DEFINING PHYSIOLOGICAL HETEROGENEITY AND OPIOID RECEPTOR REGULATION WITHIN THE VENTROLATERAL PERIAQUEDUCTAL GRAY PROVIDES A FRAMEWORK TO BETTER UNDERSTAND DESCENDING PAIN MODULATION

by

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A DISSERTATION

Presented to the Neuroscience Graduate Program at the Vollum Institute and the Oregon Health & Science University School of Medicine in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

OCTOBER 12, 2021

School of Medicine

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List of Abbreviations

aCSF	Artificial cerebral spinal fluid
ATP	Adenosine triphosphate
AMPAR	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	Analysis of variance
CFA	Complete Freund's Adjuvant
CNS	Central nervous system
COX	Cyclooxygenase
DAMGO	[D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin
DLF	Dorsolateral Funiculus
DOR	Delta opioid receptor
DMSO	Dimethyl sulfoxide
EAA	Excitatory amino acids
ED50	Half maximal dose
E/I	Excitatory/Inhibitory
eIPSC	Evoked inhibitory postsynaptic current
GABA	Gamma-aminoutyric acid
GAD	Glutamic acid decarboxylase
GBZ	Gabazine (SR-95531)
GEF	Guanine nucleotide exchange factors
GIRK	G protein-coupled inwardly-rectifying potassium channel
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate

HP	Hind paw
IPSC	Inhibitory postsynaptic current
ME	[Met^5]-enkephalin
mIPSC	Miniature inhibitory postsynaptic current
MOR	Mu opioid receptor
NDMA	N-methyl-D-aspartate
PAG	Periaqueductal gray
PNS	Peripheral nervous system
RGS	Regulators of G protein signaling
RVM	Rostral ventromedial medulla
SD	Standard deviation of the mean
SEM	Standard error of the mean
THIP	4, 5, 6, 7-Tetrahydroisoxazolo[5,4-c]pyridine 3-ol
vPAG	Ventral periaqueductal gray
vlPAG	Ventrolateral periaqueductal gray

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Acknowledgements

First and foremost, I would like to thank my advisor, Dr. Susan Ingram. It has been a transformative experience to learn how to think as a scientist and navigate academia with all of your perspective and expertise. Thank you for the countless hours spent at your office white board, lab meetings, poster sessions, etc. walking through the weeds, discussing the interesting and messy details of the cellular and circuit mechanisms we hope to illuminate. Thank you for encouraging me to broaden my scientific network and facilitating important experiences to discuss my work through the many conferences we attended. Last, thank you for providing me with the opportunity to ask the questions that I was most interested in answering and for helping me through the ambiguity.

I would also like to thank my current and former lab mates Courtney Bouchet, Sam Rios, Minghua Li, and Katie Suchland; I learned so much more from discussing our projects together and your support and friendship has been invaluable. A special thank you to Katie Suchland who was a wonderful friend when I needed one most.

Thank you to my committee: Drs. John Williams, Mary Heinricher, Tianyi Mao, and Michael Bruchas. Your collective time and efforts have shaped my approach to research. In particular, thank you to John Williams, for sharing your office rig and teaching me to patch (with the help of Dr. Erica Levitt); Mary Heinricher, for answering countless questions about descending modulation details and the pep-talk before my INRC talk; Tianyi Mao for planting the seed of interest in intrinsic firing properties; and Michael Bruchas for your interesting, thought-provoking questions during committee meetings.

Additionally, thank you to the Vollum/NGP leadership for cultivating an institute- and program-wide culture that values trainee success. In particular, thank you to Dr. Gary Westbrook for meeting with me 6 years ago to discuss why I wanted to pursue neuroscience research. Your reliable approach to supporting and advocating for trainee success has greatly benefitted my experience as a trainee with a disability, which can often complicate access to success. Thank you to Dr. Kelly Monk and Jessica Parks for setting another amazing example of how to ensure trainee support.

Graduate school also cultivated countless stellar friendships. Thank you to Emily Leff and Danielle Mathieson for all of the good times, laughs, and support. Thank you to Courtney Bouchet, and Sweta Adhikary for discussing all things opioids, electrophysiology, and for the many needed sunshine breaks in the courtyard. Thank you to my NGP class for reliably celebrating the ups and commiserating over the downs. Thank you to more senior NGP students (Alec Condon, Chris Vaaga, Kathleen Beeson, and others) for making this goal seem attainable, for all of your help along the way, and for the many interesting little discussions that reinforced my love for electrophysiology.

My educational support system was augmented by many stellar humans along my educational journey that provided additional support and encouragement. In chronological order…Thank you to Mike Butcher for the challenging labs that forced me to think critically and creatively early on, exposing my interest in designing experiments. Thank you, Diane Varga and Leisa Runyan, for being my first exposure to impressive women in science, learning from you both catalyzed my ambitions. Thank you to Dr. Kerry Thompson for discussing the neurobiological underpinnings of dystonia and supporting my independent research abroad. Thank you to the late Dr. Anthony (Ginger) Butler for driving me through the English countryside to interview countless clinicians, scientists, and patients to learn as much as possible about primary dystonias. Your kindness, general enthusiasm, and encouragement to present at my first scientific conference altered the trajectory of my life significantly. Thank you to the late Dr. Aram Nersissian; Your dedication to my access to institutional support for my disability rescued me from an often dehumanizing experience, ensuring my completion of my undergraduate degree. Furthered support in the form of letters of recommendation letters helped me attain my post-baccalaureate position and the National Science Foundation Graduate Research Fellowship. Lastly, thank you to Dr. Bruce Hope for taking a chance on a less qualified candidate. My post-baccalaureate fellowship not only revealed my love for neuroscience research but also ensured that I would be able to pursue a graduate degree. For the assistance during long perfusion days and support on every application since, I am incredibly grateful.

I would like to extend a special thank you to my family. First, my parents, Rachael and Chris, for never letting me forget that I can do “whatever [I] put my mind to”. Mom, thank you for always

helping me shed the stress, recenter around my goals, and revisit challenges with stronger clarity. Dad, thank you for forcing me to get outside, keeping me company on my walks to and from lab, and for applying your electrician knowledge base to electrophysiology concepts so we can talk circuits together. Thank you to my sister, Emmy, for your support through the many seasons of my graduate experience. Thank you to my grandma, Beth, for decades of actionable advice, support and encouragement at every peak and valley, and for ensuring that I think critically about what quality of life looks like and how to fit my professional ambitions in appropriately. Thank you to my Aunt Sarah for keeping my publications on your coffee table and always being a stellar supporter of my ability to pursue science (starting from stoking my interests in research as a kid with books on archaeology).

Finally, endless thank you's to my husband, Trevor LaSarre. Thank you for your constant encouragement and support. Thank you for keeping me grounded, getting me outside, and reminding me of the value of my efforts. Thank you for helping me cultivate a life that supports my goals. Thank you for your faith in my ability to achieve anything I aim for. For these and countless other reasons this adventure would've been much harder and less enjoyable without you. I am so lucky to have you.

I am truly grateful for the incredible support system I have accumulated in my life. Without them, I would not be here.

In loving memory of Anthony C. Dunn

Abstract

The descending pain modulatory pathway exerts bidirectional control of ongoing nociceptive inputs traveling from the body to the brain. The first primary region, the ventrolateral periaqueductal gray (vlPAG), integrates inputs from many regions associated with the processing of the nociceptive, cognitive, and affective components of pain perception. Understanding the impact of the heterogeneous neurons within the vlPAG has been defined using stimulation, pharmacology, or more current methods to selectively alter the activity of genetically identified populations. Despite a large body of work implicating the vlPAG in pain modulation, the neuron type specific activation by acute and persistent inflammation has not been identified. Furthermore, neurons have not been characterized in terms of intrinsic membrane and firing properties, which can provide a helpful framework to further define distinguishing features that influence neuronal function and implicate signal integration and transmission within a circuit. In addition to being a key integration site for descending pain modulation, the vlPAG is known to be a primary target for opioid-induced analgesia. The opioid-mediated disinhibition of pain hypothesis proposes a mechanism of action for opioid action in the vlPAG. This hypothesis posits selective MOR expression on GABAergic interneurons, however, several studies have revealed less targeted MOR expression, prompting us to determine whether MOR expression maps onto physiologically distinct neuron types.

The data in this thesis thoroughly characterizes the neuronal heterogeneity within the vlPAG in terms of intrinsic membrane and firing properties. We identified 4 distinct neuron populations: *Phasic* (103/223), *Tonic* (77/223), *Onset* (23/223), and *Random* (20/223). Subsequent evaluation of G protein coupled inwardly-rectifying K^+ channel

(GIRK) currents showed opioid-sensitive and -insensitive subpopulations in *Phasic*, *Tonic*, and *Onset* groups. Fos expression showed that the vlPAG is activated after acute CFA-induced inflammation, with a smaller population remaining strongly activated at the persistent time point. Thorough evaluation of intrinsic membrane and firing properties revealed selective activation of *Phasic* neurons seen exclusively after persistent inflammation, driven by alterations to their intrinsic firing threshold and not a change in synaptic inputs. Furthermore, we saw enhanced inhibitory tone within the vlPAG after persistent inflammation, which coupled with evidence of local GABAergic connections, suggests that increased *Phasic* firing at this time point could be contributing to vlPAG GABA tone.

Additional studies on the role of regulator of G protein signaling (RGS) proteins in μ-opioid receptor (MOR) signaling in the vlPAG, identified agonist-specific functional selectivity in $G_{i/o}$ recruitment that varied amongst effector target systems. RGS proteins expedite G protein inactivation, stopping signaling. As a result, RGS proteins are often thought to negatively regulate GPCR signaling, however, we found the opposite to be true with MOR-mediated GIRK activation. Furthermore, we found that MOR-GIRK activation preferentially signaled through G_o with DAMGO and fentanyl, but the endogenous ligand ME signaled through G_i. MOR activation triggers distinct signaling cascades in presynaptic terminals to inhibit neurotransmitter release. Furthermore, spontaneous and evoked release rely on divergent signaling cascades. With evoked release, maximum inhibition by DAMGO required G_o, fentanyl required both G_i and G_o, and ME displayed nonspecific utilization of both. With inhibition of spontaneous release ME and fentanyl require G_i and DAMGO required both.

These studies contribute to our understanding of how acute and persistent pain states differentially engage heterogenous vlPAG neurons, provide preliminary evidence for descending facilitation of pain by the vlPAG, and create a useful framework to further investigate the circuit implications of enhanced *Phasic* firing during persistent inflammation. Additionally, they have enhanced our understanding of functional selectivity in distinct receptor-effector systems in naïve rats, prompting future studies on the specificity of signaling components in achieving maximal agonist efficacy during pain states.

Chapter 1: Introduction

1.1 Modulation of Pain

Noxious stimuli evoke a sensory experience that we know as pain. The early connections in the ascending circuitry from the periphery to the spinal cord facilitate a fast motor response to remove the body from harm. The noxious signal initiated in the periphery continues up to many supraspinal structures linked to the sensory, cognitive, affective, and motivational components of the complex perceptual pain experience. The processing from these diverse regions is integrated and relayed back to the spinal cord via the descending pain modulatory pathway, where it can then influence the sensory response to ongoing stimuli. Dr. Patrick Wall explained in a 1979 review article that "Pain is better classified as an awareness of a need-state than as a sensation. It serves more to promote healing than to avoid injury. It has more in common with the phenomena of hunger and thirst than it has with seeing or hearing." This reframing of the concept of pain underscores the importance of understanding how the supraspinal circuits involved with the cognitive and affective components of pain integrate with sensory inputs to collectively impact descending pain modulation, especially during the transition from acute to chronic pain.

Nociception & ascending pain pathways

Nociception is the sensory component of pain detected and transmitted by nociceptors, a term coined by Charles Sherrington (Sherrington, 1907). Nociceptors, or primary afferents, innervate peripheral tissues such as skin and muscle allowing them to be the first to detect a noxious stimulus. The sensory machinery that detect noxious stimuli and the methods by which this sensation is transmitted as noxious to other regions of the nervous system has long been debated (see *Figure 1.1*) (Woolf and Ma, 2007). *Specificity theory* suggested that pain was its own sensory modality like hearing or vision, with distinct peripheral and central machinery that have physiological properties (ie: stimulus intensity thresholds, response ranges, adaptation rates, etc.) that shape the sensory features (Bell and Shaw, 1868, Moayedi and Davis, 2013). *Specificity theory* included the assumption that the peripheral machinery sensing the noxious stimuli give rise to pain perception through a direct connection with brain regions specifically tasked with this sensory modality, as often depicted in early anatomy figures such as that from Florentius Schuyl (Figure 1.1A) or Louis La Forge's drawing based on Descartes' description (Fig. 1.1B). *Pattern theory*, instead of focusing on the physiological specialization component of *specificity theory,* instead proposes that nociception is a product of "central summation" of a pattern of sensory inputs produced by intense/noxious stimuli onto uniform nerve endings. Extensive investigation throughout the 20th century provided evidence for spinal mechanisms of nociception as well as central, descending control over ascending primary afferent input, solidifying that these previously competing concepts work in concert to produce the sensory components of pain perception (Melzack and Wall, 1965).

Physiological diversity in nociceptive primary afferent fibers allow for a broader range of stimuli detection and transmission in the ascending pain circuit. Primary afferents can be divided into Aα/β-, Aδ-, and C-fibers. Primary afferents involved in noxious stimuli detection include 1-5 μm diameter, thinly myelinated Aδ-fibers, and smaller, 0.2-1.5 μm diameter, unmyelinated C-fibers (Bishop, 1946). Aδ-fibers are responsible for fast-onset pain (Burgess and Perl, 1967) as a result of their higher conduction velocity (9 m/s) relative to the slowly conducting C-fibers (0.2 – 1.5 m/s) (Hunt, 1954, Abraira and Ginty, 2013). Aδ-fibers are primarily temperature- and mechanosensitive with free nerve endings and some specialized nerve endings. C-fibers, which terminate in free nerve endings, are polymodal as well but largely sense chemical stimuli (ie: capsaicin or inflammatory mediators such as substance P). Though the Aα/β-fibers, with the largest myelinated axons and corresponding conduction velocity overall, primarily respond to innocuous mechanical stimuli, some continue to respond into the noxious range for mechanical stimuli or noxious heat (Todd and Koerber, 2006). In general, primary afferents fire at a rate that is directly proportional to the intensity of the noxious stimulus (Heinricher et al., 2009). Altogether, nociceptors have physical properties (axon diameter and degree of myelination) that coupled with their stimulus response profile shapes how efficiently different nociceptive signals are transmitted.

Organizationally, the pseuodunipolar primary afferents straddle the PNS and CNS, with their cell bodies in the dorsal root or trigeminal ganglia and a single axon that bifurcates into one branch that extends into the peripheral tissue and another, the central axon, that synapses onto second order neurons in the dorsal horn of the spinal cord or the trigeminal subnucleus caudalis. Structurally, C-fibers axons vary slightly in that they are

housed in bundles of varied numbers called remak bundles, which are formed by non-myelinating Schwann cells that outline the boundaries between distinct axons (Murinson and Griffin, 2004). These specialized glial cells are thought to provide trophic support to C-fibers to promote regeneration of damaged axons. Within the dorsal root, the primary afferents are housed with motor neurons, allowing for a quick removal of the body from harm (Schouenborg et al., 1995, Clarke and Harris, 2004).

In addition to this quick, harm avoidance response, the nociceptive signal continues to travel up to many different supraspinal regions involved in the processing of not only the nociceptive information but also the cognitive and affective components that concurrently shape pain perception. Nociceptive information travels to the brain through the spinothalamic, spinomesencephalic, spinoreticular, spinolimbic, spinocervicothalamic, and spinohypothalamic tracts (Willis and Westlund, 1997). This large collection of brain regions project back down to the dorsal horn of the spinal cord via the descending pain modulatory pathway. Efferent terminals of this descending pathway make direct synaptic connections with dorsal horn neurons, where they can either inhibit or facilitate any ongoing incoming nociceptive signals coming from the periphery.

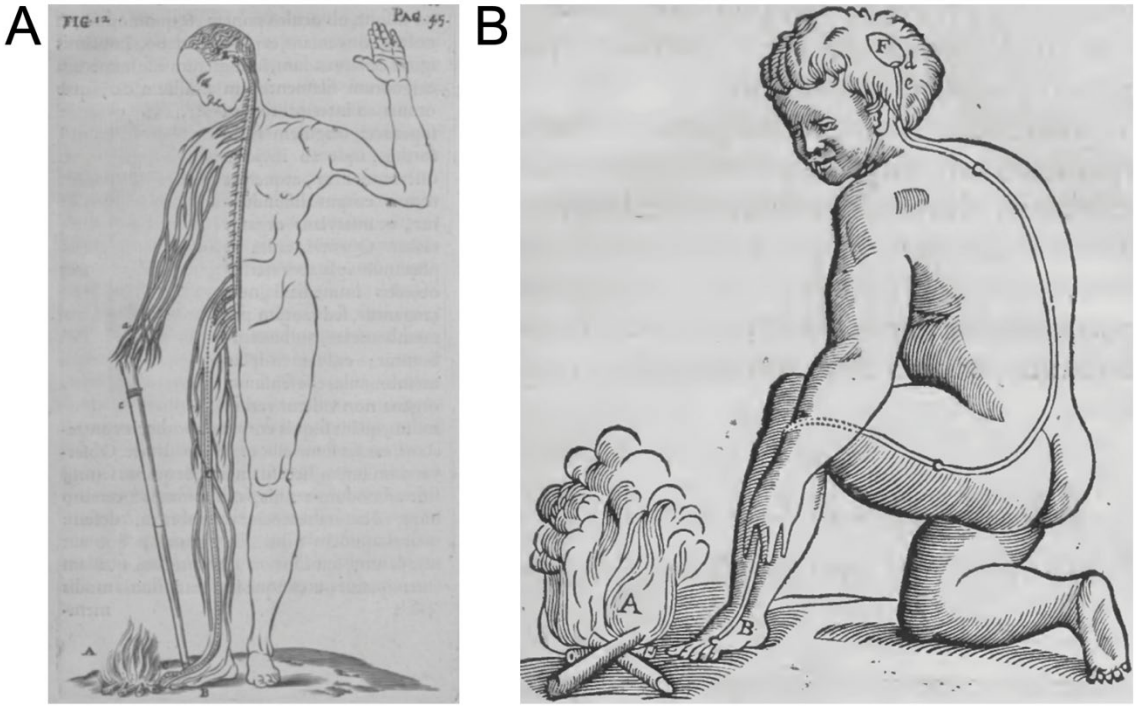

Figure 1. 1: Early understanding of ascending and descending nociceptive pathways

Line drawing of the pain system by **(A)** Florentius Schuyl and **(B)** Louis La Forge based on Descartes' description in *Treatise of Man*. The fire (*A*) is the noxious stimulus close to the foot (*B*) activating the "fibril" (or nociceptor; *C*), which opens the "pore" (*d* and *e*) where the "fibril" terminates. The open "pore" allows the "animal spirits" to flow from the cavity (*F*) back into the "fibril" allowing it to activate the muscles to move the foot away from the fire, the muscles to center focus on the fire, and the muscles to protect and attend to the injury site.

[*A* is reproduced from Descartes (1662), and *B* and text are reproduced from Descartes et al. (1664), out of copyright; translated by M. Moayedi.; adapted by K. McPherson]

Taken from: Moayedi, M., & Davis, K. D. (2013). Theories of pain: from specificity to gate control. *Journal of neurophysiology*, *109*(1), 5-12.

Descending pain modulation

The many supraspinal regions that receive nociceptive inputs from the ascending pain pathways converge at the ventrolateral periaqueductal gray (vlPAG)—the first region of the descending pain modulatory pathway. The descending pain modulatory pathway serves as a volume dial that can bidirectionally control ongoing nociceptive inputs being sent from the body to the brain. It does this through a net *descending inhibitory* or *faciliatory* output at the level of the dorsal horn of the spinal cord, where descending and ascending pathways converge. Descending projections travel to the dorsal horn of the spinal cord via the dorsolateral funiculus, funiculus being a morphological term that describes a large and regionally distinct bundle of nerve fibers. Contradictory findings have debated the extent of vlPAG efferents contained within the dorsolateral funiculus (DLF) (Basbaum and Fields, 1979, Mantyh and Peschanski, 1982). However, the vlPAG sends a dense projection to the rostroventromedial medulla (RVM) (Fields and Heinricher, 1985), that has confirmed efferents within the DLF that synapse onto dorsal horn neurons (Basbaum and Fields, 1979, Mantyh and Peschanski, 1982, Vanegas et al., 1984b). Furthermore, the vlPAG promotes descending inhibition of pain (antinociception) through the connection with the RVM (Behbehani and Fields, 1979, Gebhart et al., 1983, Prieto et al., 1983, Fields and Heinricher, 1985). The exact circuit mechanisms between the vlPAG and RVM are an ongoing area of investigation that continues to evolve (Basbaum and Fields, 1984, Lau and Vaughan, 2014, Heinricher and Ingram, 2020), however, the methods by which the RVM modulates nociception has been extensively defined.

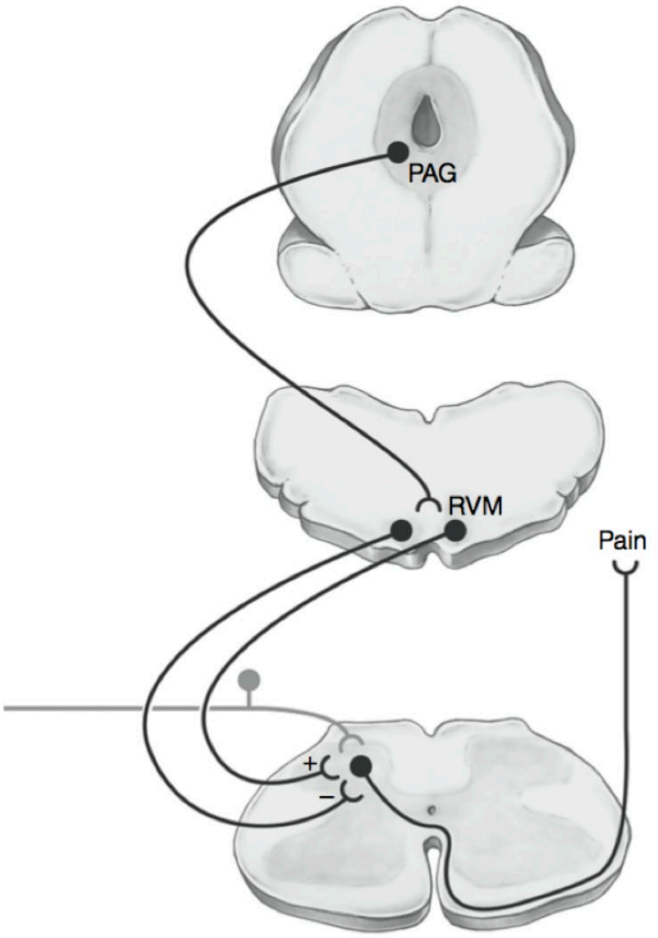

Figure 1. 2: Descending pain modulation

Functional organization the descending pain modulatory pathway, where the PAG projects to the RVM, which projects down to the dorsal horn of the spinal cord via the dorsal funiculus. The signals transmitted downstream by two functionally distinct neuron population in the RVM can then bidirectionally (+ or -) control incoming inputs arriving at the dorsal horn from noxious stimuli in the periphery that then travel up to many different brain regions. The PAG also has reciprocal connections (not shown) with many other brain regions (ie: prefrontal cortex, amygdala, BNST, and hypothalamus).

Taken from: Heinricher MM, Ingram SL (2020) Brainstem and nociceptive modulation. In: The Senses: A Comprehensive Reference ((Ed.) IBF, ed), pp 249-271: Elsevier: Academic Press.

The RVM is known to bidirectionally modulate ongoing nociceptive signals at the level of the spinal cord (see *Figure 1.2*) through a large body of meticulously executed studies evaluating the functional role of three distinct types of neurons: ON-, OFF-, and NEUTRAL-cells. These neuron types were initially characterized by their unique responses to acute noxious stimuli (Fields et al., 1983, Vanegas et al., 1984b). Recordings done *in vivo* define ON-cells by their increased firing frequency that begins just before the tail flick or paw withdrawal behavioral response to the acute noxious stimuli being applied. OFF-cells pause at the same time just before the behavioral output (the withdrawal response). NEUTRAL-cells show no changes in firing response in the presence of an acute noxious stimuli. Some of each cell type project down to the dorsal horn (Vanegas et al., 1984b, Fields et al., 1995). Enhanced OFF-cell firing promotes antinociception, observed as a suppression of nociceptive reflexes (Fields and Heinricher, 1985)—implicating them in *descending inhibition of pain*.

Conversely, ON-cells have increased activity in the presence of behavioral hyperalgesia and selective pharmacological activation of ON-cells promoted allodynia (Heinricher and Neubert, 2004, Neubert et al., 2004). Many studies using many different pain models have further defined ON-cells as pro-nociceptive (Morgan and Fields, 1994, Porreca et al., 2002, Edelmayer et al., 2009). Interestingly, additional studies expand beyond the general boundaries that most consider to be related to acute and chronic pain, implicating ON-cell activity in the pain hypersensitivity experienced by the clinical population, and rodent models, in the conjunction with acute mild stress, illness, medication overuse, and opioid withdrawal (Bederson et al., 1990, Ossipov et al., 2004, Heinricher and Ingram, 2020). Together, this collection of studies solidified the ON-cell

functional role in *descending facilitation of pain*. Although hyperexcited ON-cells promote *descending facilitation*, the antinociceptive effect of increased activation of OFF-cells overrides the facilitation, yielding a net inhibitory output for the descending circuit (Satoh et al., 1983, Hentall et al., 1984, Fields and Heinricher, 1985, Jensen and Yaksh, 1989, Heinricher and Ingram, 2020).

Altogether, the net behavioral output of the descending pain modulatory pathway is a result of the activity of these three classes of neurons within the RVM and their projections to the dorsal horn (Vanegas et al., 1984b, Fields et al., 1995). Physiological characterization of RVM neurons and their response to noxious stimuli provided a fruitful framework for defining the role these neurons play in descending modulation, **reinforcing the value of applying physiological cell type characterization and activation by acute and persistent stimuli to the vlPAG.** Furthermore, the circuit mechanism by which the vlPAG participates in the overall bidirectional nociceptive control, has been largely defined by artificial manipulation of the entire region (see *The role of the vlPAG in descending pain modulation*), **leaving a gap in our understanding of how the vlPAG is engaged by acute and persistent pain states.**

The role of the vlPAG in descending pain modulation

The vlPAG integrates excitatory and inhibitory inputs from diverse regions involved in the many facets of pain perception (Silva and McNaughton, 2019). Early observations in the cat first indicated that midbrain involvement in nociception by showing that electrolytic lesions of the midbrain could serve as sufficient anesthetic for *in vivo* recordings (Martin and Branch, 1958). Later studies identified that chemical and

electrical activation of the vlPAG in rats produced analgesia (Reynolds, 1969, Mayer et al., 1971, Mayer and Liebeskind, 1974, Akil and Liebeskind, 1975, Soper and Melzack, 1982, Jensen and Yaksh, 1989). Stimulating the vlPAG was shown to inhibit activation of dorsal horn neurons by noxious, but not innocuous, stimuli (Waters and Lumb, 1997). More specifically, vlPAG activation inhibited pain transmitted by C-fibers and left A-fiber transmission unaffected (McMullan and Lumb, 2006, Leith et al., 2007). This selective inhibition of primary afferents provides behavioral value: reducing distracting inputs from C-fibers to enhance the sensory-discriminative signal that is rapidly conducted by A-fibers (Heinricher et al., 2009).

Due to the minimal direct projections from the vlPAG to the dorsal horn, it was important to establish that the behavioral effects from vlPAG manipulation are a result of the robust output to the RVM. Inactivating the RVM with lesions or lidocaine blocked the analgesic effect of vlPAG stimulation (Behbehani and Fields, 1979, Gebhart et al., 1983, Prieto et al., 1983), and further, midbrain stimulation was only analgesic at intensities sufficient to enhance RVM OFF-cell firing (Vanegas et al., 1984a). The net outflow from the vlPAG yields activation of downstream targets, producing descending inhibition. Electrical or chemical (EAA or GABA antagonists) stimulation of the vlPAG activates both ON- and OFF-cells in the RVM, producing antinociception (Vanegas et al., 1984a, Tortorici and Morgan, 2002). Although evidence of an excitatory connection between the PAG and RVM had already been established (Behbehani and Fields, 1979), more recent studies further reinforced the antinociceptive role of vlPAG glutamate neurons using selective chemogenetic activation (Samineni et al., 2017a). Altogether, these studies show that activation of the descending system, at the level of the vlPAG,

drives antinociception by altering ascending signaling at the dorsal horn through key circuitry with the RVM.

The analgesic circuit between the vlPAG and the RVM was initially proposed to rely on the direct, excitatory connection (Basbaum and Fields, 1984), however, the additional finding of GABAergic RVM-projecting neurons increases the complexity of the circuit mechanisms mediating antinociception (Lau and Vaughan, 2014). Immunofluorescence and electrophysiology studies have shown that the vlPAG sends GABAergic afferents onto ON- and OFF-cells in the RVM (Morgan et al., 2008, Li et al., 2017). Non-GABAergic neurons (GAD-) were shown to project to both RVM populations as well (Morgan et al., 2008). In addition to the parallel projections occurring from excitatory and inhibitory vlPAG neurons to functionally distinct RVM populations, the presence of inhibitory interneurons in both regions have been proposed (Basbaum and Fields, 1984, Lau and Vaughan, 2014), further complicating the circuit mechanisms. Furthermore, these studies largely provide evidence of how the activity of neurons within the circuit and the behavioral output is altered by artificially exciting or inhibiting the vlPAG, however, they do not inform us on how pain states naturally alter vlPAG activity. **How cells within the vlPAG function intrinsically within the system and how diverse presynaptic inputs modulate this function in naïve and pain states remain open to ongoing investigation.**

Central sensitization

Central sensitization is a mechanism that fundamentally contributes to pain hypersensitivity that occurs when a noxious stimulus persists. Central sensitization is the enhanced sensory response triggered by stimuli that occurs as a result of adaptations to peripheral nociceptors induced by prolonged activity, inflammation, or nerve injury (Woolf et al., 1988, Woolf, 2011). This mechanism has been defined by a broad range of scientific findings describing cellular and circuit adaptations that increase membrane excitability, synaptic efficacy, or a reduce inhibition (Latremoliere and Woolf, 2009). Many alterations to the sensory neurons within the periphery have been identified that would further facilitate enhanced excitability yielding hyperalgesia from nociceptors and allodynia from low-threshold mechanoreceptors (Woolf, 2011). Dorsal horn neurons impacted by central sensitization often have enhanced spontaneous firing frequency, a lowered threshold for activation by peripheral stimulation, increased response to pre-sensitization suprathreshold stimuli, and a broadening of their receptive fields. Persistent inflammatory pain induced by CFA alters gene expression, neurotransmitter tone, and neuronal excitability within the spinal and supraspinal ascending and descending pain circuitry (Hylden et al., 1989, Yang et al., 2009, Zhang and Hammond, 2009, Ikeda et al., 2012, Tonsfeldt et al., 2016, Li et al., 2017), providing further evidence of pain-induced sensitization of central circuits in ways that can alter ongoing pain modulation.

Central sensitization is a mechanism that alters pain perception stemming from many changes to neurons within the CNS, the resulting pain experience is no longer tethered to the presence, magnitude, or length of the peripheral stimulus. The disconnect between the sensory input and the perceptual pain experience provides meaningful

context for understanding the lived experiences of patients with chronic pain. **This thesis in part addresses how inflammation alters the activation state of physiologically distinct neurons in the vlPAG, contributing to our understanding of central sensitization in descending modulation of pain.**

Pain assessment and terminology

Pain cannot be directly measured in rodents because pain is a perceptual experience that is shaped by the convergence of nociceptive, affective, and cognitive processing. Instead of direct pain measurements we enlist many different pain-like behaviors to indicate nociception and inferred pain, allowing for (Deuis et al., 2017). One of the most common metrics used to determine pain thresholds is paw or tail withdrawal latency, which can be elicited by the application of many different types of noxious stimuli. Mechanical stimuli (ie: force or pressure) can be applied with forceps, von Frey filaments, or pressure algometers (Anseloni et al., 2003). Mechanical hyperalgesia or allodynia can be assessed with touch, brushing, or pressure. In addition to mechanical manipulations, response to noxious temperatures are often use. The hot-plate method applies a gradual ramp in temperature to find the temperature threshold for withdrawal (Woolfe and Macdonald, 1944). Another, the Hargreaves test, rapidly heats to a predetermined noxious temperature and scores the latency to withdraw the paw (Hargreaves et al., 1988). Similarly, the tail flick test applies heat to the tail and measures the latency to induce a flick response (D'Amour and Smith, 1941).

The terminology used to describe these pain assessments varies depending on the condition of the animal and the intensity of the response to a given manipulation (ie:

noxious stimulus, therapeutic drug, etc.). Nociception, as previously described, is the detection and transmission of information relating to a noxious stimulus. Pronociception is an outcome (from a drug, circuit mechanism, etc.) that facilitates ongoing nociception, whereas antinociception is an outcome that inhibits ongoing nociception. When a drug is maximally antinociceptive, in that the animal's withdrawal latency in the nociceptive behavioral assay (ie: paw withdrawal or tail flick) goes to cut-off, it is considered analgesic—"an" meaning without and "algein" is the Greek word for "to feel pain". Hyperalgesia is a state where there is any decrease in response latency or reduction in threshold that elicits a response. Conversely, hypoalgesia is an increase in the response threshold, yielding reduced sensitivity to noxious stimuli. Lastly, a drug or circuit alteration is antihyperalgesic when the animal was previously in a hyperalgesic state and it is returned back to baseline. Thus, antihyperalgesia most accurately describes an antinociceptive manipulation when it is occurring in a hyperalgesia model.

Many of these terms are used most often when describing stimulus-evoked pain. For example, hyperalgesia measures changes in stimulus-evoked pain, because hyperalgesia requires the application of an acute stimuli to determine whether the response is exaggerated compared to baseline. Novel therapeutics derived from basic science studies have resulted in limited clinically translatable success, calling into question the conventional pain models (Mogil, 2009, Yezierski and Hansson, 2018). One key limitation is that these models are assays for stimulus-evoked pain, despite the fact that a significant portion of clinical cases of pain (and chronic pain in particular) are spontaneous pain, which varies in severity and is not linked to a noxious stimulus. Another significant concern is the distinction between nociception and pain. Both issues

have led to the use of grimace scales, burrowing assays, gait analysis, weight-bearing assessments, and automated behavioral analysis (Tappe-Theodor and Kuner, 2014, Fisher et al., 2020)—however the validity of these methods and the information that can be gained from them is still often debated (Mogil et al., 2010, Whittaker et al., 2021).

Pain-behaviors are not amongst the methods enlisted in this thesis, which primarily focuses of the physiological impact of pain conditions. However, a thorough understanding of pain models, the methods used to evaluate and substantiate them, and the terminology used to discuss these topics is key to discussing how the following studies impact our understanding of descending pain modulation and what future studies best address remaining questions.

Distinction between acute, chronic, and persistent pain models

Animal models of pain use different noxious stimuli to recapitulate clinically observed features of acute or chronic pain, such as hyperalgesia and allodynia. These features of pain sensitivity are evaluated most frequently using withdrawal latencies, or other reflexive behaviors such as limb guarding, paw licking burrowing, and reflexive grimacing. The different animal models often fall under two distinct categories: neuropathic or inflammatory pain. Neuropathic pain enlists one of many different methods, such as spinal nerve ligation, to model pain states that arise as a result of damage to sensory and spinal nerves. This thesis focuses on the effects of inflammation, specifically modeled using Complete Freund's Adjuvant (CFA), to induce a robust inflammatory response that mimics components of inflammatory pain conditions in the human population (ie: arthritis).

Acute pain is a perceptual experience that occurs from a range of injuries such as scraping your knee to having a surgical procedure. Exposure to a noxious stimulus results in acute pain though a combination of nociception and the associated supraspinal computations that occur as a byproduct of these ascending inputs. The "acute" time frame is defined by the time it takes for the injury to heal. Carrageenan in saline solution injected subcutaneously produces one acute model, with swelling that reaches the peak 3-5 h after injection and subsides by 24 h (Winter et al., 1962). Another, induced by topical application of mustard oil reaches maximal tissue damage (edema) after 30 m and are sustained for at least 2 h (Lippe et al., 1993), aligning with the time frame of another common acute noxious stimulus model capsaicin. With acute pain there can be associated hyperalgesia, or enhanced pain response to other noxious stimuli, and allodynia, pain responses to previously innocuous stimuli, which together serve as additional warnings to avoid further damage while the body heals. These associated sensory features can be "primary" meaning localized to the injury site or "secondary" meaning adjacent to the injury. Chronic pain is a prolonged pathological state that occurs when pain perception persists beyond the acute injury or in the absence of injury altogether in the instance of spontaneous pain. Neuropathic pain models are most regularly used for chronic pain as a result of irreversible effect of nerve ligation.

The studies in this thesis utilize CFA injected into the hindpaw to induce acute and persistent pain. CFA is an inactivated, dried mycobacteria (usually *M. tuberculosis*) that is emulsified in mineral oil. CFA injected subcutaneously into the plantar surface of the hindpaw creates rapid tissue inflammation and edema that induces first an acute inflammatory pain response that reaches a peak 2-6 h after injection and is maintained for

1-2 wk (Ren and Dubner, 1999). The thermal hyperalgesia and mechanical allodynia resulting from CFA injection lasts for more than 28 d, during which the animal experiences increased paw volume and decreased growth-rate and mobility (Nagakura et al., 2003). Interestingly, thermal hyperalgesia is exclusively seen in the ipsilateral (CFA-injected) hindpaw, whereas mechanical allodynia and joint hyperalgesia present first in the ipsilateral paw and then increase to a lesser extent in the contralateral paw around 10 d (Nagakura et al., 2003). Collectively, these findings make CFA a suitable model to study both acute and persistent inflammatory pain conditions. We know that acute and persistent inflammation produces implied pain behaviors (ie: paw attending, reduced withdrawal thresholds, etc.), it is important to note that we did not evaluate pain behaviors in these studies, prompting our use of "acute and persistent inflammation" to describe our model.

CFA-induced inflammation produces alterations to neuronal activity in regions within the ascending and descending pain circuits. Features of C- and Ad-fiber firing remain altered by CFA 2 and 4 d after injection into the hindpaw (Djouhri and Lawson, 1999). In the RVM, spontaneous firing is enhanced in ON-cells and reduced in OFF-cells for the first 60 m post-CFA injection, with reflex-evoked activity being unchanged in the ON-cells and the pause being slightly increased in the OFF-cells (Cleary and Heinricher, 2013). During persistent inflammation (3 or 10 d post-CFA), RVM ON- and OFF- cells had lowered thresholds for responding and increased response magnitudes (Cleary and Heinricher, 2013). Additionally, changes to GABA tone within the vlPAG and RVM are seen during persistent inflammation (5-7 d) (Tonsfeldt et al., 2016, Li et al., 2017). At this time point, the edema is still significant and animals demonstrate hyperalgesia and

allodynia from the persistent exposure to the inflammatory injury. The primary difference between chronic and persistent pain models is that in the persistent condition the injury and associated pain persists beyond the expected healing timeframe (Siddall and Cousins, 2004), instead of chronic models where the injury has healed. The studies in this thesis evaluate changes induced by acute (2 h) and persistent (5-7 d) inflammation induced by CFA injected into the hindpaw (see *Figure 1.3*).

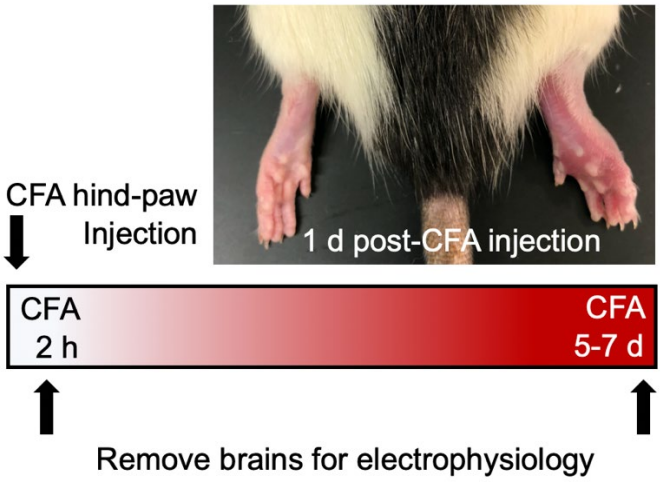

Figure 1. 3: Acute and persistent CFA-induced inflammation

CFA injected into the hind paw is used to model acute (2 h) and persistent (5-7 d) inflammation in this thesis. The hindpaw injection happens at time zero and brains are taken either at the 2 h time point or between 5 and 7 d later. The image shows the inflammatory effect in the right hindpaw 24 h after injection.

Clinical implication of persistent inflammation

An estimated 20% of adults globally experience chronic pain with an additional 10% that are newly diagnosed every year (Goldberg and McGee, 2011). There are many different forms of chronic pain pathologies that arise in the clinical population. Common causes of chronic pain include: cancer, osteo- and rheumatoid arthritis, operations and injuries, spinal damage, migraine, and fibromyalgia. Clinically categorized chronic pain

disorders also include a subset of disorders with ongoing, persistent inflammation and/or tissue damage, such as osteoarthritis (OA). OA is characterized by progressive degeneration of articular cartilage resulting in the narrowing of the joint space. This is caused by failed regeneration following stress-induced joint damage from either biomechanical, biochemical, or genetic factors, however, in the majority of patients the primary etiology is unknown. As the cartilage degenerates joint damage increases, promoting flares of local inflammation dictated by joint use/stress, features that can also be concurrently compounded by factors such as age, obesity, and altered biomechanics (Sokolove and Lepus, 2013) .

OA provides clinical examples of central sensitization resulting from persistent inflammatory pain. Normal adult human articular cartilage is both avascular and aneural, but mild and severe OA patients show both vascularization and associated innervation of articular cartilage (Arendt-Nielsen et al., 2015), demonstrating a remodeling of the sensory nervous system in this persistent inflammatory disease state. Furthermore, most joint nociceptors are silent under normal conditions. Exposure to inflammatory mediators can sensitize the nociceptors, producing prolonged neuronal discharges, enhanced response to noxious stimuli (hyperalgesia), response to innocuous stimuli (allodynia), and a widening of the receptive field (Neugebauer and Schaible, 1990). This explains why many OA patients experience hyperalgesia in regions distant from the affected joint (Kosek and Ordeberg, 2000). Despite the distinct source for the deep, aching pain described by OA patients, the degree of pain reported often misaligns with the degree of joint degeneration (Phillips and Clauw, 2011), further reinforcing the examples of central sensitization in OA pathology and progression.

Sexual dimorphism in pain conditions

Male rodents have been the default model organism for preclinical and basic science investigation until recent years, likely contributing to therapeutic diagnostic and treatment processes that disproportionately fail for women (Shansky and Murphy, 2021). Pain research provides a strong argument for the regular inclusion of female models due to disparities in the prevalence of chronic pain conditions, as well as fundamental differences in the circuits mechanisms and behavioral outcomes associated to nociception and analgesia. Females have an increased incidence of chronic pain compared to men across an array of disorders including: migraine, fibromyalgia, rheumatoid arthritis, and irritable bowel syndrome (Ruau et al., 2012, Canavan et al., 2014, Kennedy et al., 2014, Steingrímsdóttir et al., 2017). Additionally, females have many chronic pain conditions, such as endometriosis or vulvodynia, that are specific to female anatomy, further increasing the sex-specific increase in incidence. This increased prevalence does not appear to be associated with differences in aging, with the higher prevalence seen in females across all age groups including pre-pubertal (Unruh, 1996, Berkley, 1997, McGrath et al., 2000, Tsang et al., 2008, Fillingim et al., 2009, Van Hecke et al., 2013).

Overall, the high prevalence of chronic pain in females is believed to be a product of many physiological differences in nociception and therapeutic efficacy (Greenspan et al., 2007, Fillingim et al., 2009, Bartley and Fillingim, 2013). Unlike males, females experience significant fluctuations in hormone levels during and after pregnancy, after menopause, and monthly by the menstrual cycle, which can influence many different details in the multifaceted systems involved in mediating pain perception.

Mechanistically there are many possible avenues for hormones to influence pain states, such as the effects of estrogens on bone deposition and cartilage homeostasis which could influence the development or worsening of OA (Tanko et al., 2008). Postpubescent females incidence of migraine increases significantly (Stewart et al., 1992, Lipton et al., 2001). Additionally, females show a heightened inflammatory response compared to males, which can influence pain states through many different mechanisms (Straub, 2007). Sex differences have even been observed in the descending pain modulatory pathway, both in terms of circuitry, inflammatory mediators, and opioid efficacy (Loyd and Murphy, 2006, Fullerton et al., 2018) (discussed in the *MOR function in the vlPAG* section). For these reasons we analyzed our data for sex differences.

The impact of inflammation on the vlPAG

Neurons within the vlPAG have many cellular and synaptic alterations after different acute and persistent pain models. First and most simply, the vlPAG is activated by pain states. cFos, an immediate early gene transcribed to Fos protein in the presence of strong neuronal activity, is often used as an evaluative proxy to identify enhanced activation of discrete brain regions in response to a stimulus (Cruz et al., 2015). Acute and persistent CFA-induced inflammation increases Fos expression within the vlPAG (Keay and Bandler, 1993, Lantéri-Minet et al., 1995).

The vlPAG integrates dense excitatory and inhibitory inputs from many different brain regions that have altered activity during pain states, prompting the question of whether these inputs are altered during pain states. Persistent inflammation by CFA has been previously shown by our lab to enhance GABA tone in the vlPAG of female rats

(Tonsfeldt et al., 2016). The same enhanced release from GABA terminals was seen in both males and females rats after neuropathic pain (Hahm et al., 2011). Despite the often theorized population of inhibitory interneurons in the vlPAG (Lau and Vaughan, 2014), it has not been directly demonstrated that GABAergic vlPAG neurons send local projections. Interestingly, vlPAG GABA tone was increased by acute formalin injection and then reduced by local infusion of TTX, suggesting local inhibitory neurons could be contributing to the tone that is likely inhibiting other vlPAG neurons (Maione et al., 1999). However, TTX-sensitive Na^{1+} channels are also expressed in axons and terminals, which can then be inhibiting release from inhibitory afferents. Enhanced GABA release after persistent inflammation was also seen downstream in the RVM (Li et al., 2017). In addition to changes in GABA release, glutamatergic release was decreased in the vlPAG 3 and 10 d after spinal nerve ligation (Ho et al., 2013). Despite the varied pain models used in these findings, they suggest possible changes in the excitatory/inhibitory (E/I) balance. E/I balance is the proportion of excitatory and inhibitory afferent inputs onto a single neuron, which may vary amongst distinct populations of vlPAG neurons. Altered afferent release from either excitatory or inhibitory terminals can influence firing rates, and the changes to firing induced by opioid-mediated inhibition of presynaptic release (see *section 1.3: Mu-opioid receptor function in the vlPAG*), thus yielding altered engagement with downstream targets like the RVM. **Characterization of the intrinsic firing properties of vlPAG neurons in naïve rats provided the opportunity to determine alterations to distinct neuron type firing properties, and the contribution made by excitatory and inhibitory afferent inputs, after acute or persistent inflammation.**

1.2 Heterogeneity within the vlPAG

The periaqueductal gray (PAG) is a midbrain structure that surrounds the cerebral aqueduct, which can be subdivided into the dorsal, dorsolateral, lateral, and ventrolateral (vlPAG) columns, with the dorsal raphe positioned in the ventral region (see *Figure 1.4*). The dorsal and lateral columns extend the furthest along the rostral-caudal axis, with the ventrolateral column being the largest in the caudal sections, decreasing in area towards the lateral-intermediate PAG (Bandler and Keay, 1996). vlPAG neurons demonstrate many hallmarks of heterogeneity including: diverse cortical and subcortical afferent inputs, post-synaptic targets, molecular composition, receptor and channel expression, morphology, and response to stimuli (Hamilton, 1973, Heinricher et al., 1987, Barbaresi and Manfrini, 1988, Chieng and Christie, 1994a, Park et al., 2010, Heinricher and Ingram, 2020). The vlPAG is involved in many distinct behavioral circuits associated with survival (ie: threat, fear, pain) as well as vital autonomic functions like breathing, feeding, and respiration (see *Figure 1.5*) (Bandler et al., 2000, George et al., 2019, Silva and McNaughton, 2019), boosting the utility of popular genetically encoded circuit manipulation tools in understanding the implications of specific PAG afferents in distinct behaviors (Deisseroth, 2011, Fenno et al., 2011, Roth, 2016).

The broad categorization of excitatory and inhibitory neurons defined by these genetic methods does not account for the layered heterogeneity (ie: physiological, afferent inputs, efferent targets, etc.) that likely remains in these populations of neurons. For example, studies identifying GABAergic neurons in the mouse vlPAG showed a

subpopulation that uniquely expressed low-threshold spiking and opioid sensitivity (Park et al., 2010). Furthermore, little work has been done to identify whether uniform activation of molecularly distinct subsets of neurons aligns with physiological changes occurring during these different pain states (Samineni et al., 2017b). In contrast, *in vivo* studies have shown functionally distinct cells, based on firing response to noxious stimuli, display enhanced hyperexcitability after chronic neuropathic pain (Samineni et al., 2017b). This suggests that different cell types are activated by persistent pain and work in concert to effect downstream signaling at the RVM and the net output of the descending circuit, introducing more circuit complexity than how most contemporary studies are evaluating this system. **Despite the significant role that the vlPAG plays in descending modulation of pain and the analgesic effects of opioid therapeutics, the diverse functionality of this region coupled with cellular and circuit-based heterogeneity underscores the importance of investigating how distinct cell types are engaged by acute and persistent inflammation.**

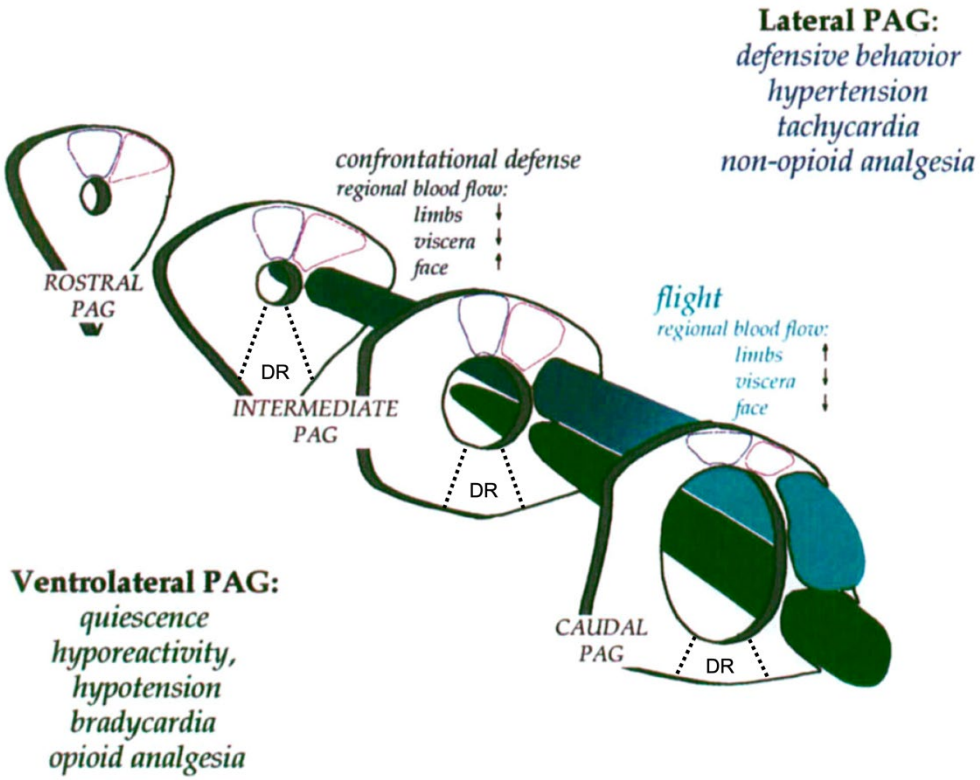

Figure 1. 4: Columnar organization of the PAG.

Distinct columns in the PAG extend along the rostral-caudal axis, with the dorsal raphe (indicated with DR) extending directly below the aqueduct. Activation of the ventrolateral PAG produces quiescence, hyporeactivity, hypotension, bradycardia, and opioid analgesia. Whereas stimulation of the lateral PAG yields defensive behavior, hypertension, tachycardia, and non-opioid analgesia.

Adapted from: Keay, K. A., & Bandler, R. (2001). Parallel circuits mediating distinct emotional coping reactions to different types of stress. *Neuroscience & Biobehavioral Reviews*, *25*(7-8), 669-678.

vlPAG connectivity

The PAG can be divided into functionally distinct columns that run along the dorsal, lateral, and ventrolateral edge of the cerebral aqueduct (Bandler and Shipley 1994), where the dorsolateral column is reduced in the caudal third of the PAG and the ventrolateral column extends through the caudal half of the PAG (Bandler et al., 1991). Due to the limitation in the spatial resolution of functional imaging studies, the PAG collectively has been determined to be activated in acute and persistent pain states in humans (Dunckley et al., 2005, Zambreanu et al., 2005, Fairhurst et al., 2007, Roy et al., 2014). More targeted stimulation or pharmacological manipulations have further distinguished the functional output of activating and inactivating each column. Stimulation or application of excitatory amino acids (EAA's) in the lateral and dorsal PAG activates defensive behaviors, whereas the same manipulations in the ventrolateral PAG are analgesic (Bandler et al., 1991, Bandler and Shipley, 1994, Bandler et al., 2000). The distinct columns within the PAG have some overlapping afferent inputs, however, many distinct projecting regions were also identified (Meller and Dennis, 1986). Interestingly, the analgesic effect of stimulating the rostral vlPAG requires the caudal vlPAG to be intact (Rhodes, 1979). This may be a result of interconnectivity across the rostral-caudal access of the vlPAG, for example with theorized GABAergic interneurons. Connectivity across distinct PAG columns has not been evaluated and local circuits within an individual column have not been functionally substantiated (Lau, 2011).

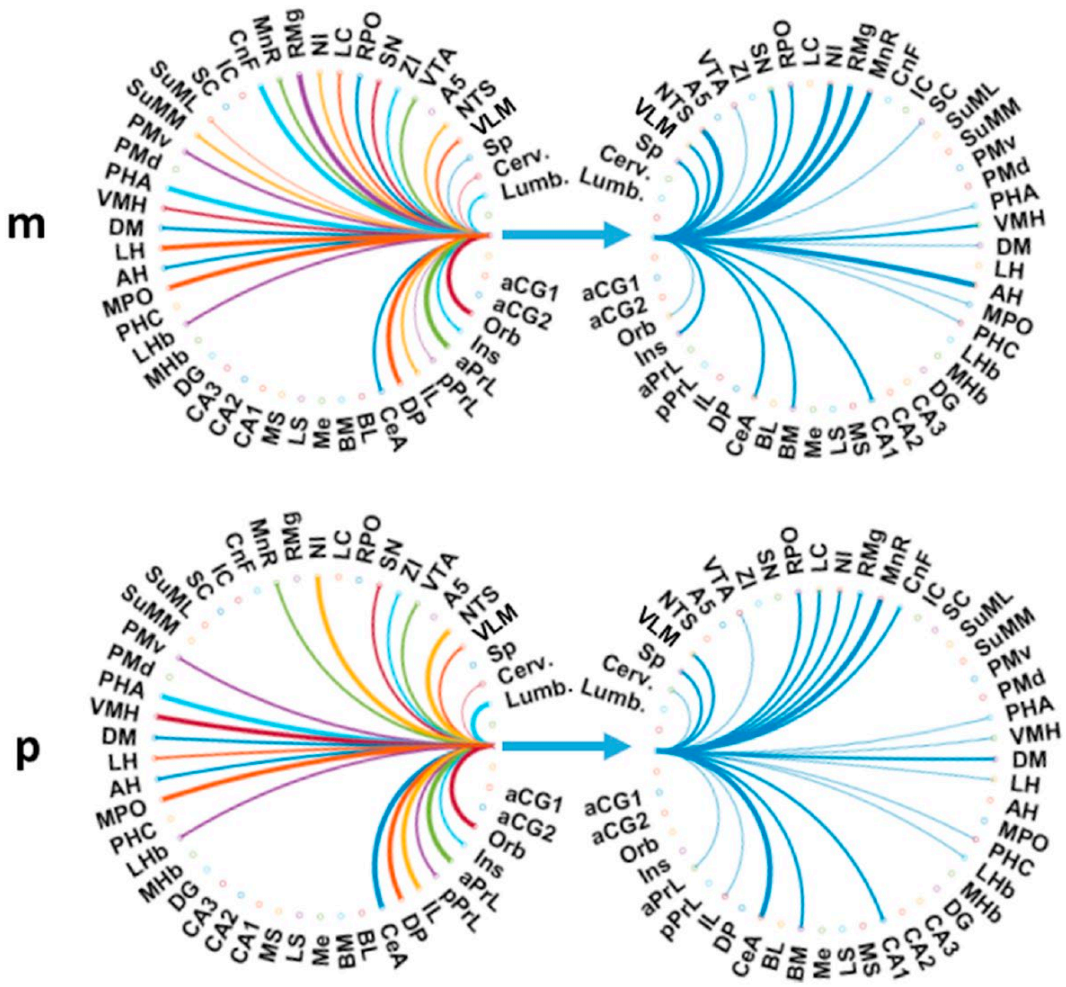

Figure 1. 5: Diverse vlPAG connectivity

The vlPAG afferent inputs (left) and projection targets (right) at the medial (m) and posterior (p) levels. Line thickness indicates the strength of the projection.

Taken from: Silva, C., & McNaughton, N. (2019). Are periaqueductal gray and dorsal raphe the foundation of appetitive and aversive control? A comprehensive review. *Progress in neurobiology*, *177*, 33-72.

The vlPAG receives inputs from many cortical and subcortical regions (see *Figure 1.5*), many of which have been associated with the nociceptive, cognitive, and affective components of pain. First, ascending nociceptive inputs to the vlPAG come through the spinothalamic, spinoparabrachial, and spinomesocenphalic tracts, with some inputs coming directly from the spinal cord to the vlPAG (Menétrey et al., 1982, Yezierski and Mendez, 1991). The spinomesencephalic tract provides direct inputs to the PAG, however, these inputs have been linked to not only nociception and analgesia, but also aversive behavior (Willis and Westlund, 1997). Additional ascending nociceptive inputs come from the parabrachial complex (Gauriau and Bernard, 2002), which receives inputs from the superficial and deep dorsal horn (Roeder et al., 2016). Forebrain regions including the medial prefrontal, agranular insular, and anterior cingulate cortices, amygdala, bed nucleus of the stria terminalis, and hypothalamus send the most significant supraspinal inputs to the PAG (Shipley et al., 1991, An et al., 1998, Floyd et al., 2000, Hao et al., 2019, Silva and McNaughton, 2019). Not only do anatomical studies show connections between these regions and the vlPAG, but studies using lesions and/or different pain paradigms have provided evidence that these regions participate in pain circuitry (Donahue et al., 2001, Ikeda et al., 2007, Starr et al., 2009, Bliss et al., 2016, Mills et al., 2018). For example, antinociception induced by morphine injected into the basolateral and medial nuclei of the amygdala, is interrupted by lesioning the vlPAG (McGaraughty et al., 2004)—emphasizing the importance of the vlPAG as an integration site for cortical inputs involved in pain modulation and opioid-mediated analgesia.

Electrical and chemical stimulation studies demonstrating the role of the vlPAG in descending pain modulation have found the functional output to be a result of the

dense projection to the RVM (Behbehani and Fields, 1979, Gebhart et al., 1983, Prieto et al., 1983), due to sparse vlPAG projections to the dorsal horn of the spinal cord (Basbaum and Fields, 1979). Furthermore, these manipulations in the vlPAG have the ability to differentially alter RVM ON- and OFF-cell activity, which produce antinociception through substantial projections to the dorsal horn via the dorsolateral funiculus (Loewy and Saper, 1978, Basbaum and Fields, 1979, Abols and Basbaum, 1981, Fields et al., 1995, Heinricher and Ingram, 2008, Heinricher et al., 2009, Zhang et al., 2015). Interestingly, female rats have nearly double the number of RVM-projecting vlPAG neurons, with less CFA-induced activation of vlPAG neurons (Loyd and Murphy, 2006), underscoring the importance of looking at sex differences in our studies in this region.

Recent studies have even shown that vlPAG projections to regions other than the RVM can be implicated in antinociception, complicating defining descending pain modulatory circuit neurons within the vlPAG by their efferents to the RVM. One example is dopamine neurons that project to the BNST (Yu et al., 2021), which interestingly has reciprocal connections with the vlPAG via GABAergic efferents (Hao et al., 2019). Another is the connection between the central medial nucleus of the thalamus, which when lesioned temporarily alleviates mechanical hyperalgesia in a neuropathic pain model (Sun et al., 2020). The vlPAG has many other efferent targets that are associated with other behaviors. GABAergic projections to the VTA have been implicated in freezing behaviors (St. Laurent and Kauer, 2020). Single-unit recordings in awake behaving animals have linked vlPAG cellular activity to threat probability evaluation (Wright and McDannald, 2019). A subpopulation of neurons involved in

freezing with distinct connectivity, molecular markers, and electrophysiological features have been identified in mice (Vaaga et al., 2020). An entire field of work has implicated this region in the acquisition, expression, and extinction of fear, anxiety, or defensive response (Borszcz et al., 1989, Fanselow, 1991, De Oca et al., 1998, McDannald, 2010, Wright et al., 2019, Wright and McDannald, 2019).

Cell-type characterization

There are many ways to characterize heterogeneity within a brain region. Early work uncovered molecularly distinct neuron populations using GAD-immunoreactivity, showing ~33% of the vlPAG cell bodies are GABAergic (Barbaresi and Manfrini, 1988, Reichling and Basbaum, 1990). Much of the interest surrounding evaluation of GABAergic neurons was motivated by the identification of a direct, excitatory connection between the vlPAG and the RVM (Behbehani and Fields, 1979), suggesting that the inhibitory neurons in the vlPAG served as an interneuron population to control the intensity of the output signal (Basbaum and Fields, 1984). To determine the functional effect of GABAergic control of vlPAG output, GABA receptor antagonists were locally infused to the vlPAG producing increase vlPAG firing, increased downstream RVM OFF-cell firing, and analgesia (Moreau and Fields, 1986, Behbehani et al., 1990b, Knight et al., 2002). It is important to note, that GABAergic inputs onto vlPAG neurons can also be a result of incoming inhibitory afferents from many different brain regions, including the lateral hypothalamus, BNST, and central amygdala.

Interestingly, new methodological tools confirmed the presumption that selective excitation of the GABAergic population within the vlPAG is hyperalgesic (Samineni and Gereau, 2017). This behavioral outcome suggests that either local GABA neurons are inhibiting neighboring glutamatergic RVM-projecting neurons that engage with downstream OFF-cells, reducing activation of these nociception-inhibiting cells. Or they are GABA neurons that project to the RVM where they can be directly inhibiting OFF-cells, producing the same outcome. Histological studies confirm that GABAergic afferents in the RVM coming from the vlPAG come in contact with both excitatory and inhibitory RVM neurons (Morgan et al., 2008).

The vlPAG also contains glutamatergic neurons that when selectively activated promote analgesia (Samineni and Gereau, 2017). This provides useful confirmation of many early studies that showed both electrical and chemical stimulation of the vlPAG is analgesic (Reynolds, 1969 and 1977; Behbehani and Fields 1979; Soper and Melzack 1982; Yaksh, 1988). Concurrent studies linked vlPAG stimulation to changes in downstream RVM activation (Venegas and Fields, 1984); 84% of ON- and OFF- cells observed, showed increased activity during PAG stimulation that was coupled with a suppression of the tail flick response. Unfortunately, selective activation of glutamatergic vlPAG neurons also enhances anxiety (Taylor et al., 2019), one of the many off-target effects precluding this stimulation target as a therapeutic option for clinical pain management. Deep brain stimulation targeting the vlPAG has been used to for treatment resistant hypertension (McBryde et al., 2011, O'Callaghan et al., 2014), however, reemphasizing the many subcircuits that utilize this region and the importance of understanding possible selective cellular activation by specific stimuli.

Dopamine (DA) neurons have also been identified within the vlPAG and despite not projecting to the RVM (Suckow et al., 2013) they have been implicated in the broader supraspinal pain circuitry and analgesia (Taylor et al., 2019, Yu et al., 2021). Interestingly, these DA neurons corelease both dopamine and glutamate at terminals in the BNST (Li et al., 2016). Additionally, selective activation of vlPAG DA neurons is antinociceptive in males only (Yu et al., 2021). Serotonergic neurons are densely populated in the dorsal raphe and extend diffusely up into the most ventral portion of the vlPAG (Crawford et al., 2010). These neurons have also been implicated in opioid-mediated analgesia (Samanin et al., 1970).

In addition to neurotransmitter content, another important distinction amongst vlPAG neurons are receptor and channel expression. The expression of different receptors and channels can significantly alter the firing features of these cells and how the circuit on the whole responds to pain and therapeutics that target pain. For the purposes of this thesis, we focus on mu-opioid receptor (MOR) expression, which will be discussed extensively in subsequent sections. Overall, MOR's are known to be expressed on a subset of vlPAG cell bodies (Chieng and Christie, 1994a). Despite evidence that MOR's are expressed on GAD+ neurons (Kalyuzhny and Wessendorf, 1998), they are also on non-GAD expressing neurons and neurons with distinct morphologies (Chieng and Christie, 1994a, Zhang and Hammond, 2009)—complicating the theorized simplicity of selective MOR expression on GABAergic interneurons in the vlPAG. Previous studies have also described the presence of many different channels (ie: GIRK, HCN, T-type calcium, TRPV1, etc.) that provide interesting avenues to further differentiate

distinct populations within this region (Chieng and Christie, 1994a, Park et al., 2010, Liao et al., 2011, Du et al., 2013, Lau and Vaughan, 2014).

Lastly, recordings done *in vivo* have that the vlPAG contains ON-, OFF-, and neutral-cells based on the same characterization used in the RVM (Heinricher et al., 1987, Samineni et al., 2017b). Having functionally distinct cell types, defined by their firing response to the application of acute noxious stimuli, has provided the ability to begin evaluating how these cells fire differently *in vivo* during pain states. Neuropathic pain induced by paclitaxel, a commonly used chemotherapy drug, resulted in not only enhanced firing of ON-cells but recruited both OFF- and NEUTRAL-cells to fire like ON-cells in response to noxious and previously innocuous stimuli. This evidence for significant alterations to the firing frequency of vlPAG neurons during persistent pain states, furthered our interest in using firing properties to evaluate how vlPAG neurons respond to stimuli and how that is altered during acute and persistent pain states.

Although these studies collectively provide many details about many features that can be used to defined heterogeneity within the vlPAG, they do not directly address the question of how distinct populations of vlPAG neurons are recruited during acute and persistent pain states. **Cellular heterogeneity coupled with our furthered understanding of many different behaviors relying on vlPAG subcircuits targeting specific cell types, highlights the importance of defining physiologically distinct neuron populations, determining which types are selectively engaged by a given noxious stimuli, and how that activation profile is altered between the acute and persistent stage.**

Electrophysiology methods to characterize cell types

Whole-cell patch-clamp electrophysiology can be used to record from acute brain slices from naïve animals and those that have experienced acute or persistent inflammation. This *in vitro* methodology allows us to evaluate the intrinsic membrane and firing properties, such as resting membrane potential (RMP), membrane resistance (R_{in}), capacitance (C), low-threshold (or rebound) spiking, spontaneous firing frequency, and features that define firing patterns (frequency and duration of firing, AP amplitude, etc.). These experiments, when done across a broad sampling of neurons, provide the information necessary to first define physiologically distinct neuron types (see *Chapter 2, methods*). Defining distinct neuronal populations allows us to categorize neurons in all conditions and then compare across conditions to determine if individual populations are selectively altered by acute or persistent inflammation.

1.3 Mu-opioid receptor function in the vlPAG

The vlPAG is a key site of opioid-induced analgesia, which has been linked to the actions of the mu-opioid receptor (MOR) (Heinricher and Morgan, 1999). MORs are expressed on both cell bodies and presynaptic terminals within the vlPAG, where they carry out different mechanisms that work in concert to promote descending inhibition by the modulatory circuit. These distinct mechanisms, described thoroughly in subsequent sections, are thought to collectively inhibit neurons. However, previously described work outlines how global inhibition of the vlPAG (for example with muscimol or baclofen) produces descending facilitation. Instead, it is excitation of the vlPAG (electrical or chemical) that yields descending inhibition. These contradictory findings were explained

in a circuit mechanism referred to as the opioid-mediated *disinhibition of pain hypothesis*. This explanation hypothesizes selective postsynaptic MOR expression on inhibitory interneurons within the vlPAG and simplifies the role of presynaptic inputs. **The studies in this thesis used physiological cell-type characterization to determine whether postsynaptic MOR expression is selective to individual cell-types, supporting the disinhibition of pain hypothesis, or expressed on distinct neuron types, providing additional information on a more complex analgesic circuit. Furthermore, we carried out functional selectivity studies to elucidate cellular mechanisms dictating agonist-specific efficacy with MOR populations expressed in distinct cellular compartments.**

MOR signaling

The mu-opioid receptor (MOR) is a G protein-coupled receptor with 7-transmembrane domains, an extracellular orthosteric binding sites for opioid ligands and allosteric binding sites for positive and negative allosteric modulators, and an internal C-terminal tail that promotes G protein binding in the presence of a ligand. Ligand binding to the orthosteric site recruits inactive $G_{i/o}$ G proteins to the c-terminal tail, which when bound undergo an exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) by guanine exchange factor (GEF) proteins. Once GTP bound, the α- and $\beta\gamma$-subunits dissociate, where they are then active and able to act on downstream effector targets. As a $G_{i/o}$-coupled receptor, MORs are considered to indiscriminately activate both G_i and G_o G proteins, however an increasing number of studies demonstrate functional selectivity in G protein coupling and subsequent activation (Masuho et al.,

2015, Masuho et al., 2020). Regulator of G Protein Signaling (RGS) proteins bind to active α-subunits driving GTP-hydrolysis to GDP. The hydrolyzed α-subunits regain their affinity for the βγ-subunits—reforming the heterotrimeric G protein and recovering the inactive G-protein substrate pool for ongoing MOR signaling.

Of the 3 major opioid receptors, MOR activation primarily promotes analgesia. The selective analgesic function of the MOR was confirmed by many studies using the MOR knockout mouse in the 1990's (Matthes et al., 1996, Kieffer, 1999). MORs are expressed both on postsynaptic cell bodies and both excitatory and inhibitory presynaptic terminals within the vlPAG. Upon local microinjection of MOR agonists to the vlPAG, these two populations of receptors, and their distinct signal transduction mechanisms, work in concert to produce analgesia (Yaksh et al., 1976, Jensen and Yaksh, 1989). Systemic morphine administration provides analgesic relief to thermal and joint hyperalgesia, as well as mechanical allodynia in rats after persistent inflammatory pain induced by CFA injected into the hindpaw (9 d) (Nagakura et al., 2003). Interestingly, RVM-retrolabeling combined with Fos immunocytochemistry revealed that both males and females showed comparable vlPAG activation after CFA, however, more RVM-projecting neurons were activated in the males (Loyd and Murphy, 2006). Additionally, systemic morphine administration reduced activation of vlPAG neurons exclusively in males. Together these striking sex differences in circuit activation by inflammatory pain and inactivation by opioids begin to address why morphine has greater efficacy in males, seen in both preclinical and clinical studies (Cepeda and Carr, 2003, Miller and Ernst, 2004, Fullerton et al., 2018). Also, a study completed in male rats showed greater opioid analgesic efficacy (paw withdrawal latency test) to DAMGO locally infused downstream

in the RVM after CFA-induced inflammatory pain, which is even greater at the persistent time point compared to acute (Hurley and Hammond, 2000). **Overall, these details inspired us to determine which physiologically distinct neuron types in the vlPAG express MORs postsynaptically, whether there are sex differences in opioid sensitivity in distinct neuronal populations, and whether opioid efficacy in the vlPAG (determined using inhibition of firing frequency) was impacted by persistent inflammation.**

Regulators of G protein signaling

Regulators of G protein signaling (RGS) are GTPase accelerating proteins (GAP) that expedite the hydrolysis of the GTP on the active α-subunit of the G protein (see *Figure 1.6*) (Hollinger and Hepler, 2002, Traynor and Neubig, 2005, Traynor, 2012). Once hydrolyzed to GDP, the α-subunit regains its affinity for the βγ-subunit and the inactive heterotrimeric protein reforms, replenishing the inactive G protein pool available to ongoing receptor activation. There are over 30 different mammalian RGS proteins, that all have a 125 amino-acid region responsible for binding the GTP-bound Gα subunit prompting GTP hydrolysis, referred to as the RGS homology (RH) domain (Hollinger and Hepler, 2002). These proteins are then divided into families based on varied functionality. The RGS proteins range from small, simplistic proteins that are primarily comprised of the RH domain, such as RGS4 and RGSZ, to larger proteins (ie: the RGS7 family) that have additional protein-protein binding cites that can guide receptor recognition, promote membrane association, and protect against degradation.

Many RGS proteins are involved in the regulation of MORs, including RGS4 (Garzón et al., 2005b, Roman et al., 2007, Leontiadis et al., 2009, Santhappan et al., 2015), RGS9-2 (Psifogeorgou et al., 2007, Papachatzaki et al., 2011, Gaspari et al., 2017), RGS19 (Wang and Traynor, 2013), and RGSz (Garzón et al., 2005a, Gaspari et al., 2018, Sakloth et al., 2019). The duration of opioid (ie: morphine) exposure impacts the association between the MOR and specific RGS proteins in the PAG (Garzón et al., 2005b). Due to the primary role of inactivating G proteins, RGS proteins are thought to negatively regulate MOR signaling, blunting the analgesic effect and contributing to tolerance (Psifogeorgou et al., 2007). However, many of these studies have been conducted in subtype specific RGS knockout mice and with functional redundancy from the many RGS isoforms these models leave open the possibility of mechanistic compensation by other RGS isoforms preforming the same hydrolysis function. The RGS model used in our studies addresses this concern by blocking all RGS hydrolysis with a single point mutation in the binding site for RGS proteins on $G\alpha_{i/o}$. Previous studies using these models uncovered functional selectivity in morphine- and methadone-induced antinociception, via G_o G proteins (Lamberts et al., 2011). **This work prompted us to investigate agonist and G protein specific recruitment in postsynaptic MOR-GIRK mechanisms as well as presynaptic MOR-mediated inhibition of release.**

Mechanisms of pre- and postsynaptic MOR signaling

Single ligand binding events can trigger the activation of many G proteins that can then open many channels, in addition to other effector targets, demonstrating a key step in GPCR signal amplification. The time course of metabotropic receptor signaling

includes a delay between ligand binding and the different cellular responses. Signal transduction is influenced by the rate limiting feature of several different binding steps, compartmental differences in receptor-effector coupling, and varied availability of necessary proteins such as the inactive G protein substrate pool that can impact the efficiency or volume of signaling.

There are many secondary effector targets activated by MOR-activated G proteins that impact cell function. MOR's expressed on postsynaptic cell bodies are often coupled to G protein-coupled inwardly-rectifying potassium channels (GIRKs) that open when bound by βγ-subunits (Logothetis et al., 1987) allowing for K^+ efflux and subsequent hyperpolarization of the neuron (see *Figure 1.6*). Concurrently, activated Gα subunits inhibit adenylyl cyclase activity, decreasing the production of cyclic adenosine monophosphate (cAMP) from ATP, which in turn reduces protein kinase A (PKA) activation. Presynaptically, MOR activation utilizes additional pathways to inhibit release of neurotransmitters into the synaptic cleft (Chieng and Christie, 1994b, Vaughan et al., 1997a).

Of the two postsynaptic mechanisms described, this thesis focuses on GIRK activation. Whole-cell voltage-clamp recordings of the GIRK current response to MOR agonist application and reversal by a MOR antagonist allow us to determine the expression of postsynaptic MOR's coupled to GIRKs. Previous studies in the vlPAG have demonstrated heterogeneity in postsynaptic MOR expression, with ~30-60% of vlPAG neurons exhibiting a GIRK current response to agonist application (Chieng and Christie, 1994a, McPherson et al., 2018). It has been suggested that MORs are selectively expressed on GABAergic interneurons within the vlPAG (Yaksh et al., 1976, Basbaum

and Fields, 1984, Reichling et al., 1988, Lau and Vaughan, 2014), however, there are several studies that contribute to a more complex opioid circuit (see *The disinhibition of pain hypothesis*).

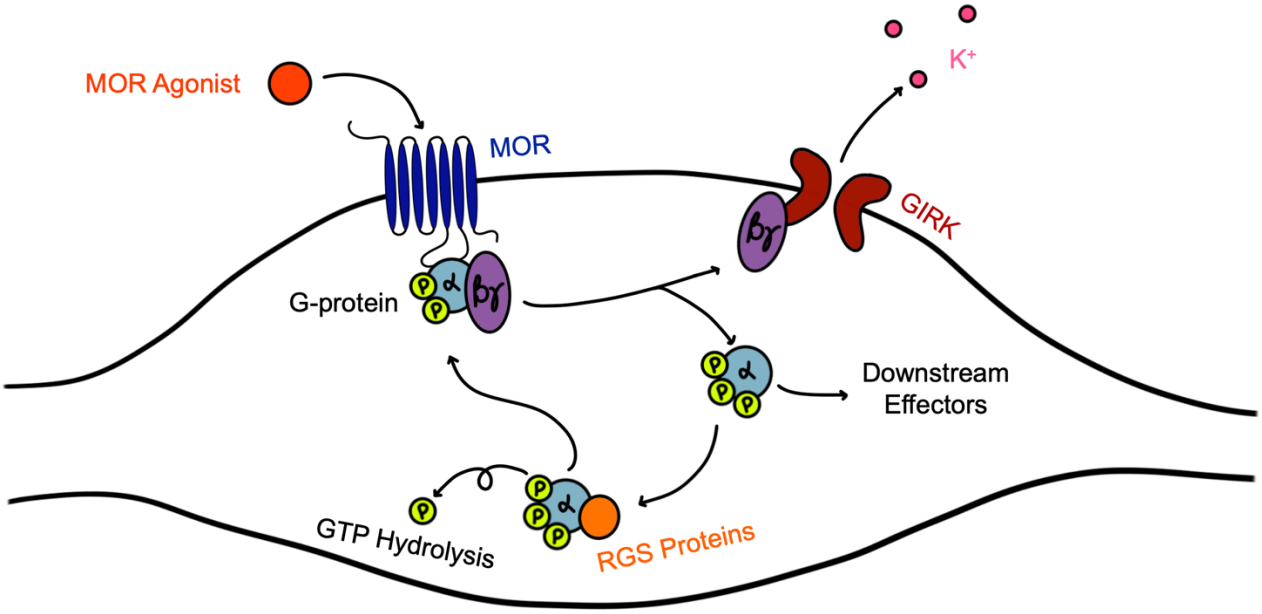

Figure 1. 6: Postsynaptically expressed MOR signaling

Schematic demonstrating simplified MOR-mediated GIRK activation. (1) Agonist binds to the extracellular ligand-binding domain, (2) conformational changes in the ligand-bound state promote inactive G protein recruitment to the intracellular C-terminal tail promoting the exchange of GDP for GTP on the α-subunit of the inactive heterotrimeric G protein, (3) once GTP bound the α- and βγ-subunits dissociate allowing for activation of downstream effector targets, such as the GIRK that is specifically activated by βγ, (4) RGS proteins expedite GTP hydrolysis on the active α-subunit, causing the now inactive α-subunit to regain affinity for the βγ-subunit, replenishing the inactive G protein substrate pool for further signaling.

Whole-cell voltage-clamp recordings can also be utilized to evaluate presynaptic release properties. Of the many different experimental protocols, spontaneous action-potential-independent miniature inhibitory (GABAergic) postsynaptic currents (mIPSCs) and electrically evoked GABA release (eIPSCs) were used to determine the mechanism for inhibition of neurotransmitter release. Pharmacological manipulations identified that MORs in GABAergic terminals couple to voltage-gated potassium channels through the phospholipase A_2 / arachidonic acid / 12-lipoxygenase cascade (Vaughan et al., 1997a). Although MORs are most effective in inhibiting presynaptic GABA release in the vlPAG, they also inhibit release from glutamatergic afferents (Vaughan 2020), but through a different mechanism (Vaughan et al., 1997a). Interestingly, other GPCRs expressed in presynaptic terminals in the vlPAG that inhibit release, such as $GABA_B$, act through a different than the MORs (Vaughan et al., 1997a).

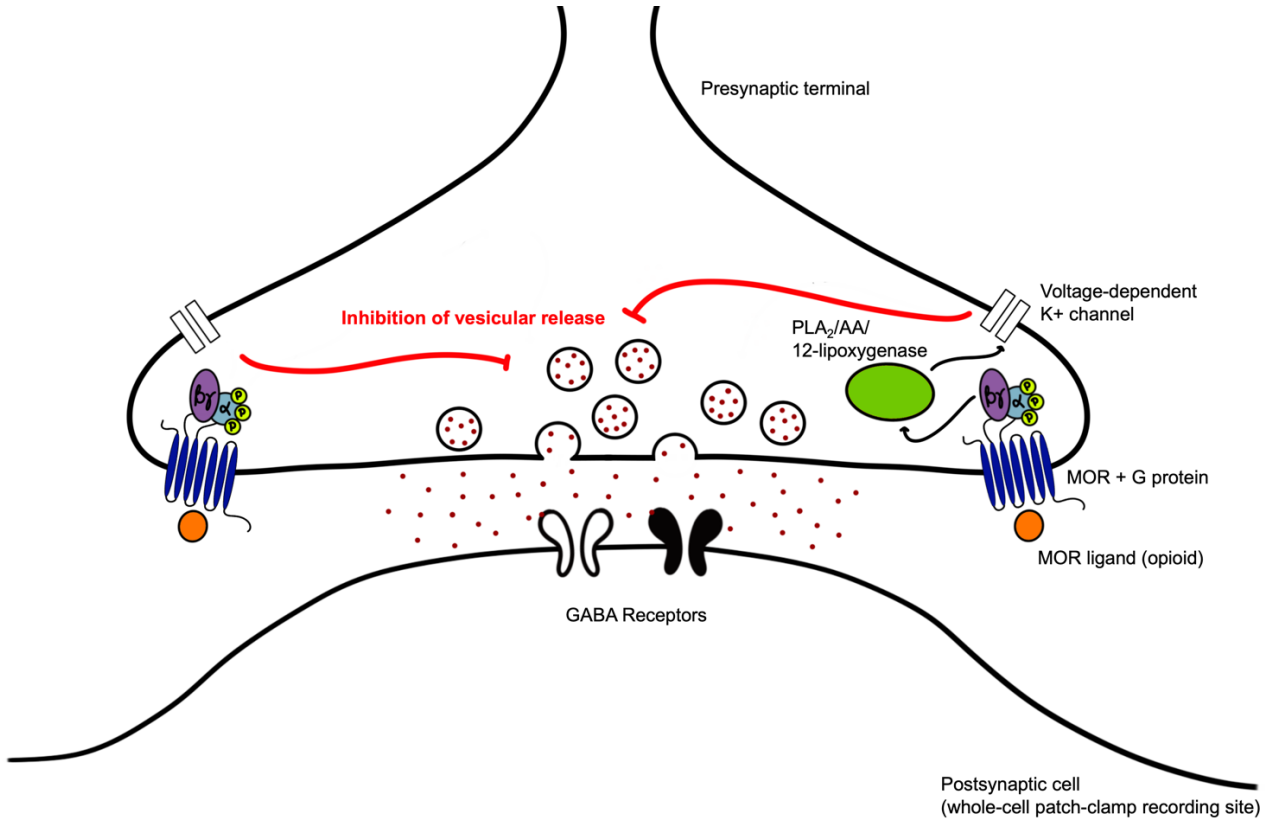

Figure 1. 7: Mechanism for MOR-mediated inhibition of presynaptic release

Simplified schematic showing MOR-mediated inhibition of presynaptic GABA release in terminals in the vlPAG. MORs couple to voltage-dependent K^+ channels in GABAergic terminals through the PLA_2/arachidonic acid/12-lipoxygenase cascade system. As a result, ligand binding at presynaptic MORs repolarize terminals, reducing vesicular release.

The disinhibition of pain hypothesis

In addition to being an important integration site for descending pain modulation, the vlPAG is a key target for opioid-induced analgesia, which is proposed to occur through a circuit mechanism referred to as the disinhibition of pain hypothesis. The disinhibition of pain hypothesis aims to reconcile how direct electrical or chemical excitation and opioid-mediated inhibition of vlPAG neurons both produce analgesia (Jensen and Yaksh, 1989). In the vlPAG, both chemical or electrical activation and opioid-mediated inhibition activate downstream nociception-inhibiting OFF-cells in the RVM. Activation of the vlPAG enhances ON- and OFF-cell activity in the RVM, however the effect was only analgesic at intensities sufficient to increase OFF-cell firing (Vanegas et al., 1984a, Tortorici and Morgan, 2002). Morphine microinjection into the vlPAG inhibits ON-cells while increasing OFF-cells spontaneous and tail-flick evoked activity (Cheng et al., 1986a, Tortorici and Morgan, 2002). The circuit mechanism proposed to account for these contradictory findings is that both manipulations activate glutamatergic RVM-projecting neurons that activate OFF-cells; stimulation does this by directly activating these glutamate output neurons, whereas opioids selectively inhibit GABAergic inputs resulting in a disinhibition of the same glutamate output neurons (see *Figure 1.7*) (Basbaum and Fields, 1984, Barbaresi and Manfrini, 1988, Reichling and Basbaum, 1990, Renno et al., 1992, Kalyuzhny and Wessendorf, 1998, Lau and Vaughan, 2014). Neurons within the vlPAG have been found to be both inhibited and activated by opioid application (Behbehani et al., 1990a), with confirmed populations of disinhibited RVM-projecting vlPAG neurons (Osborne et al., 1996, Lau et al., 2020).

Additional findings altering GABA tone or the activation of glutamatergic or GABAergic vlPAG neurons reinforces this circuit mechanism. First, microinjection of $GABA_A$ antagonists (picrotoxin and bicuculline) into the vlPAG is analgesic and local $GABA_A$ agonist (muscimol, THIP) into the vlPAG blocks the analgesic effect of locally infused opioid-mediated analgesia (Zambotti et al., 1982, Moreau and Fields, 1986, Depaulis et al., 1987). Targeted chemogenetic activation of glutamatergic neurons or inhibition of GABAergic neurons within the vlPAG is analgesic (Samineni et al., 2017a). Together these findings support that GABA receptor activation within the vlPAG blocks analgesia and can potentially contribute to *descending facilitation of pain* from the vlPAG.

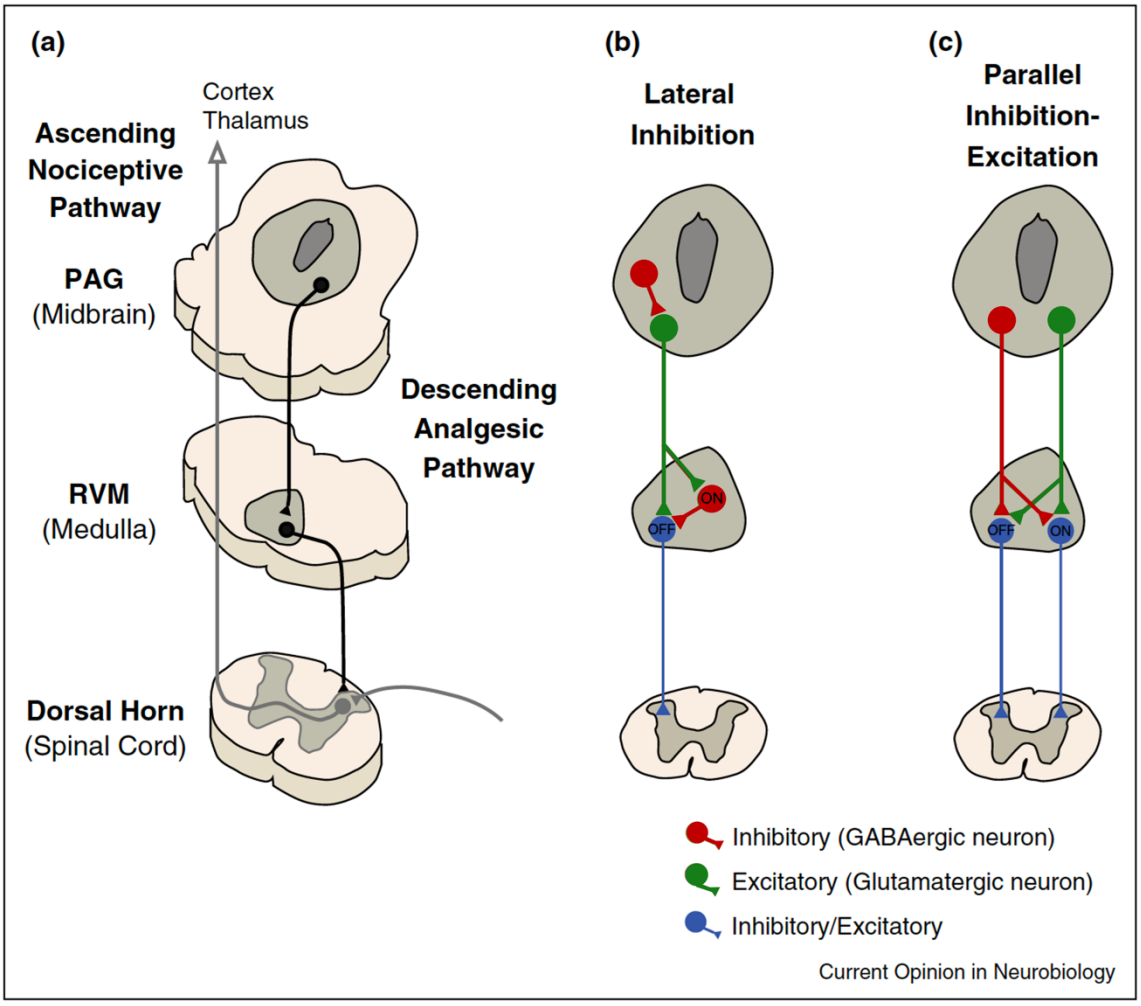

Figure 1. 8: vlPAG-RVM circuit

a) Outlines the overall ascending nociceptive and descending analgesic pathways. b) Depicts the earlier vlPAG-RVM circuit mechanism described in the 1980's (Basbaum and Fields, 1984). c) Shows the updated circuit mechanism that demonstrates both excitatory and inhibitory vlPAG populations projecting to both functionally distinct neuron populations in the RVM, that then both project to the dorsal horn.

Adapted from: Lau, B. K., & Vaughan, C. W. (2014). Descending modulation of pain: the GABA disinhibition hypothesis of analgesia. *Current opinion in neurobiology*, *29*, 159-164.

Disinhibition of glutamatergic RVM-projecting vlPAG neurons can occur either through targeted expression of postsynaptic MORs on GABAergic interneurons, which has been suggested extensively, or by opioid-mediated inhibition of GABA release from presynaptic terminals of vlPAG afferents. Opioid-mediated disinhibition of firing has been demonstrated in a modest sample of RVM-projecting vlPAG neurons (Lau et al., 2020), despite unsuccessful identification of direct functional connections between vlPAG neurons using paired recordings (Lau, 2011). MOR-mediated inhibition of presynaptic release has higher efficacy at GABAergic terminals compared to glutamate, providing evidence for a stronger E/I shift to excite vlPAG neurons in the presence of opioids (Lau et al., 2020). Furthermore, opioid-mediated activation of downstream RVM OFF-cells and subsequent antinociception (measured with tail flick latency) observed in response to systemic morphine are abolished when the non-selective EAA receptor antagonist kynurenate is applied, demonstrating the key role for glutamate afferents within the RVM in driving antinociception (Heinricher et al., 1999).

Despite clear evidence for opioid-mediated disinhibition of RVM-projecting neurons, GABAergic vlPAG neurons also project to the RVM, both neuronal populations project to both RVM ON- and OFF-cells, and MOR expression is not selective to GABAergic neurons, collectively contributing to a more complex analgesic circuit. The analgesic circuit between the vlPAG and the RVM consists of parallel descending GABAergic and glutamatergic projections onto both ON- and OFF-cells, subpopulations which can be inhibited or disinhibited by opioids depending on the compartmental expression of MOR's (on their cell bodies or presynaptic terminals) (Osborne et al., 1996, Lau and Vaughan, 2014, Heinricher and Ingram, 2020). Despite early studies that

indicated minimal GABAergic vlPAG projections to the RVM (Reichling and Basbaum, 1990, Williams and Beitz, 1990), more recent work has found evidence for more substantial GABAergic projections to the RVM (Morgan et al., 2008, Lau and Vaughan, 2014, Li and Sheets, 2018). Nearly half of a large sample of vlPAG neurons (148 out of 344) retrogradely labeled from the RVM expressed MORs and only around half of the inhibitory terminals in the vlPAG expressed MORs, demonstrating that RVM-projecting neurons can be both disinhibited and directly inhibited by opioids (Commons et al., 2000, Wang and Wessendorf, 2002). Additionally, microinjection of morphine into the vlPAG blocked glutamate evoked (iontophoresis) activation of ON-cell firing in the RVM, suggesting opioids within the vlPAG are activating GABAergic afferents onto ON-cells in the RVM (Morgan et al., 1992), expanding the circuit implication of opioid action in the vlPAG beyond just disinhibition of RVM-projecting glutamate neurons.

Afferent drive *in vivo* and agonist-specific mechanisms of action contribute to remaining questions about opioid action in the vlPAG. Interestingly, *in vivo* recordings from implanted tetrodes within the vlPAG showed almost exclusively disinhibition by morphine (Tryon et al., 2016). Similarly, $GABA_A$ antagonist (bicuculline) increased the baseline firing rate of 53% of cells *in vitro* and 74% *in vivo* (Behbehani et al., 1990b). These studies indicate that the intact circuits have even greater GABAergic afferent mediated tone in the vlPAG, which when inhibited by opioids disinhibit glutamatergic and GABAergic neurons sending projections to the RVM. Morphine has decreased antinociceptive potency measured using hot-plate latency *in vivo* when presynaptic K_v^+ channels are blocked with α-dendrotoxin, whereas fentanyl has decreased potency when GIRKs are blocked with tertiapin-Q (Morgan et al., 2020)—suggesting that agonist-

specific efficacy can rely on different components of the more complex analgesic circuit between the vlPAG and RVM. Altogether, these studies provide clear evidence for disinhibition of vlPAG→RVM neurons, while demonstrating the parallel pathways of descending glutamatergic and GABAergic vlPAG neurons that project to both ON- and OFF-cells (Lau and Vaughan, 2014). Lastly, systemic morphine administration provided analgesic relief to thermal and joint hyperalgesia, as well as mechanical allodynia in rats after persistent inflammatory pain induced by CFA injected into the hindpaw (9 d) (Nagakura et al., 2003).

1.4 Aims

This thesis focuses on characterizing cellular heterogeneity within the vlPAG to allow us to identify how opioids and acute and persistent inflammation impact the activation state of neurons within this region. Electrophysiological methodologies were enlisted to thoroughly evaluate naïve intrinsic membrane and firing properties to first determine if neuronal heterogeneity could be defined using these features. Next, we determined whether opioid sensitivity segregated amongst these physiologically distinct neurons. An additional survey was conducted after acute and persistent inflammation to determine whether these neurons experienced alterations in activity or opioid sensitivity due to the different pain states. Additional studies were conducted to investigate agonist- and neuronal compartment-specific features of MOR functional selectivity.

Chapter 2: Intrinsic membrane properties define ventrolateral periaqueductal gray neurons that are differentially activated by persistent inflammation

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The studies were supported by the National Institute of Drug Abuse (NIDA) 1 R01 DA042565 (SLI) and 1 F31 DA052114-01 (CAB). KBM was supported by the National Science Foundation Graduate Research Fellowship Program.

2.1 Abstract

The ventrolateral periaqueductal gray (vlPAG) is a key structure within the descending pain modulatory pathway and an important target for opioid-induced analgesia. This area contains heterogeneous neurons with respect to neurotransmitter and receptor expression so it is difficult to define vlPAG neurons that contribute to pain and analgesia. Characterization of intrinsic membrane properties of 371 vlPAG neurons identified 4 neuron types with distinct intrinsic firing patterns: *Phasic*, *Tonic*, *Onset,* and *Random.* The majority of the neurons sampled were *Phasic* and *Tonic*. μ-opioid receptor (MOR) expression was determined by the ability of a selective MOR agonist (DAMGO) to activate G protein-coupled inwardly-rectifying potassium channel (GIRK) currents. Within each neuronal type classification there were opioid-sensitive and -insensitive neurons in naïve rats, as well as in rats pretreated with Complete Freund's Adjuvant (CFA) to produce persistent inflammation. The presence of low threshold spikes (LTS) did not correlate with MOR-mediated GIRK currents, and MOR expression alone does not define a physiologically distinct neuron type in the vlPAG. In loose-patch recordings, MOR activation inhibited firing in nearly all spontaneously active neurons, both in naïve and persistent inflammation conditions. CFA-induced inflammation increased Fos expression at acute (2 h) and persistent inflammation (5-7 d) time points. However, persistent, but not acute, inflammation enhanced spontaneous firing and lowered firing thresholds of *Phasic* neurons, which was maintained in the absence of synaptic inputs. Taken together, persistent inflammation selectively activates *Phasic* neurons, of which only a subset are opioid-sensitive.

2.2 Introduction

The ventrolateral periaqueductal gray (vlPAG) is an important site contributing to the descending modulation of pain (Bodnar, 2000, Heinricher and Ingram, 2020). Electrical and chemical stimulation of the vlPAG induce strong antinociception in animals (Reynolds, 1969, Mayer and Liebeskind, 1974, Soper and Melzack, 1982) and pain relief in humans (Hosobuchi et al., 1977, Barbaro, 1988, Bittar et al., 2005). Direct opioid microinjections into the vlPAG also induce antinociception, implicating the vlPAG in opioid-mediated analgesia (Yaksh et al., 1976, Jensen and Yaksh, 1989, Morgan et al., 2006, Bernal et al., 2007, Morgan et al., 2020). Neurons within the vlPAG express many different neurotransmitters, neuropeptides, and receptors (Lau and Vaughan, 2014, Bagley and Ingram, 2020, Heinricher and Ingram, 2020). Current genetic strategies attempt to define analgesia circuits by targeting expression of specific markers, such as the μ-opioid receptor (MOR) or GABA and glutamate (François et al., 2017, Samineni et al., 2017a, Zhang et al., 2020), but these methods do not give insights into physiological roles of subpopulations of vlPAG neurons. The strategy we utilized in these studies was to examine intrinsic membrane properties to define functional populations of neurons that provide a framework to assess how distinct neuron types are modulated by acute and persistent inflammation, as well as opioids.

Opioid microinjections into the vlPAG disinhibit output neurons to the rostral ventromedial medulla (RVM) to produce analgesia (Basbaum and Fields, 1984, Lau and Vaughan, 2014, Lau et al., 2020). In a subpopulation of opioid-sensitive vlPAG neurons, postsynaptic MORs activate G protein-coupled inwardly-rectifying potassium channels

(GIRKs) that hyperpolarize the neurons (Barbaresi and Manfrini, 1988, Chieng and Christie, 1994a, Kalyuzhny and Wessendorf, 1998, McPherson et al., 2018), some of which are hypothesized to be locally-projecting GABAergic neurons (Basbaum and Fields, 1978, Barbaresi and Manfrini, 1988, Reichling and Basbaum, 1990, Kalyuzhny and Wessendorf, 1998). Although it is tempting to use MOR as a neuron-type specific marker, morphological features of opioid-sensitive and -insensitive neurons are similar (Chieng and Christie, 1994a). Additionally, MORs are expressed on both GABAergic and non-GABA neurons within this region (Zhang et al., 2020), both of which project to the RVM (Commons et al., 2000). Thus, a more comprehensive study is necessary to determine if opioid sensitivity defines a single functional subset of neurons in the vlPAG with distinct firing properties.

Acute noxious stimuli excite a subpopulation of vlPAG neurons (Heinricher et al., 1987, Tryon et al., 2016) and promote Fos expression in the vlPAG (Keay and Bandler, 1993, Keay et al., 2000). Chronic pain enhances activity of vlPAG neurons *in vivo* (Samineni et al., 2017b) and increased BOLD responses in the PAG are observed in humans experiencing secondary hyperalgesia (Zambreanu et al., 2005), indicating activation of PAG neurons in pain states. The intrinsic membrane firing properties of vlPAG neurons that are activated by noxious stimuli have not been characterized. Thus, these studies examined intrinsic membrane firing properties of vlPAG neurons in rats and assessed whether specific subpopulations are selectively activated by acute or persistent CFA-induced inflammation.

2.3 Materials & Methods

Animals

Female and male Long Evans rats (Harlan Laboratories and bred in house; 30-90 d postnatal for electrophysiology) were group housed with environmental enrichment squares and toy bones. Lights were on a 12 h light and dark cycle, with food and water provided *ad libitum*. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Oregon Health & Science University. Some recordings used Sprague Dawley rats (n = 70) and no strain differences were observed.

CFA treatment

Rats were lightly anesthetized with isoflurane and 100 µL of Complete Freund's adjuvant (CFA; Sigma) was injected into the left hindpaw. Rats were used 2 h or 5-7 d following injections (6 d for Fos immunohistochemistry).

Fos immunohistochemistry

Rats were deeply anesthetized with isoflurane and perfused with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde (PFA) either 2 h or 6 d after CFA injection in the hind paw. The brains were post-fixed for an additional 24 h before being transferred and incubated in 30% sucrose in PBS at 4 ºC for 2-3 d. Brains were then frozen in dry ice and stored at 80 ºC until coronal brain slices (40 µM) were cut using a Leica CM2050 S cryostat. Sections were washed in PBS, blocked in 3% normal goat serum in PBS with 0.25% Triton X-100 (PBS-Tx), and incubated for 24 h at 4 ºC with anti-Fos antibody (1:8000 dilution; Cell Signaling Technology, catalog #2250) in

blocking solution. Sections were then washed in PBS and incubated with biotinylated goat anti-rabbit secondary antibody (1:600 dilution; Vector Laboratories, catalog #BA-1000) in PBS-Tx and 1% NGS for 2 h at room temperature. After another wash in PBS, sections were incubated in avidin-biotin-peroxidase complex (ABC Elite kit, PK-6100; Vector Laboratories) in PBS containing 0.5% Triton X-100. Lastly, sections were developed in 3,3-diaminobenzidine (DAB) for 3-5 m (until uniform vlPAG staining occurred with minimal increase in background staining). Sections were then mounted onto chromalumgelatin-coated slides. After drying the slides were dehydrated using a progression of alcohol (30%, 60%, 90%, 100%, 100% ethanol) followed by Citrasolv (Fisher Scientific) before coverslipping with Permount (Sigma Aldrich). Bright-field images of immunoreactive neurons in the vlPAG were captured digitally using an Olympus BX51 fluorescence microscope at 10x magnification. Regions of interest within the vlPAG were measured and Fos labeled neurons were manually quantified by an investigator blind to the treatment conditions, to give a Fos+ nuclei/mm^2 value. The 4 sections per rat were averaged so that each rat represented an n = 1 for staining across the rostral-caudal extent of the ventrolateral column.

Electrophysiology

Rats were anesthetized with isoflurane and brains were quickly removed and immersed in ice-cold sucrose aCSF containing the following (in mM): 75 NaCl, 2.5 KCl, 0.1 $CaCl_2$, 6 $MgSO_4$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 2.5 dextrose, 50 sucrose. Coronal slices containing the vlPAG were cut 220 μm thick with a vibratome (Leica Microsystems). Slices were placed in a holding chamber oxygenated with aCSF containing the following (in mM):

126 NaCl, 21.4 $NaHCO_3$, 11.1 dextrose, 2.5 KCl, 2.4 $CaCl_2$, 1.2 $MgCl_2$, and 1.2 NaH_2PO_4, and equilibrated with 95% O_2 / 5% CO_2 at 32 °C until recording. Brain slices were placed into a recording chamber on an upright Zeiss Examiner Z1 and superfused with 32 °C aCSF. Whole-cell recordings were conducted with pipettes (3-4 MOhms) with $KMeSO_4$ internal solution containing the following (in mM): 138 CH_3KO_4S, 10 HEPES, 10 KCl, 1 $MgCl_2$, 1 EGTA, 0.3 $CaCl_2$, 4 MgATP, and 3 NaGTP, pH 7.3-7.4. A junction potential of 15 mV was corrected for at the beginning of the experiments and access was monitored throughout. During whole-cell voltage-clamp recordings, neurons were held at -70 mV. During current-clamp recordings, no holding current was used. Data acquisition was completed with Axopatch 200B Microelectrode Amplifier (Molecular Devices) at 5 kHz and low-pass filtered at 2 kHz. Currents were digitized with InstruTECH ITC-18 (HEKA), collected via AxoGraph Data Acquisition software and analyzed using AxoGraph (AxoGraph Scientific). All analyses were blind to treatment.

Neuron type characterization

We recorded from 223 neurons in the vlPAG from naïve and 148 neurons from CFA-treated male and female Long-Evans rats. A subset of neurons (n = 59) from naïve rats recorded over the same time period as the recordings from acute and chronic CFA-treated rats were used for naïve vs CFA firing frequency comparisons to control for handling, housing stress, etc. Only neurons with stable resting membrane potentials (RMP) and exhibiting action potentials that crossed 0 mV when depolarized by current step protocols were used for electrophysiological analysis. In current-clamp, 2 s long depolarizing steps from 0 pA to 120 pA in 20 pA increments, with a 1 s delay in between were used to

evaluate firing patterns of vlPAG neurons. Spontaneous firing frequency was calculated as the total number of action potentials in a 3 s trace. Firing frequency during the current steps was determined by dividing the total number of action potentials by the time spent firing. Low threshold spiking (LTS) was assessed using a 500 ms long hyperpolarizing current step (-50 pA), which either did or did not elicit a spike upon the offset of the current step.

Input resistance (R_{in}) was calculated using the steady state current response from a -10 mV hyperpolarizing step in voltage-clamp. *Resting membrane potential (RMP)* was determined by averaging the baseline traces prior to the series of depolarizing current steps. *Capacitance* values were calculated from a -10 mV hyperpolarizing voltage step using Axograph X.

GIRK currents

GIRK current responses to DAMGO were evaluated after completing the protocols used to assess intrinsic membrane properties. Neurons were held at -70 mV in voltage-clamp. After 2-3 min of a stable baseline, a maximal concentration of selective MOR agonist DAMGO (5 μM) was superfused over the slice until the outward current reached the peak. The nonselective opioid receptor antagonist naloxone (10 μM) was then superfused until a steady return baseline was achieved. GIRK currents were measured as the difference between the peak of the current compared to an average of currents measured at baseline and reversal in the presence of naloxone. A neuron was considered opioid-insensitive if there was no change in current induced by the agonist. In some experiments,

$GABA_B$-mediated GIRK currents were elicited by superfusing agonist baclofen (20 μM) followed by antagonist CGP 35348 (10 μM). GIRK current traces were analyzed blind to the neuron type and treatment.

Loose-patch recordings

Pipettes with $KMeSO_4$ internal solution (2-3 MΩ) were used to get a cell-attached loose-patch seal (Beckstead et al., 2004) to passively measure firing rates of intact, spontaneously active vlPAG neurons without altering intracellular mileau. After 2-3 min of a stable baseline, the MOR agonist DAMGO (5 μM) was superfused over the slice for 4-5 min followed by antagonist naloxone (10 μM). Traces were excluded from the data set if there was run up or run down throughout the entire recording.

Experimental Design and Statistical Analysis

All data are expressed as mean ± SEM. Data were analyzed with Prism 9 (GraphPad Software). Each electrophysiological recording from a single neuron is treated as an individual observation; however, all data sets contain recordings from at least 8 different animals. Differences between groups were assessed using ANOVA and t-tests when appropriate (significance denoted as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$).

2.4 Results

vlPAG neurons can be divided into 4 neuron types with distinct firing properties

To begin to define heterogeneity within the vlPAG, the firing pattern and associated membrane properties were evaluated. Neurons within the vlPAG of Long-Evans rats were split into 4 neuron types based on distinct firing patterns: *Phasic*, *Tonic*, *Onset*, and *Random*. Since the vlPAG is a sexually dimorphic region (Loyd and Murphy, 2006, Bernal et al., 2007, Loyd et al., 2008b, Fullerton et al., 2018), both female and male rats were used throughout the studies but we observed no notable sex differences in firing patterns or firing thresholds. Subsequently the recordings from female and male rats were combined for analysis. The 4 main firing patterns are shown in Figure 1. Of the 223 neurons in naïve rats, 103 displayed firing patterns where firing duration decreased as the stimulus intensity increased (*Phasic*). The second largest group (77/223) fired throughout each of the depolarizing steps above 20 pA (*Tonic*). Approximately 10% of the neurons fired 1-2 action potentials at the beginning of each depolarizing step (*Onset*) and another ~10% displayed randomly timed action potentials throughout (*Random*). All neurons were evaluated for intrinsic membrane properties including resting membrane potential (RMP), membrane resistance (R_{in}), capacitance, and spontaneous firing activity (Table 1). Post-hoc comparisons following One-way ANOVA analyses for membrane properties in Table 1 are only made for the two most prevalent neuron types. RMPs of *Tonic* neurons are more hyperpolarized compared to *Phasic* neurons. All neurons have a high R_{in}. Lastly, *Tonic* neurons have a significantly larger capacitance than *Phasic* neurons. These additional differences in intrinsic membrane properties further underscore the

distinction between *Phasic* and *Tonic* neuron types based on firing patterns. The topographical distribution of each neuron type was evaluated for a large subset of neurons showing no unique organization or clustering of the different types throughout the rostral-caudal axis of the vlPAG (n = 117, chi-square, p = 0.2). Previous work in the lab has used Sprague Dawley rats, so we also examined intrinsic membrane properties from this strain and observed the same neuron types in similar proportions: 57% *Phasic* (25/44), 18% *Tonic* (8/44), 11% *Onset* (5/44), and 14% *Random* (6/44).

Intrinsic firing patterns and membrane properties of Phasic and Tonic neurons

Substantial differences in firing patterns, as well as spontaneous activity and firing frequency, were noted between the two most prevalent neuron types represented in our dataset. The majority of *Phasic* neurons were spontaneously active at the resting membrane potential (71/103, 69%; Table 1), and either fired evenly spaced action potentials, or shorter, bursts of action potentials with pauses of variable duration (Fig. 2A). *Phasic* neurons increased in firing frequency from an average of 4 Hz at the RMP to 41 Hz in response to the maximal current injection (Fig. 2B); however, the firing duration decreased and action potential amplitudes became attenuated as the depolarizing steps increased in intensity (Fig. 2C). The decrease in firing duration and attenuation in action potential amplitude were also observed with repeated shorter (200 ms) current steps of 60 pA, 120 pA, and 180 pA (data not shown). In contrast, *Tonic* neurons were spontaneously active with an average firing frequency of 4 Hz (71/77, 92%; Table 1) and reached a maximal firing rate of 20 Hz in response to the 120 pA step (Fig. 2D,E). *Tonic* neurons were defined by stable firing for the full duration of each current step (Fig. 2F), which

was also observed in response to shorter, repeated current steps (data not shown). *Tonic* neurons exhibited significantly slower firing frequency in response to depolarization compared to *Phasic* neurons (Two-way ANOVA, Main effect of neuron type, $F_{(1,178)} = 70$, $p < 0.0001$).

To determine whether the firing pattern and frequency were intrinsic, independent of inhibitory and excitatory synaptic inputs, we used AMPA- and $GABA_A$-receptor blockers (NBQX, 5 μM and gabazine, 10 μM) to block the synaptic inputs onto vlPAG neurons. Both *Phasic* and *Tonic* neurons maintain the same firing patterns and firing frequency at rest and in response to a series of depolarizing current steps in the absence of synaptic inputs (Fig. 3). Collectively, these data indicate that the vlPAG has 2 main neuron types based on intrinsic membrane properties that exist in comparable proportions.

MOR-mediated GIRK currents are observed within all 4 vlPAG neuron types

To determine whether postsynaptic MORs are selectively expressed on a single neuron type in the vlPAG, the MOR agonist DAMGO (5 μM) was used to elicit GIRK currents after evaluating the firing patterns of each neuron (Fig. 4A,B). Opioid-mediated GIRK currents were elicited in some, but not all, of the *Phasic*, *Tonic*, and *Onset* neurons (Fig. 4C). All 3 *Random* neurons were opioid-sensitive but the small sample may have precluded observing opioid-insensitive neurons in this group. Opioid-insensitive neurons in each group exhibited $GABA_B$-mediated GIRK currents, consistent with GIRK channel expression in most neurons, as we noted in our previous study (McPherson et al., 2018). Average GIRK currents in the *Phasic*, *Tonic*, and *Onset* neurons were ~10 pA (Fig. 4D).

Loose-patch recordings demonstrated clear DAMGO-mediated inhibition of spontaneous firing in naïve rats (49/56 neurons were inhibited $\geq 25\%$) indicating that the small GIRK currents were sufficient to inhibit firing. Since not all *Phasic* and *Tonic* neurons express MORs, this result suggests that the majority of spontaneously active neurons express MORs, motivating the search for additional firing features that may indicate opioid sensitivity.

Neurons exhibiting low-threshold spikes (LTS) have been previously described to be linked to opioid sensitivity in the vlPAG of mice. We found that both *Phasic* and *Tonic* groups had neurons with and without LTS in response to short hyperpolarizing steps (Fig. 4E). More specifically, 6/19 opioid-sensitive *Phasic* neurons and 2/10 opioid-sensitive *Tonic neurons* showed no LTS, whereas in opioid-insensitive neurons, 7/14 *Phasic* and 1/6 *Tonic* neurons had LTS (Fig. 4F). Thus, LTS activity was not a robust indicator of opioid sensitivity in the rat vlPAG.

Persistent inflammation increases spontaneous firing and decreases firing thresholds of Phasic neurons

Defining intrinsic firing properties in naïve rats provided the framework to evaluate alterations to intrinsic properties after acute and persistent inflammation. Both acute (2 h) and persistent inflammation (6 d) induce Fos expression in the vlPAG (Fig. 5A,B). To determine the underlying activity that could be driving this increase in Fos expression we examined spontaneous firing frequencies of *Phasic* and *Tonic* neurons after acute (2 h) and persistent (5-7 d) inflammation. The firing of *Tonic* neurons remained unchanged across both post-CFA time points (Fig. 5C), while the spontaneous

firing frequencies of *Phasic* neurons approximately doubled after persistent inflammation compared to *Phasic* vlPAG neurons in naïve and acute inflammation conditions (Fig. 5D). This increase in firing was driven by approximately half of the *Phasic* neurons sampled from rats treated with CFA (5-7 d), which fired at frequencies greater than the mean + SD of *Phasic* neurons from naïve rats. Importantly, the proportions of the 4 distinct neuron types were the same between the naïve condition (n = 223) and after acute (n = 64) and persistent (n = 84) inflammation (chi-square = 12.44, df = 6, p = 0.053), suggesting that there were no firing pattern changes induced by inflammation.

To determine whether the increase in spontaneous firing of *Phasic* neurons was a result of changes to intrinsic firing properties, we examined firing threshold, R_{in}, RMP, and spontaneous firing frequencies after removing synaptic inputs (using NBQX and gabazine as described previously). In naïve rats, 14/34 *Phasic* neurons required a depolarizing current step to fire, whereas after persistent inflammation only 4/42 required a depolarizing current step and 9/42 fired during hyperpolarizing steps, resulting in a significant reduction in firing threshold after persistent inflammation (Fig. 5E). The RMP (Fig. 5F) and R_{in} (data not shown) remained unchanged across the two inflammation time points. In addition, blockade of synaptic inputs did not affect the spontaneous firing frequencies of *Phasic* neurons (Fig. 5G). Taken together, these findings substantiate that the enhanced spontaneous firing rate of *Phasic* neurons is due to alterations to the intrinsic membrane properties.

In order to determine if the most strongly activated *Phasic* neurons from CFA-treated rats were opioid-sensitive, we evaluated DAMGO-mediated GIRK currents. Both opioid-sensitive and -insensitive *Phasic* neurons were observed after persistent

inflammation (20/34 opioid-sensitive neurons in naïve and 9/15 post-CFA), with comparable GIRK current amplitudes (Fig. 5H). There was no significant correlation between spontaneous firing frequency and DAMGO-mediated GIRK currents (simple linear regression, $R^2 = 0.05$, $p = 0.41$). DAMGO-mediated inhibition of spontaneous firing after persistent inflammation was also comparable to that observed in naïve rats (49/56 naïve neurons and 34/43 neurons after CFA (5-7 d) were inhibited $\geq 25\%$). Thus, *Phasic* neurons in the vlPAG that are activated with persistent inflammation do not represent a homogenous MOR-expressing neuronal subpopulation.

2.5 Discussion

The studies presented in this paper examined intrinsic membrane properties of vlPAG neurons in naïve rats and in rats after acute (2 h) or persistent (5-7 d) inflammation. In all conditions we observed 4 types of neurons with distinct intrinsic firing patterns that did not segregate with opioid sensitivity. Despite robust Fos expression after acute inflammation, intrinsic firing properties remained unchanged until the persistent inflammation time point, where *Phasic* neurons displayed an increase in spontaneous firing frequency and lowered firing threshold that was independent of synaptic inputs.

Firing patterns and membrane properties define vlPAG neuron subtypes

We used voltage- and current-clamp step protocols (Prescott and De Koninck, 2002, Sedlacek et al., 2007, Pradier et al., 2019) to initially characterize 223 vlPAG neurons from naïve rats. *Phasic* and *Tonic* neurons comprise ~80% of the neurons in the

region and are evenly distributed across the rostral-caudal axis. *Phasic*, *Tonic*, and *Onset* firing patterns are also represented in the spinal dorsal horn, a key region in the processing of nociceptive primary afferent information (Lopez-Garcia and King, 1994, Prescott and De Koninck, 2002, Schneider, 2003). Intrinsic membrane properties of dorsal horn neurons are correlated with their low and high threshold sensory afferent inputs (Lopez-Garcia and King, 1994, Dougherty and Chen, 2016). *Phasic* neurons are effective "coincidence detectors" capable of entraining stimuli at any frequency, whereas *Tonic* neurons are ineffective at coincidence detection and appear to integrate across ongoing stimuli (Prescott and De Koninck, 2002). Future studies examining afferent inputs to *Phasic* and *Tonic* neurons within the vlPAG will determine if these firing patterns integrate incoming synaptic activity similarly.

Potential markers to further define vlPAG heterogeneity

Given the key role the vlPAG plays in opioid-induced analgesia and the hypothesized postsynaptic MOR expression on GABAergic interneurons, we considered the possibility that opioid sensitivity may map onto these functionally distinct neuron types. MORs are expressed on a subpopulation of postsynaptic cell bodies within the vlPAG and are coupled to GIRKs, producing hyperpolarization (Chieng and Christie, 1994a, McPherson et al., 2018). At least some of the MOR-expressing neurons are GABAergic neurons with hypothesized local connections (Barbaresi and Manfrini, 1988, Reichling and Basbaum, 1990, Chieng and Christie, 1994a, Kalyuzhny and Wessendorf, 1998). Opioid inhibition of GABAergic interneurons would contribute to disinhibition of RVM-projecting neurons (Lau and Vaughan, 2014, Lau et al., 2020), however, opioids

also directly inhibit a small proportion of RVM-projecting vlPAG neurons (Osborne et al., 1996). A study using fluorescence *in situ* hybridization (FISH) to assess neurotransmitter content found that 22% of MOR-expressing neurons are GABAergic, whereas 77% are glutamatergic in the mouse vlPAG (Zhang et al., 2020), also indicating that MORs do not define a single population of vlPAG neurons. Another marker that has been proposed to correlate with opioid sensitivity in the vlPAG is low-threshold spikes (LTS). Previous recordings from vlPAG neurons in mice observed DAMGO-mediated GIRK currents in 5 neurons with low-threshold spikes (LTS) compared to 4 neurons without LTS that were opioid-insensitive (Park et al., 2010). Within our larger dataset of *Phasic* and *Tonic* neurons in rats, LTS did not correlate with opioid sensitivity. In addition, our findings identified both opioid-sensitive and opioid-insensitive neurons within the *Phasic*, *Tonic*, and *Onset* groups. Altogether, these results further indicate that MOR expression is not a reliable marker of vlPAG neurons based on their intrinsic membrane properties.

Opioids produce analgesia in the PAG-RVM circuit through disinhibition (Basbaum and Fields, 1984, Lau and Vaughan, 2014) so we anticipated opioid-induced disinhibition of firing in some neurons. However, we observed little evidence of DAMGO-mediated disinhibition in our loose-patch recordings that may be explained by the fact that disinhibition appears to be more prominent in RVM-projecting neurons that are not spontaneously active (Lau et al., 2020). Our loose-patch methods only detect spontaneously active neurons, which make up 69% of *Phasic* and 92% of *Tonic* neurons and even smaller proportions of *Onset* and *Random* neurons, suggesting that opioid disinhibited neurons exist within the smaller population of quiescent neurons.

Neurotransmitter content is often used to define neuron populations within brain areas using genetic markers. Unfortunately, we have not been able to find antibodies that are specific for either GABA or glutamate neurons in the rat vlPAG that work in conjunction with biocytin-labeling of recorded neurons. Work from other labs indicates that firing patterns do not correlate with neurotransmitter content. For example, most, but not all, GABAergic neurons in mouse vlPAG displayed *Tonic*-like firing patterns (Park et al., 2010), but glutamatergic neurons also show *Tonic*-like firing (Samineni et al., 2017a). This aligns with varied intrinsic firing properties within different neurotransmitter-containing populations in the dorsal horn (Schneider, 2003). Thus, neurotransmitter content is a less informative marker than identifying other potential markers that define vlPAG neurons that respond to inflammation.

Persistent inflammation selectively activates Phasic neurons

Noxious stimuli induce Fos expression in the vlPAG (Keay and Bandler, 1993, Keay et al., 2000), and 25% of the Fos-expressing vlPAG neurons following intramuscular formalin injections are RVM-projecting (Keay et al., 2000). Typically, re-exposure to the stimulus or stimulus-associated cues are used to induce Fos; however, in this study we simply detected Fos levels induced by ongoing activity of vlPAG neurons from the earlier CFA injections (either 2 h or 6 d prior to recordings). Fos labeling indicating strong neuronal activation was detected acutely (2 h) after CFA injection and was maintained over baseline levels at the persistent (6 d) time point. In the RVM, a target region for vlPAG afferents, acute CFA increases ON-cell firing and reduces OFF-cell firing, promoting hyperalgesia, but the changes in firing resolves over hours even in

the presence of maintained hyperalgesia (Cleary and Heinricher, 2013). Additional changes in glutamate receptors and firing patterns are observed in the RVM during inflammatory pain states (Miki et al., 2002). Upstream of the vlPAG, subregions of the amygdala contain *Phasic* and *Tonic* neurons that project to the vlPAG. *Tonic* amygdalar neurons have lowered firing thresholds 24 h after CFA injection (Li and Sheets, 2018). Thus, brain areas that are upstream and downstream of the vlPAG are sensitized after CFA and suggest that afferent inputs to vlPAG may be the source of strong neuronal activation leading to Fos expression in intact rats, especially early in inflammation. The more robust Fos expression seen 2 h post-CFA injection is likely dependent on increased nociceptive inputs during the development of inflammation, that are reduced in our *in vitro* preparation. Afferent drive within the vlPAG in the intact rat has been hypothesized to yield neuronal firing changes in response to opioids that vary significantly from what is seen *in vitro* (Tryon et al., 2016).

In the absence of synaptic inputs, CFA induces increased spontaneous firing of vlPAG *Phasic* neurons after persistent, but not acute, inflammation suggesting that this is a time-dependent adaptation. In particular, a subset of the *Phasic* neurons in CFA-treated rats exhibited firing frequencies greater than the mean + SD of *Phasic* neurons in naïve rats, demonstrating that a small population of *Phasic* neurons drive this increase. Importantly, in the CFA-treated rats, vlPAG neurons displayed all 4 firing patterns in similar proportions, suggesting no inflammation-induced changes in firing patterns. In contrast to *Phasic* neurons, *Tonic* neurons exhibited no changes in firing activity after acute or persistent inflammation, reinforcing the selective activation of *Phasic* neurons. The activated *Phasic* neurons in the vlPAG may represent an ensemble of neurons

encoding continued peripheral nociceptor activation or inflammatory processing that increase spontaneous activity and lower firing thresholds (Whitaker et al., 2016, Whitaker et al., 2017, Whitaker and Hope, 2018).

The effects of MOR agonists are potentiated in the RVM after persistent inflammation (Hurley and Hammond, 2000, Schepers et al., 2008). This raised the question of whether persistent inflammation activates *Phasic* neurons that promote *descending facilitation*, but are selectively targeted by opioids. However, our results are not consistent with this mechanism as high-frequency firing *Phasic* neurons in CFA-treated rats were both opioid-sensitive and -insensitive and the degree of firing inhibition by opioids was comparable between naïve and CFA-treated rats.

Relevance to descending modulation of pain

Neurons within the vlPAG are known to have differential firing responses to acute noxious stimuli *in vivo* (Heinricher et al., 1987, Tryon et al., 2016); however, alterations in firing in persistent pain states and circuits between the PAG and RVM that contribute to the descending modulation of pain have not been fully defined. The vlPAG has been studied primarily in the context of *descending opioid-mediated inhibition* of pain (Basbaum and Fields, 1984, Morgan et al., 1991, Vanegas and Schaible, 2004). However, a growing number of studies provide evidence that the vlPAG plays a role in *facilitation* of pain (Heinricher et al., 2004, Dubový et al., 2018, Ni et al., 2019). In addition, neuroimaging in humans has implicated the PAG in pain processing and central sensitization (Dunckley et al., 2005, Zambreanu et al., 2005, Fairhurst et al., 2007, Roy et al., 2014). *In vivo* vlPAG recordings show altered spontaneous firing frequency, stimulus

response, and firing threshold in a subpopulation of vlPAG neurons after development of neuropathic pain (Samineni et al., 2017b). These neurons exhibited burst firing, suggesting that they may represent the *Phasic* neurons in this study. Chronic constriction injury (CCI) increased firing frequency of some vlPAG neurons *in vitro* as well (Du et al., 2013).

An adaptation observed with both inflammation and neuropathic pain is an increase in spontaneous GABA release in the vlPAG (Hahm et al., 2011, Tonsfeldt et al., 2016) and in the downstream RVM (Li et al., 2017). The increased GABA tone may originate from afferents coming from other brain regions or GABAergic vlPAG neurons that send local collaterals (Basbaum and Fields, 1984, Barbaresi and Manfrini, 1988, Maione et al., 1999, Lau and Vaughan, 2014). Extensive work starting in the late-1960's has established clear roles for excitatory and inhibitory neurons within the vlPAG in the descending modulation of pain. Electrical or chemical stimulation of vlPAG neurons produces analgesia (Reynolds, 1969, Mayer et al., 1971, Mayer and Liebeskind, 1974, Akil and Liebeskind, 1975, Soper and Melzack, 1982). Blocking GABAergic signaling with opioids or $GABA_A$ antagonists also elicits analgesia (Yaksh et al., 1976, Moreau and Fields, 1986, Jensen and Yaksh, 1989). Increased GABA tone contributes to *descending facilitation* of pain and reduces morphine-mediated analgesia (Zambotti et al., 1982, Moreau and Fields, 1986). More recently, chemogenetic tools have confirmed that selective activation of glutamatergic neurons in the vlPAG is analgesic and that selective activation of vlPAG GABA neurons is hyperalgesic (Samineni et al., 2017a). Therefore, it is possible that *Phasic* neurons are GABA neurons with local collaterals, producing enhanced GABA tone as a result of their increased spontaneous firing after persistent

inflammation, and ultimately promoting *descending facilitation* of pain. However, both GABAergic and glutamatergic vlPAG neurons project to pain-facilitating and pain-inhibiting neuronal populations in the RVM in the rat (Morgan et al., 2008). Further studies are necessary to unravel the complex circuits that mediate the descending modulation of pain. Identification of a specific adaptation in a functional subpopulation of vlPAG neurons provides insight into how nociceptive information produced by persistent inflammation engages the vlPAG.

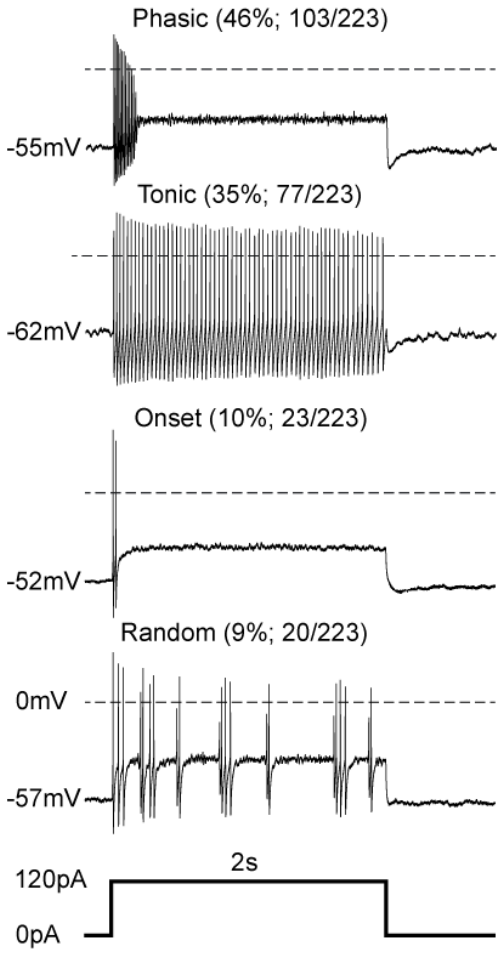

Figure 2. 1: The vlPAG contains 4 neuron types defined by distinct firing patterns.

Representative traces from vlPAG neurons from naïve rats showing 4 distinct neuron types based on their response to a 2 s long 120 pA depolarizing current step. The proportion of neurons exhibiting the firing pattern out of the total neurons recorded in naïve rats and the resulting percentage are represented in parentheses. Resting membrane potential is noted at the beginning of each trace and the dotted line represents 0 mV.

	Phasic (103 / 223)	Tonic (77 / 223)	Onset (23 / 223)	Random (20 / 223)
Resting Membrane Potential (mV)	-55 ± 0.6****	-62 ± 0.9	-52 ± 1.2	-57 ± 1.7
Membrane Resistance (GΩ)	2.4 ± 0.3	1.6 ± 0.2	1.8 ± 0.4	0.8 ± 0.1
Capacitance (pF)	42 ± 1.4****	67 ± 3.5	49 ± 4.6	64 ± 6.8
Spontaneous Firing	69% (71/103)	92% (71/77)	0% (0/23)	37% (7/20)

Table 2. 1: Electrophysiological properties of 4 neuron types in naïve rats.

The number of neurons recorded: *Phasic* (n = 103), *Tonic* (n = 77), *Onset* (n = 23), and *Random* (n = 20). Resting membrane potential ($F_{(3, 219)} = 19.1$, ****$p < 0.0001$; Tukey's multiple comparisons; *Phasic* vs. *Tonic,* ****$p < 0.0001$), Membrane resistance ($F_{(3, 219)} = 3.6$, *$p = 0.01$; Tukey's multiple comparisons test; *Phasic* vs. *Tonic,* ns), and Capacitance ($F_{(3, 219)} = 18.4$, ****$p < 0.0001$; Tukey's multiple comparisons test; *Phasic* vs. *Tonic*, ****$p < 0.0001$). Spontaneous firing is given as an overall percentage, with exact number of neurons firing at rest over the total number of that type of neuron shown in parentheses.

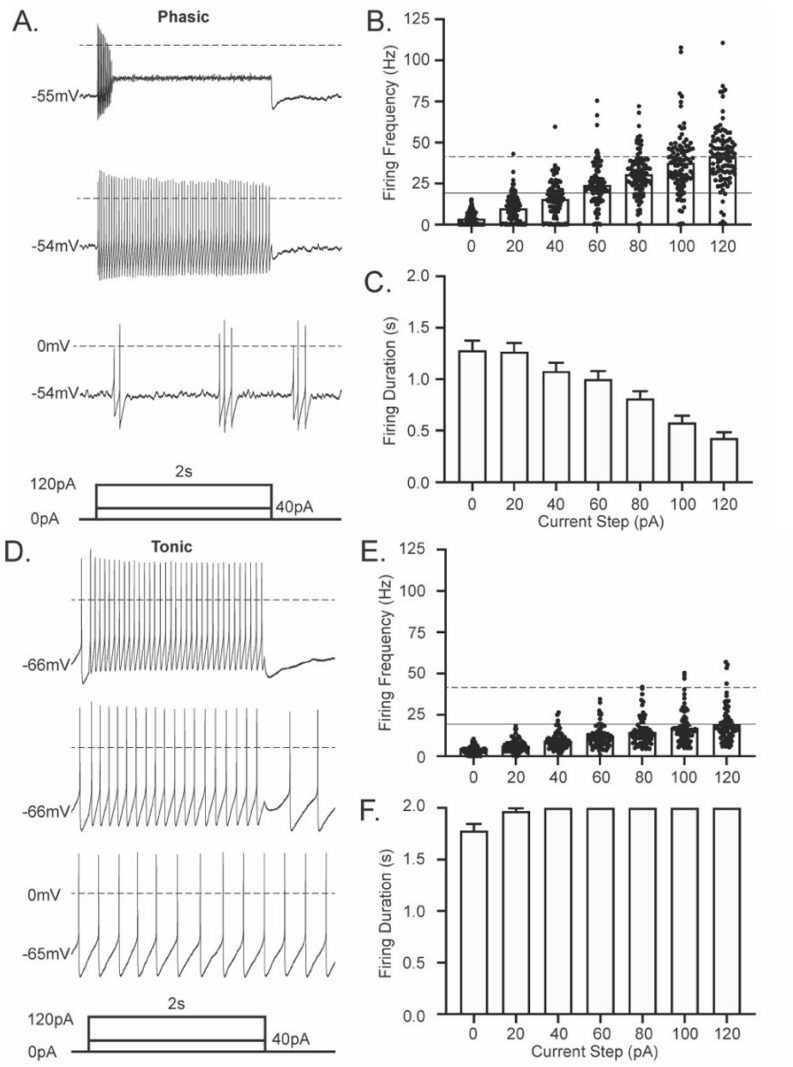

Figure 2. 2: Defining features of the 2 most common vlPAG neurons.

A. Representative trace of a recording from a *Phasic* vlPAG neuron from a naïve rat in response to 0 pA (bottom trace), 40 pA (middle trace), and 120 pA (top trace) current injections. Current injections were 2 s long after a 50 ms delay, followed by 250 ms return to baseline (current protocol schematic below the traces). **B.** Firing frequency of all *Phasic* neurons for current steps ranging from 0 pA to 120 pA in 20 pA increments (n = 103). Dashed line shows maximal firing frequency at 120 pA for *Phasic* neurons, whereas solid line shows maximal firing frequency at 120 pA for *Tonic* neurons. **C.** Total firing duration of all *Phasic* neurons throughout each of the 2 s depolarizing current steps (n = 103). **D.** Representative trace of a recording from a *Tonic* vlPAG neuron from a naïve rat in response to 0 pA (bottom trace), 40 pA (middle trace), and 120 pA (top trace) current injections. Current injections were 2 s long after a 50 ms delay, followed by 250 ms return to baseline (as shown in the current protocol schematic below the traces). **E.** Firing frequency of all *Tonic* neurons for current steps ranging from 0 pA to 120 pA in 20 pA increments (n = 77). **F.** Compiled data showing total firing duration of all *Tonic* neurons throughout each of the 2 s depolarizing current steps (n = 77).

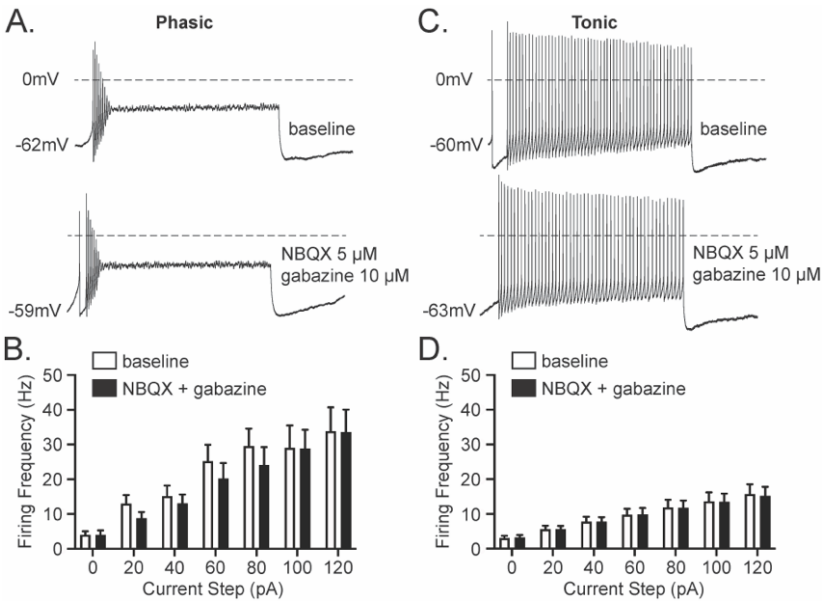

Figure 2. 3: The firing patterns of *Phasic* and *Tonic* neurons are maintained in the absence of synaptic inputs.

A. Representative traces of a recording from a *Phasic* vlPAG neuron from a naïve rat in response to 120 pA current injections before (top trace) and after synaptic blockers NBQX (5 µM) and gabazine (10 µM) (bottom trace). **B.** Compiled data showing firing frequency of all *Phasic* neurons for current steps ranging from 0 pA to 120 pA in 20 pA increments before and after synaptic blockers (Two-way Repeated Measures ANOVA, Effect of synaptic blockers, $F_{(1, 9)} = 1.3$, $p = 0.3$; $n = 10$). **C.** Representative traces of a recording from a *Tonic* vlPAG neuron from a naïve rat in response to 120 pA current injections before (bottom trace) and after synaptic blockers (top trace). **D.** Compiled data showing firing frequency of all *Tonic* neurons for all current steps before and after synaptic blockers (Two-way Repeated Measures ANOVA, Effect of synaptic blockers, $F_{(1, 10)} = 0.002$, $p = 1.0$; $n = 11$).

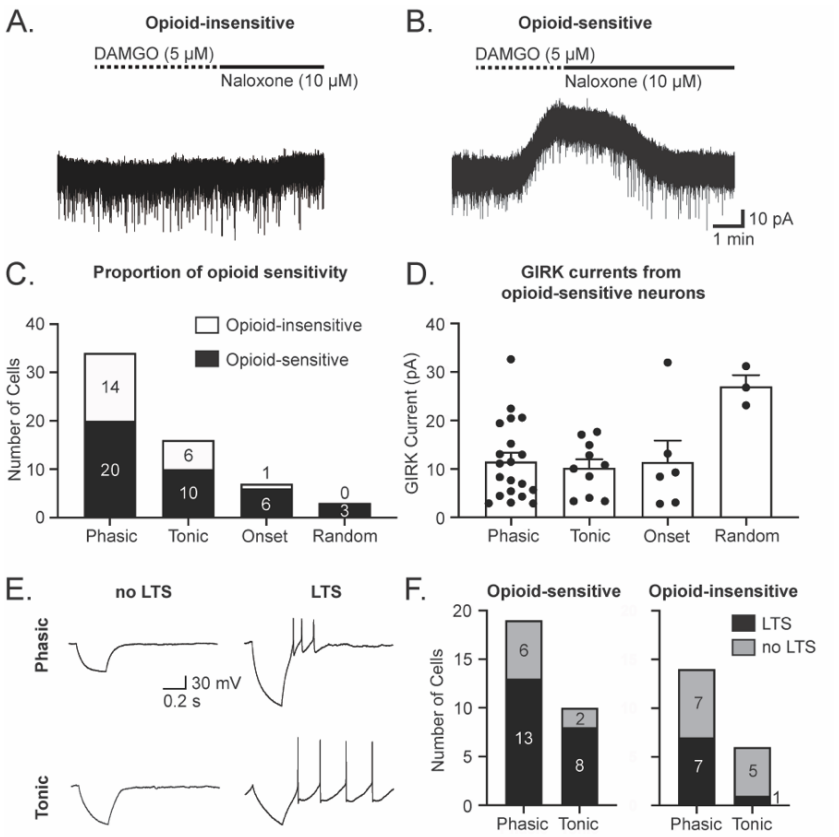

Figure 2. 4: Opioid sensitivity is not restricted to selective neuron types in the vlPAG.

A. Representative trace from a vlPAG neuron in which the selective MOR agonist DAMGO (5 μM) did not elicit an outward current (opioid-insensitive). **B.** Representative trace from a vlPAG neuron in which DAMGO (5 μM) elicited an outward current that was reversed by the MOR antagonist naloxone (10 μM) (opioid-sensitive). **C.** Number of neurons exhibiting DAMGO-mediated GIRK current responses (opioid-sensitive) compared to those that did not (opioid-insensitive) in each neuron type (n = 60). **D.** DAMGO-mediated GIRK current amplitude (pA) data for opioid-sensitive neurons in each neuron type (One-way ANOVA, main effect of neuron type, $F_{(3, 35)} = 3.9$, $p = 0.02$; Tukey's post-hoc test *Phasic vs. Random* $p = 0.01$, *Tonic vs. Random* $p = 0.01$, and *Onset vs. Random* $p = 0.04$; n = 39). **E.** Sample whole-cell traces in response to 500 ms long -50 pA hyperpolarizing current steps, demonstrating neurons with and without low threshold spikes (LTS) in both *Phasic* and *Tonic* neuron populations. **F.** The proportion of LTS and non-LTS neurons in *Phasic* and *Tonic* neuron groups, both in opioid-sensitive (left) and opioid-insensitive (right) groups.

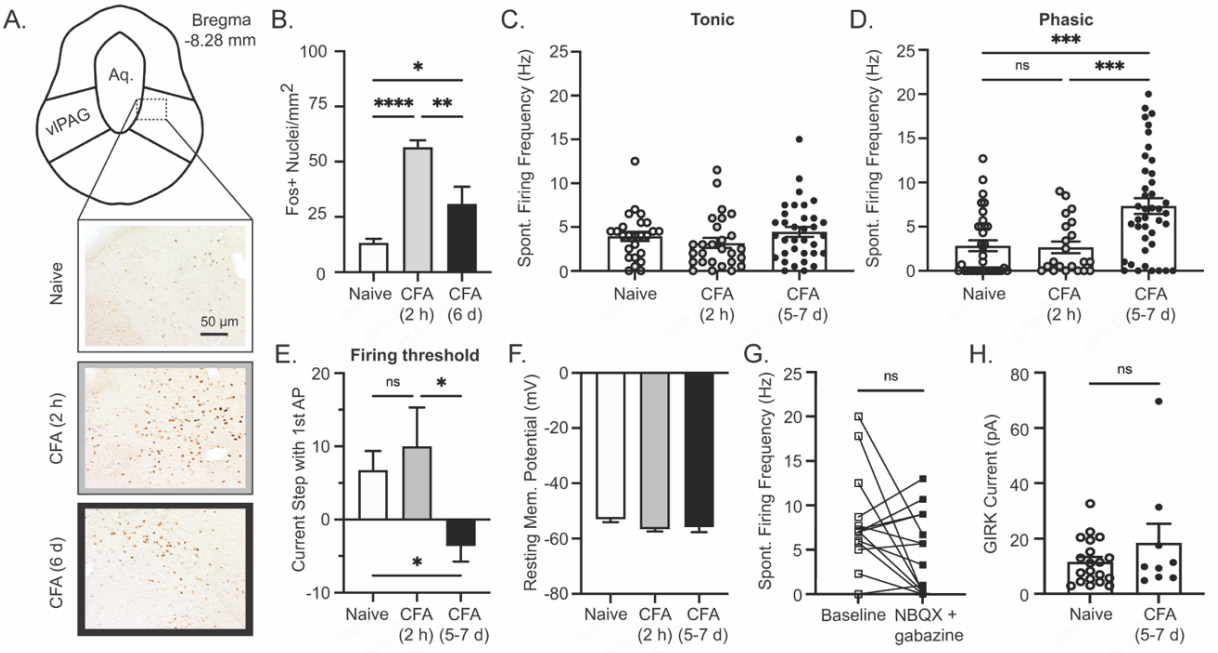

Figure 2. 5: Persistent inflammation increases spontaneous firing frequency and decreases firing thresholds of *Phasic* neurons.

A. Region of interest (vlPAG) outlined and representative image location demonstrated with dashed box. Representative images from Fos immunohistochemistry of tissue from naïve rats or rats sacrificed 2h or 6 d after CFA injection to the hind paw; cerebral aqueduct is labeled (Aq.), scale bar = 50 µm for all images. **B.** Average Fos+ nuclei/mm^2 for naïve rats and those 2 h or 6 d after CFA injections (One-way ANOVA, $F_{(2, 11)} = 26.3$, $p < 0.0001$, Tukey's post-hoc test, naïve vs CFA 2 h, ****$p < 0.0001$, naïve vs CFA 6 d, *$p = 0.03$, and CFA 2 h vs CFA 6 d, **$p = 0.01$). **C.** Spontaneous firing frequency of *Tonic* neurons after acute or persistent inflammation (One-way ANOVA, $F_{(2, 83)} = 1.3$, $p = 0.3$; naïve, n = 25, CFA (2h) n = 27, CFA (5-7 d), n = 34). **D.** Firing frequency of *Phasic* neurons shows significant increase in spontaneous firing frequency after persistent inflammation (One-way ANOVA, $F_{(2, 93)} = 11.7$, $p < 0.0001$; Tukey's post-hoc test, naïve vs CFA (5-7 d), ***$p = 0.0002$ and CFA (2 h) vs CFA (5-7 d), ***$p = 0.001$; naïve n = 34, CFA (2h) n = 21, CFA (5-7 d) n = 41) **E.** Firing thresholds (One-way ANOVA, $F_{(2, 91)} = 5.6$, $p = 0.005$, Tukey's post-hoc test, naïve vs CFA (2 h), $p = 0.8$, naïve vs CFA (5-7 d), *$p = 0.03$, and CFA 2 h vs CFA 6 d, *$p = 0.01$). **F.** Resting membrane potential of *Phasic* neurons remained stable across all conditions (One-way ANOVA, $F_{(2, 90)} = 1.4$, $p = 0.2$). **G.** Spontaneous firing frequency of *Phasic* neurons before and after synaptic blockers in CFA (5-7 d) treated rats (Paired t-test, $t_{(14)} = 1.9$, $p = 0.07$). **H.** DAMGO-mediated GIRK current amplitude comparing naïve group (data from Fig. 4D) and after CFA (5-7 d) (Unpaired t-test, $t_{(27)} = 1.3$, $p = 0.2$).

Chapter 3: Regulators of G Protein Signaling Proteins Promote Receptor Coupling to G Protein-Coupled Inwardly-Rectifying Potassium Channels

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Acknowledgements: The authors would like to thank Katherine L. Suchland and Janet D. Lowe for technical support. The studies were funded by NIH R01 DA035316 (JRT) and T32 NS007466 (KBM and ERL).

3.1 Abstract

Regulators of G protein Signaling (RGS) proteins negatively modulate presynaptic μ-opioid receptor inhibition of GABA release in the ventrolateral periaqueductal gray (vlPAG). Paradoxically, we find that G protein-coupled receptor (GPCR) activation of G protein-coupled inwardly-rectifying potassium (GIRK) channels in the vlPAG is reduced in an agonist- and receptor-dependent manner in transgenic knock-in mice of either sex expressing mutant RGS-insensitive Gαo proteins. μ-Opioid receptor agonist activation of GIRK currents was reduced for DAMGO and fentanyl but not for [met^5]-enkephalin (ME) in the RGS-insensitive heterozygous (Het) mice compared to wildtype mice. The $GABA_B$ agonist baclofen-induced GIRK currents were also reduced in the Het mice. We confirmed the role of Gαo proteins in μ-opioid receptor and $GABA_B$ receptor signaling pathways in wildtype mice using myristoylated-peptide inhibitors of $Gαo_1$ and $Gαi_{1-3}$. The results using these inhibitors indicate that receptor activation of GIRK channels is dependent on preference of the agonist-stimulated receptor for Gαo versus Gαi. DAMGO and fentanyl-mediated GIRK currents were reduced in the presence of the $Gαo_1$ inhibitor, but not the $Gαi_{1-3}$ inhibitors. In contrast, the $Gαo_1$ peptide inhibitor did not affect ME activation of GIRK currents, consistent with results in the Het mice, but the $Gαi_{1-3}$ inhibitors significantly reduced ME-mediated GIRK currents. Finally, the reduction in GIRK activation in the Het mice plays a role in opioid and baclofen-mediated spinal, but not supraspinal antinociception. Thus, our studies indicate that RGS proteins have multiple mechanisms of modulating GPCR signaling that produce negative and positive regulation of signaling depending on the effector.

3.2 Introduction

Morphine and other opioids elicit antinociception through activation of a specific set of GPCRs—the μ-opioid receptors (Matthes et al., 1996). However, μ-opioid receptor agonists also mediate adverse side effects, including tolerance, dependence and respiratory depression (Williams et al., 2013), decreasing the therapeutic value of these agonists for pain. A focus of current research has been on identifying μ-opioid receptor signaling pathways and/or circuits that mediate opioid-induced antinociception with the hope that side effects are the result of separate circuits. Several μ-opioid receptor agonists exhibit functional selectivity based on their ability to promote biased signaling via G proteins with limited recruitment of β-arrestin (Bohn et al., 1999, Raehal et al., 2005, Raehal et al., 2011, DeWire et al., 2013, Manglik et al., 2016, Koblish et al., 2017). The focus on biased signaling as a novel approach for developing μ-opioid receptor agonists that are efficacious for pain relief without serious side effects highlights the importance of understanding G protein signaling pathways.

Agonists for the μ-opioid receptor promote the exchange of GTP for GDP on inactive Gα subunits. This allows activation of the complex and subsequent dissociation of Gα and Gβγ subunits, both of which signal through multiple downstream effector proteins. Termination of G protein subunit signaling is facilitated by Regulators of G protein Signaling (RGS), GTPase-accelerating proteins (GAPs) that promote the hydrolysis of GTP on the active Gα subunits, re-establishing their affinity for Gβγ and decreasing downstream signaling to coupled effector pathways (Traynor and Neubig, 2005).

The RGS protein family is comprised of more than 30 proteins that differ in structure, function and tissue/cellular localization (Traynor and Neubig, 2005). RGS proteins are potential targets for new therapeutic drugs for pain based on their ability to regulate μ-opioid receptors. Several of the RGS family members are involved in regulation of μ-opioid receptors, including RGS4 (Garzón et al., 2005b, Roman et al., 2007, Leontiadis et al., 2009, Santhappan et al., 2015), RGS9-2 (Psifogeorgou et al., 2007, Papachatzaki et al., 2011, Gaspari et al., 2018), RGS19 (Wang and Traynor, 2013), and RGSz (Garzon et al., 2005b, Gaspari et al., 2018). These proteins regulate μ-opioid receptor signaling to effectors, including adenylyl cyclase, calcium channels and mitogen-activated protein kinase (MAPK) (Clark et al., 2003, Psifogeorgou et al., 2007), as well as μ-opioid receptor-mediated behaviors (Zachariou et al., 2003, Traynor et al., 2009, Han et al., 2010, Gaspari et al., 2014). However, redundancy and tissue expression overlap of the RGS family of proteins is a problem for interpreting knock-down or knock-out strategies for specific RGS proteins. In order to deal with this concern, knock-in transgenic mice were generated with a mutation in Gαo (G184S) that is insensitive to all RGS regulation (Goldenstein et al., 2009). Gαo subunits mediate μ-opioid receptor signaling to several effectors (Clark et al., 2003), as well as morphine and methadone-induced antinociception (Lamberts et al., 2011).

Consistent with previous studies showing that RGS proteins are negative regulators of μ-opioid receptor signaling (Clark et al., 2003, Zachariou et al., 2003, Clark et al., 2004), opioid agonist inhibition of evoked GABA release in the supraspinal vlPAG is increased in the RGS-insensitive mutant mice, along with an increased potency of morphine in the supraspinally-mediated hot-plate test (Lamberts et al., 2013). In contrast,

morphine potency is decreased in the spinally-mediated tail withdrawal test (Lamberts et al., 2013). This suggests that RGS proteins are positive regulators of some μ-opioid receptor effectors. Indeed, knockout of RGS9 compromised opioid-induced hyperpolarization in the dorsal horn of the spinal cord (Papachatzaki et al., 2011), presumably mediated by GIRK channels (Marker et al., 2004, Marker et al., 2005). Since μ-opioid receptors hyperpolarize neurons via activation of GIRK channels in the vlPAG (Chieng and Christie, 1996, Osborne et al., 1996, Ingram et al., 2008), we examined whether RGS proteins have different actions depending on the effector system. We hypothesized that μ-opioid receptor signaling to GIRK channels in the vlPAG would be compromised in the RGS-insensitive mice.

3.3 Materials & Methods

Subjects

Male and female mice heterozygous (Het) for RGS insensitivity (Goldenstein et al., 2009) and littermate WT 129S1/SvImJ male and female mice were used for all experiments comparing WT (The Jackson Laboratory, Bar Harbor, ME, USA) and Het mice. Mice were obtained from WT and Het breeding pairs to control for genetic background. Homozygous knock-in mice die before birth so WT mice were compared to Het mice. The Het mice tended to be smaller so experimenters in behavioral studies were not blind to genotype. Studies using G protein peptide inhibitors used WT mice (The Jackson Laboratory). Mice were group-housed with unlimited access to food and water. Lights were maintained on a 12-h light/dark cycle (lights on at 7:00). The Institutional Animal Care and Use Committee at Oregon Health & Science University approved all electrophysiological experimental procedures. All behavioral testing was performed

during the light phase and experimental procedures were approved by the University of Michigan Institutional Animal Care. Experiments were conducted in accordance with the US National Research Council's Guide for the Care and Use of Laboratory Animals (Council, 2011).

Electrophysiological Recordings

Mice (PN day > 25) were anesthetized with isoflurane and brains removed and placed into artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl; 21.4 $NaHCO_3$; 11.1 dextrose; 2.5 KCl; 2.4 $CaCl_2$; 1.2 $MgCl_2$; 1.2 NaH_2PO4, pH 7.35, and equilibrated with 95% O_2/5% CO_2. Brain slices containing the vlPAG were cut with a vibratome (180 – 220 μm thick) and placed in a holding chamber with oxygenated ACSF maintained at approximately 32 ºC until placed into a recording chamber. In experiments using myristoylated Gαo and Gαi peptide inhibitors, slices were incubated for at least 30 min in ACSF plus inhibitor prior to recording. Recordings were made with electrodes pulled to 2 – 3 MΩ resistance with internal solutions consisting of (in mM): either 138 potassium methylsulfate or 138 potassium gluconate; 10 HEPES; 10 KCl; 1 $MgCl_2$; 1 EGTA; 0.3 $CaCl_2$; 4 MgATP; 3 NaGTP, pH 7.4. Junction potentials of 15 mV were corrected at the beginning of the experiments. Access resistance was monitored throughout the experiments. Neurons were voltage-clamped at -70 mV. Data were collected with Axopatch 200B (Molecular Devices, Sunnyvale, CA) at 5 kHz and low-pass filtered at 2 kHz. Currents were digitized with Instrutech ITC-18, collected via Axograph Data Acquisition software and analyzed using Axograph (Axograph Scientific, Sydney, Australia). Single mice were sacrificed without opportunity to compare size so

electrophysiological studies were performed blind to genotype. Genotyping of mice was done from tail snips taken after sacrifice. In experiments using Gαo and Gαi inhibitor peptides, all mice were WT but the analyses of peak drug effects were measured blind to condition.

Behavioral tests

Experiments were conducted using adult mice between 10 and 20 weeks of age. Supraspinal antinociception was evaluated in the hot plate test and spinal antinociception was measured in the warm-water tail withdrawal assay using a cumulative dosing procedure as previously described (Lamberts et al., 2011). Briefly, mice were administered increasing doses of baclofen (intraperitoneal; i.p.) in 30 min intervals, and latency was evaluated 30 min following each injection. In the hot plate test, mice were placed on a hot plate analgesia meter (Columbus Instruments, Columbus, OH, USA) maintained at 52.0 ± 0.2°C and the latency to lick forepaw(s) or jump was measured with a cutoff time of 60 s to prevent tissue damage. In the tail withdrawal test, mice were lightly restrained and the distal tip of the mouse's tail was placed in a water bath (Fisher Scientific, Waltham, MA, USA) maintained at 50.0 ± 0.5°C. The latency to tail flick was measured with a cutoff time of 20 s.

Locomotor activity was measured using a two-compartment place-conditioning apparatus (Med Associates, Inc. St. Albans, VT). The compartments were similar in dimension (8 × 5 × 5 in.) but differed in wall color and floor texture (Compartment 1: black walls with rod flooring; Compartment 2: white walls with mesh flooring). Infrared photo-beam

sensors lining the bottom of two parallel walls recorded the beam breaks generated by mouse locomotion. Activity counts were recorded as the total number of beam breaks within 30 min, following a 30 min treatment with baclofen (3.2 or 10 mg/kg) or vehicle (sterile water). An equal number of male and female mice were placed in either compartment per treatment. Compartments were cleaned between tests.

Reagents

[D-Ala^2, N-Me-Phe^4, Gly^5-ol]-Enkephalin acetate salt (DAMGO), [Met^5]-Enkephalin acetate salt hydrate (ME) and Fentanyl citrate salt (fentanyl) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and (R,S)-Baclofen and CGP 55845 hydrochloride were purchased from Abcam (Cambridge, MA, USA). Myristoylated G protein peptide inhibitors were synthesized by GenScript (Piscataway, NJ, USA): $G\alpha o_1$ (MGIANNLRGCGLY), $G\alpha i_{1/2}$ (MGIKNNLKDCGLP), $G\alpha i_3$ (MGIKNNLKECGLT) according to sequences for mini-gene vectors designed by the Hamm laboratory (Gilchrist et al., 2002). We were unable to obtain the peptide for Gao_2 at sufficient purity to use in slice experiments. The Gαi inhibitors were combined in experiments.

Statistical Analyses

All data are expressed as mean ± SEM. Data were analyzed with Prism 6 (GraphPad Software, La Jolla, CA, USA). Each electrophysiological recording from a single neuron is treated as an individual observation; however, all datasets contain recordings from at least three separate animals. Drug effects were reversed by specific antagonists and peak drug effects were measured as an increase in current from the average of baseline and washout or presence of antagonists. A cell was considered a non-responder if there was

no change in the current induced by the agonist during wash out of the agonist or in the presence of the antagonist. Behavioral studies used 12 mice per group distributed evenly between males and females. Differences between groups were assessed using the Mann-Whitney U nonparametric test or ANOVA when appropriate. * denotes $p < 0.05$.

3.4 Results

RGS proteins are known to negatively regulate GPCR signaling by increasing hydrolysis of GTP to terminate G protein signaling. μ-Opioid receptors inhibit presynaptic GABA release in the vlPAG (Chieng and Christie, 1994b, Vaughan et al., 1997a, Vaughan and Christie, 1997, Ingram et al., 1998). We had previously observed in RGS-insensitive mice that opioid inhibition of GABA release was increased (Lamberts et al., 2013), consistent with negative regulation of μ-opioid receptor signaling. μ-Opioid receptors are Gi/o-coupled GPCRs that also couple to GIRK channels in the vlPAG with μ-opioid receptor agonists activating GIRKs in approximately 40% of vlPAG neurons (Osborne et al., 1996, Ingram et al., 2008). Interestingly, DAMGO-mediated GIRK currents in vlPAG neurons were reduced in the RGS-insensitive Het mice (14 responders/27 cells) compared to WT mice (16 responders/ 30 cells; Fig. 1A-C). A response was defined as an outward current in response to agonist application that reversed during washout of agonist or in the presence of antagonist. Neurons that did not respond with any outward currents were not included in the analyses of mean amplitude because a lack of response may be due to the loss of RGS function or no μ-opioid receptor coupling to GIRK channels in an individual neuron. Similar results were observed in locus coeruleus (LC) neurons, which express μ-opioid receptors in all

neurons (Fig. 1D) confirming that small currents in the vlPAG of RGS-insensitive Het mice were actually measured in μ-opioid receptor-expressing neurons. Recordings were made from male and female WT and Het mice but we observed no difference in the amplitude of DAMGO (5μM)-induced GIRK currents (Two-way ANOVA; Main Effect of Sex; $F_{(1,23)} = 0.32$; $p = 0.58$). Thus, male and female responses are grouped in Figure 1. These results indicate that μ-opioid receptor coupling to GIRK channels is compromised in the RGS-insensitive Gαo mutant mice.

To further characterize μ-opioid receptor agonist activation of GIRK channels in the RGS-insensitive mice, we used the selective agonist fentanyl and the nonselective μ-opioid receptor and δ-opioid receptor agonist [met^5]-enkephalin (ME). Fentanyl (1 μM)-induced currents were also smaller in the mutant mice in a similar proportion of neurons (Het: 9 responders/19 recordings and WT: 8 responders/20 recordings; Fig. 1E). In contrast, ME (10 μM)-induced currents were not reduced in the RGS-insensitive mice (Het: 8 responders/14 cells and WT: 13 responders/19 cells; Fig. 1F). This observation was not a result of δ-opioid receptor activation in the vlPAG because the selective δ-opioid receptor antagonist naltrindole (100 nM) did not significantly reduce the ME-induced current (ME alone = 12 ± 4 pA vs ME + naltrindole = 11 ± 3 pA; n = 5).

The RGS-insensitive mice have a mutation in Gαo that disrupts binding of RGS proteins to Gαo. To further confirm that the observed effects were due to Gαo signaling, we used a myristoylated peptide inhibitor synthesized according to mini-gene sequences designed by the Hamm laboratory (Gilchrist et al., 1999, Gilchrist et al., 2002). These peptides bind to the active site on Gαo subunits blocking their activation by GPCRs. The $Gαo_1$ inhibitor peptide (5 μM) blocked DAMGO-mediated GIRK currents (Fig. 2A-C).

Inhibition of $G\alpha o_1$ also reduced the GIRK currents induced by fentanyl (1 μM; Fig 2D). Because the peptide sequence to $G\alpha o_2$ could not be synthesized, it is not clear if the residual current is due to the presence of uninhibited $G\alpha o_2$ or to incomplete penetration of the $G\alpha o_1$ inhibitor peptide into the slices and incomplete blockade of the $G\alpha o_1$ proteins. Consistent with the results in the RGS-insensitive mice, the $G\alpha o_1$ inhibitor peptide had no effect on ME-induced GIRK currents (Fig. 2E). This result also provided a control for the possibility that the myristoylated peptides inhibited μ-opioid receptor activation of GIRK channels through a nonselective action. Since μ-opioid receptors couple to both Gαi and Gαo proteins, we tested the possibility that ME preferentially activates GIRK via Gai subunits. Myristoylated $G\alpha i_{1\text{-}3}$ inhibitor peptides significantly reduced the ME-induced GIRK currents (Fig. 2F) confirming ME preferentially signals to GIRK through Gαi and providing an explanation for the differences between agonists in slices from the RGS-insensitive Het mice.

$GABA_B$ receptors are also coupled to GIRK channels and the $GABA_B$ agonist baclofen (20 μM) elicits a GIRK current in most vlPAG neurons (Fig. 3A;(Chieng and Christie, 1995, Vaughan et al., 1997b). Thus, baclofen was tested in recordings as a control for neuron viability, especially in neurons that did not respond to opioid agonists. The baclofen-mediated currents were reversed by the selective $GABA_B$ antagonist CGP55845 (1 μM) and were reduced in Het mice (Fig. 3B). Baclofen-induced GIRK currents were similar in male and female mice (Two-way ANOVA; Main Effect of Sex; $F_{(1,48)} = 0.11$; $p = 0.74$), so responses were combined. We further tested baclofen with the $G\alpha o_1$ inhibitor peptide, and the $G\alpha i_{1\text{-}3}$ inhibitor peptides (Fig. 3C,D). Baclofen-induced

GIRK currents were reduced in the presence of the $G\alpha o_1$ inhibitor peptide but were unaffected by $G\alpha i_{1\text{-}3}$ inhibitor peptides.

We previously published data showing that morphine antinociception is enhanced in the RGS-insensitive mice when tested on the hot plate test but reduced when tested with the warm-water tail withdrawal test (Lamberts et al., 2013). The reduced antinociception in the warm-water tail withdrawal test was also observed for methadone (Lamberts et al., 2013) with no change in methadone antinociception in the hot plate test. These results led to the hypothesis that different μ-opioid receptor signaling pathways are critical for tests involving supraspinal (hot plate) versus spinal (warm-water tail withdrawal) circuits. Suprapinal μ-opioid receptor activation results in inhibition of GABA release in the PAG and morphine inhibition of evoked GABA release is potentiated in the RGS-insensitive mice (Lamberts et al., 2013). In contrast, we find that morphine-induced GIRK currents are reduced in the RGS-insensitive mice (WT: 12 ± 2 pA vs. Het: 6 ± 1 pA; Mann-Whitney $U_{(59, 33)} = 5$, *p = 0.01). We propose that GIRK signaling is critical for the spinally-mediated warm-water tail-withdrawal test. Thus, we hypothesized that $GABA_B$-mediated antinociception would also be differentially affected in the two behavior tests. Indeed, the baclofen dose-response curve was shifted to the right in the warm-water tail withdrawal test (Fig. 3E), similar to both morphine and methadone in our earlier study. In addition, both male and female mice responded with similar shifts in baclofen potency. The shift in the tail withdrawal test is consistent with our results demonstrating reduced baclofen-mediated activation of GIRK channels in the RGS-insensitive mice and the previously published decrease in opioid-induced

antinociception using the tail withdrawal test in RGS-insensitive mice (Lamberts et al., 2013).

We also tested baclofen in the hot plate test and observed no change in the dose-response for baclofen-mediated antinociception (Fig. 4A), similar to our finding with methadone in the previous opioid behavior study. There was no change in locomotor activity with baclofen administration (Fig. 4B). We also observed no significant difference in baclofen-mediated inhibition of evoked GABA release in the WT versus Het mice (Fig. 4C,D), consistent with the lack of shift in the baclofen dose-response curve in the supraspinally-mediated hot plate test.

3.5 Discussion

RGS proteins are widely expressed with overlapping expression patterns. The redundancy in expression has made it difficult to interpret the roles of single RGS proteins using knock-down or knock-out strategies. The knock-in mouse model that impairs binding of RGS proteins to Gαo subunits (Goldenstein et al., 2009) exhibits no changes in μ-opioid receptor binding or G protein levels (Lamberts et al., 2013) and thus has been an advantageous model for determining the effects of RGS protein signaling on μ-opioid receptor-mediated signaling pathways and behaviors. Our results using GPCR responses in vlPAG neurons from these mutant mice have determined that RGS proteins are required for optimal μ-opioid receptor and $GABA_B$ receptor activation of GIRK channels in the vlPAG. In addition, we find that not all opioid ligands signal to GIRK channels via activation of Gαo.

Role of GIRK channels in spinal antinociception

μ-Opioid receptors are expressed throughout the nervous system and opioid drugs, such as morphine, have multiple effects throughout the neuraxis. Suprapinal μ-opioid receptor modulation of antinociception is induced in the descending pain modulatory pathway from the PAG to the rostral ventromedial medulla (RVM) to the spinal cord (Heinricher and Ingram, 2008). Supraspinal morphine antinociception is dependent on μ-opioid receptors in the vlPAG (Yaksh et al., 1976, Renno et al., 1992, Lau and Vaughan, 2014) and μ-opioid receptor-mediated inhibition of GABA release from presynaptic terminals in the vlPAG (Vaughan et al., 1997a, Lau and Vaughan, 2014). Both the RGS-insensitive mice (Gαo mutant) and RGS9 knock-out mice display enhanced morphine supraspinal antinociception (Zachariou et al., 2003, Lamberts et al., 2013) as would be expected for RGS proteins acting as negative regulators of GPCR signaling; however, these mouse models have reduced morphine-mediated spinal antinociception (Papachatzaki et al., 2011, Lamberts et al., 2013). Although RGS proteins are negatively coupled to many effectors so that their actions to hydrolyze GTP facilitates rapid termination of GPCR signaling, there have been reports that RGS proteins are required for GPCR signaling (Kovoor and Lester, 2002, Papachatzaki et al., 2011, Lamberts et al., 2013). These prior reports suggested that RGS proteins exert positive regulation of μ-opioid receptor coupling to some effectors. Previous work with the RGS9 knockout mice showed reduced opioid-mediated hyperpolarization of spinal neurons compared to WT mice (Papachatzaki et al., 2011). GIRK channels play a significant role in spinal morphine antinociception (Marker et al., 2004, Marker et al., 2005) so we hypothesized that μ-opioid receptor coupling to GIRK channels would be compromised

in the RGS-insensitive Het mice. Indeed, our results indicate that μ-opioid receptor-GIRK coupling in the vlPAG and LC is reduced. In addition, GIRK coupling to $GABA_B$ receptors is compromised in the RGS-insensitive mice but presynaptic inhibition of GABA release by the $GABA_B$ receptor agonist baclofen is unaffected. This difference was reflected in the behavior data showing that the baclofen dose-response curve is unchanged in the supraspinal hot plate test but is shifted to the right in the spinally-mediated tail-withdrawal test. These results mirror our previous results with opioid inhibition of evoked IPSCs and opioid-induced antinociception in the RGS-insensitive mice (Lamberts et al., 2013). An alternative explanation for the observed differences between antinociceptive tests could be motor impairment caused by baclofen. However, we observed no significant effects of baclofen on locomotor behavior. In addition, baclofen effects on locomotor activity should have little effect on the tail withdrawal test, which is a spinal reflex, but would be expected to potentially alter complex behavioral responses in the hot-plate test. However, baclofen-mediated antinociception on the hot plate test was similar in all groups. This agrees with earlier findings that any locomotor impairment by baclofen is dissociated from antinociceptive effects measured in the hot-plate and tail withdrawal tests (Levy and Proudfit, 1977).

Heterogeneity of RGS proteins

The reliance of μ-opioid receptor-GIRK coupling on RGS binding to Gαo appears to be dependent on either location or on type of RGS protein that is expressed in certain brain areas. In contrast to the current findings, several other studies find that disruption of components of RGS protein signaling complexes increase μ-opioid receptor-GIRK

coupling (i.e., are negatively coupled). Knockdown of RGS7B, a binding partner for RGS7 increases μ-opioid receptor signaling to GIRK channels in the hippocampus (Zhou et al., 2012) and RGS protein inhibition increases $GABA_B$ receptor coupling to GIRK channels in dopamine neurons (Labouebe et al., 2007), an effect shown to be mediated by RGS2. Thus, it is clear that regulation by RGS proteins on signaling pathways is dependent on the RGS-Gα complexes that are expressed in various cell types. RGS proteins known to be expressed in the PAG include the R7 subfamily of RGS proteins (RGS6, RGS7, RGS9-2, and RGS11)(Lopez-Fando et al., 2005), RGSz (Garzon et al., 2005b, Garzón et al., 2005b, Gaspari et al., 2018), and RGS14 (Rodriguez-Munoz et al., 2007). RGS9-2 promotes μ-opioid receptor-mediated hyperpolarization in the spinal cord, presumably through enhanced activation of GIRK channels (Papachatzaki et al., 2011), so RGS9-2 may be performing a similar role in the PAG. Indeed, RGS9-2 serves as a GAP to regulate both Gαi and Gαo proteins, with a preference toward Gαo (Masuho et al., 2013). RGS7 has selective GAP activity for Gαo but it was found to negatively regulate morphine antinociception (Sutton et al., 2016) and did not co-immunoprecipitate with the μ-opioid receptor in PAG tissue (Garzon et al., 2005a) and thus may not be a likely candidate for μ-opioid receptor-GIRK coupling in the PAG. Similar increases in opioid antinociception were observed with knockdown of other R7 subfamily members (Garzon et al., 2003) and RGSz (Garzon et al., 2005b).

Role of RGS proteins in functional selectivity

The GAP activity of RGS proteins affects maximal responses of partial agonists more than full agonists at the μ-opioid receptor as predicted by the collision-coupling

model (Clark et al., 2003, Clark et al., 2008). This model also predicts that potency of both partial and full agonists should be reduced to similar extents (Clark et al., 2008). μ-Opioid receptor coupling to inhibition of evoked GABA release in the vlPAG is consistent with this prediction as EC50 concentrations of morphine and ME were enhanced in the RGS-insensitive mice (Lamberts et al., 2013). Interestingly, we observed different results using maximal concentrations of DAMGO, fentanyl and ME to activate μ-opioid receptor coupling to GIRK channels in WT and RGS-insensitive mice. Maximal GIRK currents elicited by DAMGO and fentanyl were reduced in the Het mice, whereas ME-evoked currents were unaffected. Further experiments with selective inhibitors of Gαo and Gαi provided evidence that DAMGO and fentanyl selectively activate Gαo proteins, but ME utilizes Gαi to couple to GIRK channels. Previous studies have shown that RGS proteins have different binding affinities for Gα subunits (Talbot et al., 2010, Masuho et al., 2013) so it is possible that different G protein/RGS complexes are bound in the presence of different μ-opioid receptor agonists. RGS9-2 promotes μ-opioid receptor coupling to $G\alpha i_3$ in the striatum in the presence of morphine but Gαq in the presence of fentanyl (Han et al., 2010, Psifogeorgou et al., 2011), and it negatively modulates morphine analgesia but positively modulates actions of fentanyl, methadone, and oxycodone (Gaspari et al., 2017). In any case, the differential activation of Gαi by the peptide ME versus activation of Gαo by other opioid agonists may provide a means to manipulate pathways preferentially used by endogenous μ-opioid receptor ligands.

In summary, our studies using the RGS-insensitive mouse model have elucidated key aspects in RGS modulation of μ-opioid receptor signaling in the vlPAG. Although RGS proteins negatively modulate μ-opioid receptor inhibition of evoked GABA release

(Lamberts et al., 2013), they positively modulate μ-opioid receptor-GIRK coupling. In addition, RGS complexes differentially affect opioid agonists as ME-mediated GIRK currents were unaffected in the RGS-insensitive mice. This is presumably due to the preference for a ME-bound μ-opioid receptor for Gαi proteins. Further study of the role of RGS proteins in agonist functional selectivity looking at multiple signaling pathways may elucidate selective targets for manipulating biased signaling at μ-opioid receptors.

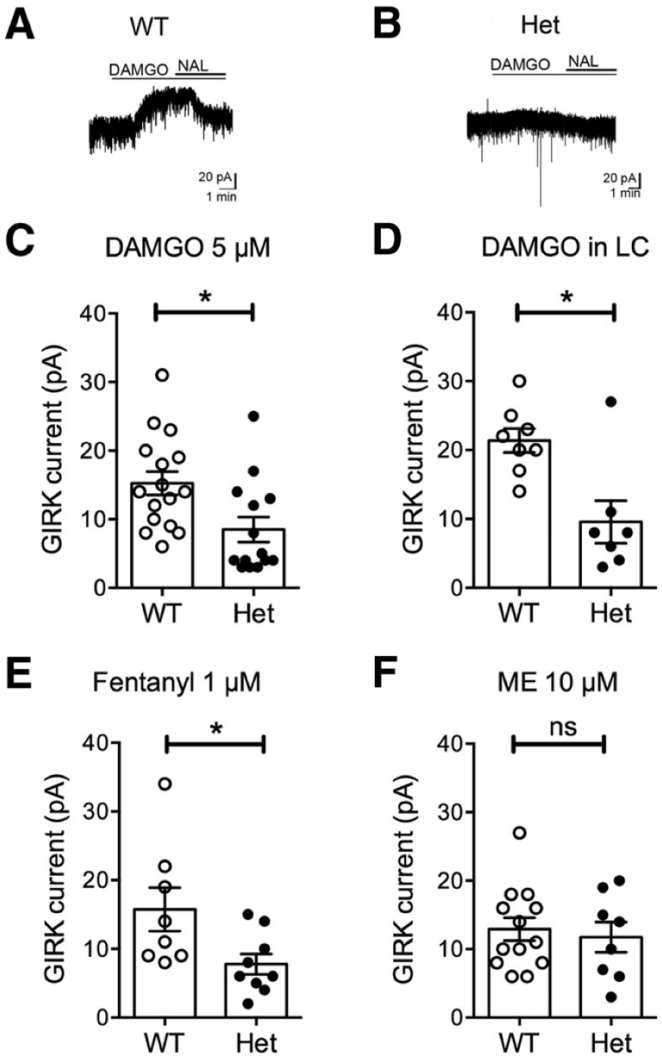

Figure 3. 1: Opioid agonist activation of GIRK channels is differentially affected in the RGS-insensitive mice.

A. Representative trace of a recording from a vlPAG neuron from a WT mouse where the selective opioid agonist DAMGO (5 µM) elicited an outward current that was reversed by the µ-opioid receptor antagonist naloxone (NAL; 10 µM). **B**. Representative trace from a vlPAG neuron from a RGS-insensitive heterozygous (Het) mouse. **C**. Compiled data for DAMGO in WT and Het mice showing reduced GIRK currents in Het mice (Mann-Whitney $U_{(313,152)} = 47$, *p = 0.006). **D**. Compiled data from recordings in locus coeruleus (LC) neurons showing decreased currents in Het mice (Mann-Whitney $U_{(85, 35)} = 7$, *p = 0.01). **E.** Compiled data for fentanyl (1 µM) activation of GIRK currents in vlPAG neurons, also with reduced GIRK currents in Het mice (Mann-Whitney $U_{(95, 58)} = 13$, *p = 0.03). **F.** [Met^5]-enkephalin (ME)-induced GIRK currents were similar amplitude in WT and Het mice (Mann-Whitney $U_{(148, 84)} = 48$, p = 0.76).

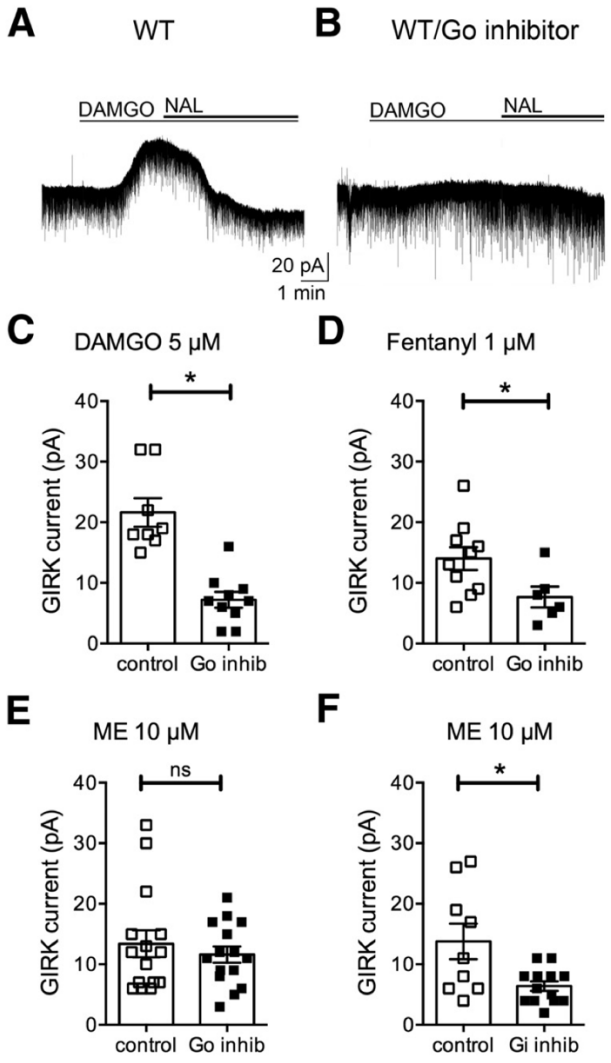

Figure 3. 2: Specific inhibitor peptides for Gα proteins reduce opioid-induced GIRK currents in an agonist-dependent manner.

A. Representative trace of a vlPAG neuron recording from a WT mouse showing the outward current in response to DAMGO (5 µM) that is reversed by naloxone (NAL; 10 µM). **B**. DAMGO-mediated GIRK current from a vlPAG neuron from a WT mouse in the presence of the $G\alpha o_1$ inhibitor peptide (5 µM). **C**. Compiled data showing $G\alpha_{o1}$ inhibitor peptide (5 µM) inhibition of DAMGO-mediated GIRK currents (Mann-Whitney $U_{(115, 56)} = 1$, $^*p < 0.0001$). **D**. Compiled data showing $G\alpha_{o1}$ inhibitor peptide (5 µM) inhibition of fentanyl-mediated GIRK currents (Mann-Whitney $U_{(105,31)} = 10$, $^*p = 0.03$). **E**. Compiled data showing that the $G\alpha_o$ inhibitor peptide (5 µM) does not affect GIRK currents mediated by ME (Mann-Whitney $U_{(237, 229)} = 109$, $p = 0.88$). **F.** The $G\alpha_{i1-3}$ inhibitor peptide (5 µM) reduces ME-induced GIRK currents (Mann-Whitney $U_{(134, 119)} = 28$, $^*p = 0.04$).

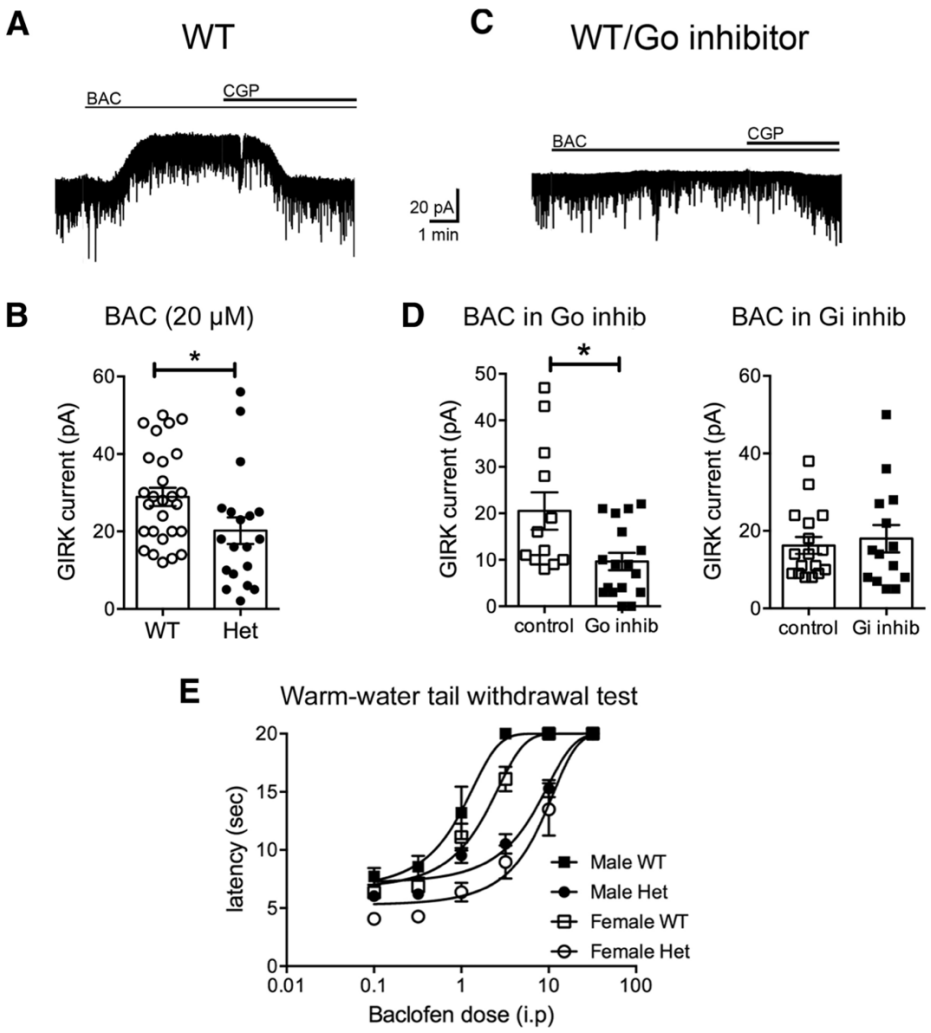

Figure 3. 3: $GABA_B$ receptor agonist baclofen uses $G\alpha_o$ in vlPAG neurons and baclofen-mediated antinociception is diminished in RGS-insensitive mice.

A. Baclofen (BAC; 20 µM) increases an outward current in WT mice that is reversed with the selective $GABA_B$ antagonist CGP55845 (CGP; 1 µM). **B.** Baclofen-mediated currents are decreased in RGS-insensitive Het mice (Mann-Whitney $U_{(743,338)} = 148$, *p = 0.01). **C.** Representative trace showing reduced baclofen-mediated current in the presence of the $G\alpha_{o1}$ inhibitor peptide (5 µM). **D.** Compiled data showing that the Gαo inhibitor peptide decreases baclofen-mediated GIRK currents (Mann-Whitney $U_{(232,203)} = 50$, *p = 0.02) but the $G\alpha_{i1-3}$ inhibitor peptide (5 µM) does not reduce baclofen-induced GIRK currents (Mann-Whitney $U_{(332,263)} = 142$, p = 0.99). **E**. Baclofen dose-response curve for antinociception using the warm water tail withdrawal test showing a significant shift to the right in the RGS-insensitive Het male (EC50 = 3.1 mg/kg; 95% CI, 2.3 – 3.9 mg/kg) and female mice (EC50 = 5.5 mg/kg; 95% CI, 4.7 – 6.4 mg/kg) compared to WT male (EC50 = 0.50 mg/kg; 95% CI, 0.4 – 0.6 mg/kg) and female mice (EC50 = 1.0 mg/kg; 95% CI, 0.7 – 1.2 mg/kg; $F_{(3,116)} = 63$, p < 0.0001, n = 6 per group). Measurements were made 30 min after each baclofen dose.

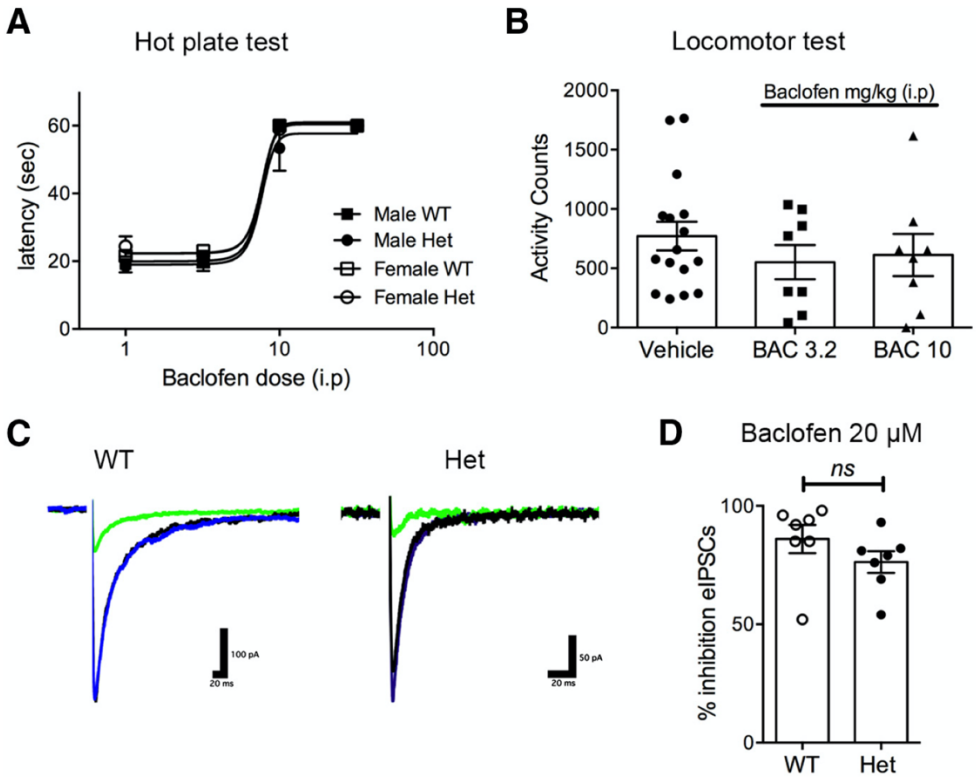

Figure 3. 4: Baclofen dose-response curve on the supraspinally-mediated hot plate test is similar in both genotypes.

A. Dose-response curve for baclofen-mediated antinociception using the hot plate test showing no difference between genotypes or sex (n = 6 per group). **B**. Baclofen-treated WT mice showed no loss of locomotor activity in a novel environment. Locomotor activity was recorded for 30 min, following a 30 min treatment of baclofen (3.2 or 10 mg/kg; n=8 per dose) or vehicle (V, water; n = 18; $F_{(2,29)} = 0.68$, p = 0.52). Each bar ± SEM represents an equal number of male and female mice. **C**. Representative evoked IPSCs showing inhibition by baclofen (20 µM, green traces) compared to control (blue traces) and the $GABA_B$ antagonist CGP55845 (1 µM; black traces) in recordings from WT and Het mice**. D**. Summary data for percent inhibition showing no significant difference between genotypes (Mann-Whitney $U_{(67,38)} = 10$, p = 0.07).

Chapter 4: Additional Data

4.1 No sex-differences in intrinsic firing properties, opioid sensitivity, or impact of persistent inflammation on spontaneous firing frequency of *Phasic* neurons

Neuronal heterogeneity was characterized in a large sample of vlPAG neurons, which provided the opportunity to also identify any sex differences in the prevalence of the neuron types. However, all 4 types (*Phasic*, *Tonic*, *Onset*, and *Random*) were seen in comparable proportions in both males (n = 101) and females (n = 121) (data not shown; *Phasic* 49M and 56F, *Tonic* 37M and 38F, *Onset* 8M and 15F, *Random* 7M and 12F; Chi-square = 2.142 p = 0.5435); the two most common types were graphed and analyzed separately to ensure that the less common Onset and Random neuron groups did not skew the statistics for the larger groups (Figure 1.4A). We also wanted to determine whether postsynaptic MOR expression was different between males and females. Using GIRK currents as a method to measure postsynaptic MOR activation, we found that there were no sex differences in opioid sensitivity in either *Phasic* or *Tonic* populations (Figure 4.1B, C). We confirmed that the enhanced spontaneous firing frequency of *Phasic* neurons seen in the combined groups was maintained in males and females alone (Figure 4.1D). RMP is a membrane property that can influence firing threshold, which we found to be lowered after persistent inflammation in our data set that combined males and females, prompting us to confirm that a change in RMP is not contributing to the enhanced spontaneous firing frequency of *Phasic* neurons after persistent inflammation in either sex. Our data demonstrates that enhanced Phasic firing after persistent (5-7 d) CFA-induced inflammation is not a byproduct of altered RMP in males or females.

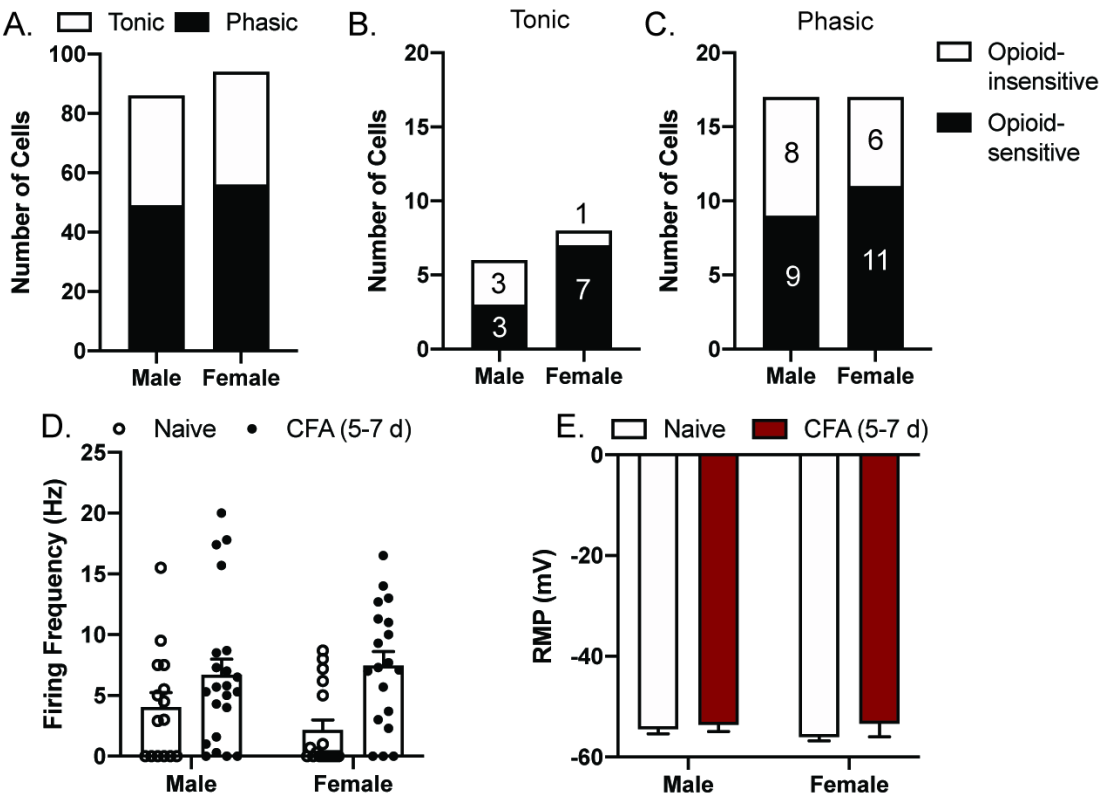

Figure 4. 1: No observed sex-differences in intrinsic firing properties, opioid sensitivity, and CFA-induced activation of *Phasic* neurons.

A. Sex differences in proportions of neuron types of naïve rats (*Phasic* 49M and 56F, *Tonic* 37M and 38F; Fisher's exact test, p = 0.7634). **B.** Proportion of opioid-sensitive and -insensitive neurons (measured with MOR-mediated GIRK currents) in *Tonic* neuron population showed no significant differences in opioid sensitivity between males and females (Chi-square p = 0.2448). **C.** No significant differences in opioid sensitivity of *Phasic* neurons between males and females (Chi-square p = 0.7283). **D.** Selective enhanced firing frequency in *Phasic* neurons after persistent inflammation is seen in both males and females (Two-way ANOVA significant effect of treatment **p=0.0012, NS effect of sex p=0.6334, NS interaction p=0.2611). **E.** *Phasic* neurons from naïve and CFA-treated (5-7 d) rats show no sex differences in resting membrane potential. Two-way ANOVA confirmed no effect of treatment ($F_{(1, 132)} = 1.781$, $p = 0.1843$) or sex ($F_{(1, 132)} = 0.2517$, $p = 0.6168$), with no significant interaction ($F_{(1, 132)} = 0.4008$, $p = 0.5278$) are seen in resting membrane potential after persistent inflammation in either males or females (naïve n = 49 (M), 54 (F), CFA 5-7d n = 23 (M), 10 (F)).

4.2 Persistent inflammation alters afferent drive onto *vlPAG* neurons

As a large integration site receiving many excitatory and inhibitory afferents, vlPAG neurons are likely to receive heterogeneous synaptic inputs. The impact these synaptic inputs have on spontaneous firing frequency was evaluated both using whole-cell recordings and the loose-patch method enlisted to observe firing over a longer period without interrupting the intracellular milieu. For the whole-cell recordings, a series of 2s long depolarizing current steps, starting with a 3s long A baseline was obtained until the firing frequency maintained a steady state over 3-5 m, then $GABA_A$ and glutamate receptor antagonists (Gabazine 10 uM and NBQX 5uM) were washed on for 5-7 m to observe how firing was altered by removing synaptic inputs. If a neuron receives excitatory-dominant synaptic inputs the blockers will inhibit firing and if the neuron receives inhibitory-dominant inputs the synaptic blockers will activate firing (Figure 4.2). Removing synaptic inputs produced both inhibition and activation, with naïve neurons demonstrating excitatory-dominant afferent inputs (Figure 4.3A, B and 4.4A, B). In naïve rats both excitatory- and inhibitory-dominant E/I balance were seen in the *Phasic* and *Tonic* neuron groups (Figure 4.3A, B). Interestingly, after persistent inflammation there was a significant enhancement in the number of inhibitory-dominant neurons that were activated by removing synaptic inputs (Figure 4.4B). This aligns with previous studies published from the lab showing that there is enhanced GABA tone within the vlPAG after persistent inflammation in male rats (Tonsfeldt et al., 2016).

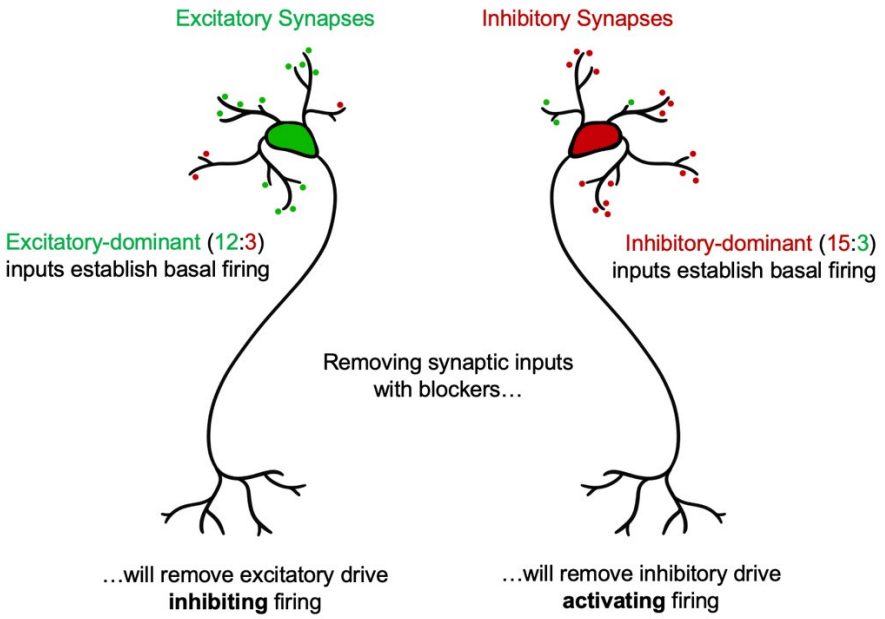

Figure 4. 2: Excitatory/Inhibitory balance dictates firing response to removing synaptic inputs

vlPAG neurons receiving more excitatory inputs than inhibitory inputs are considered excitatory-dominant, and as a result removing synaptic inputs will inhibit their spontaneous firing frequency. Conversely, neurons receiving more inhibitory inputs than excitatory inputs are considered inhibitory-dominant, and removing their synaptic inputs should disinhibit or activate their spontaneous firing frequency.

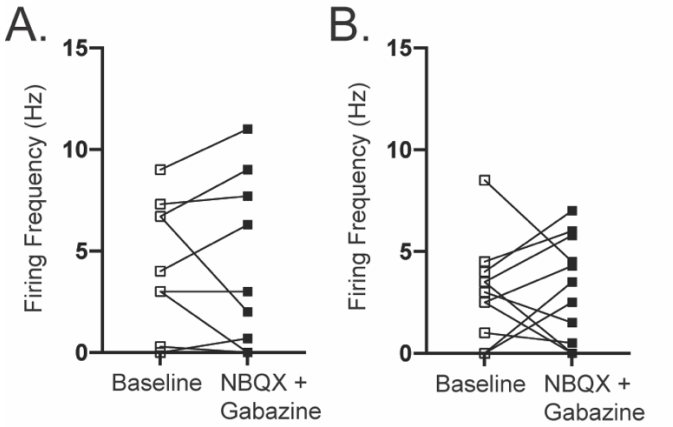

Figure 4. 3: The excitatory/inhibitory balance onto *Phasic* and *Tonic* neurons varies

A. Firing frequency of *Phasic* neurons before and after synaptic blockers reveals differential E/I balance onto individual neurons (5 activated, 3 inhibited, 3 unchanged) with no overall effect of removing synaptic inputs (paired t-test, $t_{(9)} = 0.04$, $p = 0.9676$).
B. Firing frequency of *Tonic* neurons before and after synaptic blockers reveals differential E/I balance onto individual neurons (6 activated and 5 inhibited) with no overall effect of removing synaptic inputs (paired t-test, $t_{(10)} = 0.2869$, $p = 0.7801$).

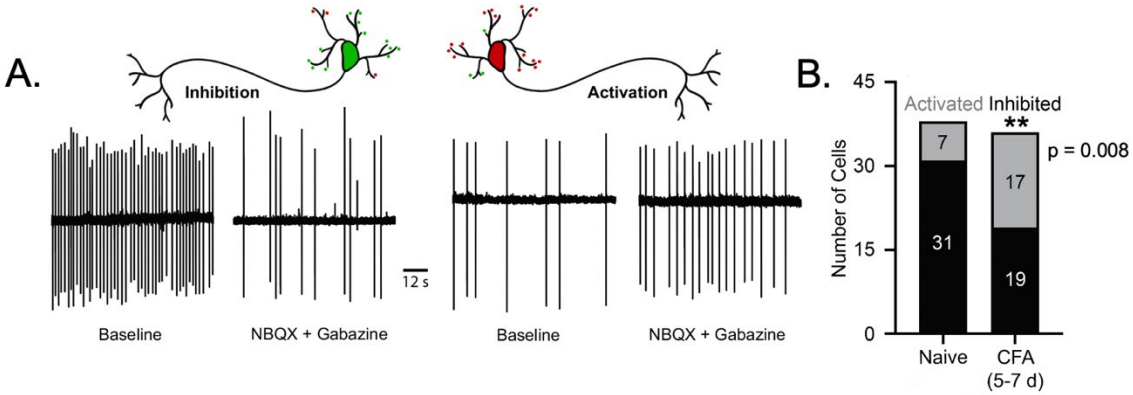

Figure 4. 4: Persistent inflammation increases incidence of inhibitory-dominant synaptic inputs onto vlPAG neurons

A. Sample loose-patch traces showing inhibition (left) and activation (right) of spontaneous firing that demonstrate excitatory-dominant (left) and inhibitory-dominant (right) neuronal responses to synaptic blockers. **B.** Quantified proportion of neurons activated or inhibited by removing synaptic inputs. 31/38 neurons from naïve rats were excitatory-dominant and those exposed to persistent inflammation showed a significant increase in inhibitory-dominant neurons (Chi-square **p = 0.008).

Chapter 5: Discussion

4.1 Intrinsic membrane and firing properties—a framework for defining cellular diversity

Characterizing intrinsic membrane and firing properties is a common approach to defining neuronal heterogeneity (Prescott and De Koninck, 2002, Sedlacek et al., 2007, Van Aerde and Feldmeyer, 2015, Pradier et al., 2019). We used voltage- and current-clamp protocols to determine the threshold, frequency, and pattern of firing of 223 vlPAG neurons from naïve rats. Confirming that these properties were maintained in the absence of synaptic inputs reinforced their intrinsic nature. Despite many sexually dimorphic features within the vlPAG, *Phasic* and *Tonic* neurons are observed in comparable proportions in males and females (Figure 4.1A). These neuron types defined by intrinsic properties serves as a useful framework to apply other features of neuronal heterogeneity. Our primary interests were determining whether opioid sensitivity mapped onto these distinct neuron types (see *section 4.2*) and if the vlPAG is activated broadly or selective to neuron type after acute or persistent inflammation. The characterization of intrinsic properties in naïve animals allows for evaluation of intrinsic property alterations induced by persistent inflammation (Li and Sheets, 2018, Adke et al., 2021). Using the same electrophysiology protocols with 62 neurons after 2 h CFA and 80 neurons after 5-7 d CFA, we were able to observe selective activation of *Phasic* neurons that occurred after persistent inflammation only, in both male and female rats (Figure 4.1D). RMP is a common intrinsic membrane property that can influence spontaneous firing frequency;

however, we see that RMP does not contribute to enhanced firing of *Phasic* neurons after persistent inflammation in the male or female population (Figure 4.1E).

Neuronal firing properties and response to noxious stimuli have been used to define important, functionally distinct neurons within another region of the descending pain modulatory pathway, the RVM (Fields et al., 1983, Vanegas et al., 1984b). Landmark papers demonstrated that not only were there neurons that responded differently to the application of a noxious stimulus *in vivo*, serve as a framework for countless follow up studies that used this neuron type distinction to define additional features of these neurons—ultimately shaping our understanding of the functional role that they play in descending pain modulation. These findings include: opioid sensitivity (Heinricher et al., 1992), response to excitatory and inhibitory afferent input (Heinricher and Roychowdhury, 1997, Heinricher et al., 1999), ability for ON- and OFF-cells to inhibit or facilitate ongoing nociception (Basbaum and Fields, 1979, Mantyh and Peschanski, 1982), and that that acute and persistent inflammation had distinct impacts on the firing properties of these neurons, both at rest and in response to an acute noxious stimulus (Cleary and Heinricher, 2013).

Two key topics of interest for vlPAG neuronal characterization that can contribute to our understanding of the impact of enhanced *Phasic* firing frequency by persistent inflammation include neurotransmitter content and projection target. The most widely used tools to determine the role of excitatory and inhibitory neuronal populations are genetic mouse models. Chemogenetic manipulation of these populations *in vivo* found that activation of glutamatergic vlPAG neurons is analgesic, confirming vlPAG activation studies (Reynolds, 1969, Mayer et al., 1971, Mayer and Liebeskind, 1974, Akil

and Liebeskind, 1975, Soper and Melzack, 1982, Jensen and Yaksh, 1989); whereas, activating GABAergic vlPAG neurons is hyperalgesic, similarly to how GABA agonists within this region reverse opioid-mediated analgesia (Zambotti et al., 1982, Moreau and Fields, 1986, Depaulis et al., 1987). Unfortunately, the dense expression of $GAD_{65/67}$ on processes within the vlPAG enhances background signal, distorting cell body specific expression. Pilot studies done in collaboration with Dr. Sue Aicher's lab, demonstrated the difficulty of confirming in our hands distinct GAD+ and GAD- cell bodies with dense $GAD_{65/67}$ expression on afferent processes within the vlPAG. Methodologies such as RNAscope have improved specificity (Zhang et al., 2020) and reduce background signal from expression in the processes as a result of the thin tissue slices. However, there are methodological limitations to obtained these thin slices after *in vitro* slice recordings from much thicker slices while also preserving the biocytin dialyzed into the cell during the recording. Future advancements to post-hoc tissue processing may circumnavigate these limitations.

Studies utilizing genetic mouse models have shown that firing patterns do not fully correlate with neurotransmitter content, which would make this additional data likely not refine our interpretation of our current data. A study evaluating GABAergic neurons in a genetically defined mouse model observed 31/33 GABAergic neurons had a *Tonic*-like firing pattern with the other 2/33 showing a *Phasic*-like pattern (Park et al., 2010), however, *Tonic*-like firing was also seen in glutamatergic neurons (Samineni et al., 2017a). These proportions expanded across our large data set lead us to believe that GABAergic neurons are likely to be present in both our *Tonic* and *Phasic* groups. Future studies utilizing single-cell PCR will allow us to determine whether neurotransmitter

content map onto *Phasic* and *Tonic* neuronal populations (Bosch et al., 2013). Although we anticipate some degree of mixed expression, it will be most interesting to determine whether the most strongly activated *Phasic* neurons are predominately glutamatergic or GABAergic. Single-cell PCR will also allow us to determine whether the most strongly activated *Phasic* neurons express MORs. However, if *Phasic* neurons contain both GABAergic and glutamatergic neurons that are both activated by persistent inflammation, this additional data does not alter the interpretation of our current findings.

Projection target is another important feature that can theoretically increase our understanding of how *Phasic* neurons activated by persistent inflammation impact descending pain modulation. The vlPAG contributes to the overall output of the descending pain modulatory pathway at the level of the dorsal horn of the spinal cord through its connection with the RVM (Behbehani and Fields, 1979, Gebhart et al., 1983, Prieto et al., 1983). The RVM-projecting population contains both GABAergic and non-GABAergic neurons (Morgan et al., 2008). In the mouse, both *Tonic*-like (7/12) and *Phasic*-like (5/12) neurons project to the RVM with comparable density, with low-threshold spiking, MOR-expressing GABAergic (*Tonic*-like) neurons in particular not projecting to the RVM (Park et al., 2010). It also suggests that it is likely that both the *Phasic* and *Tonic* neuronal populations that we observed contained RVM-projecting neurons, including those that expressed MORs. Despite female rats having more RVM-projecting neurons than males, persistent inflammation activated significantly more RVM-projecting neurons in males than females (Loyd and Murphy, 2006). Despite our data showing comparable *Phasic* neuronal activation by persistent inflammation (Figure 4.1D), if more of these neurons are RVM-projecting in male rats, that could result in

distinct implications for enhanced *Phasic* firing on the descending modulatory circuit between males and females.

Future studies confirming mixed *Phasic* and *Tonic* projections to the RVM in rats would be marginally useful because it would still not tell us if they both project to ON- and OFF-cells, which, coupled with data on neurotransmitter content, could completely change the interpretation of the data. However, with the tools currently available, the most interesting point would be whether the RVM-projecting population contains the *Phasic* neurons most strongly activated by persistent inflammation, suggesting a targeted activation of the descending circuit. It is important to also note that due to reciprocal connections with other brain regions, vlPAG neurons that do not project to the RVM may still impact the signal being relayed to the RVM during ongoing signaling (ie: from persistent inflammation). The implication of the data presented in this thesis would be greatly enhanced by the ability to determine which population of RVM neurons (ON- or OFF-) vlPAG *Phasic* neurons project to. Selective MOR expression on RVM ON-cells may allow for eventual advancements in tools allowing us to transfect retrogradely transported channelrhodopsin/halorhodopsin using MOR-cre rat lines. This would first allow us to identify ON-cell projecting vlPAG neuron types (*Phasic*, *Tonic*, or both). Then, if they contain *Phasic* neurons, we could activate them *in vivo* in naïve rats to determine the behavioral response to *Phasic* neuron activation and inhibit them after persistent inflammation to solidify whether reversing *Phasic* neuron activation is a useful therapeutic target. There is of course the possibility that *Phasic* neurons project to both ON- and OFF-cells complicating these studies and subsequent interpretation. Furthermore, expression profiles for OFF-cell targeting using similar methods have not

been defined. These future experiments demonstrate how the findings in this thesis will continue to contribute to our understanding of the circuit in persistent inflammatory states as new tools are developed.

4.2 Postsynaptic MOR expression does not correlate with intrinsic properties

MOR agonists can either inhibit or disinhibit vlPAG neurons in multiple ways depending on post- and presynaptic compartmental expression. A subset of vlPAG neurons postsynaptically express MORs that activate GIRK channels, hyperpolarizing neurons (Chieng and Christie, 1994a, Lau et al., 2020), which can also inhibit their firing frequency (see *Chapter 2*). Presynaptic MOR activation inhibits neurotransmitter release from vlPAG afferents (Chieng and Christie, 1994b, Vaughan et al., 1997a, Vaughan and Christie, 1997). If a vlPAG neuron receives more excitatory inputs than inhibitory inputs ("excitatory-dominant") inhibition of neurotransmitter release can inhibit firing, whereas if the neuron receives more inhibitory inputs ("inhibitory-dominant") inhibition of neurotransmitter release can disinhibit *or* activate firing. The impact of presynaptic MOR activation on postsynaptic neuronal firing is further influenced by the enhanced efficacy of MOR agonists to inhibit firing at GABA compared to glutamate terminals (Lau et al., 2020). Both inhibitory-dominant and excitatory-dominant neurons are observed in the *Phasic* and *Tonic* populations (Figure 4.3A, B), providing evidence that MOR agonists can inhibit and disinhibit/activate neurons within both populations.

Opioid-mediated disinhibition of pain is only one component of parallel pathways between the vlPAG and the RVM that contribute to descending pain modulation (Lau and Vaughan, 2014). Glutamatergic, RVM-projecting vlPAG neurons are thought to be

tonically inhibited by GABA inputs and disinhibition of pain specifically refers to the disinhibition (or activation) of these neurons by targeted inhibition of GABA release (Basbaum and Fields, 1984, Lau and Vaughan, 2014). These GABAergic inputs are known to be comprised of incoming vlPAG afferents, as well as GABAergic vlPAG neurons that have been hypothesized to send local collaterals (Basbaum and Fields, 1984, Barbaresi and Manfrini, 1988, Maione et al., 1999, Lau and Vaughan, 2014). MOR expressing vlPAG neurons in the mouse are 22% GABAergic and 77% glutamatergic (Zhang et al., 2020), complicating the assertion that MORs are selectively expressed on GABAergic neurons. Recent electrophysiology studies demonstrated RVM-projecting neurons are in fact disinhibited by opioid superfusion (Lau et al., 2020). However, RVM-projecting GABAergic and non-GABAergic neurons were also found to express MORs on their cell bodies (Osborne et al., 1996, Commons et al., 2000), providing evidence that RVM-projecting neurons can also be directly inhibited. Furthermore, a large portion of GABAergic axon terminals that express MORs came in contact with GABAergic dendrites within the vlPAG, suggesting a mechanism for disinhibiting GABAergic RVM-projecting neurons as well (Commons et al., 2000). Altogether these findings underscore the two parallel pathways between the vlPAG and RVM, which become increasingly complicated when taking into consideration which neuron types they synapse on to within the RVM. The existence of these parallel pathways complicates our ability to understand the impact enhanced *Phasic* firing has on the overall output of the descending modulatory circuit, which cannot be resolved by defining neurotransmitter content and projection target.

Other mouse studies have found MOR expression on *Tonic*-like, GABAergic neurons can be predicted by the presence of an intrinsic firing feature called a low-threshold spike (LTS), which is an action potential that occurs during the offset of a hyperpolarizing current step (Park et al., 2010). This study found 5 neurons with LTS had opioid-mediated GIRK currents, where 4 neurons without LTS did not. Not only did we find postsynaptic MOR expression to not be isolated to an individual neuron type (ie: GABAergic interneuron) but that in a larger data set LTS no longer predicted opioid sensitivity. More specifically, *Phasic* and *Tonic* neuronal populations had both LTS-expressing and non-LTS neurons, but these intrinsic firing features did not coincide with opioid-mediated GIRK currents.

Many different acute and chronic pain assays have shown that the median effective dose for morphine in female rodents is roughly twice the dose needed for males to achieve comparable antinociception (Kepler et al., 1989, Cicero et al., 2002, Ji et al., 2006, Loyd and Murphy, 2006, Loyd et al., 2008b, Posillico et al., 2015). This could be a result of males having significantly higher MOR-expression within the vlPAG compared to cycling females, with the lowest MOR-expression measured in proesterus females (Loyd et al., 2008b). Sexual dimorphism in opioid action and the descending pain modulatory circuit motivated us to evaluate whether our two most common neuron types have differing opioid sensitivity between males and females. Within our data set we found comparable numbers of opioid-sensitive *Tonic* and *Phasic* neurons between the male and female groups (Figure 4.1B, C), with comparable GIRK amplitudes (Unpaired t-test, $t_{(26)} = 1.642$, $p = 0.1126$). Our experiments not detecting enhanced MOR expression in males could either be a result of neurons with postsynaptic MORs coupled

to other effector targets (and not GIRK), enhanced MOR immunolabeling being influenced by presynaptic MOR expression as well, or our sampling not being high enough to observe these differences.

The potency of DAMGO injected into the RVM is enhanced by CFA-induced persistent inflammation in male rats (Hurley and Hammond, 2000), motivating us to determine whether opioid-mediated inhibition of vlPAG firing frequency is altered by persistent inflammation. Using loose-patch recordings to assess firing frequency over longer firing windows (1-3 m compared to the 2-3 s used in experiments to assess firing patterns) we determined that spontaneously active neurons are inhibited by DAMGO application that was reversed by naloxone. Recent studies found that disinhibited neurons were not spontaneously active (Lau et al., 2020), accounting for us not observing disinhibition of firing our loose patch recordings that select for spontaneously active neurons. The same experiments conducted after 5-7 d CFA showed a comparable degree of inhibition of spontaneous firing frequency, suggesting that opioid efficacy measured by inhibition of spontaneous firing frequency is not altered by persistent inflammation.

4.3 The vlPAG as a 2nd locus of bidirectional top-down control

The descending pain modulatory pathway bidirectionally modulates ongoing nociception through RVM afferents at the dorsal horn of the spinal cord. Descending inhibition and facilitation refer to the net output of this pathway, which can exert an antinociceptive or pronociceptive role. Much of these functional characterization studies have focused on the role of these distinct RVM neuron populations. However, an

increasing number of studies implicate the vlPAG in descending facilitation as well (Vanegas and Schaible, 2004, Heinricher and Ingram, 2020).

The RVM has been broadly demonstrated to exert bidirectional control over nociception through many studies using stimulation, pharmacology, and pain models. Descending inhibition primarily relies on increased OFF-cell firing, which can present as enhanced spontaneous firing or decreased stimulus-induced pause duration, as well as a reduction in ON-cell firing. The most obvious example of this can be seen with opioid agonists delivered locally to the RVM, which produce a targeted inhibition of ON-cells that selectively express MORs (Heinricher et al., 1992), as well as a disinhibition of OFF-cells by reducing GABA-mediated synaptic potentials (Pan et al., 1990). Similar to MOR agonists, $GABA_A$ antagonist bicuculline increases the activity of OFF-cells by blocking the tail flick induced OFF-cell pause (Heinricher et al., 1994, Heinricher and Tortorici, 1994).

Conversely, descending facilitation is achieved by enhancing ON-cell activity, which is often seen alongside reductions in OFF-cell activity. Selective pharmacological activation of RVM ON-cells, with EAA, cholecystokinin, neurotensin, etc. increases hyperalgesia (Heinricher and Neubert, 2004, Neubert et al., 2004, Heinricher and Ingram, 2020). Acute inflammation enhances the spontaneous activity of ON-cells and suppression of pain-inhibiting OFF-cells, producing hyperalgesia, and lowered response threshold after persistent inflammation (Cleary and Heinricher, 2013). Bicuculine-mediated disinhibition of the dorsomedial nucleus of the hypothalamus, a key region associated with emotional stressors, promotes hyperalgesia through enhanced ON- and decreased OFF-cell activity. Blocking the ON-cell activation alone is sufficient to prevent

the hyperalgesic effect (Martenson et al., 2009). This study demonstrates how regions upstream of the RVM contributes to descending facilitation.

The vlPAG has primarily been implicated in descending inhibition of pain due to the analgesic effect of vlPAG stimulation and locally applied opioid agonists. Electrical and chemical stimulation of the vlPAG produces comparable analgesia, which has been correlated with changes in the activity of downstream RVM neurons (Reynolds, 1969, Mayer et al., 1971, Mayer and Liebeskind, 1974, Akil and Liebeskind, 1975, Soper and Melzack, 1982, Jensen and Yaksh, 1989). Interestingly, persistent inflammation induced by CFA in the hindpaw enhanced the analgesic efficacy of PAG stimulation in the ipsilateral paw (Morgan et al., 1991). Similarly to systemic administration, morphine microinjected into the PAG depresses glutamate-evoked firing of ON-cells (Morgan et al., 1992) and increases OFF-cell firing by removing the GABA-mediated tail flick induced OFF-cell pause (Cheng et al., 1986a, Tortorici and Morgan, 2002), suggesting two distinct circuit components of descending inhibition of pain promoted by opioids. The vlPAG is also responsible for relaying opioid-mediated descending inhibition from other supraspinal regions (McGaraughty et al., 2004).

A more modest collection of papers makes a convincing argument that the vlPAG also contributes to descending facilitation. Descending facilitation can be achieved through artificial manipulation of the vlPAG, such as local capsaicin infusion or blocking P/Q-type calcium channels (Knight et al., 2002, McGaraughty et al., 2003). Earlier studies looking at the effects of non-steroidal anti-inflammatory drugs (NSAIDs) applied directly to the PAG, resulted in the inhibition of acute nociceptive processing *in vivo*, increased withdrawal latencies, and depressed C-fiber-evoked activity (Carlsson et al.,

1986, Tortorici and Vanegas, 1995, Vanegas et al., 1997). NSAIDs block a an enzyme called cyclooxygenase (COX) that catalyzes the production of prostaglandins, which are key components of the inflammatory response (Ricciotti and FitzGerald, 2011). Microinjection of prostaglandin E2 into the PAG significantly decreased paw withdrawal latency (pronociceptive/ hyperalgesic), showing both activated RVM ON-cells and suppressed RVM OFF-cell firing (Heinricher et al., 2004). Furthermore, cyclooxygenase (COX)-1-derived prostaglandins in the PAG exerts tonic facilitatory control selective to C-fiber-mediated spinal nociception (Leith et al., 2007).

Although inflammatory mediators mediate descending facilitation in response to acute and persistent pain states. (COX)-1 expression is increased in the vlPAG after persistent inflammation induced by CFA injected into the intra-articular space of the knee (Drake et al., 2016). Furthermore, blocking the prostaglandin EP3 receptor in the vlPAG produces antinociception through altered activation of C-fibers, as seen in acute paradigms. However, the breadth of this effect is increased to include C-fibers secondary to the stimulus/injury site (responsible for secondary hyperalgesia) and A-fibers, demonstrating an expansion of the downstream control of vlPAG-mediated descending facilitation in persistent inflammation models (Drake et al., 2016). Additionally, neuropathic pain alters the spontaneous firing frequency, stimulus response, and firing threshold of vlPAG neurons *in vivo* (Samineni et al., 2017b). Altogether, these studies indicate that the inflammatory response promotes endogenous descending facilitation in acute and persistent pain models.

Inflammatory and neuropathic pain models induce increased spontaneous GABA release in the vlPAG and in the downstream RVM (Hahm et al., 2011, Tonsfeldt et al.,

2016, Li et al., 2017). Enhanced vlPAG GABA tone may originate from GABAergic vlPAG neurons that send local collaterals or from incoming inhibitory vlPAG afferents. Selective activation of vlPAG GABA neurons is hyperalgesic (Samineni et al., 2017a). Activation of vlPAG GABA receptors also produces hyperalgesia and negates the analgesic effect of opioids (Zambotti et al., 1982, Moreau and Fields, 1986, Depaulis et al., 1987). GABA tone within the RVM can contribute to hyperalgesia by reducing OFF-cell firing (Heinricher et al., 1991, Heinricher and Tortorici, 1994). Our loose-patch recordings revealed an enhanced proportion of neurons receiving inhibitory-dominant synaptic inputs, resulting in activation of spontaneous firing after blocking both excitatory and inhibitory inputs, after persistent inflammation (Figure 4.4A, B). This data provides additional evidence of enhanced GABA tone in the vlPAG induced by 5-7 d CFA and shows that this GABA tone impacts the spontaneous firing in a subset of neurons. Despite being unable to determine neuron type with the loose-patch method, the enhanced firing frequency of *Phasic* neurons at this same time point suggests that they are not they cells being inhibited by increased GABA tone. Furthermore, the enhanced GABA tone can originate from incoming afferents *or* GABA neurons within the vlPAG sending local collaterals, providing a possible circuit mechanism by which enhanced *Phasic* firing contributes to increased GABA tone through local inhibitory connections. If confirmed, this would implicate enhanced firing of *Phasic* neurons in descending facilitation of pain after persistent inflammation.

Categorizing vlPAG neurons based on intrinsic membrane and firing properties revealed a targeted activation of *Phasic* neurons by persistent inflammation and provides a framework for future studies to further investigate the implication of this selective

activation on descending pain modulation. Identifying the neurotransmitter content of *Phasic* neurons is likely to show *Phasic* neurons are comprised of subpopulations of GABAergic and glutamatergic cells, however, the most strongly activated subset in the presence of persistent inflammation may be a homogenous population. To best address this with the tools currently available the lab will conduct single-cell PCR on neurons after collecting intrinsic firing properties. The resulting data from these experiments does not account for one key feature that substantially limits our interpretation of the mechanism by which these neurons impact the descending circuit, which is whether they synapse onto ON- or OFF-cells in the RVM. This limitation underscores the importance of finding a method to pharmacologically reverse the enhanced *Phasic* firing after persistent inflammation to the levels seen in naïve rats. Once determined *in vitro*, this same pharmacological approach can be applied *in vivo* to evaluate the effect of inhibiting vlPAG *Phasic* neurons on RVM ON- and OFF-cell firing as well as the overall impact on nociception using withdrawal thresholds.

4.4 RGS proteins and agonist and effector target specific $G_{i/o}$ recruitment reveal functional selectivity in MOR signaling

RGS proteins are generally thought to negatively regulate GPCR signaling, due to their role in hydrolyzing and thus inactivating GTP-bound G proteins. In fact, the RGS-insensitive mouse model used in the studies in this thesis, previously showed both increased opioid-mediated inhibition of presynaptic GABA release within the vlPAG accompanied with increased morphine antinociception using the hot plate test (Lamberts et al., 2011). These findings support that RGS proteins negatively modulate MOR

inhibition of evoked GABA release (eIPSCs), influencing supraspinal nociception. One study in this thesis (see *Appendix A*) built upon these findings to determine whether there is evidence for functional selectivity in the form of agonist-specific recruitment of G_i or G_o G proteins. Maximal DAMGO-mediated inhibition of eIPSCs required G_o; maximal fentanyl-mediated inhibition of eIPSCs required both G_o and G_i; whereas, ME sufficiently inhibited release with either (Bouchet et al., 2021). Comparable evaluation was conducted with mIPSCs to determine whether the mechanisms involved in spontaneous release differed from that of evoked release. First, inhibition of mIPSCs was unaltered in the RGS-insensitive mice. Maximal DAMGO-mediated inhibition of mIPSCs required both G_o and G_i, and G_i was necessary for maximal ME- and fentanyl-mediated inhibition of mIPSCs. Altogether, these findings indicate that RGS proteins differentially impact MOR-mediated inhibition of mIPSCs and eIPSCs and that agonist-specific $G_{i/o}$ recruitment influence MOR-mediated inhibition of spontaneous and evoked GABA release.

An additional mechanism by which MOR activation promotes analgesia is through GIRK-activation. Interestingly, we found (see *Chapter 3*) that RGS proteins positively modulate MOR-mediated GIRK activation in the vlPAG (McPherson et al., 2018). Follow up experiments looking at agonist-specific $G_{i/o}$ recruitment identified functional selectivity differences in MOR-GIRK signaling. In particular, maximal GIRK currents induced by DAMGO and fentanyl required G_o G proteins, compared to ME, which required G_i. RGS proteins playing a facilitatory role in MOR-GIRK signaling is counterintuitive, as RGS proteins inactivate G proteins which activate GIRK channels. However, a "kinetic scaffolding" model outlines the necessity of rapid turnover of G

proteins to replenish the inactive G protein substrate pool for rapid recoupling and activation by the receptor (Clark et al., 2003, Zhong et al., 2003a). This model suggests that RGS proteins serve as a key component in receptor and effector coupling, enhancing the efficiency of the signal transduction pathway. Distinct actions of RGS proteins, and agonist-specific G protein recruitment, in pre- and postsynaptic MOR signaling provides another way in which compartment-specific MOR signaling may affect the analgesic circuit. Future studies on acute and persistent inflammation induced alterations in the RGS actions in pre- and postsynaptic MOR signaling will further our understanding of how RGS-mediated positive and negative modulation of compartment-specific MOR signaling influences pain states.

4.5 Conclusion

The neurobiological underpinnings of chronic pain are complex, affecting many neuron types, brain regions, and circuits. The results in this thesis establish a framework of neuronal categorization within the vlPAG that illuminated selective activation of a single neuron type by persistent, and not acute, inflammation. The targeted enhancement of *Phasic* neuron firing coupled with the increased proportion of inhibited vlPAG neurons implies a potential mechanism for increased local inhibition during persistent inflammation, which if confirmed would implicate *Phasic* neurons in descending facilitation. This same neuron type categorization reinforced that MOR expression is not selective to a single vlPAG neuron type and that MOR expression does not define the most strongly activated subpopulation of *Phasic* neurons after persistent inflammation. Additional work on MOR signaling demonstrates several examples of distinct agonist and effector target specific functional selectivity. The present results developed a useful framework for further assessment of neurons that are selectively altered by persistent pain states, support the parallel pathways from vlPAG to RVM that are important in opioid-mediated analgesia, and demonstrate functional selectivity in MOR signaling in the vlPAG.

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Appendix A: RGS-insensitive mice define roles of μ-opioid receptor (MOR)-$G\alpha_o$ and $G\alpha_i$ subunit coupling in inhibition of presynaptic GABA release.

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This work was supported by National Institutes of Health National Institute on Drug Abuse [Grant R01DA035316 JRT].

3.1 Abstract

Regulators of G protein signaling (RGS) proteins modulate signaling by G protein-coupled receptors (GPCRs). Using a knock-in transgenic mouse model with a mutation in Gαo that does not bind RGS proteins (RGS-insensitive), we determined the effect of RGS proteins on presynaptic mu opioid receptor (MOR)-mediated inhibition of GABA release in the ventrolateral periaqueductal gray (vlPAG). The MOR agonists [D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin (DAMGO) and met-enkephalin (ME) inhibited evoked inhibitory postsynaptic currents (eIPSCs) in the RGS-insensitive mice compared to wildtype (WT) littermates, respectively. Fentanyl inhibited eIPSCs similarly in both WT and RGS-insensitive mice. There were no differences in opioid agonist inhibition of spontaneous GABA release between the genotypes. To further probe the mechanism underlying these differences between opioid inhibition of evoked and spontaneous GABA release, specific myristoylated Gα inhibitors for $G\alpha o_1$ and $G\alpha i_{1\text{-}3}$ that block receptor-G protein interactions were used to test the preference of agonists for MOR-Gα complexes. The $G\alpha o_1$ inhibitor reduced DAMGO inhibition of eIPSCs but $G\alpha i_{1\text{-}3}$ inhibitors had no effect. Both $G\alpha o_1$ and $G\alpha i_{1\text{-}3}$ inhibitors separately reduced fentanyl inhibition of eIPSCs, but had no effects on ME inhibition. $G\alpha i_{1\text{-}3}$ inhibitors blocked the inhibitory effects of ME and fentanyl on mIPSC frequency, but both $G\alpha o_1$ and $G\alpha i_{1\text{-}3}$ inhibitors were needed to block the effects of DAMGO. Finally, baclofen-mediated inhibition of GABA release is unaffected in the RGS-insensitive mice and in the presence of $G\alpha o_1$ and $G\alpha i_{1\text{-}3}$ inhibitor peptides suggesting that $GABA_B$ receptors expressed on presynaptic terminals are coupled to different G proteins than MORs.

3.2 Introduction

Regulators of G protein signaling (RGS) proteins accelerate the hydrolysis of GTP to GDP, terminating G protein signaling. These proteins comprise a large family of proteins that differ in structure and function and are expressed in various tissues with overlapping distributions (Traynor and Neubig, 2005). RGS proteins bind to active G proteins to regulate both temporal and spatial signaling to downstream effectors (Hollinger and Hepler, 2002, Neubig, 2015). In addition, RGS proteins recognize specific Gα proteins (Masuho et al., 2020) highlighting the importance of understanding RGS-Gα interactions within discrete neural circuits. Specific RGS protein knockout mouse models have been generated to probe regulation of GPCR signaling by RGS proteins but there is evidence of strong compensation by redundant RGS proteins in various knockout lines (Grillet et al., 2005). To circumvent this issue, we use a mutant mouse line that has a knock-in mutation in the Gαo subunit (G184S) that does not bind to any RGS proteins (RGS-insensitive) (Goldenstein et al., 2009).

Mu-opioid receptors (MORs) are GPCRs that typically couple to inhibitory G proteins, including Gαo and Gαi subunits (Gaibelet et al., 1999). However, opioid analgesia is dependent on MOR coupling to Gαo, but not Gαi (Lamberts et al., 2011, Lamberts et al., 2013). In addition, different MOR agonists preferentially bind MORs coupled to specific G protein subunits (Massotte et al., 2002, Clark et al., 2006). This differential coupling constitutes one determinant of functional selectivity of opioid agonists and differential activation of G proteins by MOR agonists could have important impacts in understanding opioid-mediated behaviors. The ventrolateral periaqueductal gray (vlPAG) is a supraspinal site for opioid-induced analgesia. MORs expressed

postsynaptically on a subpopulation of vlPAG neurons are coupled to GIRK channels that hyperpolarize the cells (Chieng and Christie, 1994a, Ingram et al., 2007, Ingram et al., 2008). We observed in our prior studies that MOR coupling to GIRK channels was reduced in the RGS-insensitive mice (McPherson et al., 2018) indicating that RGS proteins support signaling to some effectors, in contrast to their well-known negative regulation due to their GTPase accelerating activity (Clark et al., 2003, Clark et al., 2008, Lamberts et al., 2013). High efficacy synthetic agonists DAMGO and fentanyl were less effective in the RGS-insensitive mice but the GIRK currents induced by the peptide agonist met-enkephalin (ME) were unaffected (McPherson et al., 2018). These effects were further confirmed using selective peptide inhibitors of Gαo and Gαi subunits showing that ME-induced GIRK currents could be inhibited only with the Gαi peptide inhibitor. Taken together, these results support the idea that different opioid agonists recruit or prefer receptors bound to specific G proteins, similar to observations in cell lines (Milligan et al., 1990a, Moon et al., 2001, Clark and Traynor, 2006). However, the loss in MOR coupling to GIRK channels in the RGS-insensitive mice does not explain the enhanced analgesia observed in these mice (Lamberts et al., 2013) so we have continued to examine presynaptic MOR signaling in the vlPAG.

MORs expressed on presynaptic terminals are coupled to phospholipase A2 resulting in inhibition of neurotransmitter release (Vaughan et al., 1997a, Ingram et al., 1998). RGS proteins negatively regulate presynaptic MORs that inhibit GABA release (Lamberts et al., 2013) but the G proteins that are involved in presynaptic MOR signaling have not been identified previously. In these studies, we have examined several MOR agonists for their ability to activate MOR signaling via Gαo or Gαi, using the RGS-

insensitive mice to further define MOR signaling in GABAergic terminals within the vlPAG. Based on observations that RGS-insensitive mice display enhanced antinociception, we hypothesized that MOR inhibition of presynaptic release is enhanced in these mice. Further, we expected to find differences between agonists in the presence of the selective Gαo and Gαi inhibitor peptides.

3.3 Materials & Methods

These studies used male and female heterozygous (RGS-insensitive Het) mice for a mutation in the Gαo protein (G184S) that is insensitive to RGS protein binding (Goldenstein et al., 2009) and wildtype (WT) 129S1/SvImJ littermates. Homozygous knock-in mice die *in utero*, so WT mice were compared with Het mice. WT mice were used in the studies assessing the effect of G protein peptide inhibitors. Mice were group housed with unlimited access to food and water. Lights were maintained on a 12 h light/dark cycle (lights on at 7:00 A.M.). Mice were sacrificed and cellular recordings were conducted during the light phase of this cycle. The Institutional Animal Care and Use Committee at Oregon Health & Science University approved all experimental procedures. Experiments were conducted in accordance with the United States National Research Council *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2011).

Electrophysiological recordings

Mice (postnatal day >25) were anesthetized with isoflurane, brains were removed, and brain slices containing the vlPAG were cut with a vibratome (180–220 μm thick) in

sucrose cutting buffer containing the following (in mM): 75 NaCl, 2.5 KCl, 0.1 $CaCl_2$, 6 $MgSO_4$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 2.5 dextrose, 50 sucrose and placed in a holding chamber with artificial cerebral spinal fluid (ACSF) containing the following (in mM): 126 NaCl, 21.4 NaHCO3, 11.1 dextrose, 2.5 KCl, 2.4 CaCl2, 1.2 MgCl2, and 1.2 NaH2PO4, pH 7.35, equilibrated with 95% O2/5% CO2 until moved into a recording chamber. In experiments using myristoylated Gαo and Gαi peptide inhibitors, slices were incubated for at least 30 min in ACSF plus inhibitors (1-10 μM) before recording. Recordings were made with electrodes pulled to 2–4 MOhm resistance with an internal solution consisting of the following (in mM): 140 CsCl, 10 HEPES, 10 KCl, 1 MgCl2, 1 EGTA, 0.3 CaCl2, 4 MgATP, and 3 NaGTP, pH 7.4. Junction potentials of 5 mV were corrected at the beginning of the experiments. Access resistance was monitored throughout the experiments. Neurons were voltage-clamped at -70 mV. Data were collected with Axopatch 200B microelectrode amplifier (Molecular Devices) at 5 kHz and low-pass filtered at 2 kHz. Currents were digitized with InstruTECH ITC-18 (HEKA), collected via AxoGraph data acquisition software and analyzed using AxoGraph (Axograph Scientific). The Het mice tend to be smaller, so experimenters were not blind to genotype; however, data analysis was done blind to genotype. In experiments using Gαo and Gαi inhibitor peptides, all mice were WT, but the analyses of peak drug effects were measured blind to slice treatment.

Reagents

[D-Ala(2),N-Me-Phe(4),Gly(5)-ol]-enkephalin (DAMGO), met-enkephalin acetate salt hydrate (ME) and fentanyl citrate salt (fentanyl) were obtained from Sigma-Aldrich, and

(*R*,*S*)-baclofen and CGP 55845 hydrochloride were purchased from Abcam. Myristoylated G protein peptide inhibitors were synthesized by GenScript (Piscataway, NJ), as follows: Gαo1 (MGIANNLRGCGLY), Gαi1/2 (MGIKNNLKDCGLP), and Gαi3 (MGIKNNLKECGLT) according to sequences for mini-gene vectors designed by the Hamm laboratory (Vanderbilt University Medical Center, Nashville TN; (Gilchrist et al., 2002). We were unable to obtain the peptide for $G\alpha o_2$ at sufficient purity (<60%) to use in slice experiments. The Gαi inhibitors were combined as a cocktail.

Statistical analyses

All data are expressed as mean & standard deviation (SD). Data were analyzed with Prism 9 (GraphPad Software). Each electrophysiological recording from a single neuron is treated as an individual observation because the vlPAG contains heterogenous cell populations; however, all datasets contain recordings from at least three separate animals. Drug effects were reversed by specific antagonists, and peak drug effects were measured as an increase in current from the average of baseline and washout or the presence of antagonists. Differences between groups were assessed using Student's t-test or ANOVA when appropriate (significance is denoted as $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$).

3.4 Results

Opioid inhibition of evoked GABA release

In order to test the hypothesis that RGS proteins affect opioid signaling in presynaptic terminals, we compared the ability of several opioid agonists to inhibit evoked GABAergic inhibitory postsynaptic currents (eIPSCs) in vlPAG neurons of

wildtype (WT) and RGS-insensitive Het mice. The studies used concentrations of opioid agonists that were consistent with our previous study examining opioid activation of GIRK channels in the vlPAG (McPherson et al., 2018). For comparison, we also tested maximal concentrations of each agonist so we could assess the efficacy of each agonist at inhibiting presynaptic GABA release (Fig. 1). The maximal % inhibition was the same for all three opioid drugs. Het mice had similar effects to WT mice at the maximal concentration (mean ± SD; DAMGO: 54 ± 5%, n = 6; ME: 67 ± 28%, n = 5; fentanyl: 55 ± 10%, n = 4). For opioid inhibition of eIPSCs, sub-maximal concentrations of each agonist were compared in WT and RGS-insensitive Het mice. The selective MOR agonist DAMGO inhibited eIPSCs 37% more in neurons from the RGS-insensitive Het mice (95% CI = 54 to 73; Fig 1A,B). The nonselective agonist ME inhibited the eIPSCs 88% more in cells from the RGS-insensitive Het mice (95% CI = 38 to 52; Fig. 1C). These results are consistent with our prior report showing an increase in morphine and ME inhibition of eIPSCs in RGS-insensitive Het mice (Lamberts et al., 2013). In contrast, fentanyl inhibited GABAergic eIPSCs similarly in neurons from both WT and RGS-insensitive Het mice (Fig. 1D).

In a subset of experiments, a paired pulse protocol (2 stimuli, 50-100 ms apart) was used to examine the probability of GABA release from presynaptic terminals in WT and RGS-insensitive Het mice. Paired-pulse ratios (PP ratio = P2/P1) for eIPSCs in slices from Het mice (0.6 ± 0.2; n = 8) were lower than WT mice (1.1 ± 0.1; n = 12; $t_{(18)} = 2.2$, *p = 0.04). A lower PP ratio indicates a higher release probability in the RGS-insensitive Het mice. This change in release probability could be due to changes in endogenous opioid tone, so we tested whether an endogenous opioid tone could be measured in slices

from either the WT or RGS-insensitive Het mice using naloxone (5 – 10 μM). Spontaneous IPSCs (in the absence of tetrodotoxin) were similar in the absence and presence of naloxone for both genotypes (% change ± SD in naloxone for WT: 103 ± 20%; One sample t-test, $t_5 = 0.4$, $p = 0.7$ and Het: 95 ± 19%; One-sample t-test, $t_6 = 0.8$, $p = 0.5$) indicating a lack of endogenous opioid tone in either genotype.

In order to determine whether the difference between the agonists in the RGS-insensitive mice was due to a preference for Gαo versus Gαi subunits coupling to MORs in presynaptic terminals, selective peptide inhibitors of each subunit binding sites were tested. Inhibitor peptides corresponding to the carboxy terminal amino-acids of the Gα subunit compete for binding to the receptor, inhibiting activation of the G proteins (Gilchrist et al., 2002). The effect of DAMGO was reduced in the presence of the Gαo inhibitor but the Gαi inhibitor had no effect (Fig. 2A). Adding all of the peptide inhibitors together essentially abolished DAMGO-mediated inhibition of eIPSCs. Neither the Gαo or Gαi peptide inhibitors significantly reduced ME inhibition (Fig. 2B); however the combined inhibitors also significantly reduced ME inhibition of eIPSCs. Finally, both Gαo and Gαi inhibitor peptides superfused alone reduced fentanyl inhibition (Fig. 2C). These data suggest that DAMGO preferentially activates MOR-Gαo in presynaptic terminals in the vlPAG, but ME and fentanyl are less selective.

Male and female mice were used throughout the studies. DAMGO recordings from WT mice had approximately equal numbers of recordings from male and female mice with similar inhibition in both sexes (males: 49 ± 10% (SD); n = 8 versus females: 41 ± 10% (SD), n = 7; $t_{(13)} = 1.4$, $p = 0.19$). In addition, no noted differences were

observed with the other agonists. The lack of sex differences is consistent with our prior study (McPherson et al., 2018).

Opioid inhibition of spontaneous GABA release

The change in PP ratio indicated that the RGS-insensitive Het mice have altered evoked GABA release so we were interested in whether spontaneous release (in the presence of TTX) was altered in the knock-in mouse line. Inter-event intervals of mIPSCs measured in the presence of NBQX (5 μM) and TTX (500 nM) were similar in WT (0.33 ± 0.22 s (SD), n = 27) and Het mice (0.24 ± 0.15 s (SD), n = 27; $t_{(52)} = 1.6$, $p = 0.1$). All three opioid agonists inhibited spontaneous mIPSC frequency to similar degrees in both the WT and Het mice (Fig. 3). The mIPSC amplitude, as well as rise and decay kinetics (data not shown) were also not different between agonists indicating that the opioid modulation of mIPSC frequency was due to presynaptic modulation of release. There were no sex differences in the amount of inhibition induced by any of the 3 opioid agonists (data not shown). These results suggest that either RGS proteins have little impact on opioid modulation of spontaneous release in presynaptic terminals or that inhibition of spontaneous release is not dependent on Gαo since the RGS-insensitive knock-in mutation is specific for Gαo. In order to test whether MOR-Gαi coupling is involved in opioid inhibition of spontaneous GABA release in WT mice, we examined the effects of the specific Gαo and Gαi peptide inhibitors. Neither of the inhibitors superfused alone reduced inhibition of mIPSC frequency by DAMGO (Fig. 4A), but the inhibitors applied to slices together reduced DAMGO-mediated inhibition by 83% (95% CI = -5 to 27) compared to control. In contrast, inhibition by ME and fentanyl was

reduced in the presence of the Gαi inhibitors but unaffected in the presence of the Gαo inhibitor peptide (Fig. 4B,C). These results are consistent with results in the RGS-insensitive Het mice which have a mutation specifically in Gαo that perturbs RGS protein binding and subsequent GTP-hydrolysis and indicate that MOR-Gαi coupling is important for the inhibition of spontaneous GABA release.

$GABA_B$-mediated inhibition of GABA release is unaffected by $G\alpha_o$ or $G\alpha_i$ inhibitors

Our previous study found no difference in the amount of inhibition of evoked GABA release induced by a maximal concentration of the $GABA_B$ agonist baclofen (20 μM) between slices from WT and RGS-insensitive Het mice (McPherson et al., 2018). Because RGS proteins have less influence on high efficacy agonists, especially at maximal concentrations (Clark et al., 2008), we repeated the studies using a lower concentration of baclofen (5 μM). This concentration of baclofen also inhibited evoked GABA release to a similar extent in slices from the two genotypes (Fig. 5A). Consistent with these results, incubation of slices in the Gαo and Gαi inhibitors did not alter the responses to either concentration of baclofen (5 μM or 20 μM; Fig. 5B and 5C, respectively). Baclofen was typically tested on the same cells before or after an opioid response that was affected by either the Gαo or Gαi inhibitor indicating that these peptide inhibitors were effective in blocking binding of the Gα subunits in a given experiment and providing positive controls.

Both concentrations of baclofen were also tested for inhibition of spontaneous release of GABA (Fig. 6). The data show that baclofen inhibition of mIPSC frequency is similar in both the WT and RGS-insensitive Het mice and the inhibition is unaffected by

the Gαo and Gαi peptide inhibitors. Similar results were obtained at both 5 and 20 μM concentrations of baclofen.

3.5 Discussion

These studies used a transgenic knock-in mutant mouse model with a mutation in Gαo (G148S) that blocks RGS protein binding (Goldenstein et al., 2009). The advantage of this model is that it is unbiased with regard to RGS protein subtypes because compensatory expression of RGS proteins can obscure RGS regulation in knockout mice (Grillet et al., 2005). Opioid analgesia is substantially reduced in Gαo knockout mice (Lamberts et al., 2011) providing evidence that MOR couples to Gαo in analgesia pathways. Consistent with the knockout data, RGS-insensitive Het mice display enhanced supraspinal morphine analgesia (Lamberts et al., 2011, Lamberts et al., 2013). Since MOR inhibition of GABA release in the vlPAG is important for the analgesic actions of opioids (Moreau and Fields, 1986, Vaughan and Christie, 1997, Budai and Fields, 1998, Bobeck et al., 2014), we expected that inhibition of GABA release by opioid agonists would be increased in the RGS-insensitive Het mice. Importantly, we observed differences between MOR agonists in the RGS-insensitive Het mice suggesting that RGS regulation plays a role in functional selectivity of MOR agonists. In addition, MORs activate different Gα subunits to inhibit evoked and spontaneous GABA release. Finally, $GABA_B$-mediated inhibition of GABA release is not regulated by RGS proteins binding to Gαo subunits and are unaffected by peptide inhibitors of either Gαo or Gαi subunits.

We first examined opioid inhibition of evoked GABA release in the vlPAG. DAMGO and ME, but not fentanyl, inhibited GABA release more in the RGS-insensitive mice. Since sub-maximal concentrations were used for each of the agonists, the lack of increase with fentanyl in the recordings from RGS-insensitive Het mice was not attributed to a ceiling effect. These data are consistent with recent data showing that inhibition of RGS4 in the PAG enhanced morphine, but not fentanyl, antinociception . There is evidence that RGS protein GTPase accelerating activity is more evident with low compared to high efficacy MOR agonists (Clark et al., 2008, Morgan et al., 2020); however, the maximal inhibition by all agonists was comparable. Thus, the differences between agonists in inhibiting GABA release in the two genotypes are likely due to a different mechanism, such as the ability of fentanyl-bound MORs to couple to Gαi.

MORs activate pertussis-toxin (PTX)-sensitive Gαo and Gαi subunits (Williams et al., 2013). Analgesia induced by morphine (Parenti et al., 1986, Lutfy et al., 1991, Shah et al., 1994) and DAMGO (Sanchez-Blazquez and Garzon, 1988) is reduced in the presence of PTX. In order to probe the signaling of specific G proteins in vlPAG presynaptic terminals further, we used myristoylated peptide inhibitors of Gαo and Gαi subunits. Incubation of slices with the Gαo peptide inhibitor reduced the inhibition by DAMGO and fentanyl, but not ME. Gαo inhibition of Ca^{2+} channels is more potent than Gαi (Hescheler et al., 1987), and there are differences in coupling between Gα subunits and effectors (McKenzie and Milligan, 1990, Milligan et al., 1990a, Milligan et al., 1990b, Moon et al., 2001). Thus, it is reasonable that inhibition by DAMGO and fentanyl was reduced by the Gαo peptide inhibitor since evoked release is dependent on voltage-gated Ca^{2+} channels (Hubbard et al., 1968). Incubation of slices with the Gαi peptide

inhibitors reduced fentanyl, but not DAMGO or ME, inhibition of eIPSCs. These results indicate that coupling to Gαi subunits is equally effective at inhibiting eIPSCs in the vlPAG, and that DAMGO and fentanyl form different MOR complexes in presynaptic terminals. Combining the peptide inhibitors reduced the effects of both DAMGO and ME compared to incubating slices in either inhibitor alone. Together these results suggest that there is redundancy and competition between the peptide inhibitors and G proteins for the receptor binding sites. It is interesting to note that the endogenous peptide ME is less sensitive to both peptide inhibitors given alone compared to DAMGO and fentanyl suggesting that ME-bound MORs couple equally well to $G\alpha o_1$ and $G\alpha i_{1\text{-}3}$ subunits. The data highlight the importance of G protein subunit expression and levels as a factor in MOR coupling to effectors (Connor and Christie, 1999).

A surprising finding in these studies was the difference in G protein subunits involved in MOR inhibition of spontaneous GABA release. Inhibition by all three opioid agonists was similar in both the WT and RGS-insensitive Het mice. Consistent with these results was the lack of effect of the Gαo inhibitor peptide on opioid inhibition of mIPSC frequency. Instead, the $G\alpha i_{1\text{-}3}$ inhibitor peptides applied alone decreased inhibition by ME and fentanyl without affecting DAMGO-mediated inhibition. However, DAMGO inhibition was reduced in the presence of both inhibitors. This pattern supports the results with DAMGO on evoked release and further suggests that DAMGO preferentially couples to MOR-Gαo subunits (Laugwitz et al., 1993, Chakrabarti et al., 1995, Clark et al., 2006). The data presented here indicate that opioid inhibition of spontaneous release is mediated by Gαi subunits, explaining why opioid inhibition of spontaneous GABA release was unaffected in the RGS-insensitive Gαo mouse model. Thus, these studies are

not able to determine if RGS proteins regulate MOR inhibition of spontaneous release. The molecular mechanisms involved in MOR regulation of spontaneous release are not completely understood but there is data to support direct G protein βγ subunit regulation of release machinery (Zurawski et al., 2016, Zurawski et al., 2019).

$GABA_B$ receptors also readily inhibit evoked and spontaneous GABA release in the vlPAG (Vaughan et al., 1997a). In the RGS-insensitive Het mice, baclofen inhibited both evoked and spontaneous GABA release similarly to WT mice. Since the Gαo and Gαi peptide inhibitors were ineffective at blocking baclofen inhibition, even when applied together, we are not able to make a statement regarding the ability of RGS proteins to modulate $GABA_B$ signaling. The results are interesting considering data that $GABA_B$ coupling to voltage-gated Ca^{2+} channels is abolished by PTX (Connor and Christie, 1998). However, $GABA_B$-Gi protein coupling has different structural features compared to other GPCR classes (Shen et al., 2021). Thus, the peptide inhibitors used in this study may not bind to the $GABA_B$ receptor to block G protein binding. Alternatively, Gαz is a G protein with 60% sequence homology to the Gi family (Tsu et al., 1997), is densely expressed in the vlPAG and couples to MOR (Garzon et al., 1998, Garzon et al., 2005b, Gaspari et al., 2018). The Gαz residues that bind to MOR have not been identified so it is not clear whether the peptide inhibitors block Gαz coupling to MOR or $GABA_B$ receptors.

The descending pain modulatory pathway is sexually dimorphic (Loyd and Murphy, 2006, Loyd et al., 2008a, Loyd and Murphy, 2014) and there is evidence that MOR agonists are more efficacious in males than females (Fullerton et al., 2018). There were no sex differences in either genotype in opioid agonist inhibition of evoked and

spontaneous GABA release. Thus, sex differences in opioid signaling are not explained by RGS-mediated regulation of signaling, at least via Gαo subunits. This is consistent with the lack of sex differences in MOR coupling to GIRK channels in the WT and RGS-insensitive Het mice (McPherson et al., 2018).

Our results showing enhanced MOR inhibition of presynaptic GABA release by several opioid agonists in the RGS-insensitive mice, in addition to morphine which we examined in our previous paper (Lamberts, et al., 2013), provide a mechanism for the increase in opioid antinociception on the supraspinal hot plate test observed in RGS-insensitive mice (Lamberts, et al., 2013). There is substantial evidence that opioid inhibition of GABA release in the vlPAG activates descending pain modulatory circuits that produce analgesia (Cheng et al., 1986b, Moreau and Fields, 1986). We previously reported that postsynaptic MOR coupling to GIRK channels is reduced in RGS-insensitive mice (McPherson, et al., 2018), possibly through a scaffolding function of RGS proteins (Zhong et al., 2003b). Although it is tempting to argue that postsynaptic MORs in the vlPAG do not play a role in opioid-induced antinociception, an equally valid interpretation is that MOR coupling to GIRK channels opposes antinociceptive circuits and removal of this MOR signaling supports opioid analgesia in the RGS-insensitive Het mice (Lamberts et al., 2013). Indeed, blocking both GIRK channels and presynaptic MOR signaling decreases morphine antinociception . As discussed above, Morgan and colleagues also found that inhibition of RGS4 in the vlPAG enhanced opioid-induced antinociception suggesting that RGS4 may play an important role in regulating presynaptic MOR signaling through Gαo in the vlPAG. However, RGS gene expression in the PAG includes RGS4, RGS7, RGS8, RGS10, RGS17 and RGS20

(https://alleninstitute.org/legal/citation-policy/ Allen Brain Atlas), and these RGS proteins bind preferentially to different G proteins (Masuho et al., 2020, Morgan et al., 2020). Thus, additional RGS proteins may also regulate opioid analgesia through MOR coupling in the PAG.

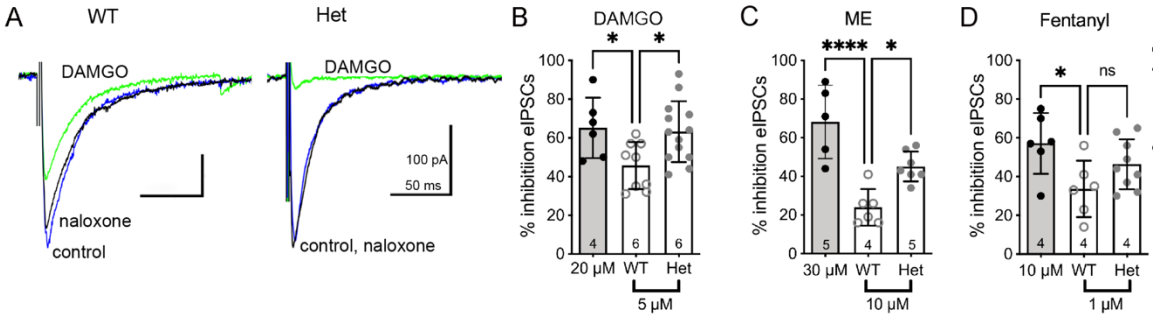

Figure A. 1: Opioid agonist inhibition of evoked IPSCs are differentially affected in RGS-insensitive mice.

A. Representative traces depicting inhibition of eIPSCs by DAMGO (5 µM) in wildtype (WT) and RGS-insensitive (Het) mice. The inhibition is reversed by naloxone. **B.** Combined experiments of % inhibition (± SD) by a maximal DAMGO concentration (20 µM; gray bar) and a sub-maximal concentration (5 µM) in WT compared to Het mice (One way ANOVA; $F_{(2, 25)} = 4.9$, $p = 0.02$; Dunnett's, *$p < 0.05$). **C.** Combined experiments of % inhibition (± SD) by a maximal met-enkephalin concentration (30 µM; gray bar) and a sub-maximal concentration (10 µM) in WT compared to Het mice (One way ANOVA; $F_{(2, 15)} = 17.7$, $p = 0.0001$; Dunnett's, *$p < 0.05$; ****$p < 0.0001$). **D.** Combined experiments of % inhibition (± SD) by a maximal fentanyl concentration (10 µM; gray bar) and a sub-maximal concentration (1 µM) in WT compared to Het mice (One way ANOVA; $F_{(2, 18)} = 4.1$, $p = 0.03$; Dunnett's, *$p < 0.05$). Symbols in bars denote recordings and numbers denote number of animals used in each group.

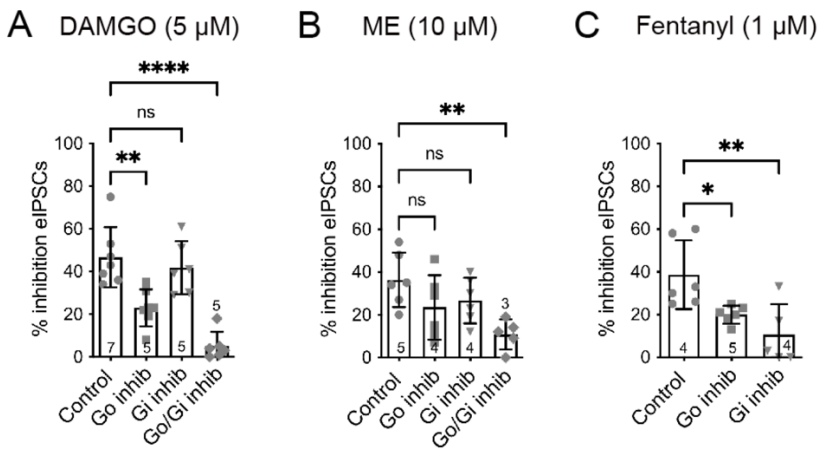

Figure A. 2: MOR agonists differentially activate Gα subunits to inhibit evoked GABA release.

A. DAMGO (5 μM)-mediated inhibition of eIPSCs in the absence (control) and presence of Gαo peptide inhibitor and Gαi peptide inhibitors (One way ANOVA, $F_{(3, 22)} = 19.1$, $p = 0.0001$; Dunnett's, **$p < 0.001$, ****$p < 0.0001$). **B.** ME (10 μM)-mediated inhibition of eIPSCs in absence and presence of inhibitors (One way ANOVA, $F_{(3, 19)} = 4.2$; $p = 0.02$; Dunnett's, **$p < 0.001$). **C.** Fentanyl (1 μM)-mediated inhibition of eIPSCs in absence and presence of inhibitors (One way ANOVA, $F_{(2, 14)} = 7.3$, $p = 0.007$, Dunnett's, *$p < 0.05$, ** $p < 0.001$). Symbols in bars denote recordings and numbers denote number of animals used in each bar.

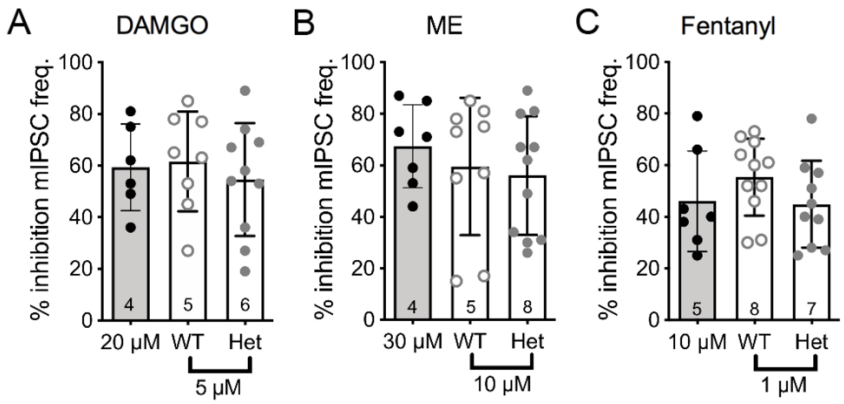

Figure A. 3: Opioid inhibition of GABAergic mIPSCs is not altered in RGS-insensitive mice.

A. Combined experiments of % inhibition (± SD) by a maximal DAMGO concentration (20 µM; gray bar) and a sub-maximal concentration (5 µM) in WT compared to Het mice (One way ANOVA; $F_{(2, 21)} = 0.3$, $p = 0.8$). **B.** Combined experiments of % inhibition (± SD) by a maximal met-enkephalin concentration (ME 30 µM; gray bar) and a sub-maximal concentration (10 µM) in WT compared to Het mice (One way ANOVA, $F_{(2, 24)} = 0.5$, $p = 0.6$). **C.** Combined experiments of % inhibition (± SD) by a maximal fentanyl concentration (10 µM; gray bar) and a sub-maximal concentration (1 µM) in WT compared to Het mice (One way ANOVA; $F_{(2, 25)} = 1.2$, $p = 0.3$). Symbols in bars denote recordings and numbers denote number of animals used in each bar.

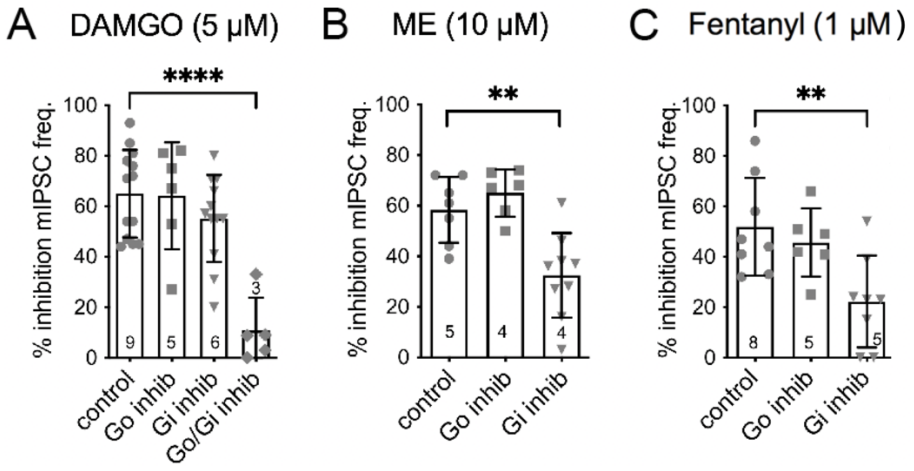

Figure A. 4: MOR-Gαi coupling is more important for inhibition of spontaneous GABA release.

A. Inhibition of mIPSCs by DAMGO (5 μM) is unaffected by Gαo and Gαi inhibitors (One way ANOVA; $F_{(3, 32)} = 12.6$, $p = 0.0001$; Dunnett's, ****$p = 0.0001$). **B.** Inhibition by ME is reduced in the presence of Gαi inhibitors, but not by the Gαo inhibitor ($F_{(2, 19)} = 11.8$, $p = 0.001$, Dunnett's, **$p < 0.01$). **C.** Inhibition by fentanyl is reduced in the presence of Gαi inhibitors, but not by the Gαo inhibitor ($F_{(2, 19)} = 6.2$, $p = 0.01$, Dunnett's, **$p < 0.01$). Symbols in bars denote recordings and numbers denote number of animals used in each bar.

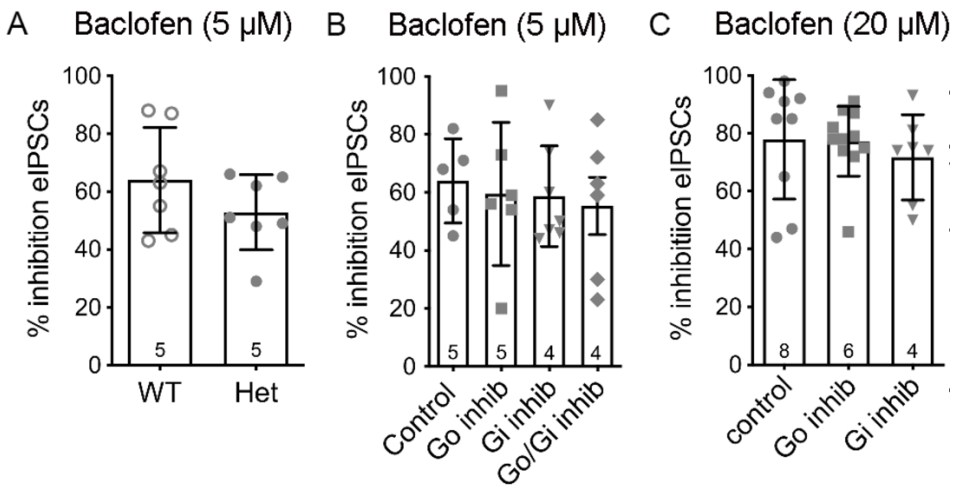

Figure A. 5: Baclofen-mediated inhibition of evoked GABA release is not affected in slices from RGS-insensitive mice or by Gαo/i peptide inhibitors.

A. Baclofen (5 µM) inhibition is similar in wildtype (WT) and RGS-insensitive (Het) mice ($t_{(12)} = 1.3$, $p = 0.2$). **B.** Baclofen (5 µM)-mediated inhibition is not altered in the presence of peptide inhibitors ($F_{(3, 20)} = 0.2$, $p = 0.9$). **C.** Baclofen (20 µM)-mediated inhibition is not altered by the peptide inhibitors ($F_{(2, 24)} = 0.3$, $p = 0.7$). Symbols in bars denote recordings and numbers denote number of animals used in each bar.

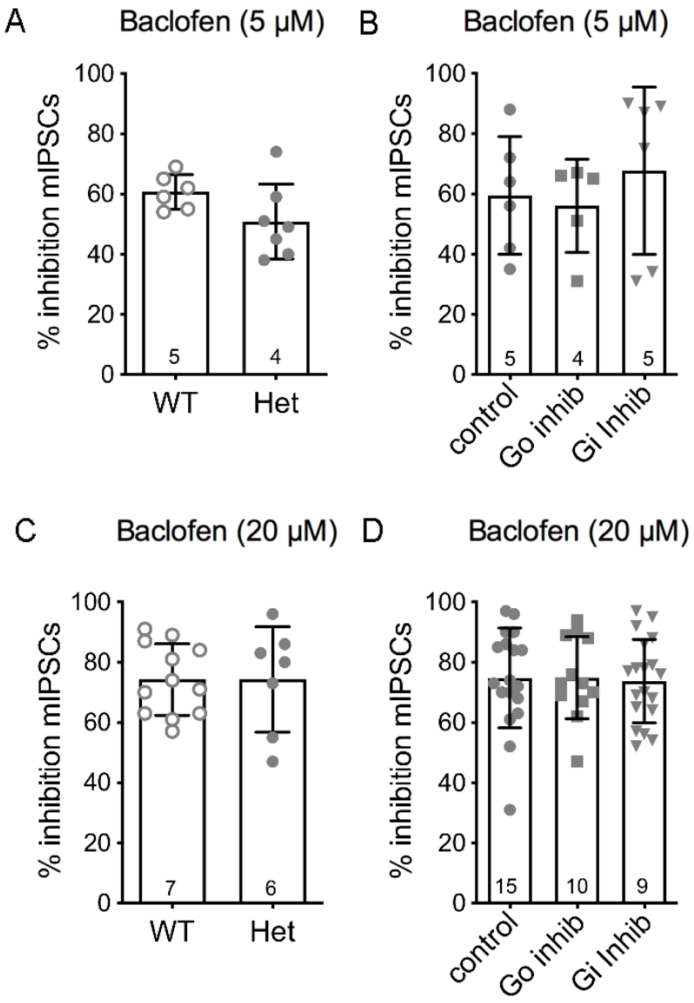

Figure A. 6: Baclofen-mediated inhibition of spontaneous GABA release in slices is not affected in RGS-insensitive mice or in the presence of Gαo/i peptide inhibitors.

A. Baclofen (5 µM) inhibition is similar in wildtype (WT) and RGS-insensitive (Het) mice ($t_{(11)} = 1.8$, $p = 0.1$). **B.** Baclofen (5 µM)-mediated inhibition is not altered in the peptide inhibitors ($F_{(2, 14)} = 0.4$, $p = 0.7$). **C.** Baclofen (20 µM) inhibition is similar in wildtype (WT) and RGS-insensitive (Het) mice ($t_{(17)} = 0.005$, $p = 1.0$). **D.** Baclofen (20 µM)-mediated inhibition is not altered in the peptide inhibitors ($F_{(2, 46)} = 0.03$, $p = 1.0$). Symbols in bars denote recordings and numbers denote number of animals used in each bar.