HR*, **Ross SE*** (2016) Semi-intact ex vivo approach to investigate spinal somatosensory circuits. *eLife,* 5: e22866. PMC5214752

   b. Cai X, Kardon AP, Snyder LM, Kuzirian MS, Minestro S, de Souza L, Rubio ME, Maricich SM, **Ross SE***. (2016) Bhlhb5::flpo allele uncovers a requirement for Bhlhb5 for the development of the dorsal cochlear nucleus. *Dev Biol.* 414(2):149-60. PMC4930277

   c. Cai X, Huang H, Kuzirian MS, Snyder LM, Matsushita M, Lee MC, Ferguson C, Homanics GE, Barth AL, **Ross SE***. (2016) Generation of a KOR-Cre knockin mouse strain to study cells involved in kappa opioid signaling. *Genesis.* 54(1):29-37. PMC4747253

   d. Huang H, Kuzirian MS, Cai X, Snyder LM, Cohen J, Kaplan DH and **Ross SE*** (2016) Generation of a NK1R-CreER Knockin Mouse Strain to Study Cells Involved in Neurokinin 1 Receptor Signaling. *Genesis.* 54(11):593-611. PMC5241089

3. **Somatosensory Integration: current and future directions** A main focus of my lab is to understand how neural circuits integrate somatosensory information, particularly pain and itch. Recently, we took an optogenetic approach to study the neural circuit basis for wind-up, which is one mechanism through which sensory input is amplified in the spinal cord. Our studies reveal that a specific population of excitatory neurons (defined genetically using the *Nts-cre* allele) are necessary and sufficient for wind-up (*Pain*, in press). These findings suggest that wind-up is mediated through a polysynaptic circuit of spinal interneurons. A second (and often under-appreciated are where somatosensory integration occurs is the skin, which communicates to sensory neurons. Our work revealed that optogenetic activation of keratinocytes is sufficient to excite primary afferents whereas optogenetic inhibition of keratinocytes reduces primary afferent excitability. These findings suggest that the skin is in constant communication with sensory neurons, where it plays a key role in the modulation of sensory input (*eLIFE* 4: e09674). We also continue to test the latest tools and technologies, providing insight into both their utility and limitations (*J. Neurosci 36:107690*). Finally, we are working to develop new and better ways to analyze mouse behavior. For instance, we recently devised a new method that takes advantage of the fact that scratching is a stereotyped behavior that is audible. Thus, we developed an approach that uses supervised learning to extract scratching behavior from acoustic recordings (*PLoS One **12**(7)*). This approach will provide an inexpensive and scalable means to quantify itch in a mouse's home cage over extended periods of time.

   a. Hachisuka J, Omori Y, Chiang MC, Gold MS, Koerber HR* and **Ross SE*** (2018) Wind-up in lamina I spinoparabrachial neurons: a role for reverberatory circuits. *Pain* 159(8): 1484-1493 PMC6053328

   b. Baumbauer KM, DeBerry JJ, Adelman PC, Miller RH, Hachisuka J, Lee KH, **Ross SE**, Koerber HR, Davis BM, Albers KM. (2015) Keratinocytes can modulate and directly initiate nociceptive responses *eLIFE* 4:e09674. PMC4576133

   c. Saloman JL, Scheff NN, Snyder LM, **Ross SE**, Davis BM and Gold MS. (2016) Gi-DREADD expression in peripheral nerves produces ligand-dependent analgesia, as well as ligand-independent functional changes in sensory neurons. *J Neurosci.* 36(42):10769-81. PMC5083007

d. Elliot P, G'Sell M, Snyder LM, **Ross SE**, Ventura V. (2017). Automated acoustic detection of mouse scratching *PLoS One* ***12****(7): e0179662.* PMC5497976

4. **Neural Development: Bhlhb5 and Prdm8 form an obligate repressor complex.** As a postdoctoral fellow in Dr. Mike Greenberg's laboratory, I studied the function of transcription factors in neural development and function, with a particular focus on members of the Atonal superfamily *(Neuron 39: 13; Science 303: 2011).* Towards this end, I generated a number of knockout and knock-in mice for the transcription factors *Bhlhb4* and *Bhlhb5*.

   This work led to the discovery Bhlhb5 forms a repressor complex with the PR/SET domain protein, Prdm8 *(Neuron 73: 292*). I found that Bhlhb5 binds to sequence-specific DNA elements and then recruits Prdm8, which mediates the repression of target genes. This interaction is critical for repressor function since mice lacking either Bhlhb5 or Prdm8 have strikingly similar cellular and behavioral phenotypes, including axonal mis-targeting by neurons of the dorsal telencephalon and abnormal itch behavior. I also discovered that Cadherin-11 functions as target of the Prdm8/Bhlhb5 repressor complex that must be repressed for proper neural circuit formation to occur. These findings suggest that Prdm8 is an obligate partner of Bhlhb5, forming a repressor complex that directs neural circuit assembly in part through the precise regulation of Cadherin-11.

   One of the key functions of the Bhlhb5/Prdm8 repressor complex is for the survival of B5-I neurons, a subtype of spinal inhibitory interneuron that is required normal itch sensation (*Neuron* 65: 886). This discovery became the foundation for the initial studies as I began my own research program at the University of Pittsburgh.

   a. **Ross SE**, Greenberg ME, Stiles CD. Basic helix-loop-helix factors in cortical development. *Neuron*. 2003 39(1):13-25. PMID: 12848929

   b. Brunet A, Sweeney LB, Sturgill JF, Chua KF, Greer PL, Lin Y, Tran H, **Ross SE**, Mostoslavsky R, Cohen HY, Hu LS, Cheng HL, Jedrychowski MP, Gygi SP, Sinclair DA, Alt FW, Greenberg ME. (2004) Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. *Science*. 303(5666):2011-5. PMID: 14976264

   c. **Ross SE**, McCord AE, Jung C, Atan D, Mok SI, Hemberg M, Kim TK, Salogiannis J, Hu L, Cohen S, Lin Y, Harrar D, McInnes RR, Greenberg ME. (2012) Bhlhb5 and Prdm8 form a repressor complex involved in neuronal circuit assembly. *Neuron*. 73(2):292-303. PMC3269007

   d. **Ross SE**, Mardinly AR, McCord AE, Zurawski J, Cohen S, Jung C, Hu L, Mok SI, Shah A, Savner EM, Tolias C, Corfas R, Chen S, Inquimbert P, Xu Y, McInnes RR, Rice FL, Corfas G, Ma Q, Woolf CJ, Greenberg ME. (2010) Loss of inhibitory interneurons in the dorsal spinal cord and elevated itch in Bhlhb5 mutant mice. *Neuron*. 65(6):886-98. PMC2856621

5. **Cell Fate Decisions: Wnt signaling blocks adipogenesis** As a graduate student in Dr. Ormond MacDougald's laboratory, I explored how individual cells respond to their environment by investigating molecular mechanisms through which extracellular factors regulate lineage specification of multipotent progenitors. My work led to the discovery that Wnt signaling acts as a determination switch, regulating the cell fate decision between fat cells and muscle cells *(Science, 289: 950)*, and I identified some of the transcriptional targets that mediate this developmental switch *(Mol. Cell. Biol. 22: 5989)*. In a separate line of investigation, I discovered that the transcription factor C/EBPα (which is mutated in acute myeloid leukemia) is regulated through phosphorylation in response to extracellular growth factors *(Mol. Cell. Biol. 19: 8433*). In addition, I showed that ERK-mediated phosphorylation inhibits C/EBPα function and thereby blocks the differentiation of blood cell progenitors into granulocytes *(Mol. Cell. Biol. 24: 675)*. Thus, my graduate studies uncovered two important molecular mechanisms through which extracellular signals regulate cell fate.

   a. **Ross SE,** Erickson RL, Hemati N, MacDougald OA. (1999) Glycogen synthase kinase 3 is an insulin-regulated C/EBPalpha kinase. *Mol Cell Biol*. (12):8433-41. PMC84944

   b. **Ross SE**, Hemati N, Longo KA, Bennett CN, Lucas PC, Erickson RL, MacDougald OA. (2000) Inhibition of adipogenesis by Wnt signaling. *Science*. 289(5481):950-3. PMID: 10937998

   c. **Ross SE**, Erickson RL, Gerin I, DeRose PM, Bajnok L, Longo KA, Misek DE, Kuick R, Hanash SM, Atkins KB, Andresen SM, Nebb HI, Madsen L, Kristiansen K, MacDougald OA. (2002) Microarray analyses during adipogenesis: understanding the effects of Wnt signaling on adipogenesis and the roles of liver X receptor alpha in adipocyte metabolism. *Mol Cell Biol*. (16):5989-99. PMC133961

d. **Ross SE**, Radomska HS, Wu B, Zhang P, Winnay JN, Bajnok L, Wright WS, Schaufele F, Tenen DG, MacDougald OA. (2004) Phosphorylation of C/EBPalpha inhibits granulopoiesis. *Mol Cell Biol*. 24(2):675-86. PMC343788

D. Research Support

ONGOING RESEARCH SUPPORT

R01 AR063772 07 — Ross (PI) 04/01/18 – 01/31/23 6 calendar months
Investigating the neural circuits of itch

R01 NS096705 03— Koerber (PD/PI) 09/01/16 – 08/31/21 3 calendar months
Molecular genetic dissection of the spinal microcircuits of wind-up (Role: MPI)

R01 EY029323 01— Demb (PI) 09/01/16 – 08/31/21 0.5 calendar months
Molecular genetic dissection of the spinal microcircuits of wind-up (Role: co-PI)

ADRC (Pilot Project) — Ross (PI) 04/01/19 – 03/31/20 0 calendar months
The role of neurovascular dysfunction in the development of Alzheimer's Disease

COMPLETED RESEARCH SUPPORT

CTSI (Pilot project) — Ross (co-PI) 7/1/17 – 6/30/18 0 calendar months
Kappa antagonists to treat pain

AMRF — Ross (PI) 07/01/16 – 06/30/17 0.6 calendar months
Molecular genetic approaches to understand the ontogeny of the dorsal cochlear nucleus

Fight for Sight 07/01/15 – 06/30/16 0.6 calendar months
Intersectional genetic strategies define a large population of monostratified wide-field amacrine cells

CTSI (Pilot project) — Ross (co-PI) 07/01/15 – 06/30/16 0 calendar months
Understanding the amplification of pain

Rita Allen Foundation Award in Pain 09/01/12 – 08/31/15 1.8 calendar months
Investigating the neural circuits of itch and pain

R21 AR064445 — Ross (PI) 4/1/13 – 3/31/15 1.8 calendar months
Using dual intersectional genetics to understand and modulate itch

CMRF (Pilot project) — Ross (PI) 7/1/12 – 6/30/14 0.6 calendar months
Understanding the neural mechanisms of itch

CTSI (Pilot project) — Ross (PI) 7/1/12 – 6/30/13 0 calendar months
Understanding the neural circuits of nociception

Whitehall Research Grant — Ross (PI) 1/1/13 – 12/31/13 2.4 calendar months
Dissecting the neural mechanisms of itch and scratching behavior

PHS Fellowship Supplemental Form

OMB Number: 0925-0001
Expiration Date: 3/31/2020

Introduction

1. Introduction to Application (for Resubmission applications)

Fellowship Applicant Section

2. * Applicant's Background and Goals for Fellowship Training — Background and Goals

Research Training Plan Section

3. * Specific Aims — Specific Aims
4. * Research Strategy — Research Strategy
5. * Respective Contributions — Respective Contributions
6. * Selection of Sponsor and Institution — Selection of Sponsor and Institution
7. Progress Report Publication List (for Renewal applications)
8. * Training in the Responsible Conduct of Research — Responsible Conduct of Research

Sponsor(s), Collaborator(s), and Consultant(s) Section

9. Sponsor and Co-Sponsor Statements — Sponsor and Co-Sponsor Statements
10. Letters of Support from Collaborators, Contributors, and Consultants — Letters of Support

Institutional Environment and Commitment to Training Section

11. Description of Institutional Environment and Commitment to Training — Institutional Environment

Other Research Training Plan Section

Vertebrate Animals

The following item is taken from the Research & Related Other Project Information form and repeated here for your reference. Any change to this item must be made on the Research & Related Other Project Information form.

Are Vertebrate Animals Used? [X] Yes [ ] No

12. Are vertebrate animals euthanized? [X] Yes [ ] No

If "Yes" to euthanasia

Is method consistent with American Veterinary Medical Association (AVMA) guidelines? [X] Yes [ ] No

If "**No**" to AVMA guidelines, describe method and provide scientific justification

13. Vertebrate Animals — Vertebrate Animals

Other Research Training Plan Information

14. Select Agent Research — Select Agent Research
15. Resource Sharing Plan — Resource Sharing Plan
16. Authentication of Key Biological and/or Chemical Resources

PHS Fellowship Supplemental Form

Additional Information Section

17. Human Embryonic Stem Cells

* Does the proposed project involve human embryonic stem cells? [ ] Yes [X] No

If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s), from the following list: http://stemcells.nih.gov/research/registry/. Or, if a specific stem cell line cannot be referenced at this time, please check the box indicating that one from the Registry will be used:

[ ] Specific stem cell line cannot be referenced at this time. One from the registry will be used.

Cell Line(s):

18.. Alternate Phone Number:

19. Degree Sought During Proposed Award:

Degree:	If "other", please indicate degree type:	Expected Completion Date (month/year):
OTH: Other	MD/PhD	05/31/2025

20. Field of Training for Current Proposal:

160 Neurosciences & Neurobiology

21. Current or Prior Kirschstein-NRSA Support? [ ] Yes [X] No

If yes, please identify current and prior Kirschstein-NRSA support below:

Level	Type	Start Date *(if known)*	End Date *(if known)*	Grant Number *(if known)*

22. Applications for Concurrent Support? [X] Yes [ ] No

If yes, please describe in an attached file: Concurrent Support

23. * Citizenship:

U.S. Citizen U.S. Citizen or Non-Citizen National? [X] Yes [ ] No

Non-U.S. Citizen With a Permanent U.S. Resident Visa

With a Temporary U.S. Visa

If you are a non-U.S. citizen with a temporary visa applying for an award that requires permanent residency status, and expect to be granted a permanent resident visa by the start date of the award, check here: [ ]

24. [ ] Change of Sponsoring Institution Name of Former Institution:

PHS Fellowship Supplemental Form

Budget Section

All Fellowship Applicants:

25. * Tuition and Fees: [ ] None Requested [X] Funds Requested:

Year 1	$63,359.00
Year 2	$65,893.00
Year 3	$68,529.00
Year 4	
Year 5	
Year 6 *(when applicable)*	
Total Funds Requested:	$197,781.00

Senior Fellowship Applicants Only:

	Amount	Academic Period	Number of Months
26.Present Institutional Base Salary:			

27. Stipends/Salary During First Year of Proposed Fellowship:

	Amount	Number of Months
a. Federal Stipend Requested:		

	Amount	Number of Months
b. Supplementation from other sources:		

Type (e.g., sabbatical leave, salary)	
Source	

Appendix

28. Appendix

Applicant's Background and Goals for Fellowship Training

A. Doctoral Dissertation and Research Experience

For details regarding publications, presentations, and awards produced during these experiences please see **Biosketch.**

Undergraduate Research Assistant | Advisor: Dr. Bart De Jonghe, PhD 2012-2016
University of Pennsylvania, Philadelphia, PA

Identification of neural circuits involved in chemotherapy-induced anorexia and malaise

In my first research lab, I worked in the De Jonghe Lab on a series of projects which sought to uncover the neural mechanisms mediating chemotherapy-induced nausea and anorexia. Using immunohistochemistry and RT-PCR, my earliest experiments identified brain regions activated in response to cisplatin administration, including the nucleus tractus solitarius (NTS), lateral parabrachial nucleus (lPBN), central amygdala (CeA) and bed nucleus of the stria terminalis (BNST). Consistent with prior studies implicating glutamate receptor signaling in inhibitory feeding pathways, we found dose-dependent increases in AMPA and NMDA glutamate receptor expression in these regions. To identify the connectivity between these brain regions and elucidate their role in food intake behaviors, we used neural tracing techniques and chemogenetics and found cisplatin activates direct projections from the NTS to the lPBN, as well as calcitonin gene related peptide (CGRP)-positive projections from the lPBN to the CeA, and found that inhibition of lPBN to CeA projections attenuated cisplatin-induced anorexia and body weight loss. My contributions to this work, which spanned more than four years, included experimental design, behavioral experiments on rats and mice, stereotaxic surgeries, as well as tissue harvesting and processing. I was also extensively involved in data analysis and preparation of manuscripts.

Skills Acquired: PCR, ELISA, immunohistochemistry, stereotaxic surgeries, chemogenetics, food intake behavior, experimental design, statistical analysis, preparation of manuscripts

Achievements and Awards: One first-author publication (*Physiology and Behavior)*, and three co-authored publications *(Physiology and Behavior; Journal of Neuroscience).* Presented posters at two conferences at the University of Pittsburgh, for both of which I received travel awards. Gave a University Scholars talk and a poster presentation at the University of Pennsylvania.

Research Specialist | Advisor: Dr. Bart De Jonghe PhD; Dr. Matthew Hayes, PhD 2016-2017
University of Pennsylvania, Philadelphia, PA

Discovery of a neural circuit for the suppression of pain during hunger

In my position as a research specialist, I worked on a number of collaborative projects for the De Jonghe Lab, Hayes Lab, and other groups. Most notably, I worked on a highly collaborative project investigating the effect of hunger on pain responses in rodents. Using chemogenetics and pain behavioral paradigms, we discovered that hunger attenuates behavioral responses and affective properties of inflammatory pain without altering acute nociceptive responses, through activity of AgRP-expressing neurons converging in the PBN. Strikingly, activity in AgRP PBN neurons blocked the behavioral response to inflammatory pain as effectively as hunger or analgesics. The anti-nociceptive effect of hunger is mediated by neuropeptide Y (NPY) signaling in the PBN. This work bridged my former research experience in food intake dysregulation and current interest in pain mechanisms. My primary contribution to this project involved conducting rodent experiments and injections, tissue collection and processing, and optimizing an immunohistochemistry protocol for co-staining AgRP and NPY in mouse brain sections.

Skills Acquired: Rodent pain behaviors, stereotaxic surgeries, optogenetics, chemogenetics

Achievements and Awards: one co-authored publication *(Cell).*

MD/PhD Rotation Student | Advisor: Dr. Michael Gold June-August 2017
University of Pittsburgh, Pittsburgh, PA

Investigating the role of gut microbiome changes in chemotherapy-induced peripheral neuropathy

Paclitaxel is a chemotherapy commonly paired with carboplatin in the treatment of a variety of solid tumor cancers. Under this regimen, patients report an array of debilitating peripheral neurological symptoms including chemotherapeutic-induced peripheral neuropathy (CIPN) often experienced as a numbness and tingling in the hands and feet which develops into debilitating pain. In my first lab rotation in the Pittsburgh Center for Pain Research, I worked in the Gold Lab to study the mechanisms underlying paclitaxel-induced

peripheral neuropathy and cognitive impairments. We hypothesized that the cognitive impairments caused by chemotherapy were due, in part, to changes in the gut microbiome, based on prior studies linking microbiome dysregulation to cognitive function. I contributed to this project by administering a paclitaxel/carboplatin cocktail to male and female rats and testing thermal and mechanical pain responses periodically over two months. I then measured memory through open field tests and anxiety through open field tests. Rats were then euthanized, and fecal samples and peripheral nerves were collected. Sex was found to be an important biological variable in this study, as my experiments showed female mice experienced significant increases in mechanical and thermal hyperalgesia as well as anxiety while males did not. This finding was consistent with human data indicating women report more severe symptoms of paclitaxel chemotherapy, indicating that rats were a viable model for paclitaxel-induced cognitive impairments.

Skills Acquired: Rodent memory and cognition behaviors, rodent pain behaviors, statistical analysis

Achievements and Awards: Presented a poster at the University of Pittsburgh MSTP Retreat

MD/PhD Rotation Student | Advisor: Dr. Sarah Ross, PhD **June-August 2018**
University of Pittsburgh, Pittsburgh, PA

Characterization of primary afferent phenotypes implicated in sexual pleasure and pelvic pain signaling

In my second lab rotation in the Pittsburgh Center for Pain Research, I worked in the Ross Lab to investigate a new frontier in somatosensory signaling: sexual pleasure. I used immunohistochemistry, neuroanatomical tracing, and a viral/genetic approach to visualize and characterize genital corpuscles, a specialized form of afferent which had not yet been identified in the mouse. I visualized two broad categories of primary afferents: a significant population of unique, clustered terminals within the glans of the mouse penis and clitoris, resembling genital corpuscular receptors described in prior literature, as well as free nerve endings extending to the epidermis. The genital corpuscular receptors are VGlut2 positive, identifying a genetic target by which these cells can be optogenetically or chemogenetically targeted to elucidate their role in sexual reward pathways. This project expanded the research interests of the Ross Lab and led to my identification of Dr. Ross as my thesis mentor.

Skills Acquired: Mouse genetics, neural tracing techniques (both viral and non-viral), literature review

Achievements and Awards: Presented a poster at the University of Pittsburgh MSTP Retreat

Doctoral Dissertation in the Lab of Dr. Sarah Ross

The work outlined in this proposal will contribute to a substantial amount of my proposed dissertation. My goal is to understand how MOR+ RVM spinal projections facilitate pain. I will utilize viral tracing, FISH, slice electrophysiology, along with acute and persistent pain assays to assess the functional role of MOR+ RVM spinal projections in the facilitation of pain. These cutting-edge techniques will help address key unanswered questions in the organization of the RVM and the circuitry underlying facilitation of pain. With the guidance of my sponsor, Dr. Ross, co-sponsor, Dr. Koerber, and my consultants **(Accomplishing Proposal),** I will develop the skills and expertise in anatomy, electrophysiology, and behavior that are necessary for me to complete the proposed project and eventually become an independent investigator. Thus, the projects outlined in this fellowship application will align closely with my dissertation.

I have independently developed the ideas behind this project and have consulted members in our lab and beyond for the training and resources necessary to successfully complete the proposed experiments. Furthermore, my previous research experiences have equipped me with many of the technical skills and research techniques I plan to utilize in my proposal. Thus, I believe that my proposed dissertation timeline is realistic and reasonable. Funding for this proposal will support my general research interest of understanding the mechanisms underlying pain signaling and will provide me with the opportunity to continue towards my long-term goal of improving quality of life and treatment options for patients suffering from chronic pain.

B. Training Goals and Objectives

My long-term career goal is to become a physician-scientist at an academic medical center, specializing in anesthesiology. I hope to apply my clinical practice towards generating and addressing important clinical questions with basic neuroscience research. Specifically, I would like to understand the underlying circuitry linking peripheral and central nervous system pathways in chronic pain. I hope to spend 70% of my time as the primary investigator of a basic science lab, 20% of my time in clinical practice, and 10% of my time involved in teaching and public outreach initiatives.

My interest in neuroscience research began in my freshman year of college at the University of Pennsylvania, when I became a research assistant for Dr. Bart De Jonghe, who studied the circuitry underlying chemotherapy-induced nausea and vomiting (CINV). I gained expertise in a variety of exciting research techniques, learned how to ask important research questions, and contributed to scientific communication through publications and poster presentations. While volunteering in a cancer unit and through working with cancer patients in my leadership roles in Colleges Against Cancer, I saw firsthand how CINV devastated people's lives and was lacking in treatment options. CINV not only diminished quality of life but was the most significant contributor to poor treatment outcomes and declining nutritional states which led patients to succumb to cancer. I quickly realized how important my research could be in informing the development of novel therapeutics for CINV, and that a career as a physician scientist would allow me to apply basic science research to improving the lives of my patients. During a collaborative project investigating competing hunger and pain signaling, I discovered a new passion for pain research. This led me to the University of Pittsburgh MSTP, and the Pittsburgh Center for Pain Research, a premier center where a collaborative group of investigators and clinical faculty study pain.

My passion for pain research is well aligned with my clinical interests in anesthesiology. I was immediately drawn to anesthesiology as early as my first course of medical school, Medical Anatomy, during which I attended an outstanding lecture on peripheral nerve anatomy and blockade by Dr. Steve Orebaugh, an attending anesthesiologist who specializes in nerve block procedures. From then on, I took advantage of every opportunity I was given to learn more about the field of anesthesiology. I took the Peripheral Nerve Block Mini-Elective course and was appointed as coordinator for the Anesthesiology Interest Group. I shadowed Dr. Orebaugh and other anesthesiologists, where I learned how anesthesiologists work to reduce patient suffering, improve quality of life, and minimize the need for opioid medications. Through a variety of clinical experiences, I met many patients who suffered from chronic pain and struggled with opioid dependence, and was frustrated by our lack of treatment options for these patients. I realized that working as an academic anesthesiologist would give me a unique opportunity to study pain circuits in more detail in order to change the lives of the patients I would care for. Thus, completion of this proposal will enhance our understanding of the circuitry underlying pain signaling and may translate to better therapies for patients suffering from pain.

In order to achieve these long-term goals, I will take advantage of the training proposed to expand my skillset in a broad range of techniques to investigate neural circuits underlying a broad range of somatosensory phenomena, especially pain. While my previous research experiences have equipped me with a collection of valuable technical skills, it is through my MD/PhD training with Dr. Ross that I will hone these skills and expand my technical and intellectual repertoire. Gaining an in-depth understanding of these approaches will provide me with the foundation I need to succeed as an academic anesthesiologist and independent investigator. Specifically, I plan to apply this proposal towards my development in three major areas: **1) expansion of technical skills, 2) scientific experimental design, and 3) development of scientific communication skills.**

1) Expansion of technical skills. My first training goal is to develop expertise in the techniques outlined in this proposal as well as other emerging approaches in neuroscience research. My objective is to develop sufficient understanding and experience using these approaches to prepare me for running my own lab where I will need to set up, operate, and troubleshoot experiments independently. Stereotaxic Surgeries: **Aims 1-3** all involve stereotaxic injections into the RVM. While I have extensive experience with stereotaxic surgeries in the rat, I am less experienced in performing surgeries in the mouse. Multiple Ross Lab members have expertise in mouse stereotaxic injections; I will consult Eileen Nguyen, a graduate student in the Ross Lab with experience successfully targeting the RVM with stereotaxic injections **(Letter of Support).** Fluorescent in situ hybridization (FISH): **Aim 1** involves the use of fluorescent in situ hybridization (FISH). Eileen Nguyen,

a graduate student in the Ross Lab with experience successfully performing FISH, will train me in this technique **(Letter of Support)** so that I can apply FISH towards enhancing our understanding of the cell types in the RVM which express MOR. Slice electrophysiology: One priority of my graduate training is to become a skilled electrophysiologist so that I can use this expertise as an independent investigator. The experiments proposed in **Aim 2A-B** were designed with this goal in mind. Specifically, I will learn to use electrophysiology to investigate how MOR+ RVM projections influence interneurons with assistance from the lab, particularly from Dr. Kelly Smith, a postdoctoral fellow in the Ross Lab **(Letter of Support)**. Confocal Microscopy: **Aim 2B** proposes to use confocal microscopy to reconstruct interneuron morphology. The Ross Lab has access to a confocal microscope and Neurolucida system for cell reconstruction. I will receive training in these techniques from Dr. Zachary Wills, a principal investigator in the Department of Neurobiology who provides training for all researchers who use the confocal microscope **(Equipment; Microscopes).** Pain behavioral models: **Aim 3** proposes to use mouse models of both acute and persistent pain to investigate the role of MOR+ RVM projections in pain facilitation. I will receive training in these behavioral paradigms from Dr. Tayler Sheahan, a postdoctoral fellow in the Ross Lab **(Letter of Support)**. I will also take the Pain Models course (MSNBIO 2623) taught by Dr. Ross**.** Statistical analysis. The ability to analyze and interpret the data generated from experiments is essential for an independent investigator to generate hypotheses and heed scientific rigor. While I have some experience in statistical analysis, I will take an elective upper-level biostatistics class (BIOST 2042) to gain expertise in R Studio.

3) Scientific experimental design. My next goal is to cultivate my ability to identify gaps in scientific knowledge, select specific problems to pursue, and rigorously formulate hypotheses and approaches to solve them. These skills will better prepare me to be an independent investigator, capable of identifying clinical and basic science problems for study as well as the strengths and limitations of various experimental designs. Through my preparation of this proposal, Dr. Ross has already taught me an incredible amount about this process, and will continue to work with me to plan future directions arising from my work and innovate the relevant experimental paradigms. Biannual meetings with my thesis committee will further this training, as my committee members emphasize critical evaluation of one's own experiments.

4) Development of scientific communication skills. A critical skill for success as an independent investigator is the ability to communicate effectively in written and oral formats. Dr. Ross is an excellent orator and writer, and I look forward to her mentorship in not just the conceptual but also the communicative aspects of doing science. To cultivate my presentation skills, I will present data in weekly lab meetings, where Dr. Ross and other members of the lab will provide critiques and feedback. I will also present data annually within the Pittsburgh Center for Pain Research, and regularly in formal journal clubs and research in progress talks for the CNUP, where I will receive feedback from a wide range of faculty and trainees. I will also present my findings in poster sessions and/or symposia at the annual meetings of the Society for Neuroscience. To prepare for talks at these meetings, the Ross lab will critique my presentations slide by slide. To cultivate my writing, I will write manuscripts for submission to peer-reviewed journals. Each manuscript will go through iterations of feedback and revision at each stage with Dr. Ross and contributing authors. All of these experiences will provide a solid foundation for a career of publications and presentations of scientific work.

C. Activities Planned Under this Award

	Year 1	Year 2	Year 3	Year 4
Research				
Aim 1	70%	0%	0%	0%
Aim 2A	0%	60%	0%	0%
Aim 2B	0%	28%	83%	0%
Aim 3	0%	0%	0%	85%
Coursework				
• Graduate Courses • Center for Neuroscience Journal Club	15%	2%	2%	0%
Medical Training				
• Longitudinal Clinical Clerkship (LCC)	5%	0%	5%	0%
Seminars				
• Pittsburgh Center for Pain Research Seminar • Pittsburgh Center for Pain Research Journal Club • MSTP Workshops	2%	2%	2%	2%
Meetings				
• Individual Meetings with Dr. Ross • Ross Lab Meetings	5%	5%	5%	5%
Conferences				
• Society for Neuroscience (SFN) • American Academy of Pain Medicine (AAPM) • International Association for the Study of Pain (IASP)	3%	3%	3%	8%

Research: I seek to complete **Aims 1-3** by the end of Year 4. I anticipate most of my research time will be dedicated to **Aim 2** as I develop the skills necessary to perform slice electrophysiology. I will receive training from Dr. Smith, a postdoctoral fellow in the Ross Lab and Dr. Koerber, my co-sponsor, in slice electrophysiology techniques **(Respective Contributions: Accomplishing Proposal).** During Years 3-4, I will complete any remaining manuscripts in preparation.

Coursework: As an MSTP student, I have already taken courses offered in medical school and the MSTP Program which fulfill the Neurobiology, Grant Writing, and Statistics course requirements for the CNUP graduate program. Therefore, I plan to complete my course requirements by the end of Year 1. This includes CNUP Journal Club, Proseminar, and 6 credits of electives, for which I have decided to take Molecular Pharmacology (MSMPHL 3360), Neurobiology of Disease (MSNBIO 2112), and a higher-level statistics course (BIOST 2042) to gain expertise in R Studio. I will also take courses offered by the PCPR to enrich my pain knowledge, including Mechanisms of Pain (MSNBIO2622) taught by Dr. Gold, and Pain Models (MSNBIO2623) taught by my sponsor, Dr. Ross. In the spring of Year 1, I will also take the Ethics course offered by the MSTP program. I plan to complete my reprint exam by the end of Year 1. I plan to complete my comprehensive exam by the end of Year 2 and advance to candidacy by the beginning of Year 3.

Medical Training: I will participate in the Longitudinal Clinical Clerkship (20 half-days) in Year 1 and Year 3 in an anesthesiology subspecialty in order to maintain continuity in my clinical training. I will return to medical school after Year 4.

Seminars: The CNUP and PCPR host frequent research seminars given by invited faculty, Current Research On Pain (CROP) talks given by trainees, and the CNUP and PCPR Journal Clubs. I plan to regularly attend

a variety of seminars in order to enhance my knowledge about neuroscience and pain, network with critically analyze papers, and present my own work in a CROP talk by the end of Year 1. to share my findings with other faculty in the PCPR and receive feedback. I also attend monthly MSTP Workshops which will provide me with professional development opportunities.

Meetings: Throughout all four years I will attend weekly Ross Lab meetings to discuss lab business and share project results. I will also meet one-on-one with Dr. Ross for one hour each week to discuss my progress and troubleshoot. While I am working on **Aim 2,** I will meet with Dr. Koerber weekly to discuss my electrophysiology training. Drs Ross and Koerber have offices a few doors down from my desk, so we will have frequent impromptu meetings more often. To discuss my career development, I will meet with my career advisor, Dr. Alan Sved, and my physician-scientist mentor, Dr. Ajay Wasan, once a semester **(Letter of Support)**.

Conferences: I will present at a conference each year of my graduate training, at the Society for Neuroscience (SFN), the International Association for the Study of Pain (IASP), or the American Academy of Pain Medicine (AAPM).

Specific Aims

Chronic pain is a debilitating condition which severely impacts quality of life. Unfortunately, our current treatment options are limited by adverse effects, and the specific neural circuits that modulate pain remain incompletely understood. Specifically, descending modulation of pain through neurons in the **rostral ventromedial medulla (RVM)** occurs via ON-cells, which facilitate pain, and OFF-cells, which inhibit pain[1–4], but many details of this circuitry remain unclear. In this model, morphine, a mu-opioid receptor (MOR) agonist, is thought to suppress pain, at least in part, through two mechanisms: 1) direct inhibition of ON-cells, which express MOR[5–7] and 2) indirect activation of OFF-cells, via a mechanism of disinhibition[8,9]. The proportion of RVM spinal projections responsive to MOR is increased in persistent inflammatory pain states[10], and opioid-induced hyperalgesia is partially mediated by the RVM[11], suggesting these neurons underly a pro-nociceptive circuit. However, the precise role MOR+ neurons play in pain facilitation is unknown. Thus, there is a major gap in knowledge regarding the underlying circuitry by which the RVM facilitates pain and contributes to chronic pain states.

Until recently, we lacked the tools to clearly visualize and selectively manipulate MOR+ spinal projections *in vivo*. With the recent development of the Oprm1^{cre} mouse, MOR+ RVM spinal projection neurons can be selectively targeted and modulated for the first time, both *in vitro* and *in vivo,* to uncover the anatomy and physiology of these neurons and determine their role in acute pain behaviors and persistent pain states. Thus, we are now poised to **test the hypothesis that MOR-expressing RVM neurons that project to the spinal cord will innervate the dorsal horn (Aim 1), inhibit post-synaptic interneurons in the dorsal horn (Aim 2), and facilitate pain (Aim 3) (Figure 1).**

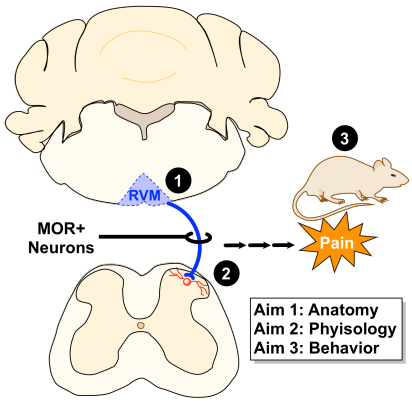

Figure 1: Hypothesis. spinally projecting MOR+ RVM neurons inhibit interneurons in the superficial dorsal horn to facilitate pain.

Aim 1: Determine the spinal targets and neurochemical phenotypes of MOR+ RVM neurons. To test the specific hypothesis that GABAergic MOR+ RVM neurons innervate the dorsal horn, I will trace these neurons with an adeno-associated virus (AAV) containing a fluorescent probe into the RVM of Oprm1^{cre} mice and will visualize fluorescent terminals throughout the neuraxis with immunohistochemistry (IHC). To determine the neurochemical phenotype of MOR+ RVM spinal projections, I will inject the retrograde neural tracer Fluoro-Gold into the dorsal horn and perform fluorescence *in situ* hybridization (FISH) in the RVM to co-stain with the cell type-specific markers Vgat, VGlut2, and Tph, and Oprm1 to determine which spinally projecting RVM neurons are GABAergic, glutamatergic, or serotonergic, and express MOR. Consistent with previous studies tracing RVM spinal projections, I expect to identify MOR+ GABAergic spinal projection neurons that synapse in laminae I and II.

Aim 2A: Identify the MOR+ RVM inputs received by interneurons in lamina I and II of the dorsal horn. In order to test the specific hypothesis that MOR+ RVM neurons inhibit dorsal horn interneurons, I will use optogenetics and the Oprm1^{cre} mouse to insert a cre-dependent channelrhodopsin (ChR2) into MOR+ RVM neurons. Then, I will use slice electrophysiology to quantify light-evoked inhibitory or excitatory post-synaptic currents (IPSCs or EPSCs) within dorsal horn interneurons. I expect to observe light-evoked IPSCs in dorsal horn interneurons, indicating inhibition. **Aim 2B: Explore the electrophysiological, neurochemical and morphological phenotype of dorsal horn interneurons receiving input from MOR+ RVM projections.** I using the preparation described in **Aim 2A**, I will characterize responsive interneurons by firing pattern and pharmacological responsivity. Post-hoc avidin-neurobiotin staining and confocal microscopy will be used to reconstruct the morphology of recorded cells. Consistent with inhibitory interneurons, I expect these interneurons to exhibit tonic firing and display an islet or central morphology.

Aim 3: Elucidate the functional role of MOR+ RVM spinal projections in behavioral assays of pain. To test the specific hypothesis that MOR+ RVM spinal projections facilitate pain, I will use a chemogenetic approach to selectively activate and inhibit MOR+ RVM spinal projections and measure the impact on nocifensive behavior evoked with chemical (capsaicin), mechanical (Von Frey), and thermal (Hargreaves) stimuli, as well as on a spared nerve injury (SNI) model of chronic neuropathic pain. I expect inhibition of MOR+ RVM spinal projections to attenuate chronic pain, while excitation of these neurons will exacerbate acute and SNI-induced chronic pain.

The results of these experiments will further validate (or potentially challenge) our current model of descending pain modulation by the RVM. Uncovering the underlying circuitry by which MOR+ RVM spinal projections facilitate pain will advance the pain field and pave the way for the development of novel pain therapeutics. Finally, the proposed experiments will provide me with excellent training in genetic techniques, electrophysiology, and behavioral paradigms which will enable me to achieve my long-term goal of becoming a physician-scientist.

Research Strategy

SIGNIFICANCE

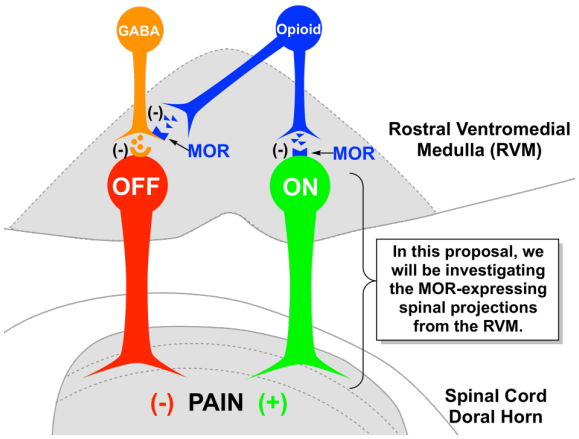

Figure 2: Model by which MOR signaling in the RVM inhibits ON-cells and activates OFF-cells. Dissecting the underlying circuitry using pharmacological approaches is complicated because MOR signaling modulates the activity of several distinct cell types. In this proposal, I will use the MOR-cre mouse 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.

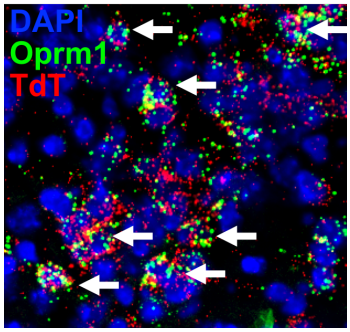

Figure 3: FISH performed by Eileen Nguyen of the Ross Lab validating the Oprm1-cre mouse.

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.**

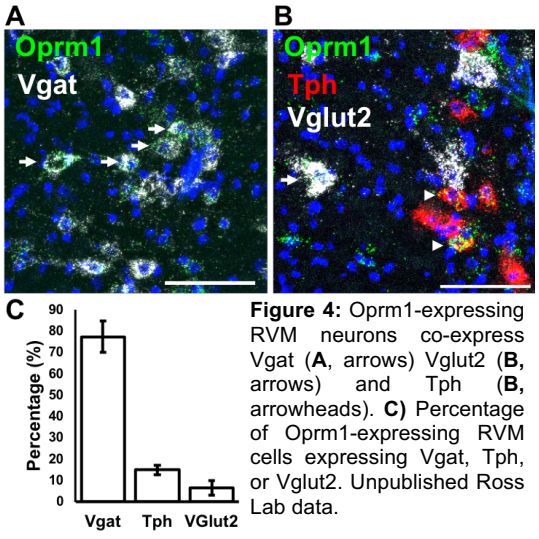

Figure 4: Oprm1-expressing RVM neurons co-express Vgat (**A**, arrows) Vglut2 (**B,** arrows) and Tph (**B,** arrowheads). **C)** Percentage of Oprm1-expressing RVM cells expressing Vgat, Tph, or Vglut2. Unpublished Ross Lab data.

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

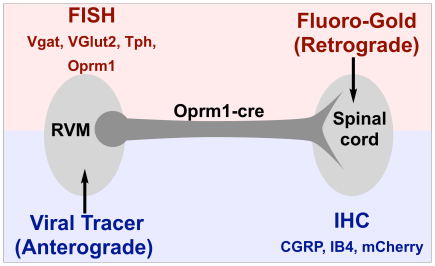

Figure 5: In experiment 1A **(Red)**, MOR+ spinal projections will be retrogradely traced from the spinal cord to the RVM; FISH will then be performed in RVM sections. In experiment 1B **(Blue)**, MOR+ RVM spinal projections will be anterogradely traced from the RVM; IHC will be performed on spinal cord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

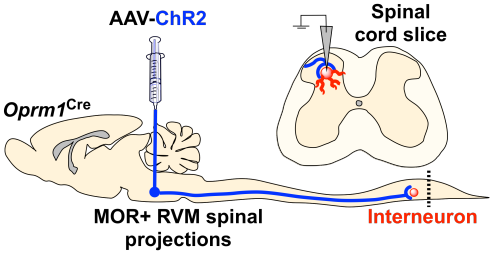

Figure 6: Schematic of **Aim 2A**. Using the Oprm1^{cre} mouse and a virus containing ChR2, I will optogenetically activate the MOR+ spinal projections from the RVM and record the inputs received by spinal interneurons.

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

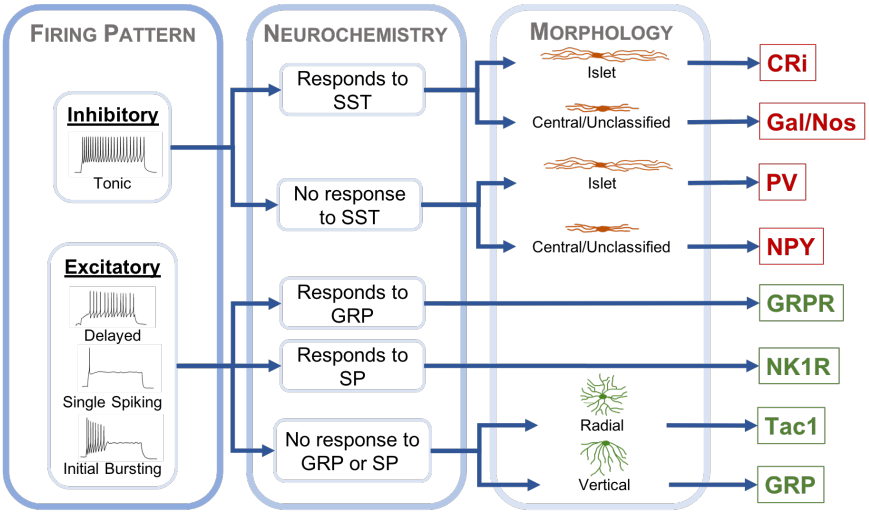

Figure 7: Interneurons exhibiting a light-evoked current will be classified by this scheme*. Inhibitory neurons subtypes: SST, somatostatin; GRP, gastrin-releasing peptide; SP, substance P; CRi, calretinin inhibitory; Gal/Nos, galanin/nitric oxide synthase; PV, parvalbumin; NPY, neuropeptide Y. Excitatory 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.

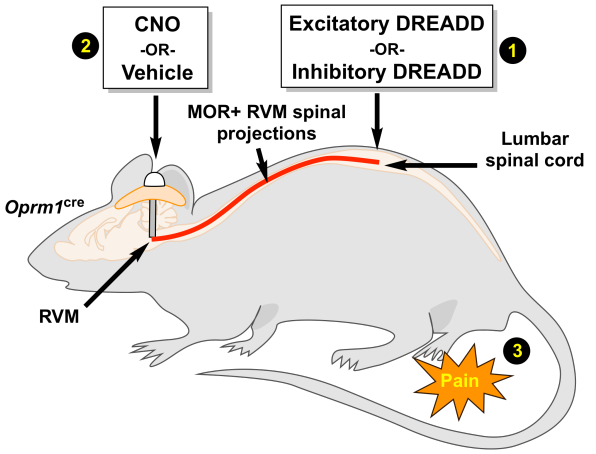

Figure 8: Schematic of **Aim 3. 1)** I will chemogenetically target MOR+ RVM spinal projections by injecting a retrograde AAV containing the gene for a DREADD receptor into the lumbar spinal cord of Oprm1^{cre} mice. **2)** I will activate the DREADD through injections of CNO into the RVM. **3)** After 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.

Respective Contributions

Preparing Proposal

I selected Dr. Sarah Ross as my thesis mentor after completion of two research rotations in the Pittsburgh Center for Pain Research. During medical school, I met with Sarah Ross periodically to discuss possible projects which would combine my research interests and experience with areas of growth and training opportunities to ultimately prepare me to become an independent researcher. Specifically, we were interested in the circuitry underlying the pain modulatory effects of the rostral ventromedial medulla. Through reviewing the literature and discussing with Dr. Ross, I developed the hypothesis that mu-opioid receptor-expressing neurons in the RVM facilitate pain through synapsing on interneurons in the dorsal horn. I then reached out to Dr. H. Richard Koerber, a renowned expert in electrophysiology. He was excited to collaborate on this project and agreed to be my co-sponsor, as his expertise would provide me with invaluable guidance and training.

I wrote the first draft of every section of this proposal, with the exception of Letters of Support and the Sponsor and Co-Sponsor Statement, which was written by Dr. Ross and Dr. Koerber. I then obtained feedback from Dr. Ross and Dr. Koerber, who all gave me impactful and valuable critiques which aided in the development of the final proposal. The process of preparing this proposal has provided experience and a wealth of knowledge in grant writing that I will benefit from greatly as I continue to write grants, publications and communicate science to a wider audience.

Accomplishing Proposal

I have prior experience with immunohistochemistry and mouse behavioral experiments from my previous research experiences and have consulted a postdoctoral fellow in the Ross Lab, Dr. Tayler Sheahan **(Letter of Support)** for technical assistance in the completion of the stereotaxic injections required for **Aims 1-3** as well as the behavioral assays proposed in **Aim 3.** In addition to offering technical assistance, Dr. Sheahan will provide feedback on analysis of my behavioral data obtained from the pain behavioral assays proposed. In addition, I will also receive training in the FISH experiments proposed in **Aim 1** from Eileen Nguyen, an MSTP graduate student in the lab **(Letter of Support).** Eileen is an additional lab member who has experience with stereotaxic surgeries, specifically into the RVM and so will be an invaluable resource should I require additional technical assistance. For **Aim 2,** I will learn electrophysiology in slice by receiving direct training from Dr. Kelly Smith, a postdoctoral fellow in the Ross Lab **(Letter of Support)**. When I begin my training, I will be using practice mice and working directly with Dr. Smith, with the goal that I will be conducting the proposed experiments independently. I will also have the support of my co-sponsor, Dr. Koerber, another principal investigator within the PCPR for additional assistance and training with electrophysiology. Additionally, **Aims 1-3** all require the *Oprm1*^{cre} knock-in mouse, which has been generously provided by Dr. Richard Palmiter, a renowned expert in the development and use of genetic tools for the study of mouse behaviors **(Letter of Support).** Dr. Palmiter has provided valuable information regarding the development and validation of this mouse line. Should questions arise about the clinical manifestations or management of chronic pain, I have consulted Dr. Ajay Wasan, a specialist in chronic pain management who applies medical informatics to the investigation of pain treatment outcomes. He will serve as both a clinical consultant for my proposal and as a physician-scientist mentor for my career development **(Letter of Support).**

Throughout my training, I will continue to have weekly one-on-one meetings with Dr. Ross where we discuss the results of my project and troubleshoot experiments. In addition, I will be attending weekly lab meetings, during which I will present findings with the Ross Lab. I will be primarily responsible for writing up our findings as a manuscript, and plan to present our discoveries at research conferences, such as Society of Neuroscience, American Academy of Pain Medicine, and International Association for the Study of Pain. Manuscripts and posters will be drafted by me with guidance from Dr. Ross and Dr. Koerber in a collaborative process.

Finally, I have assembled a team of mentors including my sponsor, Dr. Ross, my co-sponsor, Dr. Koerber, my clinical consultant and physician-scientist mentor, Dr. Ajay Wasan, as well as my career advisor, Dr. Alan Sved. This involved group of mentors consists of accomplished investigators positioned at diverse stages of their careers who will provide me with outstanding academic and professional development.

Selection of Sponsor and Institution

Sponsor: When selecting a thesis mentor, I sought out a principal investigator who was established and well-funded, had a strong record for training MSTP students, and has a commitment to mentorship, collaboration, and creative thinking. **Dr. Sarah Ross**, an Associate Professor in the Department of Neurobiology and active faculty member in the Pittsburgh Center for Pain Research, is the best possible mentor for an aspiring physician scientist. As demonstrated by her excellent publication record, the Dr. Ross uses diverse methodologies to study fundamental questions about neural circuitry, including mouse genetics, imaging techniques, anatomical studies, behavior, and electrophysiology. These multifaceted approaches speak to Dr. Ross's rigor as a scientist. All of the graduate students in her lab have received NRSA support. She also shows enthusiasm for training aspiring physician scientists, as demonstrated by the recent successful dissertation defense by Michael Chiang, an MSTP student who will be returning to medical school in the fall. There is an additional MSTP student in the lab, which fosters a unique longitudinal mentorship environment through which I work closely with more senior MSTP students on a similar career path. Furthermore, Dr. Ross employs a variety of strategies to aid her trainees in their development as well-rounded independent investigators – she encourages creative but rigorous scientific approaches, scientific communication, and professional development. For these reasons Dr. Ross was named an honoree for the 2018 William E. Brown Outstanding MSTP Mentor Award. Taken together, Dr. Ross will certainly be a strong mentor to support me as I move towards becoming a physician scientist.

Co-sponsor: Before the development of this proposal, I sought out faculty who would best complement the mentorship of Dr. Ross and expand my intellectual and technical repertoire. **Dr. H. Richard Koerber** is a highly respected Professor in the Department of Neurobiology and an active faculty member in the Pittsburgh Center for Pain Research. Dr. Koerber is a highly respected, experienced scientist in the pain field and has mentored several successful trainees. He is an expert electrophysiologist with decades of experience performing carefully designed electrophysiology experiments in somatosensory circuits. Drs. Ross and Koerber have a strong collaborative relationship, co-authoring on numerous publications and serving as co-investigators on an R01. He has been an invaluable resource in the development of this proposal and promises to be an excellent co-mentor throughout my postdoctoral training. Furthermore, as he is in an advanced career stage, he can offer me career mentorship that will complement that of Dr. Ross. Thus, Dr. Koerber is well-qualified to supplement my scientific and professional development.

Institution: The main factors important to me when selecting an institution were a collaborative and distinguished neuroscience program with a community of accomplished research faculty, excellent clinical training, and a well-established MSTP program with a strong history of alumni success. The **University of Pittsburgh (Pitt) and Carnegie Mellon University (CMU) Medical Scientist Training Program (MSTP)** is a joint MD/PhD program which gives me the opportunity to acquire excellent training in a broad range of skills and to tailor my training to my interests and long-term goals **(Additional Educational Information)**. The School of Medicine ranks among the nation's top medical schools and affiliates with University of Pittsburgh Medical Center, which is among the nation's most distinguished and competitive anesthesiology residency programs. To maintain clinical continuity, I will complete two Longitudinal Clinical Clerkships (LCCs), both within the Department of Anesthesiology. For my graduate studies, I have the pleasure of working within the Center of Neuroscience at the University of Pittsburgh (CNUP) which is an inter-institutional and multidisciplinary training program that includes over a hundred training faculty at both Pitt and CMU. During my fourth year of medical school, I will have the option to complete the MSTP Postdoctoral Fellowship, a 5-month experience of nearly 100% dedicated research time. Therefore, the Pitt-CMU MSTP is the best fit for me given my research interest in neuroscience and clinical interest in anesthesiology.

Pittsburgh Center for Pain Research (PCPR): I was especially drawn to the Pitt-CMU MSTP because of the Pittsburgh Center for Pain Research (PCPR). The PCPR brings together 41 faculty members spanning departments in Anesthesiology, Medicine, Neurobiology, Pharmacology, and Psychiatry to advance pain research in a collaborative and integrative environment. This will prove critical as I explore avenues to integrate both basic and clinical research. I believe the integration of basic and clinical research within the PCPR will positively impact my future as a physician scientist. Regarding networking and collaborations, the PCPR hosts frequent networking events and monthly seminars of distinguished scholars from throughout the country, many of whom I have had the opportunity to meet. The PCPR will actively contribute to my training through weekly pain journal clubs, monthly Current Research on Pain (CROP) talks given by trainees, and other course offerings including Mechanisms and Clinical Presentations of Pain. The PCPR provides travel awards, research prizes, and has an NIH-funded T32 grant to support 2 predoctoral and 2 postdoctoral trainees.

Training in the Responsible Conduct of Research

A top priority for my graduate training is conducting ethically responsible research. The University of Pittsburgh and Carnegie Mellon University MSTP has provided me with courses, seminars, and professional development workshops which will aid in my development into an ethical and thoughtful physician scientist. Over the course of my eight-year MSTP training, I will complete over 100 hours of ethics training. The format of my ethical training is as follows, in order of completion: 1) medical school lectures and online coursework, 2) Medical Scientist Training Program (MSTP) ethics coursework, 3) MSTP workshops, and 4) graduate school ethics seminars and online coursework. **For each of these components, I will address the format, subject matter, level and form of faculty participation, duration of instruction, and frequency of instruction**.

1) University of Pittsburgh School of Medicine: Ethics, Law and Professionalism Course in Patient, Physician, and Society block (Completed 12/2017): This course met weekly for a 50-minute lecture from the course directors, Dr. Julie Childers and Dr. Joseph Yanta, and for a 50-minute small-group discussion led by a School of Medicine faculty member. The course was 20 weeks in duration. Topics discussed included ethical recruitment and use of human subjects in research and ethical approaches to delivering patient care, among many others. These topics were reviewed with online content before small group discussions.

2) MSTP Professional Development 2 Course (Completed 08/2018): In the summer of 2018, I completed this course as part of the Pitt MSTP curriculum. The course met eight times for 90-minute sessions that combined didactic lecture and small-group discussion led by the program director, Dr. Richard Steinman. Topics included rigor and reproducibility, falsification/fabrication of data, data documentation and ownership, and mentor/mentee relationships.

3) MSTP Workshops (Started 06/2017, ongoing): The MSTP program holds monthly gatherings for its entire student body. Each session is 90 minutes long and involves a combination of lecture and round-table discussion. Topics vary widely, but each session includes ethics as a learning objective.

4) University of Pittsburgh IACUC Animal Research Online Classes (Last Completed 06/2018 and ongoing): The University of Pittsburgh IACUC provides a series of online training modules that are required for individuals performing research at the university. I first had to complete these modules when I started in the MSTP in June of 2017 and I have regularly re-completed the modules. Topics include use of rodent subjects in research, laboratory safety, research integrity, and environmental health and safety.

Training to be Completed During the Fellowship Period

Ethics for Medical Scientists: In the spring of my first year of graduate school (2020), I will take a specialized ethics course offered by the Pitt MSTP designed to teach ethics topics relevant to aspiring physician scientists. This course consists of five two-hour long group sessions led by Dr. Richard Steinman and Dr. Karen Schmidt, the Director of Responsible Conduct of Research Center at Pitt. During these sessions, students will learn about applying analytical methods systematically to the evaluation of ethical dilemmas. Students will then apply these methods within the context of biomedical ethics cases. This course will provide with me a framework based upon current methods and principles in ethics and will translate conceptual methodologies into practical skills for evaluating ethical dilemmas.

University of Pittsburgh Responsible Conduct of Research Training Center: The Clinical and Translational Sciences Institute at the University of Pittsburgh offers weekly, one-hour seminars on a wide variety of topics taught by faculty members from throughout the University of Pittsburgh. I will attend at least eight of these seminars during my graduate school training.

MSTP Workshops: (see above)

Role of Dr. Sarah Ross in Responsible Conduct of Research Training:

Throughout my graduate school training, I have had and will continue to have constructive conversations with Dr. Ross regarding ethical issues in the conduct of research. In addition, the experience of performing research in her lab and our close mentor/mentee relationship will allow for robust training in the responsible conduct of research.

Sponsor and Co-Sponsor Statements

A. Research Support Available

ONGOING RESEARCH SUPPORT

Sarah Ross

R01 AR063772 06 — Ross (PI) 04/01/18 – 01/31/23
Investigating the neural circuits of itch

R01 NS096705 02 — Koerber (PD/PI) 9/01/16 – 8/31/21
Molecular genetic dissection of the spinal microcircuits of wind-up (Role: co-PI)

R01 EY029323 01 — Demb (PD/PI) 9/16/16 – 9/15/21
Functional circuitry of the long-range connections in the retina (Role: co-PI)

ADRC — Ross (co-PI) 4/01/16 – 3/01/20
The role of neurovascular dysfunction in the development of Alzheimer's Disease

H. Richard Koerber

R01 NS096705 02 — Koerber (PD/PI) 9/01/16 – 8/31/21
Molecular genetic dissection of the spinal microcircuits of wind-up (Role: co-PI)

R01 AR069951-02 — Koerber (MPI) 4/01/16 – 3/31/21
Characterization of Epithelial-Neural Communication

B. Sponsor's/Co-Sponsor's Previous Fellows/Trainees

Dr. Sarah Ross:

I take mentorship very seriously and work very hard to ensure the success of all my trainees. Two of the four students that have trained in my lab have already completed their graduate studies and the other two are on a track to complete within 4 – 4.5 years. **Lindsey Snyder** defended in July, 2017, with 8 peer-reviewed publications, including a first-author report in *Neuron*. She is currently pursuing postdoctoral training with Dietrich Stephan at the University of Pittsburgh. **Michael Chiang**, an MD-PhD student, defended July, 2019 with 4 peer-reviewed publications and a fifth, the major work from his thesis (available at *BioRxiv*), to be resubmitted next month first-author publication, having received strong reviews from *Neuron*. He is now returning to medical school. **Catherine Ruff**, who is beginning her third year, has made a major discovery about the regulation of cortical blood flow by long range inhibitory interneurons that express NK1R. She has already received two poster prizes for her work at internatisnal conferences. **Eileen Nguyen**, who is beginning her second year, received an NRSA to investigate the neural basis of morphine-induced itch. Since this project required a lot of breeding (and waiting for mice) she developed a back-up project studying the RVM neurons that express the kappa opioid receptor (putative OFF-cells). Now she has extremely exciting data from both projects and will likely graduate with two major first-author papers. All four of my current and previous graduate students received NRSAs and then went on to be highly successful with their scientific projects.

My first post-doc, **Junichi Hachisuka**, developed a novel preparation to study spinal circuitry (*Elife*) and used this preparation to investigate wind-up (*Pain*) and the neural circuits of cold (under review at *Pain,* available at *BioRxiv*). Based on his postdoctoral success with co-mentorship from me and Rick Koerber, he was hired as tenure-track faculty at the University of Glasgow (senior lecturer), which he started July, 2019. My second post-doc, **Marissa Kuzirian** received an NRSA, published several papers, and is now an Executive Associate at Pittsburgh Life Sciences Greenhouse.

Dr. H. Richard Koerber:

I have been the primary supervisor for 14 postdocs and 3 Ph.D. students, including an MD/PhD student.

9 of my former postdocs currently hold academic appointments (Karoly Mirnics, University of Nebraska; Jeffrey Woodbury, University of Utah, Michael Jankowski; Cincinnati Children's Hospital; Jeffrey Lawson, Fairmont

State; Sabrina McIlwrath, University of Kentucky; Kristofer K Rau, University of Louisville; Kyle Baumbauer, University of Connecticut; Bin Feng**,** University of Connecticut; Junichi Hachisuka, University of Glasgow).

4 of my former postdocs currently hold industry positions (Maggie Wright, Ultragenyx; Colleen Cassidy LifeX; Peter Adelman, Afinini; Amy Ritter Bristol-Myers Squibb).

One of my former Ph.D. students (Deepak Soneji) is currently Neurology Resident at UPMC.

C. Training Plan, Environment, Research Facilities

C.I *Training Plan*

Ruby Holland's goal is to become physician-scientist, where she will combine an independent research program to study pain and clinical work aimed at helping patients who suffer from pain. For her thesis work, she plans to investigate the descending neurons in the brainstem that enable top-down control of pain, and the pathological changes that occur within these circuits that may lead to chronic pain. The training described here is designed to allow Ruby to obtain all the technical and professional skills necessary to help enable this goal.

Rick Koerber and I are ideal co-mentors for Ruby because we are scientific partners, and we more or less run a joint lab with a joint lab meetings and daily interactions. In particular, Rick and I have an extensive and ongoing collaboration that includes four published papers, two additional manuscripts under review (uploaded to BioRxiv), an R21 (now completed) and an R01. As a testament of our success as a training team, our co-mentorship of Junichi Hachisuka resulted in his recent attainment of a faculty position as a senior lecturer at the University of Glasgow with the spinal cord group.

I am an expert in both pain and molecular genetic approaches to study neural circuit function, including the use of Cre alleles and viruses to target and manipulate specific neuronal populations. In my last eight years at the University of Pittsburgh, I have already developed a track record as an outstanding mentor to students and postdocs alike. Attesting to this, I was recently short-listed for William E. Brown Outstanding MSTP Mentor Award. Rick Koerber has been studying neural circuits of pain for the last 40 years, and has extensive experience with anatomy and electrophysiological recordings. Thus, for Ruby's training, I will be primarily responsible to ensure the success of Aims 1 (viral labeling, FISH, immunohistochemistry) and 3 (acute and persistent pain models), whereas Rick will be primarily responsible to ensure the success of Aim 2 (electrophysiology in slice). Together, our years of experience and expertise investigating somatosensory circuits will provide excellent mentorship for Ruby as she studies the neural circuits of descending modulation.

The training plan that Ruby and I have discussed has three key components: 1) mentored research training; 2) broad exposure to neuroscience and related fields through seminars, journals clubs, and attendance at national and international meetings; and 3) professional development activities.

1) Mentored research training

Ruby's training will consist largely of mentored research experience. Rick Koerber and I are very excited about her project, which we believe will provide important new insight into the neural circuits through which pain is modulated by descending circuitry in the RVM. In particular, Ruby will target the subset of MOR-expressing RVM neurons that project to the spinal cord, which are putative ON-cells, using a newly developed Oprm1-cre allele that was developed in the Palmiter lab **(Letter of Support)** and targeted viral delivery. The targeting of specific descending projections in this way will allow her to visualize and characterize a defined population of neurons (putative ON-cells), ascertain their targets in the spinal cord through optogenetics and electrophysiology, and then manipulate their activity in vivo (both activating and inhibiting) to provide cause-and-effect evidence for a role of these neurons in the modulation of pain. This combination of approaches will provide Ruby with many opportunities to expand her technical and methodological training, including viral labeling, stereotaxic surgeries, FISH, immunohistochemistry, cell quantification, electrophysiology, optogenetics in vitro, and behavioral experiments in vivo.

Ruby's training will be gained through the course of performing experiments, overseen primarily by myself and Rick Koerber. Rick and I are both highly invested in Ruby's success and have ample time for one-on-one training that is critical for becoming a skilled neuroscientist. Technical training will be under our supervision and also will involve others in my group including Eileen Nguyen (expertise targeting the RVM; **Letter of Support**), Kelly Smith (expertise in electrophysiology; **Letter of Support**) and Tayler Sheahan (expertise in behavior; **Letter of Support**). In addition, I will meet with Ruby for at least one hour per week to discuss recent data, relevant literature and plans for future experiments. Ruby will also present her research findings at our weekly lab

meetings (joint with the Koerber lab), thereby giving her practice in oral communication and organization of data into representative figures. This weekly meeting encourages the lab members to think broadly about other projects and to share their experiences.

2) Broad training in neuroscience and biology:

Intramural Interactions: Beyond her mentored research experience, Ruby will take a broad range of courses including statistics, ethics, and two pain-specific classes (Mechanisms of Pain taught by Michael Gold, and Pain Models, taught by me). She will attend seminars (~1/week), including 12 Pain Center Seminars, as well as a wide array of other neuroscience seminars that are advertised through the Pittsburgh Brain Institute. Ruby will regularly have the opportunity to meet with and even act as a host for seminar speakers of particular interest to her. She will present her research in several different formats to different audiences on an annual basis. These include a work-in-progress presentation to the Pain Center, a poster at Brain Day, a poster at the MSTP retreat, and a short-talk at an annual 3-day CNUP (graduate program) retreat. Ruby will also participate in the Pain Journal Club, which meets weekly to discuss a paper.

Extramural Interactions: Part of Ruby's training entails attending at least one extramural conference a year in order to diversify her professional and scientific exposures. I let trainees pick which meeting they would like to attend. Next year, she may wish to attend the Keystone meeting (there are concurrent meetings on Pain and Somatosensation), which I have helped organize, or the International Association for the Study of Pain (IASP), which will be held in Amsterdam. I like to bring trainees to meeting that I attend so that I can introduce them to my network of colleagues. I also encourage all of my trainees to apply for the North American Pain School (NAPS, a week-long boot camp), which is an outstanding experience.

3) Professional Development

Ethics: Ruby will participate in the bimonthly Clinical and Translational Science Institute's "Responsible Conduct of Research" (RCR) workshops that include lectures by experience faculty and case study-centered group discussions on multiple aspects of research ethics. These workshops provide trainees at all levels with an opportunity to interact and discuss issues such as (but not limited to) mentoring, research involving animal and human subjects, managing conflict of interest, and publication. We take training of our students in the ethical conduct of research seriously and frequently raise relevant issues at group meetings, as well as during one-on-one interactions with lab members.

Clinical: Ruby will participate in two LCC rotations (20-week clinical rotations for one half-day per week) to provide 1) direct experience balancing both clinical and scientific work and 2) develop valuable clinical skills in Ruby's field of interest, anesthesiology, during a scientifically rigorous part of her overall training. The LCC will provide Ruby with the clinical context for her science research as she begins to think about integrating her research and clinical work, a crucial aspect of her planned career as a physician scientist performing translational research. Ruby will also have frequent professional development meetings with Ajay Wasan **(Letter of Support)**, who combines an active research program and clinical practice as the director of the Pain Clinic. As an academic anesthesiologist, Dr. Wasan will be a fantastic role model and mentor for Ruby.

Communication in science: Through the Professional Development courses offered by the MSTP, Ruby has received formal instruction on scientific analysis of literature, writing and presenting, and networking skills. Ruby will actively gain oral communication experiences through lab meetings, journal clubs, poster presentations at local (CNUP and MSTP retreats) and national/international meetings (e.g., SfN, and IASP), and work-in-progress meetings through the Pittsburgh Center for Pain Research Pain. She will also learn skills in networking and establishing collaborations at these extramural meetings. To ensure that Ruby actively develops her written communication skills, Ruby will be expected to write or contribute written work from the lab in the form of book chapters, review articles, or primary papers. Ruby will develop her knowledge of and critically analyze the scientific literature through her active participation in journal clubs as well as assisting me review manuscripts several times per year. Lastly, Ruby will receive experience assuming a teaching role to undergraduate students within the lab.

Feedback: Ruby will meet with me daily through informal, impromptu interactions to briefly update me on his experimental progress. Weekly meetings will entail overall research progress, data analysis and literature discussions. More importantly, Ruby will benefit from the more serious meetings that I will schedule with her several times per year to provide constructive feedback for her scientific development as a graduate student.

C.II Environment

Ross Laboratory*:* My personal philosophy is that graduate advisors demonstrate unwavering support for their trainees. Science offers the thrill of discovery. For students early in their careers, I create a supportive environment to keep students motivated when encountering hardships. I believe that inspiring students in this manner builds their confidence to pursue visionary experiments that lead to breakthrough discoveries.

Koerber Laboratory: With forty years of experience in graduate training, I provide steady guidance to students and endeavor to share my expertise with trainees so that they can develop into rigorous scientists.

PCPR: Ruby will receive outstanding training as a member of the Pittsburgh Center for Pain Research (PCPR). Our Pain Center is made up of eight core faculty along with their trainees. These faculty members, in addition to Rick and myself include; Michael Gold, Brian Davis, Kathy Albers, Rebecca Seal and Brad Taylor. Most of these investigators, including Rick and myself have contiguous space on the 14th floor of the Biomedical Sciences Tower (BST). Center faculty and trainees meet weekly for journal club and twice monthly for work-in-progress talks. Ruby will present in both of these forums every year. In addition, the PCPR has an outstanding monthly seminar series in which leaders in the field of pain are invited to speak, and Ruby will have lunch with each of these speakers. We also offer two popular graduate courses, *Mechanisms and Clinical Presentation of Pain* as well as *Pain Models: Rationale, Testing and Interpretation*, which Ruby will take. Finally, the PCPR faculty are collegial and highly interactive, which is fostered by being in contiguous space and virtually daily interactions, including an annual skating party and numerous happy hours. For more details about the PCPR and its activities, see *http://pcpr.pitt.edu.*

CNUP: Ruby will be completing her thesis within the Center for Neuroscience at the University of Pittsburgh (CNUP), a well-established graduate program with over 100 faculty and 80 students spanning 20 departments across The University of Pittsburgh and Carnegie Mellon. Activities include an annual three-day retreat in the fall as well as Brain Day (local retreat) in the spring.

Physical environment: The Koerber and Ross labs are on the same floor as 5 other members of the Pain Center. On the floor there is also a conference room, shared offices for trainees, and a kitchen/eating area, locker rooms with showers, and two large relaxation areas with outstanding views of the city (east and west). These spaces foster wellbeing and create an ideal environment for learning and scientific exchange. Every lunchtime can be an impromptu meeting or think-tank between scientists from all backgrounds and at all levels. Ruby regularly interacts with these investigators and their students and postdocs, gaining valuable feedback on her project as well as mentoring advice.

C.III Research Facilities

Ruby will have access to all the equipment and facilities necessary to carry out her project.

The **Ross lab** occupies approximately 1,000 sq. ft. of space on the 14th floor of the Biomedical Science Tower (BST). The lab owns two electrophysiology rigs fully equipped with the following: air table, upright microscopes (Olympus), CED power1401-3 interface with associated Signal 5 software, an Axoclamp 2B microelectrode clamp, AC & DC amplifier, Sutter or Scientifica micromanipulators, temperature control units, XM10-IR Olympus CCD camera, Lumencor SOLA LED light source. We have a Leica CM1950 cryostat, Leica vibratome, small animal sterotaxic, dissection hood, Leica dissecting microscope, an RNAscope FISH oven, and two epifluorescent microscopes, -20C and -80C freezers, tissue cell culture room and perfusion room. Shared equipment on our floor includes a Nikon A1R fast-scanning confocal microscope (housed in the Ross Lab), cold room, a warm room, cell culture facilities, sterilization apparatus, dry ice, liquid nitrogen, and ultracentrifuges.

The **Koerber lab** occupies 1200 sq. ft of space on the 14th floor of the Biomedical Science Tower.

Electrophysiology: The Koerber lab has 3 complete electrophysiology recording rigs with each one equipped with an isolation platform, an upright fluorescent microscope (2 Leicas, 1 Olympus), One rig has an Axon Instruments Digadata 1322A with Axoclamp software, An Olympus BX51WI upright fluorescent microscope with a Sutter Instr. DG-4 light source with computer controlled shutters and multiple cubes for emitting blue and green light for activation of channel Rhodopsin and Archrhodopsin and a Hamamatsu ORCA-ER camera. The others have CED mini 1401 digital interfaces with appropriate software including Spike and Signal, all have an Axopatch 200B or Axoclamp 2B electrometer, a Narishige or Siskiyou 4 axis micromanipulator, appropriate AC & DC amplifiers, A computer controlled 50mW blue laser (473 nM) (Laserglow, Toronto) for optogenetic activation of cutaneous afferents. Two Yale University thermal stimulator and controller. Two Aurora Scientific mechanical

stimulators controlled by DACs from the CED 1401s. Each setup also has oscilloscopes, computers and monitors. I also have two dissecting stations complete with Zeiss Stemi 2000 dissecting microscopes with digital cameras and monitors for instructional purposes.

Histology and molecular biology: Leica CM 3050 cryostat, a Micron sliding microtome, Lica DMR fluorescent microscope, a RITEGA 2000B digital camera and computer/ monitor and Q-imaging software. Bio-Rad CFX Connect Real-Time PCR System, Thermal cycler, vacufuge, refrigerated centrifuge, Polytron homogenizer, thermomixer, spectrometer, western blotting and PAGE gel apparatuses, Gel dryer.

Animal Facilities: Mice are housed in a recently constructed vivarium facility in Biomedical Science Towers 3 within the University of Pittsburgh Division of Laboratory Animal Resources (DLAR). The facility has state of the art caging systems, rooms for quarantine and for breeding specialized populations, and a suite for BSL-2 work. This facility is fully accredited by the American Association for Accreditation of Laboratory Animal Care and is connected by a bridge to the BST where the Ross laboratory is located. This facility is professionally staffed by a manager and animal care technicians who provide basic animal care and is overseen by a primary veterinarian, Dr. Beth Ahner, and a back-up veterinarian, Dr. Edwin Klein. The Ross lab maintains a large breeding colony (including the Oprm1-cre allele to be used in this study) that is maintained by a full-time staff member.

Animal Behavior Core Adjoining the animal housing area is an animal behavioral core with full-time support staff. This core facility contains all of the equipment for a very large number of pain behavioral assays including Hargreave's apparatus, Von Frey testing, and high-speed video cameras that are required for this proposal.

Computing: Ruby has her own personal desktop that is networked behind the University of Pittsburgh firewall and are supported by dedicated IT staff in the Department of Neurobiology.

D. Number of Fellows/Trainees to be Supervised During the Fellowship

Dr. Sarah Ross: The Ross lab has two graduate students (Catherine Ruff and Eileen Nguyen) and two post-doctoral fellows (Taylor Sheahan, and Kelly Smith), as well as a third postdoc (Charles Warwick), who works jointly in the Koerber and Ross labs.

Dr. H. Richard Koerber: The Koeber lab has one graduate student (Joseph Salsovik) and one postdoctoral fellow (Charles Warwick), who works jointly in the Koerber and Ross labs.

E. Applicant's Qualifications and Potential for a Research Career

Co-Sponsor: Sarah E. Ross, PhD.; Associate Professor of Neurobiology

Ruby is a truly exceptional student. I would easily rank her in the top 1% of students that I have seen at the University of Pittsburgh and at Harvard Medical School. She knows exactly where she wants to go in life and is very focused on achieving her goals. I am positive that Ruby is PI material, and I committed to giving her the training and opportunities that she needs to help launch her towards the goal of becoming a physician scientist.

Ruby worked for four years as an undergraduate and an additional year as a research technician with Bart De Jonghe and Matthew Hayes (University of Pennsylvania) studying the circuitry in the brainstem that mediates chemotherapy-induced nausea. Her work contributed to five peer-reviewed publications, including a first-author publication, as well as a study in *Cell* on the role of the parabrachial nucleus in suppressing hunger in the context of pain. As attested by her letters of recommendation, Ruby was one of the brightest and hardest working students at Penn.

Ruby selected the University of Pittsburgh for her MD-PhD training because of the strength of the Pittsburgh Center for Pain Research and her desire to study the brainstem circuitry that modulates pain. I was thrilled when she selected my lab for graduate training because I could feel the spark and passion that Ruby has for research.

In my lab, I encourage my trainees to work on whatever scientific problem they find most exciting and to develop their own thesis projects independently. Towards this end, Ruby and I met multiple times over this past school year while Ruby was completing her second year of medical school to discuss her ideas for a thesis project. Ruby is drawn to the circuitry of the RVM because of her familiarity with the brainstem. Through her previous research experiences and fruitful discussions with multiple members of my lab, Ruby developed an interest in MOR signaling in the RVM. The approaches detailed in Ruby's proposal were inspired by the preliminary work of Eileen Nguyen (a second-year MD-PhD student in my lab) who had made some exciting discoveries through her research on putative OFF-cells and developed surgical strategies to selectively target the RVM with precise stereotaxic injections. Just at this time, we had imported the Oprm1-cre allele (developed by the Palmiter lab)

for another project. When these factors aligned, Ruby recognized an opportunity to use this new genetic tool to advance the field of pain by studying MOR+ RVM spinal projections in the RVM (putative ON-cells), and selected this project for dissertation work. After meeting with me several times to discuss her aims, Ruby wrote the entire NRSA application on her own, and then made minor modifications based on feedback from Rick Koerber, Michael Gold, Bart De Jonghe, several trainees in my lab, and myself.

Since Ruby is an MSTP student, she has outlined experiments that she can accomplish within a 4-year framework. I am confident that Ruby will make rapid progress towards these aims because she is a very hard worker with a lot of experience and a history of highly productive endeavors, including numerous papers.

Rick and I are very excited about Ruby's proposal because it has all the features that are appealing to a mentor who wants to ensure success for their trainees: it is a very *safe project* with lots of *opportunity to learn* new approaches together with huge *potential for big discoveries*. The project is a feasible yet compelling learning opportunity because all of the experiments proposed therein, while challenging, involve techniques that are currently working in our labs — Eileen Nguyen is currently using AAVr strategies to retrogradely label RVM projection neurons that target the spinal cord and cannulas implanted into the RVM to deliver CNO **(Letter of Support)**; Kelly Smith, a postdoc in my lab with years of electrophysiology experience from Brett Graham's lab, is currently performing pharmacological analysis and post-hoc reconstruction of spinal cord neurons recorded in slice **(Letter of Support)**; finally, Taylor Sheahan, a postdoc with years of behavioral experience from Rob Gereau's lab is currently analyzing the behavioral consequences of manipulating neurons in the spinal cord **(Letter of Support)**. Thus, in addition to mentorship from me and Rick Koerber, she will also get direct, hands-on training as she learns an array of news skills including anatomy, physiology, and behavior.

Ruby's project is exciting in terms of its research potential because she is studying a very important population — putative ON-neurons — in a new way, using tools and strategies that will allow her to visualize (aim 1), manipulate in vitro (aim 2) and in vivo (aim 3) the RVM neurons that send descending projections to the spinal cord to facilitate pain. If her hypothesis is correct (confirming the current model of ON-cell modulation of pain), her experiments will have provided a new level of granularity to the underlying circuitry; if her hypothesis is incorrect (challenging the current model of ON-cell model), her discoveries have the potential to change textbook dogma, eventually leading us towards more accurate models of descending modulation.

I am delighted to provide mentorship to Ruby as she undertakes the experiments enumerated in this proposal and I am committed to her success. As evidence of this, even though I am still relatively junior, my mentorship of MSTP students has already been recognized by virtue of being short-listed for the *William E. Brown Outstanding MSTP Mentor Award*. Thus, I believe that with my guidance and support, Ruby has the potential to develop into a highly successful physician scientist who will have a positive impact on science, medicine, and the community around her. Ruby has all of the attributes, personal and professional, of a first-rate scientist and I give her my enthusiastic support for this NRSA award, which would provide her with the recognition that would help launch her career.

Co-Sponsor: H. Richard Koerber, PhD; Professor of Neurobiology

To characterize Ruby Holland as a whip-smart student and a diligent worker would be an understatement. As an undergraduate at Penn, she had a first author paper and contributed to four others. Her letters say it all: Ruby is outstanding.

Understanding the specific circuitry in the spinal cord that is modulated by ON-cells remains a major gap in the field. I believe that the experiments that Ruby has articulated in Aim 2 (which I will supervise) will begin to address this gap by identifying the cell type(s) that receive direct input from MOR-expressing RVM neurons that project to the dorsal horn and by characterizing this input through optogenetic approaches. With my 40+ years of expertise in electrophysiology and anatomy in the dorsal horn, I am extremely well-suited to supervise this aspect of her training.

I am delighted to co-sponsor Ruby for this proposal, which I find very interesting, and to provide her with mentorship. Already, Ruby has shown great potential for a successful career as an independent physician scientist and has surrounded herself with a mentoring team, including her physician-scientist mentor Dr. Ajay Wasan, and an environment with the proper expertise, support, and dedication to her success. Ruby is a highly meritorious candidate for this F-award.

University of Pittsburgh

School of Medicine
Department of Neurobiology
Pittsburgh Center for Pain Research

July 24, 2019

Re: NRSA Support Letter

Dear Ruby,

I am happy to provide guidance and support for any experiments involving fluorescent in situ hybridization. As you may know, I have experience from my time as a postbaccalaureate fellow at the NIH and as a graduate student in Sarah's lab performing and perfecting this technique. Also, I have successfully performed RNAscope experiments in the brain and stereotaxic injections into the RVM. I would be happy to help you with your experiments involving the RVM.

I look forward into the learning about the insights you'll make into RVM circuitry and have no doubt that you will be able to successfully apply stereotaxic injections of virus and fluorescent in situ hybridization to this project. Good luck with your application!

Sincerely,

Eileen Nguyen
MD/PhD Student
University of Pittsburgh

University of Pittsburgh

School of Medicine
Department of Neurobiology
Pittsburgh Center for Pain Research

200 Lothrop St., BSTWR E1402
Pittsburgh, PA 15213
Tel: (412) 648-9590
Fax: (412) 648-1441
E-mail: ksmith5@pitt.edu

Kelly Smith
Post-Doctoral Fellow

July 22, 2019

Re: F31 Support Letter

Dear Ruby,

It would be my pleasure to offer support in your proposed experiments. Electrophysiology will be a great technique to add to your experimental repertoire and I am excited to contribute to this aspect of your project.

My graduate work in Dr Brett Grahams lab at the University of Newcastle (Australia) gave me extensive experience in whole cell patch clamp electrophysiology in spinal cord slices and I will be happy to provide support during these experiments. Specifically, my graduate work used whole cell patch clamp electrophysiology in combination with pharmacology, optogenetics and *post hoc* analysis of neuron morphology to understand sensory processing in the spinal cord. I have continued using these techniques in Dr Ross's lab and I am very excited to be able to offer you training and support in these techniques as well as data analysis and interpretation.

I am really excited to see you project progress, I think it will give us valuable insight into how sensory information is processed within the central nervous system and will be the first work to show specific connectivity of MOR+ RVM neurons in the dorsal horn. Further, the experimental techniques proposed in your grant will be valuable as you continue further into your career.

Working with you so far has been an absolute pleasure and I look forward to helping you throughout your degree.

Sincerely,

Kelly Smith, PhD
Pittsburgh Centre for Pain Research
University of Pittsburgh

University of Pittsburgh

School of Medicine
Department of Neurobiology
Pittsburgh Center for Pain Research

200 Lothrop St., BST E1402
Pittsburgh, PA 15213
Tel: (412) 648-8182
Fax: (412) 648-1441
E-mail: tayler.sheahan@pitt.edu

Tayler Sheahan
Postdoctoral Scholar

July 29, 2019

Re: NRSA Support Letter

Dear Ruby,

I am delighted to assist with rodent injury models and behavioral testing, as well as chemogenetic approaches proposed in your fellowship. Moreover, I can also provide mentorship to you as you develop your skills in this fellowship. I think working together will be a positive and productive experience.

Based on your progress thus far, it is clear that you are a skilled scientist and possess the patience and dedication required to succeed at behavioral experiments. As you know, from my graduate work in Rob Gereau's lab at Washington University in St. Louis, I have extensive experience in and routinely use several of the techniques you propose in your grant including mouse pain models and pain behavioral assays. I've also been using chemogenetic approaches regularly since joining the Ross Lab. This will provide us with many opportunities for you to continue to learn these skills from me. Should you encounter challenges with behavioral assay data analysis and interpretation, I am happy to assist you in trouble-shooting.

After reading your NRSA proposal, I am excited to discuss the discoveries you make regarding the descending modulation of pain mediated by mu opioid-expressing RVM spinal projections, as these will certainly move the field forward. Importantly, I believe that learning rodent behavioral testing and chemogenetic strategies will allow you to continue to ask meaningful questions about spinal circuitry as your progress in your career.

Sincerely,

Tayler Sheahan

Tayler Sheahan, PhD
Pittsburgh Center for Pain Research | Department of Neurobiology
University of Pittsburgh

University of Pittsburgh

School of Medicine
Department of Anesthesiology

UPMC Pain Medicine
5750 Centre Avenue, Suite 400
Pittsburgh, PA 15206
412-665-8030
Fax: 412-665-8033
wasanad@upmc.edu
www.pain.pitt.edu

Ajay D. Wasan, MD, MSc
Professor of Anesthesiology and Psychiatry
Vice Chair of Pain Medicine

8/2/19

Ruby A. Holland
MSTP Student
E1401 Biomedical Science Tower
University of Pittsburgh

Dear Ruby,

I would be pleased to serve as a physician-scientist mentor for your career development and as a clinical consultant for your F31 on descending pain modulation by the rostral ventromedial medulla with Dr. Sarah Ross. I have great respect for Sarah's scientific work and her mentoring abilities, and I think that your project has significant promise.

As a physician-scientist and the Vice Chair for Pain Medicine in the Department of Anesthesiology and Perioperative Medicine at UPMC, I have successfully integrated a career involving clinical trials and medical informatics to study pain treatment outcomes while specializing clinically in the care of patients with chronic pain. I look forward to seeing your project on the RVM develop, as it will have a major impact on how we understand chronic pain. Additionally, I am more than happy to offer advice and guidance on your career trajectory, as I know you plan to pursue a similar path combining neuroscience research and clinical practice in anesthesiology. I look forward to having you observe in the clinic whenever it is convenient for you. I also look forward to seeing your project develop. Please feel free to contact me at any time should you have any questions about treatment and mechanisms of chronic pain as your work progresses.

Sincerely,

A. D. Wasan

HHMI
HOWARD HUGHES MEDICAL INSTITUTE

19 July 2019

Dear Ms. Holland,

I write this letter of support to confirm that I recently provided the Ross lab with the Oprm1-Cre mice that were generated in my lab. These mice will be a very useful tool to visualize and manipulate the neurons that express MOR, thereby enabling the studies described in your F30 application, studying the role of mu-opioid receptor expressing neurons in the RVM in the modulation of nociception. In this mouse, an Oprm1-IRES-Cre cassette is knocked into the first coding exon. Although this genetic tool is not yet described in a publication, we have nevertheless validated correct targeting by Southern blotting and the expression pattern in the parabrachial nucleus resembles that determine by Allen Institute *in situ* hybridization. Thus, I anticipate that the Oprm1-Cre mice will allow you to visualize and manipulate MOR-expressing neurons in the RVM, which are likely to be involved in descending pain modulation.

I wish you success in your grant application.

Sincerely,

Richard D. Palmiter, Ph.D.
Investigator
Howard Hughes Medical Institute

University of Washington
1959 N.E. Pacific Street, Health Science Building, J-661
Seattle, Washington 98195-7370
206.543.6064 • Fax 206.543.0858

Additional Educational Information (by Richard Steinman MD PhD, Director MSTP, Steinman@pitt.edu)

A. University of Pittsburgh-Carnegie Mellon University MSTP Structure. MSTP students in Pittsburgh complete a MSTP-specific enrichment curriculum beyond the standard courses in medical and graduate school. This consists of 3 summer research rotations, 3 summer professional development courses, a 3-semester weekly journal club featuring research papers consistent with the coincident SOM curriculum, a 4-week case-based ethics course, a monthly program-wide workshop, a 40-week longitudinal clinical clerkship (1/2 day/week) during the graduate years, a 11 day Junior Hospitalist service and yearly special events such as the two-day MSTP Scientific Retreat.

B. Laboratory Research Rotations. Research rotations begin the summer prior to the start of medical school. In addition to developing manuscripts and presenting at scientific meetings based on their rotation results, all students turn in a written scientific report that is reviewed by MSTP leadership and present their work at the annual MSTP Scientific Retreat. The choice of thesis laboratories by students is informed by their rotation history and by discussion with their individual Career Advisors (who follow them longitudinally in the program).

C. Professional Development. Students take three successive 10-week long Professional Development Courses during summers prior to starting graduate school. The first course (PD1) focuses on scientific writing and introduces students to biomedical software and to key methods used by different disciplines to approach scientific problems. The PD2 course focuses on scientific design and career development strategies, with particular emphasis on reproducibility and biostatistics. The PD3 course focuses on grant review and writing.

D. Training in Reproducibility in Science. The PD2 course focuses on optimizing reproducibility of findings, to power experiments, and analyze data with appropriate statistical testing. Topics for classes include problems arising from non-reproducible work, optimal experimental and reagent documentation and handling, the ARRIVE guidelines for animal work, measurement validity and sources of error, robust hypothesis testing, and a series of sessions on biostatistics including customized problem solving tied to student data.

E. Biomedical and ethical expertise. During MS1 and 2 years, students build biomedical knowledge through a 3-semester MSTP literature review course in which student's present papers after formal consultation with local faculty experts in the field of that paper. During the G1 year of graduate school, MSTP students take a month-long, weekly, case-based research ethics course. Throughout both medical school and graduate school, all MSTP students meet monthly for student-arranged seminars that pose scientific, logistical, clinical and/or ethical dilemmas. These workshops are presented by students and/or guest faculty experts.

F. Clinical and Research Integration. This is a central focus to better model the physician scientist career.

F.1 Clinical Activities during the Research Years. Prior to starting graduate school, all MSTP students complete 8 weeks of required clinical core clerkships. This front-loads requirements once students re-enter medical school post thesis and enables research engagement in MS3 and 4. MSTP students are required to complete a (credited) minimum of two 20-week long Longitudinal Clinical Clerkships during graduate school. For each LCC, students spend a half day per week with a clinician scientist receive one-on-one clinical mentoring by a clinician scientist in an area of interest chosen by the student with guidance from the MSTP LCC director, Paul Monga, MD. Student objectives for the LCC and write-ups at the end are reviewed by MSTP leadership.

F.2 Transition from Graduate to Clinical Years. After the student's thesis defense but prior to returning to medical school, students take the MSTP required Junior Hospitalist Service, also known as the LCC3. A master clinician mentors the returning students for 11 days as they examine, discuss, diagnose and plan treatment for surrogate patients presenting with common outpatient or inpatient ailments.

F.3 Research during Clinical Years. Our students continue their research focus after re-entry to medical school generally in four ways: (1) MS3 and MS4 students continue to plan and execute MSTP Workshops that feature research topics and research challenges to be discussed with MSTP peers. (2) Students complete formal reflective and goal-oriented self-assessment evaluations during twice-yearly Career Advisor meetings. (3) Students average 2.8 new publications during the MS3 and MS4 years (at least one first authored), averaging 5-7 papers upon graduation. (4) Most students elect to take 1-2 Research Elective months during their MS4 year to extend findings of thesis work and/or to build skillsets in a translational area. Another novel feature of our MSTP, the Postdoctoral Fellowship, provides support for 5 months of postdoctoral research prior to residency for MSTP students graduating in December (25% of graduates in recent years). Applications

address research hypotheses and aims, career development aims, planned deliverables, mentor fit and intellectual goals.

G. Monitoring and Evaluating Student Progress. Prior to matriculation, the Program Director assigns each new student a Career Advisor based on matching research interests who help orient and guide the students throughout their careers. Most of a trainee's time in the graduate program is spent in research training under the guidance of their research mentors, program leadership, and eventually their doctoral dissertation committee. To customize advice and resource allocation, all MSTP students complete and share *individual development plans* with the Director and with their Career Advisor. The form allows students to identify specific skills that they want to develop, set technical, intellectual and professional goals, and identify how goals will be achieved and measured. Resources to reach goals and obstacles that could compromise success are enumerated and discussed. Progress toward goals is regularly reviewed with the Advisor and new goals are set.

H. Career Counseling. To better reflect the student's educational experience to prospective residency programs, the MSTP creates an executive summary which describes student evaluations, honors, presentations and participation in the combined degree training, rewarding students who altruistically give their time and demonstrate prowess in working in groups. Six months to one year before completing their doctoral program, students meet with the Program Director and the Career Advisory committee to discuss postgraduate training, residencies, fellowships, and faculty positions and non-academic based positions. Many of the faculty are MD/PhDs and are capable of participating in career planning for third- and fourth-year medical students.

I. Program Duration and Outcomes. Over the past 6 years, our time from enrollment to graduation has averaged 7.6 years (8.1 years in the prior 5-year period). The Pittsburgh MSTP has 170 alumnae. 89% of graduates from the past 15 years are in the academic pipeline (either still in training or in academic positions). Senior MSTPs in 2018 averaged 9.6 papers with 4.5 first-authored (median: 10 total, 4.5 first-authored).

J. Ruby Holland is a superb member of our MSTP who matriculated into the MSTP program in June 2017 and is in her G1 year as a graduate student in the Center for Neuroscience at the University of Pittsburgh (CNUP) Graduate Program. She is pursuing her doctorate in the laboratory of Dr. Sarah Ross, a well-accomplished expert in central nervous system mechanisms of sensory integration, particularly pain and itch. Ruby's outstanding performance in the MSTP to date is described in the **Letter of Recommendation** from the MSTP Director.

Ruby completed her MS1 and MS2 coursework and passed USLME Step 1 on April 2019. She completed the MSTP Professional Development courses, 2 laboratory rotations (1 in the laboratory of Dr. Ross), 3 Research Basis of Medical Knowledge courses, and 4-week clinical rotations in Obstetrics/Gynecology and Pediatric Internal Medicine prior to beginning graduate training. Ruby formally entered the CNUP Graduate Program in July 2019. The coursework Ruby must complete for the CNUP consists of a total of 4 course requirements including Neurobiology Seminar Series, Journal Club, and six hours of electives, for which Ruby plans to take Molecular Pharmacology and Neurobiology of Disease, and 1 semester of teaching assistant experience in an undergraduate neuroscience course. MSTP students are excused from the foundational Neurobiology courses, Grant Writing, and Ethics course requirements because of material covered through comparable courses in the MSTP/medical school curriculum.

The CNUP has several program milestones with timelines adjusted for expeditious completion by MSTP students: Ruby will complete her Comprehensive Exam in August 2020, where she will prepare an R01-style grant and defend the proposal in front of pre-selected training faculty. Following this, Ruby will formulate her thesis committee and will propose the research aims that she will complete to fulfill her PhD in December 2020 to her dissertation committee. As a formal PhD candidate, Ruby's progress will be monitored at regular, biannual committee meetings as well as in biannual MSTP Career Advisor Meetings where her updated Individualized Development Plans will be reviewed. Ruby expects to complete Longitudinal Clinical Clerkships in anesthesiology subspecialties during graduate school. She expects to defend her PhD and return to medical school in September 2023, putting her on track to complete her remaining medical school clerkships and graduate from our program in May 2025. For the terms of the fellowship proposed, Ruby plans to complete an additional 48 months of research, followed by clinical work for 20 months (of which 0 months would be under the purview of this F30)**.** Should Ruby elect the route in which she graduates medical school in December of 2024, she will then undertake the 5-month MSTP Postdoctoral Fellowship which is not included in the time of covered support requested in the current application.

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 $CaCl_2$, 10 $MgSO_4$, 1.25 NaH_2PO_4,26 $NaHCO_3$, 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, rhe 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[52,53]*:* 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^{cre} 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[34], 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^{cre} 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^{cre} mice needed.

Aim 2A: A power analysis was performed based on previous experiments performing optogenetic stimulation and slice electrophysiology[45]. 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^{cre} 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[39]. 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^{cre} mice.

Aim 3: A power analysis was performed based on previous work from our lab using chemogenetics to manipulate pain behaviors[55]. 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^{cre} mice will be used per group for a total of 36 Oprm1^{cre} mice.

Total mice used:

Oprm1^{cre} mice: 6 (Aim 1) + 4 (Aim 2A) + 20 (Aim 2B) + 36 (Aim 3) = **66 Oprm1^{cre} 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.

Select Agent Research

The proposed experiments involve the use of **tetrodotoxin (TTX),** a select agent listed by the U.S. Department of Agriculture (USDA) and Department of Health and Human Services (DHHS)/Center for Disease Control and Prevention (CDC). We possess **less than 100 mg** of TTX at any time, which is exempt from regulation according to the USDA. Possession and use of exempt quantities of Select Agents are monitored by the responsible biosafety officers within the Department of Environmental Health and Safety (EH&S) within the University of Pittsburgh. Our lab is an approved facility by EH&S to handle, store and use TTX.

We will be using TTX at the University of Pittsburgh, in the Department of Neurobiology, on the 14th floor of the Biomedical Science Tower. We handle TTX with extreme care: we have a dedicated person who orders, stores, logs and tracks TTX for the entire lab. All laboratory members involved in research with select agents have received specific training in laboratory safety and the proper use and disposal of these agents. Each investigator will only obtain an aliquot of TTX for research at a time and sign for the aliquot. Aliquots are prepared under a fume hood with appropriate personal protective equipment, including gloves, lab coat, and mask. For disposal of used or excess TTX, the appropriate deactivation procedure will be performed, witnessed by an EH&S representative, before being disposed as hazardous waste. This proposal does not involve the transfer of TTX; however, for transfer of Select Agents, federal guidelines are followed, including filing of USDA import permits (where applicable), CDC/APHIS forms and other permits (where applicable).

Resource Sharing Plan

We are committed to the rapid dissemination of knowledge generated from the work in this proposal. Protocols, animals, and resources will be made available to other researchers upon request. Data generated during the training period will be shared through posters and/or presentations at the Pittsburgh Center for Pain Research (PCPR) work-in-progress meetings, Center for Neuroscience at the University of Pittsburgh (CNUP) annual retreats, as well as national and international meetings such as the Society for Neuroscience and the International Association for the Study of Pain.

All manuscripts will contain complete methodological details to enable others to fully replicate the experimental protocols, and raw data will be made available after publication to those requesting such information. Our goal is to publish our data by completing experiments outlined in this application to provide valuable insight into the functional role of mu-opioid receptor-expressing neurons in the RVM on pain facilitation.

Applications for Concurrent Support

Ruby Holland recently received a diversity supplement, which is part of Dr. Ross's active R01AR063772 grant. The research plan for the supplement builds on studies of the parent grant that investigate the mechanisms through which KOR signaling modulates itch. The NIH diversity supplement totals $238,465 for the 09/01/2019–01/31/2023 period. In the event that the F31 is funded, the applicant will relinquish the diversity supplement.

PHS Assignment Request Form

OMB Number: 0925-0001
Expiration Date: 3/31/2020

Funding Opportunity Number: PA-19-195

Funding Opportunity Title: Ruth L. Kirschstein National Research Service Award (NRSA) Individual Predoctoral Fellowship (Parent F31)

Awarding Component Assignment Request *(optional)*

If you have a preference for an awarding component (e.g., NIH Institute/Center) assignment, use the link below to identify the appropriate short abbreviation and enter it below. All requests will be considered; however, assignment requests cannot always be honored.

Awarding Components: https://grants.nih.gov/grants/phs_assignment_information.htm#AwardingComponents

	First Choice	Second Choice	Third Choice
Assign to Awarding Component:	NINDS		
Do Not Assign to Awarding Component:			

Study Section Assignment Request *(optional)*

If you have a preference for study section assignment, use the link below to identify the appropriate study section (e.g., NIH Scientific Review Group or Special Emphasis Panel) and enter it below. Remove all hyphens, parentheses, and spaces. All requests will be considered; however, assignment requests cannot always be honored.

Study Sections: https://grants.nih.gov/grants/phs_assignment_information.htm#StudySection

	First Choice	Second Choice	Third Choice
Assign to Study Section: *Only 20 characters allowed*			
Do Not Assign to Study Section: *Only 20 characters allowed*			

PHS Assignment Request Form

List Individuals who should not review your application and why *(optional)* *Only 1000 characters allowed*

Identify Scientific areas of expertise needed to review your application *(optional)*
Note: Please do not provide names of individuals

	1	2	3	4	5
Expertise: *Only 40 characters allowed*					

OMB Number: 0925-0001
Expiration Date: 03/31/2020

PHS Human Subjects and Clinical Trials Information

Please complete the human subjects section of the Research & Related Other Project Information form prior to completing this form.

The following items are taken from the Research & Related Other Project Information form and displayed here for your reference. Any changes to these fields must be made on the Research & Related Other Project Information form and may impact the data items you are required to complete on this form.

Are Human Subjects Involved	[ ] Yes [X] No
Is this Study Exempt from Federal Regulations?	[ ] Yes [ ] No
Exemption Number:	[ ]1 [ ]2 [ ]3 [ ]4 [ ]5 [ ]6 [ ]7 [ ]8

If No to Human Subjects

Does the proposed research involve human specimens and/or data? [ ] Yes [X] No

If Yes, provide an explanation of why the application does not involve human subjects research.

Skip the rest of the PHS Human Subjects and Clinical Trials Information Form.

If Yes to Human Subjects

Add a record for each proposed Human Subject Study by selecting 'Add New Study' or 'Add New Delayed Onset Study' as appropriate. Delayed onset studies are those for which there is no well-defined plan for human subject involvement at the time of submission, per agency policies on Delayed Onset Studies. For delayed onset studies, you will provide the study name and a justification for omission of human subjects study information.

Other Requested Information

Study Record(s)

Delayed Onset Study(ies)