Functional and anatomical characterization of descending periaqueductal gray (PAG) projections and their role in pain modulation

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Functional and Anatomical Characterization of Descending Periaqueductal Gray (PAG) Projections and their Role in Pain Modulation

by

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A dissertation presented to
The Graduate School
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

May 2022
St. Louis, Missouri
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This work was funded by the NINDS R01NS106953 to Robert W. Gereau, the NIDDK K01DK115634 to Vijay K. Samineni, and the NINDS F31NS103472 and the Medical Scientist Training Program (MSTP) Grant T32GM07200 to Jose G. Grajales Reyes. Thanks to the Neuroscience Scholars Program (NSP): Preparing the Next Generation of Neuroscience Leaders R25NS089462-07 for supplemental funding directed towards professional development opportunities. Lastly, thanks to Washington University School of Medicine in St Louis (WUSM), to the Neuroscience Graduate Program at WUSM, and to the WUSM Anesthesiology Department for an extraordinary time and training.

Jose G. Grajales Reyes

Washington University in St. Louis

May 2022
Dedicated to my family, my parents and my brother. Thanks for being my champions and for your unwavering support throughout this journey. I would have not gotten here without you.
ABSTRACT OF THE DISSERTATION

Functional and anatomical characterization of descending periaqueductal gray (PAG) projections and their role in pain modulation

by

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Doctor of Philosophy in Biology and Biomedical Sciences

Neurosciences

Washington University in St. Louis, 2022

Professor Robert W. Gereau IV, Chair

Endogenous analgesic pathways embody a potential target for the development for chronic pain therapies. Previous studies have demonstrated the role of the ventrolateral periaqueductal gray (vlPAG) in descending pain modulation. It has been proposed that tonic GABAergic neurotransmission at the level of the vlPAG serves to inhibit efferent excitatory projections that mediate descending analgesia. Disinhibition of these projection neurons allows subsequent activation of rostral ventromedial medulla (RVM) neurons that inhibit nociception at the level of the spinal cord. However, lack of cell-type specificity in these studies has prevented the determination of the role of specific subsets of vlPAG neurons in analgesia. We have utilized recently developed genetic tools to selectively manipulate and monitor the activity of subclasses of vlPAG neurons to identify the circuit components critical for analgesia. Our data suggest that increasing the activity of glutamatergic (excitatory) vlPAG neurons or decreasing the activity of GABAergic (inhibitory) vlPAG neurons results in increases in sensory thresholds in uninjured animals, while ameliorating hyperalgesia associated with chronic pain states. In addition,
preliminary anatomical analysis of vlPAG circuitry suggested that excitatory glutamatergic neurons composed the majority of efferent projections onto the RVM. We therefore hypothesized that vlPAG excitatory projections to the RVM are partially responsible for the antinociception and analgesia observed after stimulation of glutamatergic neurons in the vlPAG and proposed that such manipulation could result in analgesia during chronic pain states. Population-specific fiber photometry revealed evoked calcium transients in both glutamatergic and GABAergic vlPAG neurons after a variety of acute sensory stimuli, suggesting engagement of both populations during acute nociception. However, anatomical tracing confirmed that the nearly all neurons projecting from vlPAG to the RVM are glutamatergic. Optogenetic stimulation of these glutamatergic vlPAG-RVM neurons results in elevation of thermal thresholds in uninjured animals and had no effect on anxiety-like behaviors. In addition, we demonstrated that stimulation of glutamatergic vlPAG-RVM neurons is sufficient to reverse heat hyperalgesia associated with the induction of an inflammatory state but had minimal effect in conditions of neuropathic injury. Our results provide further support for the GABA disinhibition hypothesis, highlighting the role of descending glutamatergic neurotransmission at the level of the RVM as a key component of endogenous analgesic pathways. This work was supported by funds from the Medical Scientist Training Program (MSTP) Grant T32 GM07200 and the NINDS F31NS103472.
Chapter 1: Introduction

1.1 The impact of chronic pain in society and public health

It has been estimated that 100 million adults in the United States suffer from chronic pain, accounting for an expenditure of approximately 600 billion dollars in pain related patient care.\textsuperscript{1–3} Current pharmacological treatments for pain management are sub-optimal, and adverse effects limit their use.\textsuperscript{4} In order to address this issue many potential therapeutic targets have been identified, both in the peripheral (PNS) and central nervous system (CNS), to develop new approaches to treat pain. However, the identification of novel targets has not translated to the successful development of new pharmaceuticals that represent better alternatives to our standard of care.\textsuperscript{5} Examples of such efforts include attempts to target the peripheral nervous system via nociceptor specific treatment or centrally mediated pathways such as novel opioid-based approaches. Most of these have failed or not met expectations.\textsuperscript{6} In brief, the success rate of advancing preclinical targets into clinic is low. There are a number of possible reasons for this poor translation including the low fidelity of animal models in terms of how they reflect human disease and our poor understanding of how some of these drugs are acting in the CNS.

1.2 Current approaches to study pain

The International Association for the Study of Pain defines pain as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage”.\textsuperscript{7} Therefore, the animal models we use to study pain should represent both the sensory and emotional aspects of the patients experience. However, it has been difficult to model the full experience of pain in rodents, due to the inherent limitation of animal models in which the subject cannot express or transmit their emotional state, limiting researchers to rely on the reflexive
component of nociception to guide their research. Recent developments in experimental paradigms have started to include non-reflexive measurements such as conditioned place preference/aversion, burrowing, natural behaviors and even social interaction as possible variables we could utilize to further assess the level of “pain” an animal might feel and how that can be altered by drugs or manipulations in our studies.8–14

In contrast, some might argue that current pharmacological treatments for pain are effective and represent avenues for improvement, ultimately representing possible guides for the development of novel therapies. As a result, an argument could be made for refining our pharmacological approaches in pre-clinical studies as a way to increase our rate of discovery and translation, in addition to the use of more appropriate animal models.

1.3 Re-evaluating analgesic mechanisms: A lesson from progress in opioid pharmacology

Opioids revolutionized the treatment of pain when they were first introduced in the 1600’s, with use of opium as an analgesic.15 Derivatives such as morphine and heroin were later introduced in the 1900’s and represent a breakthrough in pain management due to their potency and their long-term impact on the field of medicine.15 Opioids and their derivatives represent a series of agents which have extremely potent acute analgesic effects but ones that come with negative side-effects associated with their chronic use and abuse potential. Unfortunately, the limited development of new and safer opioids has restricted the use of opioids in modern medicine. The increased use of opioid drugs has contributed to an abuse epidemic which has compelled pain scientists to develop novel approaches to treat patients who are addicted to opioids as well as patients who are in need of potent analgesics but might not benefit from the use of opioids.16,17 The downsides of using opioid derivates in the clinic are multiple, and include abuse potential, tolerance, sedation,
constipation, and nausea.\textsuperscript{18} In summary, abuse of opioids comprises a major public health problem and has contributed to the current opioid epidemic, which now defines the search for new alternatives for pain management.

This newly found motivation to search for new therapeutic avenues has led researchers to study old drugs including opioids, in order to improve these and develop the next generation of safe and potent analgesics. An example of such development is the use of biased ligands of the G-protein coupled µ-opioid receptor (MOR).\textsuperscript{19–21} G-protein coupled receptors (GPCRs) are membrane proteins composed of seven transmembrane domains that act as ligand-activated receptors and typically play an important role in modulating neurotransmission in the CNS/PNS. It has been shown that GPCRs can engage multiple signaling cascades that act via secondary messengers and initiate long-lasting processes in neurons that induce changes at a cellular level. As part of the several cascades that GPCRs engages when activated, it has been shown that different ligands that activate GPCRs have a bias for specific signaling cascades. In regard to MOR signaling, upon GPCR activation, two main signaling cascades can be recruited either simultaneously or in a biased manner. G-protein mediated signaling has been shown to be responsible for the analgesic effects of MOR agonists such as morphine, while β-arrestin signaling mediates most of the negative side-effects such as respiratory depression and constipation.\textsuperscript{22} As a result, new MOR ligands have been developed which preferentially bias its signaling through the G-protein cascade and limits the impact of β-arrestin signaling.\textsuperscript{23,24}

Although there have been some promising results in the development of novel analgesic therapeutics such as MOR biased ligands, there is an urgent need to develop new compounds in order to keep up with the demands of personalized medicine and the heterogeneity of pain as a disease. Revisiting some of the basic mechanisms engaged by known analgesic compounds has
the potential to inform us on viable approaches for the successful development of novel pain therapeutics. When thinking about a system that is extremely effective in the treatment of pain, it is impossible not to go back to the opioid system. The mechanism of opioid-induced analgesia is a complex one, its composed of multiple CNS sites and convoluted intracellular signaling.\textsuperscript{25–30} In order to further understand how opioids work and hopefully use this to develop safer and more effective therapies for the treatment of pain, it is necessary to go back and study the circuits in the CNS/PNS that are targeted by opioids to tease these apart and find potentially novel druggable targets.\textsuperscript{30}

1.4 Potential avenues for the development of novel therapeutics

Endogenous analgesic pathways represent viable targets for the development for chronic pain therapies. The CNS has been shown to have the capacity to exert pain relief in chronic pain patients when engaged by alternative methods such as the placebo effect, mindfulness, fear or pleasure.\textsuperscript{27,31–34} As a result, it’s imperative we revisit the basic principles which govern such endogenous analgesic mechanisms and attempt to understand how CNS circuitry can be manipulated to either serve as an alternative to pharmacotherapy or become an adjuvant to current therapies.

1.5 Understanding nociceptive pathways

The IAPS defines nociception as “the neural processes of encoding and processing noxious stimuli”.\textsuperscript{7} Such process utilizes nociceptors or receptors in sensory neurons that encode for noxious information and relays this information into the CNS via nociceptive neurons located in the dorsal root ganglion. Ultimately, this process converts sensory stimuli into information that gets
processed and interpreted initially at the level of the spinal cord, but ultimately reaching sites like the cingulate/somatosensory/insular cortex, thalamus, among others, for further processing and to engage either facilitatory or inhibitory descending modulation of nociceptive information.\textsuperscript{35} Ascending sensory pathways provide the brain with spatial and modality-specific properties of sensory signals, while descending pain pathways modulate this input and result in attenuation or potentiation of nociception.\textsuperscript{36–42}

Extensive work has been done studying the ascending and descending nociceptive pathways, delineating a subset of brain nuclei involved in nociception, also known as the pain matrix.\textsuperscript{43} This collection of nociceptive CNS centers represents a network of brain nuclei that work together to assign value, valence and salience to sensory states such as the central amygdala, parabrachial nucleus, hypothalamus, and the periaqueductal gray amongst others.\textsuperscript{36} Interestingly, all these different CNS sites share some common anatomical pathways (i.e. spinoparabrachial and spinomesencephalic pathways) but differ greatly in their role during nociception. As a result, it is important to understand how these components of the pain matrix work as a whole if we wish to attempt and target some components of this circuit for development of novel therapeutics.

Initially, nociceptive signals are carried into the CNS via nociceptive neurons in the dorsal root ganglion (DRG). These primary sensory afferents and their corresponding somas are classified in terms of their conduction velocity and size, respectively. C-type fibers are known as classical nociceptors which have a slow-conduction velocity due to their unmyelinated state and are responsible for the manifestation of dull pain. These C-type fibers express thermosensitive receptors that mediate responses to heat and cold. Unmyelinated afferents as also include low- and high-threshold mechanoreceptors. A-type sensory afferents have larger diameter cell bodies and myelinated axons. A-Delta afferents are thinly myelinated and respond to heat and noxious
mechanical stimuli (fast/sharp pain). A-Beta afferents have more heavily myelinated axons and are mostly responsive to low threshold mechanical stimuli, although a subpopulation of these respond to high threshold mechanical stimuli and are classified as nociceptors. The largest sensory afferent neurons are A-Alpha fiber afferents which have thickly myelinated axons and mediate proprioception.

The central processes of primary afferent DRG neurons synapse on second-order neurons in the dorsal horn of the spinal cord. C- and A-delta fiber DRG neurons that respond to noxious stimulation, or nociceptors, project to Laminae I and II of the spinal cord, whereas A-beta and A-alpha afferents target deeper laminae of the dorsal horn. Spinal projection neurons relay nociceptive information to higher CNS centers for further processing.\(^{36}\)

The main role of ascending sensory tracts is to provide the brain with spatial/temporal resolution and the modality specific information of sensory input coming from the periphery. Thus, the organization of these tracts follows patterns that direct the information to relevant brain nuclei like the thalamus, periaqueductal gray, parabrachial nucleus, medulla, amygdala, hypothalamus, among others.\(^{36,37,43–45}\) Ascending pathways vary in several major aspects such projection targets and the distribution routes of sensory information, but most importantly they seem to be the primary source of direct nociceptive information for important centers like the PAG (Spinomesencephalic tract), thalamus (Spinothalamic tract), parabrachial/amygdala (Spinoparabrachial tract) and hypothalamus (Spinohypothalamic tract).

Descending pathways from the brain to the spinal cord modulate the activity of neurons in the spinal dorsal horn and can result in either attenuation or potentiation of nociceptive signals. Descending modulation of pain happens in a top-to-bottom manner and usually takes place in nuclei that are downstream of centers which have already been engaged within the matrix.\(^{38}\)
However, when focusing on descending analgesia, descending modulation has the end result of modulating incoming information from the periphery at the level of the spinal cord, with the goal of halting nociceptive information from entering the matrix, thus resulting in antinociception or analgesia.\textsuperscript{37}

1.6 Descending hubs for nociceptive modulation

Descending pathways from the brainstem to the spinal dorsal horn are known to effectively modulate pain in rodents and humans\textsuperscript{46–50}. Targeted manipulation of these systems has the potential to provide an effective therapy for chronic pain with limited adverse effects\textsuperscript{48,49}. A detailed understanding of endogenous descending pain pathways, including the identification of the specific cell populations that are involved, could provide a foundation for the development of such therapies.

In order to understand the mechanisms underlying descending modulation of pain, a systematic approach has been taken to manipulate single sites in the CNS of model organisms to interrogate whether these are involved in what is known as stimulation-produced analgesia (SPA). In the late 1960’s the periaqueductal gray (PAG) was identified as a single site that could mediate analgesia in rodents when stimulated.\textsuperscript{47} More recent studies have uncovered multiple sites in the CNS that are potentially also involved in descending inhibition of nociception.\textsuperscript{51–53} In brief, we have come to learn that sites such as the periaqueductal gray, ventromedial medulla, reticular formation, and hypothalamus amongst others, are all potentially linked in this descending branch of the pain matrix largely due to the demonstration of SPA after manipulating the activity of these centers via a variety of methods.\textsuperscript{52}

1.7 The role of the periaqueductal gray (PAG) in nociception
The periaqueductal gray (PAG) surrounds the cerebral aqueduct and is composed of a heterogeneous population of neurons. The PAG serves as a processing hub for many aspects of physiology and behavior, including autonomic function, motivated behaviors, and nociception. The PAG has a unique structure which is composed of interconnected columns of neurons that project in a rostro-caudal direction and are divided by their anatomy and function into: dorsomedial, dorsolateral, lateral, ventrolateral and ventral PAG. Functional PAG heterogeneity, in terms of its anatomy, has been described extensively and it is thought that more dorsal aspects of the PAG predominantly mediate defensive and autonomic behaviors, while lateral and ventral aspects mediate nociception and motivated behaviors.

Initial reports of effective analgesia following electrical stimulation of the PAG in animal models prompted investigation into the role of the PAG in nociception. It was speculated early on that the analgesia evoked from PAG stimulation was not due to a generalized blockade of sensory information. The effect of periventricular PAG electrical stimulation in these experiments was short lived and reversible, focused and robust. Similar observations had previously been made following microinjection of morphine into the PAG, possibly indicating a common underlying mechanism that

![Diagram of electrode and PAG](image)

**Figure 1.1. Effects of Periaqueductal gray (PAG) electrical stimulation.** Cartoon representation of the effects of PAG electrical stimulation in animal models and humans. Shaded area represents the ventrolateral PAG.
Table 1.1. Effects of Periaqueductal gray (PAG) electrical stimulation

<table>
<thead>
<tr>
<th>Reference</th>
<th>Species</th>
<th>Sex</th>
<th>Naive vs Pain Model</th>
<th>Type of Stimulation</th>
<th>Site of Stimulation</th>
<th>Paw Withdrawal Latency (PWL)</th>
<th>Paw Withdrawal Threshold (PWT)</th>
<th>Others</th>
<th>Assessment</th>
<th>Site of Assessment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reynolds, 1969</td>
<td>Rat</td>
<td>Male</td>
<td>Naive</td>
<td>Electrical</td>
<td>Midbrain Central Gray</td>
<td>Unilateral</td>
<td>Increased</td>
<td>Awake laparotomy did not produce evoked pain responses</td>
<td>Analgesimeter</td>
<td>Tail &amp; hindpaw</td>
</tr>
<tr>
<td>M注射剂 &amp; L associés, 1974</td>
<td>Rat</td>
<td>Male</td>
<td>Naive</td>
<td>Electrical</td>
<td>Periventricular Gray Matter</td>
<td>Unilateral</td>
<td>-</td>
<td>Pinch analysis</td>
<td>Analgesimeter</td>
<td>Tail &amp; hindpaw</td>
</tr>
<tr>
<td>Lewis et al., 1977</td>
<td>Rat</td>
<td>Male</td>
<td>Naive</td>
<td>Electrical</td>
<td>PAG</td>
<td>Unilateral</td>
<td>-</td>
<td>Increased trigeminal stimulation threshold</td>
<td>Analgesimeter</td>
<td>Tail &amp; hindpaw</td>
</tr>
<tr>
<td>Richardson &amp; Alsé, 1977</td>
<td>Human</td>
<td>Male/Female</td>
<td>Known etiology pain</td>
<td>Electrical</td>
<td>PAG</td>
<td>Unilateral</td>
<td>-</td>
<td>Reduced pain score</td>
<td>Analgesimeter</td>
<td>Tail &amp; hindpaw</td>
</tr>
<tr>
<td>Fardin et al. 1984</td>
<td>Rat</td>
<td>Male</td>
<td>Naive</td>
<td>Electrical</td>
<td>vPAG/vlPAG</td>
<td>Unilateral</td>
<td>Increased</td>
<td>vPAG naloxone blocks SPA effects, while systemic naloxone had no effect in comparison to intrathecal naloxone blocked SPA</td>
<td>Analgesimeter</td>
<td>Tail &amp; hindpaw</td>
</tr>
<tr>
<td>Lee et al. 2012</td>
<td>Rat</td>
<td>Male</td>
<td>SNI</td>
<td>Electrical</td>
<td>vPAG</td>
<td>Unilateral</td>
<td>Increased</td>
<td>Reduced spontaneous behavior</td>
<td>Analgesimeter</td>
<td>Tail &amp; hindpaw</td>
</tr>
<tr>
<td>Ning et al. 2016</td>
<td>Rat</td>
<td>Male</td>
<td>Naive</td>
<td>Electrical</td>
<td>vPAG</td>
<td>Unilateral</td>
<td>Increased</td>
<td>Hargreaves</td>
<td>Analgesimeter</td>
<td>Tail &amp; hindpaw</td>
</tr>
<tr>
<td>Sims-Williams et al. 2016</td>
<td>Human</td>
<td>Male/Female</td>
<td>Discomfort-related pain</td>
<td>Electrical</td>
<td>PAG</td>
<td>Unilateral</td>
<td>-</td>
<td>Reduced VAS pain score</td>
<td>Analgesimeter</td>
<td>Tail &amp; hindpaw</td>
</tr>
<tr>
<td>Huntall et al. 2016</td>
<td>Human</td>
<td>Male/Female</td>
<td>Spinal cord injury</td>
<td>Electrical</td>
<td>Central Gray</td>
<td>Bilateral</td>
<td>-</td>
<td>Reduced VAS pain score</td>
<td>Analgesimeter</td>
<td>Tail &amp; hindpaw</td>
</tr>
</tbody>
</table>
produces analgesia upon either electrical stimulation of the PAG or morphine microinjection into the PAG.  

A number of electrical stimulation experiments have suggested potential applications of PAG-mediated SPA and expanded understanding of the optimal conditions for SPA. Generally, the effects of PAG SPA are more robust for noxious heat and mechanical stimulation in various pain models, suggesting modality specific effects of PAG SPA. Consistently, inflammatory pain models seem to respond well to PAG SPA. However, some reports have also shown effects of SPA in neuropathic injury models. In addition, SPA appears to involve opioid signaling since SPA is sensitive to administration of opioid receptor antagonists. Other types of stimulation-induced analgesia such as stress-induced analgesia, that are also mediated at the level of the PAG may also involve opioid signaling. 

The effects of PAG SPA observed in animal models can be recapitulated in humans. Several studies have shown that deep-brain stimulation (DBS) in the human PAG provides pain relief to patients who suffer from chronic pain conditions. Although different methodologies were used, the results of human studies coincide with animal models. Patients report a general decrease in pain scores recorded in a variety of conditions such as chronic intractable pain, deafferentation pain and spinal cord injury, among others. In addition, patients with PAG DBS also exhibited a phenomenon that had been previously described in animals, tolerance to stimulation, a well-known phenomenon in opioid therapy. 

The previously discussed findings all suggest an important role of the PAG in nociception, but do not fully address whether the PAG is necessary for nociception, chronic pain or SPA. Initial studies used electrolytic lesions in the PAG to determine the necessity of the PAG in morphine-induced analgesia. In these studies, partial lesions of the PAG resulted in an increase in the
threshold of current needed for SPA in acute nociception, suggesting that an intact PAG as required for optimal SPA. Moreover, these lesion studies provided an understanding of the role of the PAG in the descending branch of the pain matrix. Lesions caudal to the PAG do not abolish PAG SPA and suggest that PAG-induced analgesia is also mediated through ascending pathways that do not involve descending tracts. More recent work, has also shown that more circumscribed lesions that are restricted to the ventral columns of the PAG, the vlPAG, recapitulate the effects of global PAG lesions by diminishing the analgesic effect of morphine in the context of pain while having little effect on naïve nociceptive thresholds. It is important to note that just as blockade of opioid signaling at the level of the PAG affects stress or fear induced analgesia, lesions to the vlPAG have similar effects.

Focusing on functional units of the ventrolateral PAG (vlPAG), pharmacology experiments have revealed bi-directional control of nociception. It is thought that certain agents that result in activation of PAG neurons produce analgesia, similar to the effects of SPA. It is also possible to alter nociceptive thresholds and induce hyperalgesia by injecting GABA. These pharmacology studies suggest a proportional relationship between net increase of PAG neuron firing and analgesia.
## Table 1.2. Effects of Periaqueductal gray (PAG) lesions

<table>
<thead>
<tr>
<th>Reference</th>
<th>Species</th>
<th>Sex</th>
<th>Naive vs Pain Model</th>
<th>Type of lesion</th>
<th>Site of lesion</th>
<th>Unilateral vs Bilateral</th>
<th>Acute or Chronic</th>
<th>Paw Withdrawal Latency (PWL)</th>
<th>Paw Withdrawal Threshold (PWT)</th>
<th>Others</th>
<th>Assessment</th>
<th>Site of Assessment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dostrovsky &amp; Deakin, 1977</td>
<td>Rat</td>
<td>Male</td>
<td>Acute</td>
<td>Electrolytic</td>
<td>PAG</td>
<td>Unilateral/midline</td>
<td>Aute</td>
<td>-</td>
<td>-</td>
<td>Lesion blocked morphine induced analgesia and convulsia</td>
<td>Tail-flick</td>
<td>Tail</td>
</tr>
<tr>
<td>Rhodes, 1979</td>
<td>Rat</td>
<td>Male</td>
<td>Acute</td>
<td>Electrolytic</td>
<td>PAG</td>
<td>Bilateral</td>
<td>Aute</td>
<td>-</td>
<td>-</td>
<td>Rostral PAG/SPA threshold increased after spinal PAG lesions</td>
<td>Tail-flick</td>
<td>Tail</td>
</tr>
<tr>
<td>Bednaba &amp; Fields, 1979</td>
<td>Rat</td>
<td>Male</td>
<td>Naive</td>
<td>Electrolytic</td>
<td>NRM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Area lateralized to be at least 500um in diameter; next to the NRM to abolish phasic reactive injection effects</td>
<td>Tail-flick</td>
<td>Hindlimb</td>
</tr>
<tr>
<td>Rydenhag &amp; Anderson, 1981</td>
<td>Rat</td>
<td>Male</td>
<td>Naive</td>
<td>Microinjection using fine forceps</td>
<td>DLF - Th1 or lower</td>
<td>Bilateral</td>
<td>Aute</td>
<td>-</td>
<td>-</td>
<td>Morphine increases tail-flick latency</td>
<td>Tail-flick</td>
<td>Tail</td>
</tr>
<tr>
<td>Prieto et al 1983</td>
<td>Rat</td>
<td>Male</td>
<td>Naive</td>
<td>Electrolytic</td>
<td>NRM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Area lateralized to be at least 500um in diameter; next to the NRM to abolish phasic reactive injection effects</td>
<td>Tail-flick</td>
<td>Tail</td>
</tr>
<tr>
<td>Morgan et al 1989</td>
<td>Rat</td>
<td>Male</td>
<td>Naive</td>
<td>Electrolytic</td>
<td>NRM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Reduced number of animals that showed SPA</td>
<td>Radiation source (tail-flick) &amp; hot plate</td>
<td>Tail &amp; hindpaw</td>
</tr>
<tr>
<td>Xin &amp; Xu-Gai, 1990</td>
<td>Rat</td>
<td>Male</td>
<td>Naive</td>
<td>Electrolytic</td>
<td>NRM</td>
<td>-</td>
<td>Aute</td>
<td>-</td>
<td>-</td>
<td>Reduced number of animals that showed SPA</td>
<td>Tail-flick</td>
<td>Tail</td>
</tr>
<tr>
<td>Burgess et al 2002</td>
<td>Rat</td>
<td>Male</td>
<td>Sham</td>
<td>Spinal Nerve Ligation (SNL)</td>
<td>Saporin</td>
<td>RVM</td>
<td>Bilateral</td>
<td>Chronic</td>
<td>Increase</td>
<td>SNL animals killed with DMSH recovered tail and thermal mechanical thresholds within a week after SNL injury</td>
<td>Hargreaves &amp; von Frey Filaments</td>
<td>Hindpaw</td>
</tr>
<tr>
<td>McGaraughty et al 2004</td>
<td>Rat</td>
<td>Male</td>
<td>Naive</td>
<td>Electrolytic</td>
<td>PAG/vPAG</td>
<td>Bilateral</td>
<td>Aute</td>
<td>-</td>
<td>-</td>
<td>PAG lesions blocked the effects of morphine injection into the BLA and MeA, regarding the behavioral response and RVM ON-OFF cell firing</td>
<td>Tail-flick</td>
<td>Tail</td>
</tr>
<tr>
<td>Flores et al 2014</td>
<td>Rat</td>
<td>Male</td>
<td>Naive</td>
<td>6-OHDA</td>
<td>PAG</td>
<td>Unilateral</td>
<td>Aute</td>
<td>-</td>
<td>-</td>
<td>PAG lesions blocked the anesthetic effect of opiates in the hot plate and tail-immersion test</td>
<td>Tail-flick</td>
<td>Tail</td>
</tr>
<tr>
<td>Mendes &amp; Ribeiro, 2009</td>
<td>Mouse</td>
<td>Male</td>
<td>Naive</td>
<td>Electrolytic</td>
<td>dPAG</td>
<td>Bilateral</td>
<td>Aute</td>
<td>-</td>
<td>-</td>
<td>Lesions had no effect on formalin-induced nociceptive behavior, but seemed to be ameliorated in the direct plus maze</td>
<td>Formalin</td>
<td>Hindpaw</td>
</tr>
<tr>
<td>Vicente-Romero et al 2015</td>
<td>Guinea Pig</td>
<td>Male</td>
<td>Naive</td>
<td>Ibotenic acid</td>
<td>vPAG</td>
<td>Bilateral</td>
<td>Aute</td>
<td>-</td>
<td>-</td>
<td>Locomotion block fear-induced antinociceptive behavior</td>
<td>Hot plate</td>
<td>Genitals</td>
</tr>
</tbody>
</table>
Global activation of neurons by delivery of glutamate or any agonist of ionotropic glutamate receptors to the PAG results in an elevation of sensory thresholds and/or analgesia.\(^{68,85,86}\) Local delivery into the PAG of agents that globally inhibit neurotransmission such as sodium channel blockers (i.e. lidocaine), have similar effects.\(^{87,88}\) The consideration of such results brings up an interesting concept, either global activation or inhibition of PAG neurons produces analgesia. These observations provide insight into the complexity of the mechanisms via which the PAG contributes to descending pain modulation.

Injection of an \(\mu\)-opioid receptor (MOR) agonist into the PAG results in elevation of sensory thresholds and/or analgesia.\(^{63,68,89–93}\) Since inhibitory cells within the PAG express MOR receptors, this result suggests that MOR agonists produce analgesia by effecting disinhibition of PAG output.\(^{29,30}\) Consistently, antagonism of GABA receptors, which also would result in disinhibition of PAG output, also produces analgesia.\(^{55,84,94}\) In addition, vlPAG injection of a number of other neuroactive ligands including angiotensin III, galanin, substance p and calcitonin-related gene peptide have been shown to be anti-nociceptive.\(^{95–98}\)

**Figure 1.3. Effects of Periaqueductal gray (PAG) pharmacological manipulation.** Cartoon representation of the effects of PAG delivery of a variety of pharmacological agents in animal models. Shaded area represents the ventrolateral PAG.
<table>
<thead>
<tr>
<th>Reference</th>
<th>Species</th>
<th>Sex</th>
<th>Naive vs Pain Model</th>
<th>Compound</th>
<th>Type of Compound</th>
<th>Site of Injection</th>
<th>Unilateral vs Bilateral</th>
<th>Acute vs Chronic</th>
<th>Paw Withdrawal Latency (PWL)</th>
<th>Paw Withdrawal Threshold (PWT)</th>
<th>Others</th>
<th>Assessment</th>
<th>Site of Assessment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lewis and Gibbels, 1977</td>
<td>Rat</td>
<td>Male</td>
<td>Naive</td>
<td>Phentolamine</td>
<td>MDR agonist</td>
<td>PAG</td>
<td>Unilateral</td>
<td>Acute</td>
<td>Increase</td>
<td>-</td>
<td>-</td>
<td>Hot plate &amp; Tail flick</td>
<td>Electrical trigeminal stimulation threshold</td>
</tr>
<tr>
<td>Behbehani &amp; Fields, 1979</td>
<td>Rat</td>
<td>Male</td>
<td>Naive</td>
<td>Glutamate</td>
<td>-</td>
<td>PAG</td>
<td>Unilateral</td>
<td>Acute</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Hot plate &amp; Tail flick</td>
<td>Electrical trigeminal stimulation threshold</td>
</tr>
<tr>
<td>Fung &amp; Sato, 1984</td>
<td>Rat</td>
<td>Male</td>
<td>Naive</td>
<td>Carbamazepine</td>
<td>Voltage-gated sodium channel blocker</td>
<td>PAG</td>
<td>Unilateral</td>
<td>Acute</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>No Effect</td>
<td>Biting behavior</td>
</tr>
<tr>
<td>Monta &amp; Fields, 1986</td>
<td>Rat</td>
<td>Male</td>
<td>Naive</td>
<td>Pentobarbital</td>
<td>-</td>
<td>PAG</td>
<td>Unilateral</td>
<td>Acute</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Tail-flick, radiant heat source</td>
<td>Tail</td>
</tr>
<tr>
<td>Carisi et al, 1990</td>
<td>Rat</td>
<td>Male</td>
<td>Naive</td>
<td>Glutamate</td>
<td>MDR agonist</td>
<td>vPAG</td>
<td>Unilateral</td>
<td>Acute</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Tail-flick, radiant heat source</td>
<td>Tail</td>
</tr>
<tr>
<td>Marnone et al, 1998</td>
<td>Mouse</td>
<td>Male</td>
<td>Naive</td>
<td>(S)-3RSACPD</td>
<td>Group I &amp; II mGluR agonist</td>
<td>vPAG + MDR agonist</td>
<td>Unilateral</td>
<td>Acute</td>
<td>Increase</td>
<td>-</td>
<td>Effect blocked by preadministration of mGluR antagonist (S)-3RSACPD and pretreatment of MDR agonist (S)-3RSACPD</td>
<td>Hot plate</td>
<td>-</td>
</tr>
<tr>
<td>Burgess et al, 2002</td>
<td>Rat</td>
<td>Male</td>
<td>Naive</td>
<td>Lidocaine</td>
<td>Sodium Channel Blocker</td>
<td>IRM</td>
<td>Bilateral</td>
<td>Acute</td>
<td>Increase</td>
<td>Increase</td>
<td>Same effect on POS 3.6, 9.12</td>
<td>Hargreaves &amp; von Frey Filaments</td>
<td>Hindpaw</td>
</tr>
<tr>
<td>Yu et al, 2003</td>
<td>Rat</td>
<td>Male</td>
<td>Naive</td>
<td>CGRP</td>
<td>CGRP Receptor agonist</td>
<td>PAG</td>
<td>Unilateral</td>
<td>Acute</td>
<td>Increase</td>
<td>Increase</td>
<td>Effect blocked by antagonist co-admin CGRP 8,32</td>
<td>Hot plate &amp; Randall-Selitto</td>
<td>Hindpaw</td>
</tr>
<tr>
<td>Hemmricher et al, 2004</td>
<td>Rat</td>
<td>Male</td>
<td>Naive</td>
<td>GluR</td>
<td>MDR agonist</td>
<td>vPAG</td>
<td>Unilateral</td>
<td>Acute</td>
<td>Decrease</td>
<td>-</td>
<td>No Effect</td>
<td>Hargreaves</td>
<td>Hindpaw</td>
</tr>
<tr>
<td>Roan et al, 2004</td>
<td>Rat</td>
<td>Male</td>
<td>Naive</td>
<td>Substance P</td>
<td>Nk1 Receptor agonist</td>
<td>PAG</td>
<td>Unilateral</td>
<td>Acute</td>
<td>Increase</td>
<td>Increase</td>
<td>Effect Blocked Following Administration of Nk1 Receptor Agonist, Spetzler</td>
<td>Hot plate &amp; Randall-Selitto</td>
<td>Hindpaw</td>
</tr>
<tr>
<td>Morgan et al, 2008</td>
<td>Rat</td>
<td>Male</td>
<td>Naive</td>
<td>Bicuculline</td>
<td>GABA receptor antagonist</td>
<td>vPAG</td>
<td>Unilateral</td>
<td>Acute</td>
<td>Decrease</td>
<td>-</td>
<td>No Tolerance observed</td>
<td>Hargreaves</td>
<td>Hindpaw</td>
</tr>
<tr>
<td>Guo et al, 2006</td>
<td>Rat</td>
<td>Male</td>
<td>Naive</td>
<td>CFA</td>
<td>Hydroxyflavone</td>
<td>vPAG + MDR agonist</td>
<td>Unilateral</td>
<td>Acute</td>
<td>Increase</td>
<td>-</td>
<td>Administration of boc-ERG, both pre and post-CFA, alleviates hypersensitivity</td>
<td>Hargreaves</td>
<td>Hindpaw</td>
</tr>
<tr>
<td>Loyd et al, 2008</td>
<td>Rat</td>
<td>Male</td>
<td>Naive</td>
<td>Morphine</td>
<td>MDR agonist</td>
<td>vPAG</td>
<td>Unilateral</td>
<td>Acute</td>
<td>Increase</td>
<td>-</td>
<td>Estrogen cycle dependent</td>
<td>Hargreaves</td>
<td>Hindpaw</td>
</tr>
<tr>
<td>Loyd et al, 2008</td>
<td>Rat</td>
<td>Male</td>
<td>Naive</td>
<td>Morphine</td>
<td>MDR agonist</td>
<td>vPAG</td>
<td>Unilateral</td>
<td>Chronic</td>
<td>Increase</td>
<td>-</td>
<td>Tolerance observed</td>
<td>Hot plate</td>
<td>-</td>
</tr>
<tr>
<td>Pellegina-Dhahamet et al, 2009</td>
<td>Rat</td>
<td>Male</td>
<td>Naive</td>
<td>Angiotensin 1 B</td>
<td>Angiotensin 1 B Receptor Agonist</td>
<td>vPAG</td>
<td>Unilateral</td>
<td>Acute</td>
<td>Increase</td>
<td>-</td>
<td>Tail-flick</td>
<td>Hot plate &amp; Randall-Selitto</td>
<td>Tail</td>
</tr>
</tbody>
</table>

Table 1.3. Effects of Periaqueductal gray (PAG) pharmacological manipulation
Table 13. Effects of Periaqueductal gray (PAG) pharmacological manipulation

<table>
<thead>
<tr>
<th>Study</th>
<th>Species</th>
<th>Sex</th>
<th>naive</th>
<th>Treatment</th>
<th>Region</th>
<th>Approach</th>
<th>Acute/Narc</th>
<th>Effect</th>
<th>Control Group/Effect</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maione et al. 2009</td>
<td>Rat</td>
<td>Male</td>
<td>Naive</td>
<td>Capsaicin + TrpV1 agonist</td>
<td>vlPAG</td>
<td>Unilateral</td>
<td>Acute</td>
<td>No Effect</td>
<td>-</td>
<td>Low dose DAMGO</td>
</tr>
<tr>
<td>Morgan et al. 2009</td>
<td>Rat</td>
<td>Male</td>
<td>Naive</td>
<td>MK-801</td>
<td>vlPAG</td>
<td>Unilateral</td>
<td>Acute</td>
<td>No Effect</td>
<td>-</td>
<td>Administration of MK-801 did not block the development of morphine tolerance</td>
</tr>
<tr>
<td>Yang et al. 2011</td>
<td>Rat</td>
<td>Male</td>
<td>Naive</td>
<td>Oxytocin receptor antagonist</td>
<td>PAG</td>
<td>Unilateral</td>
<td>Acute</td>
<td>-</td>
<td>-</td>
<td>Increased threshold</td>
</tr>
<tr>
<td>Zhang et al. 2015</td>
<td>Rat</td>
<td>Male</td>
<td>Naive</td>
<td>Galanin</td>
<td>PAG</td>
<td>Bilateral</td>
<td>Acute</td>
<td>Increase</td>
<td>Increase</td>
<td>Effects blocked by Galanin receptor antagonist injection, Mrg7. Effects also blocked by co-administration of MAP, a selective CAMKII inhibitor</td>
</tr>
<tr>
<td>Tonnfeldt et al. 2016</td>
<td>Rat</td>
<td>Male</td>
<td>CFA + morphine</td>
<td>DS2 Positive allosteric modulator for δ-Containing GABA A receptor</td>
<td>vlPAG</td>
<td>Unilateral</td>
<td>Acute</td>
<td>-</td>
<td>-</td>
<td>Hot Plate &amp; Randall-Sellin</td>
</tr>
<tr>
<td>Umano et al. 2017</td>
<td>Rat</td>
<td>Male</td>
<td>Formain</td>
<td>PNU-120596 α7 NAChR PAM</td>
<td>vlPAG</td>
<td>Unilateral</td>
<td>Acute</td>
<td>-</td>
<td>-</td>
<td>Reduced nocifensive behavior</td>
</tr>
</tbody>
</table>

**Legend:**
- vlPAG: Ventrolateral PAG
- DAMGO: D-amino-2-phosphonopropionic acid
- TrpV1 agonist: Transient Receptor Potential V1 agonist
- MOR agonist: Mu opioid receptor agonist
- α7 NAChR: α7 nicotinic acetylcholine receptor
- α7 NAChR PAM: α7 nicotinic acetylcholine receptor positive allosteric modulator
- MAP: MAP, a selective CAMKII inhibitor
- CFA: Complete Freund's adjuvant
- Morphine
- DS2: DS2 positive allosteric modulator for δ-containing GABA A receptor
- PNU-120596: α7 NAChR PAM
- Hargreaves: Plantar threshold
- Hot Plate: Hot Plate
- Tail Stimulation: Tail Stimulation
- Tail: Tail
In addition, injection of GABA or GABA receptor modulators into the vlPAG, a manipulation that would be expected to increase inhibition within the PAG and decrease PAG output, has been shown to be pro-nociceptive.\textsuperscript{84,99,100} Other molecules that inhibit PAG neurons such as metabotropic glutamate receptor (mGluR 2/3) agonists and inflammatory mediators like PEG2 are also pro-nociceptive.\textsuperscript{86,101} In brief, pharmacological manipulations that increase PAG output result in analgesia. Conversely, manipulations that increase GABAergic tone in the PAG produce hyperalgesia, perhaps via GABA receptor activation in the same subset of neurons that are disinhibited by opioids.

1.8 The GABA disinhibition hypothesis

The GABA disinhibition hypothesis proposes that tonic inhibitory (GABAergic) neurotransmission at the level of the vlPAG serves to inhibit excitatory PAG output projections, attenuating descending analgesic mechanisms.\textsuperscript{46,85,102–104} Disinhibition of vlPAG excitatory neurons that project to the rostral ventromedial medulla (RVM) is thought to allow subsequent activation of RVM cells that project to the dorsal horn of the spinal cord and inhibit nociceptive information processing, resulting in analgesia.\textsuperscript{28} This model explains how, depending on the subpopulation of PAG neurons that are affected, inhibition or activation can result in analgesia. For example, inhibition of PAG GABAergic neurons, which occurs when morphine is injected into the PAG, relieves inhibition on PAG projection neurons resulting in analgesia.\textsuperscript{44,104}

Altered vlPAG neuronal transmission has been described in chronic pain states. The GABA disinhibition hypothesis provides a framework within which the potential factors that contribute to the dysregulation of circuits in the PAG during chronic pain can be identified. In neuropathic injury models, it has been shown that the firing frequency of PAG neurons the frequency of
spontaneous IPSCs (inhibitory post-synaptic currents) and the magnitude of GABA tonic currents are all enhanced. Conversely, a hypoglutamatergic state has been described in neuropathic injury conditions, wherein a decrease in spontaneous EPSCs (excitatory post-synaptic currents) and a decrease in the frequency of evoked EPSCs is observed in PAG neurons. In brief, under chronic pain conditions, the vlPAG exhibits reduced glutamatergic and enhanced GABAergic neurotransmission. It has been hypothesized that these neuroplastic changes in the PAG contribute to the development and maintenance of chronic pain.

In addition to these maladaptive synaptic changes that occur in the PAG under chronic pain conditions, PAG neuronal responses to noxious stimuli are also affected. The PAG consists of ON and OFF cells similar to the ON and OFF cells described in the medulla. ON and OFF cells, are named based on their responses when a noxious heat stimulus is applied to the tail of a mouse. When heat is applied to the tail, ON cells rapidly increase their firing frequency while OFF cells rapidly decrease their firing frequency in relationship to their withdrawal response. In conditions of chronic pain such as chemotherapy-induced neuropathic injury, single unit recordings from the PAG reveal an increased spontaneous and evoked unit firing after noxious stimuli.

![Figure 1.4](image)

**Figure 1.4. Effects of chronic pain in Periaqueductal gray (PAG) physiology.** Cartoon representation of the effects of chronic pain in PAG GABAergic neurotransmission. Shaded area represents the ventrolateral PAG.

Although it is clear from the above studies the PAG plays a significant role in pain processing, important questions remain. For example, is there a specific population of vlPAG neurons that are the target of this increased GABAergic tone in
chronic pain states? Is the increased GABAergic tone of the vlPAG that is observed in the context of chronic pain driven exclusively by local inhibitory interneurons or is it mediated from centers such as the central amygdala? Is reversal of this hyper-GABAergic state in the PAG a potential therapeutic approach for chronic pain?

It was originally proposed that the majority of GABAergic terminals within the PAG and RVM were derived from local interneurons within these nuclei.\textsuperscript{102,109} Moreover, it has been shown via electron microscopy and histology that at the level of both the PAG and RVM, the dispersion and density of GABAergic terminals suggests a local inhibitory network.\textsuperscript{103} In addition, a dense and extensive network of projections between PAG columns suggests a local network that could be formed at least partially by PAG GABAergic neurons.\textsuperscript{60} Therefore most of the current information suggests that GABAergic neurons in the PAG are interneurons. However, a few studies have reported that GABAergic PAG neurons project to the RVM.

1.9 Descending PAG to RVM Projections

Although, GABAergic neurons have been shown to comprise a minority of the PAG projections to the RVM, it has been conclusively demonstrated that these two sites are connected.\textsuperscript{92,102,109–117} The PAG can facilitate or inhibit spinal nociceptive transmission via its projections to the rostral ventromedial medulla (RVM).\textsuperscript{42,88,102,103,118} Descending, glutamatergic projections from the vlPAG to the RVM are hypothesized to mediate the effect of the PAG on spinal nociceptive transmission and analgesia.\textsuperscript{42,53,66,69,85,91} As previously described, it is thought that a subset of vlPAG GABAergic neurons are tonically active and act locally to inhibit glutamatergic output neurons that project to the RVM, thereby regulating the descending analgesic mechanism originating from the PAG.\textsuperscript{37,44,94,102,103} As a result, inhibition of tonically active vlPAG
GABAergic neurons is hypothesized to be the mechanism underlying opioid mediated analgesia.\textsuperscript{30,93} The PAG has also been shown to project to many other nociceptive centers. Connections with the amygdala, parabrachial nucleus, ventral tegmental area, hypothalamus and spinal cord highlight how the PAG’s extensive connectivity with centers involved in both ascending and descending processing of nociception suggest potential mechanisms by which the PAG can modulate SPA.\textsuperscript{59,92,110,112,114,117,119–122}

1.10 The role of the rostral ventromedial medulla (RVM) in nociception

As a downstream partner of the periaqueductal gray (PAG), the rostral ventromedial medulla (RVM) has been shown to modulate the processing of nociceptive information in the spinal dorsal horn.\textsuperscript{41,42,88,103,123–125} Anatomical analysis of descending PAG projections to the RVM primarily demonstrate monosynaptic connections between the lateral and ventrolateral columns of the PAG and subdivisions of the RVM, including the nucleus Raphe Magnus (RMg).\textsuperscript{110–112,114} Physiology experiments have also confirmed a connection between the PAG and the RVM via paired recordings of RVM neurons with PAG stimulation, suggesting direct control of the PAG over RVM neuron firing.\textsuperscript{85,94,126} The PAG to RVM projections have been suggested to be excitatory, although some propose that inhibitory output from the PAG helps maintain

\textbf{Figure 1.5. Anatomical characterization of Periaqueductal gray (PAG) circuitry.} Cartoon representation of experimentally confirmed PAG afferent and efferent projections. Neuronal subtypes represented as glutamatergic (blue), GABAergic (magenta), dopaminergic (cyan), and serotonergic (red). Shaded area represents the ventrolateral PAG.
the GABA tone at the level of the RVM.\textsuperscript{85,112} The presence of an inhibitory projection from the PAG to the RVM has been debated.\textsuperscript{102,103,109} Current evidence, supports the hypothesis of local GABAergic tone in the PAG which regulates excitatory projections to the RVM.\textsuperscript{102,103,112,123}

The RVM’s importance in descending modulation of pain was initially described using similar approaches to those used in the study of the PAG. The RVM receives inputs from areas related to nociception including the somatosensory cortex, zona incerta, parabrachial nucleus, thalamus and PAG, amongst others (see results section).\textsuperscript{127} As a result, the RVM represents another important hub of descending processing of nociceptive information. Electrical stimulation in the RVM has been shown to result in antinociception.\textsuperscript{65,128–130} Similar to effects shown in the PAG, opioid delivery into the NRM results in analgesia.\textsuperscript{130} Additionally, other pharmacological manipulations of the NRM neurons, such as blocking sodium channels or injecting Trk-B receptor agonists induces analgesia in conditions of chronic pain.\textsuperscript{88,131}

It has been shown that lesions in the NRM disrupt ventral midbrain SPA, increasing the threshold required for analgesia.\textsuperscript{85,132} In addition, RVM lesions also diminish the effects of systemic opioid administration.\textsuperscript{78} However, lesions in the RVM are probably a bit more complex than in the PAG. It has been reported that lesions in RVM are not responsible for the induction of hyperalgesia after neuropathic injury, but for its maintenance.\textsuperscript{88} This idea proposes that the RVM, while being able to induce analgesia via SPA and opioid administration, plays a larger role in the maintenance of chronic pain. This observation suggests that the RVM would be a viable target for the treatment of chronic pain.
<table>
<thead>
<tr>
<th>Reference</th>
<th>Species</th>
<th>Sex</th>
<th>Nuclei</th>
<th>Tracer</th>
<th>Volume</th>
<th>Direction</th>
<th>Specificity</th>
<th>Unilateral vs Bilateral</th>
<th>Inputs</th>
<th>Outputs</th>
<th>Others</th>
</tr>
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<tbody>
<tr>
<td>Wetselaar &amp; Buijsema, 1981</td>
<td>Cat</td>
<td>-</td>
<td>NRM</td>
<td>Horseradish peroxidase (HRP)</td>
<td>2-0.5µL</td>
<td>Retrograde</td>
<td>Midline</td>
<td>Unilateral</td>
<td>vPAG/IPAG, PAG</td>
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<tr>
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<td>dmPAG/IPAG (IPAG=EPAG)</td>
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<td>Bilateral</td>
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<td>Rat</td>
<td>PAG</td>
<td>Fluorogold</td>
<td>60µL</td>
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<td>Bilateral</td>
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<td>Bilateral</td>
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<td>Male</td>
<td>RVLM</td>
<td>Cholera toxin subunit B (CTB)</td>
<td>250µL</td>
<td>Retrograde</td>
<td>Unilateral</td>
<td>Bilateral</td>
<td>vPAG/IPAG, PAG</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Wang et al, 2014</td>
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<td>Male</td>
<td>LVM</td>
<td>Biocytin coated beads (BDN)</td>
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<td>Midline</td>
<td>Bilateral</td>
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<td>Bilateral</td>
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</tr>
<tr>
<td>Francois et al, 2017</td>
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<td>Male</td>
<td>RVM</td>
<td>Cholera toxin subunit B (CTB)</td>
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<td>Midline</td>
<td>Bilateral</td>
<td>vPAG/IPAG, PAG</td>
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<tr>
<td>Urbanova et al, 2017</td>
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<td>Male</td>
<td>RVM</td>
<td>Cholera toxin subunit B (CTB)</td>
<td>300µL</td>
<td>Retrograde</td>
<td>Midline</td>
<td>Bilateral</td>
<td>vPAG/IPAG, PAG</td>
<td>-</td>
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<tr>
<td>Umani et al, 2017</td>
<td>Rat</td>
<td>Male</td>
<td>RVM</td>
<td>Fluorogold</td>
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<td>Midline</td>
<td>Bilateral</td>
<td>vPAG/IPAG, PAG</td>
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<tr>
<td>Namuti et al, 2018</td>
<td>Mouse</td>
<td>Male &amp; Female</td>
<td>VTA</td>
<td>Fluorogold</td>
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<td>Retrograde</td>
<td>Midline</td>
<td>Bilateral</td>
<td>vPAG/IPAG, PAG</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Wang et al, 2019</td>
<td>Rat</td>
<td>Male</td>
<td>vPAG</td>
<td>AA V:Cholera toxin (CT)</td>
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<td>Anterograde</td>
<td>Midline</td>
<td>Bilateral</td>
<td>vPAG/IPAG, PAG</td>
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<tr>
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<td>Mouse</td>
<td>Male</td>
<td>PAG</td>
<td>Cholera toxin subunit B (CTB)</td>
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<td>Midline</td>
<td>Bilateral</td>
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<td>vPAG/DRN</td>
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<td>Bilateral</td>
<td>vPAG/IPAG, PAG</td>
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</table>
In addition to functional similarities between the PAG and the RVM, it’s important to highlight that ON-OFF cells were initially described in the RVM.\textsuperscript{133–136} On cells, rapidly increase their firing frequency approximately 1s after the tail-flick response. Meanwhile, Off cells rapidly decrease their firing frequency right after the tail-flick response.\textsuperscript{39,125} As PAG ON/OFF cells, RVM neurons also display altered (elevated) firing patterns after chronic pain induction.\textsuperscript{137} As in the PAG, these two functionally divergent population of neurons seem to be differently involved in nociception. Interestingly, both of these populations express opioid receptors and markers of inhibitory neurons.\textsuperscript{125}

Ultimately, RVM to spinal cord projections represent the last leg for descending modulation of nociceptive processing from the PAG. This has been extensively shown by both anatomy and physiology studies that not only show the sufficiency of the RVM to inhibit nociceptive information at the level of the spinal cord, but also suggest serotonin as an important neurotransmitter in this process.\textsuperscript{65,123,124,129}

1.11 The role of the spinal cord in nociception

Lastly, at the bottom of the descending branch of the pain matrix, is the spinal cord. Easily confused for just a mere cord to transmit information, the spinal cord is both the location where nociceptive information initially gets processed and where it can get modulated by descending circuits.\textsuperscript{37,38,42} Here, distinct inputs coming from a variety of dorsal root ganglion neurons and can be sorted into different laminae to transmit modality specific information to the appropriate ascending tracts.\textsuperscript{35}

The concept of nociception being process at the level of the spinal cord is an old one and has been characterized as a gate that had some sort of selectivity to what kind of information passes
through, while adapting and modulating nociception accordingly. The gate theory states that sensory information from both small diameter and large diameter nociceptors compete via a set of two different interneurons in the spinal cord.\textsuperscript{138,139} In brief, it proposes that large diameters fibers can silence small diameter afferents (nociceptors) when entering the spinal cord. Meanwhile, under no opposition, small fiber afferents allow for the gate to remain open to all types of information.\textsuperscript{139}

All this is of interest due to mounting evidence that morphine and PAG/RVM SPA effects can be blocked by spinal cord delivery of agents such as lidocaine or modulating monoamine or adrenergic systems, hinting at an interaction all of these circuits.\textsuperscript{140–144} Moreover, both the PAG and RVM have been reported to modulate spinal cord circuitry in what could be described as a fiber-specific fashion. It has been thoroughly shown that spinal cord neurons that receive C-type fiber inputs are preferentially the subset of neurons that are suppressed during PAG or RVM stimulation.\textsuperscript{42,64,118,145} All of these facts suggest that not only the descending pain modulation branch of the pain matrix is capable of suppressing pain, it can also do it in specific way to avoid blocking all sensory information which could be detrimental.

1.12 Recent progress in our understanding of descending pain modulation

As previously described, pharmacology and electrical stimulation have extensively described the role of the PAG and RVM in descending pain modulation, but due to the lack of cell-type specificity, the identity and definitive role of the neurons responsible for stimulation-produced analgesia remains unclear. In addition, electrical stimulation of the vlPAG can result in simultaneous activation of diverse neuron populations and passing fibers. Therefore, limitations such as the inability to target or manipulate specific neuronal subpopulations have resulted in a lack of understanding of their role in descending pain modulation and ultimately hindered the
possibility of targeting subsets of vlPAG neurons for pain management in a clinical setting.\textsuperscript{66,87,94,108,140} Moreover, this information would be useful for developing novel therapies that are effectively targeted to distinct components or neuronal subsets of this important endogenous pain control system.

Harnessing novel techniques such as chemo- and opto-genetics, in combination with genetic mouse models, have allowed us to selectively manipulate neuronal circuits with specific population resolution and finally interrogate the role of these in nociceptive processing. Now, we are able to selectively manipulate distinct vlPAG neuronal populations, precisely defining their role in vlPAG-mediated regulation of nociception and analgesia.

The principle of chemogenetics consists of the utilization of custom engineered G-protein coupled receptors, Designer Receptors Exclusively Activated by Designer Drugs (DREAD), to manipulate the excitability state of neurons in an intact circuit. Human muscarinic acetylcholine receptors were mutated to be solely activated by an otherwise inert ligand, clozapine-N-oxide (CNO), that can be delivered systemically.\textsuperscript{146} In conjunction with a genetic expression system that allows DREADD expression in subsets of neurons based on pre-determined gene promoters, we can then manipulate the activity of whatever subset of neurons we desire. There are two main version of DREADDS: (1) an hM3Dq version which signals through Gq protein signaling and induces increased levels of intracellular calcium and cAMP, and (2) an hM4Di version which signals through Gi protein signaling and induces potassium (G protein-activated inwardly rectifying K\textsuperscript{+}, GIRK) currents and lowers levels of cAMP.\textsuperscript{146} In brief, selective expression of hM3Dq in a subset of neurons allows us to engage in signaling that will result in increased neuronal excitability, while activating hM4Di would inhibit neurons, often via GIRK currents.
On the other hand, optogenetics relies on the principle of using non-mammalian opsins expressed in selective subsets of neurons to alter their ion concentrations and thus depolarizing or hyperpolarizing neurons at will. The most utilized opsin is Channelrhodopsin 2 (ChR2), an algae opsin that serves as a cation channel and allows us to drive neuronal firing following tissue delivery of 473nm light.\textsuperscript{147,148} In contrast to chemogenetics, optical stimulation of neuronal ensembles provides a slight advantage in that we can drive their activity following deliberate patterns like ones recorded from neurons in an \textit{in-vivo} setting in addition to more temporally precise control.

Using the GABA disinhibition hypothesis as our starting point, it’s important to assess the role of both excitatory and inhibitory neurotransmission in the PAG. A precise understanding of descending analgesic circuitry, neuronal subpopulations and the mechanism by which these can modulate chronic pain states may direct future research focused on novel targeted therapies for such disorders in patients. As a result, previous work from our laboratory has attempted to isolate the role of both excitatory and inhibitory PAG neurons.\textsuperscript{149}

We selectively expressed hM3Dq and hM4Di in inhibitory (Vgat+) and excitatory (Vglut2+) vlPAG neurons. Our results showed that stimulation of glutamatergic neurons (hM3Dq-Vglut2) results in elevations of naïve sensory thresholds, while inhibition of glutamatergic neurons (hM4Di-Vglut2) results in a decrease of naïve sensory thresholds (less pain). Conversely, stimulation of GABAergic neurons (hM3Dq-Vgat) results in a decrease of naïve sensory thresholds (more pain), while inhibition of GABAergic neurons (hM4Di-Vgat) results in an increase of naïve sensory thresholds. In brief, we have shown that as had been proposed by others,\textsuperscript{104} increasing PAG GABAergic tone is pro-nociceptive, while facilitating glutamatergic PAG output is anti-nociceptive.\textsuperscript{149,150}
Confirming evidence from others has also shown administering CNO to animals that expressed the hM3Dq DREADD in either excitatory (Vglut2+) or dopaminergic (TH+) PAG neurons, results in anti-nociception. In addition, it has been proposed that although both of these populations, Vglut2+ and TH+, are capable of inducing an anti-nociceptive state, stimulation of Vglut2 PAG neurons is accompanied by anxiogenic effects.

Similar results have been shown regarding vIPAG GABAergic neurons, and how they are posed to modulate anxiety-like behaviors. Similar work has shown that both vIPAG inhibitory (Vgat+) and excitatory (Vglut2+) neurons project to the ventral tegmental area (VTA), and that these projections are responsible for the aversiveness featured as part of animal models of headache.

These approaches have also translated to the more caudal part of this circuit and helped dissect RVM projections to the spinal cord. Although not as extensive as work done in the PAG, important strides have been made in describing both GABAergic and serotonergic NRM neurons that project to the spinal cord. In brief, it has been shown that stimulation of both of these populations result in pro-nociception, in the case of tryptophan hydroxylase positive neurons (serotonergic) they may induce long-lasting sensitization. In contrast, inhibitory GABAergic
Table 1.5. Functional characterization of Periaqueductal gray (PAG) circuitry

<table>
<thead>
<tr>
<th>Reference</th>
<th>Species</th>
<th>Sex</th>
<th>Naive vs Pain Model</th>
<th>Type of Manipulation</th>
<th>Nuclei</th>
<th>Specificity</th>
<th>Manipulation</th>
<th>Unilateral vs Bilateral</th>
<th>Withdrawal Latency (WL)</th>
<th>Withdrawal Threshold (WT)</th>
<th>Others</th>
<th>Assessment</th>
<th>Site of Assessment</th>
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<td>Male</td>
<td>Naive</td>
<td>Opto</td>
<td>RVM</td>
<td>TPH2 Cre</td>
<td>CMr2 - stim</td>
<td>Decreased</td>
<td>Decreased</td>
<td>-</td>
<td></td>
<td>Hot Plate</td>
<td>Hindpaw</td>
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<td>Male</td>
<td>Naive</td>
<td>Opto</td>
<td>vTPAG</td>
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<td>Gq - DREADD</td>
<td>Decreased</td>
<td>Decreased</td>
<td>-</td>
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<td>Opto</td>
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<td>Naive</td>
<td>Chemo</td>
<td>TH Cre</td>
<td>vGAT Cre</td>
<td>CSN2 - stim</td>
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<td>vGAT Cre</td>
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<td>Tdyn</td>
<td>eNpHR3.0; inhibit</td>
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<td>Aversion</td>
<td>Condensed Place Aversion</td>
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<td>Sham</td>
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<td>PFC to vPAG</td>
<td>CAMK2a</td>
<td>Gq - DREADD</td>
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<td>Increased</td>
<td>-</td>
<td>-</td>
<td>Ach. block of vF</td>
<td>Hindpaw</td>
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neurons have been shown to synapse onto enkephalin expressing spinal cord neurons and thus mediating descending facilitation instead of inhibition.\textsuperscript{123}

Lastly, novel approaches allow one to record from selective populations of neurons using genetically encoded calcium indicators (GECIs). This approach uses the expression of GaMP6s in specific cell-types and using an implanted optical fiber, we can measure changes in intracellular calcium activity as a proxy for neuronal activation. In brief, and for the first time, we have been able to take a sneak-peek into serotonergic neurons in both the vPAG and the RVM during acute nociception\textsuperscript{154}. Not to our surprise, both populations seem to be engaged in both heat and acute pinch stimuli, confirming all that the field have been working on for decades, and just highlighting the importance of serotonin expressing cells even further.

1.13 Concluding Remarks

Recent advances in genetics and biophysics have allowed us to take the field of neuroscience further that we even imagined. For example, until recently it was practically impossible to study specific populations of neurons and to ask what role they played in a specific behavioral or physiological scenario with the spatial and temporal resolution that we have nowadays. As a result, the field of neuroscience has started using these novel tools to dissect everything from neuronal biochemistry to the circuits in which these cells are engaged. To our interest, various groups have started revisiting distinct parts of descending circuitry in order to better understand the basic mechanisms of such phenomenon with hopes of discovering new approaches or druggable targets that lead to the development of new therapies for chronic pain.

In terms of descending modulation of pain, and the focus of this dissertation, one of the biggest questions pending regarding PAG circuitry revolves around the role of excitatory versus
inhibitory identity of PAG neurons and how these play a role in descending pain modulation. We hypothesize that PAG SPA is modulated largely by descending glutamatergic (excitatory) projections to the nucleus raphe magnus in the caudal medulla. We propose that manipulation of these neurons is sufficient to alter naïve nociceptive thresholds and to ultimately induce analgesia in a variety of pain models.

In brief we propose that descending excitatory neurotransmission from the PAG represents an important hub of relevance in the study of endogenous neuronal circuits of pain control. Therefore, we believe that further study of such powerful endogenous system could represent the key that we are so desperately looking to develop the new generation of pain therapies, either via pharmacological or genetic approaches.
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Chapter 2: Functional and anatomical characterization of descending periaqueductal gray (PAG) projections and their role in pain modulation

2.1 Abstract

Endogenous analgesic pathways embody an alternative target for the development for chronic pain therapies. Previous studies have demonstrated the role of the ventrolateral periaqueductal gray (vlPAG) in descending pain modulation. It has been proposed that tonic inhibitory (GABAergic) neurotransmission at the level of the vlPAG serves to inhibit efferent excitatory projections that mediate descending analgesia. Disinhibition of these projection neurons allows subsequent activation of rostral ventromedial medulla (RVM) neurons that inhibit nociception at the level of the spinal cord. However, the lack of cell-type specificity in these studies has prevented the determination of the role of specific subsets of vlPAG neurons in analgesia. We have utilized recently developed genetic tools to selectively manipulate and monitor the activity of subclasses of vlPAG neurons to identify the circuit components critical for analgesia. Our data suggest that increasing the activity of glutamatergic (excitatory) or decreasing the activity of GABAergic (inhibitory) vlPAG neurons results in increases in sensory thresholds of uninjured animals, while ameliorating hyperalgesia associated with chronic pain states. In addition, anatomy experiments suggest that excitatory glutamatergic neurons compose the majority of vlPAG efferent projections onto the RVM. Therefore, we hypothesized that excitatory vlPAG-RVM projections are partially responsible for the antinociception and analgesia observed after stimulation of vlPAG neurons and propose that such manipulation is sufficient to induce antinociception and analgesia during chronic pain states. Population-specific fiber photometry, a proxy for monitoring in-vivo neuronal activity, revealed comparable evoked calcium transients in both glutamatergic and GABAergic vlPAG neurons after a variety of acute sensory stimulations, suggesting engagement
of both populations during acute nociception. However, anatomical tracing confirmed that nearly all neurons projecting from vlPAG to the RVM are glutamatergic. Optogenetic stimulation of these glutamatergic vlPAG-RVM neurons resulted in elevation of thermal thresholds of uninjured animals and had no effect on anxiety-like behaviors. In addition, we have demonstrated that stimulation of glutamatergic vlPAG-RVM neurons is sufficient to reverse heat hyperalgesia associated with the induction of an inflammatory state but had little effect in conditions of neuropathic injury. Our results provide experimental evidence supporting the GABA disinhibition hypothesis, highlighting the role of descending glutamatergic neurotransmission at the level of the PAG-RVM as a key component of endogenous analgesic pathways. This work was supported by funds from the Medical Scientist Training Program (MSTP) Grant T32 GM07200 and the NINDS F31NS103472.
2.2 Introduction

It has been reported that as many as 100 million Americans suffer from chronic pain in the United States, leading the reasons of why patients seek medical care and representing a burden of approximately 600 billion dollars.\textsuperscript{1,2} In addition, chronic pain has been associated with a reduction in quality of life, and debilitating co-morbidities that include anxiety, depression and opioid dependence, among others.\textsuperscript{1} However, alternatives for the treatment of chronic pain are few, and mostly consists in medications with considerable side-effects and questionable long-term efficacy. Opioids have been available for several centuries, but their use has surged significantly in the past decades due to the development of opioid derivatives with high analgesic potency.\textsuperscript{3} As a result, an epidemic has developed and has brought to light some of the limitations and dangers of opioids as the standard of care for pain management (i.e. tolerance and abuse potential, amongst others).\textsuperscript{3–5} As a result, the search for alternatives to treat chronic pain using non-opioid or non-pharmacological approaches has led us to revisit the concept of nociception and endogenous modulation, in hopes that a deeper understanding will lead to the development of novel, safer and more effective approaches.

Endogenous mechanisms of analgesia have been studied for decades, shedding light behind a variety of mechanisms behind how the central nervous system (CNS) can induce anti-nociceptive states or provide analgesia in both humans and animal models.\textsuperscript{6–11} The current dogma defines a descending modulatory system that can inhibit nociceptive processing at the level of the spinal cord, and one that is largely responsible for endogenous modulation of nociception in a variety of experimental conditions.\textsuperscript{8,12–19} The GABA disinhibition hypothesis proposes that a major mechanism behind endogenous analgesia relies on the disinhibition of descending excitatory output neurons at various brainstem sites such as the periaqueductal gray (PAG) and the rostral
ventromedial medulla (RVM). It is thought that local inhibitory control at the level of the PAG modulates the activity of brainstem descending excitatory projections that can induce analgesia via recruitment of medullary neurons that project to the spinal cord and can inhibit nociception.\(^{20-25}\)

The PAG has been shown to be involved in multiple types of endogenous analgesia such as stimulation-produced analgesia (SPA), opioid-induced analgesia, and fear/stress-induced analgesia, amongst others.\(^{26,27}\) More specifically, electrical stimulation and lesion studies have highlighted the relevance of the ventrolateral aspect of the PAG in descending analgesia.\(^{11,22,28-40}\) The vlPAG has been shown to possess a large number of opioid-sensitive neurons, and contains a large density of RVM-projecting neurons.\(^{16,41-44}\) An extensive body of work suggests vlPAG modulation of rostral ventromedial medulla neurons is essential for engagement of endogenous analgesic pathways. Yet, the role of distinct PAG neuronal subpopulations and the GABA disinhibition hypothesis have not been able to be fully addressed, largely due to technical limitations. However, due to recent advances in genetics and novel biophysical tools that allow us to manipulate neuronal circuits, such as chemo- and opto-genetics, we can now revisit this important postulate and interrogate important aspects of endogenous analgesic mechanisms that could prove useful for the development of new therapeutic alternatives for pain management.

Recent work from our group has shown that stimulation of glutamatergic (Vglut2+) or inhibition of GABAergic (Vgat+) vlPAG neurons, is sufficient to increase naïve sensory withdrawal thresholds.\(^{45,46}\) Conversely, inhibition of glutamatergic (Vglut2+) or stimulation of GABAergic (Vgat+) vlPAG neurons results in a decreased of naïve sensory withdrawal thresholds.\(^{45}\) In brief, we have shown that glutamatergic and GABAergic PAG neurons represent a bi-directional and divergent system for the control of nociception, confirming the proposed role
of these two populations, not only in terms of neurotransmitter expression but in regard to their function. Our findings support the concept of the GABA disinhibition hypothesis and solidify our belief that inhibitory control at the level of the PAG is an important modulator for endogenous analgesia.

Nevertheless, much has yet to be shown or thoroughly addressed regarding the mechanism behind such a circuit, for example: (1) How are these neural ensembles recruited during nociceptive processing? (2) What is the identity of descending PAG-RVM projections? (3) Is stimulation of PAG-RVM projections antinociceptive or analgesic? (4) Could there be any negative side-effects of stimulating PAG-RVM neurons? Here we utilize a variety of novel genetic tools to address these questions, as an attempt to refine our current understanding of the PAG and its role in descending modulation of nociception.
2.3 Materials and Methods

2.3.1. Experimental Models and subject details

All experiments and animal procedures were submitted and approved by the Animal Care and Use Committee of Washington University School of Medicine. Mice were housed on a 12-hour light/dark cycle (6:00am/pm) and received ad libitum access to food and water. For the duration of these experiments, male littermates (2 - 5 per cage) between 8-12 weeks old were utilized from the following strains: *Slc32a1*<sup>tm2Lowl</sup> (Vgat-ires-Cre), *Slc17a6*<sup>tm2Lowl</sup> (Vglut2-ires-Cre), *Gt(ROSA)26Sor*<sup>em9(CAG-tdTomato)Hze</sup> (Ai9), FVB-Tg(GadGFP)<sup>45704Swn/J</sup> (GAD67-GFP) and C57BL/6<sup>47,48</sup>. Mouse genotyping was done in our laboratory following Jackson laboratories protocols and/or oligonucleotide sequences provided. Behavioral studies were performed with heterozygous animals back-crossed to a C57BL/6J background. Homozygous animals were used for photometry and anatomical experiments. A subset of female animals were used for anatomical tracing using CTB.

2.3.2. Method Details

2.3.2.1. Stereotaxic Surgeries

Prior to surgery, mice were transferred into an anesthesia induction chamber (2% isoflurane), to sedate them and administer Buprenorphine SR (0.1mg/kg) as part of their pre-operative care, while facilitating their placement in the stereotaxic apparatus. Once secured in the stereotaxic apparatus, eye ointment was used to prevent eye dryness and subcutaneous sterile saline (1.0 mL) was administered to help avoid dehydration during the procedure. The pre-shaved surgical site (head) was disinfected with iodine and ethanol before a midline incision was made to
expose the skull. Anatomical landmarks, bregma and lambda, were identified and the skull was leveled both in the anterior/posterior (AP) and medio/lateral (ML) axis. To gain access to the brain, 1mm holes were drilled at the desired coordinates for appropriate delivery of tracers or viruses. We used two delivery methods for our experiments which included using a neurosyringe or Nanoject loaded with a glass capillary and a selection of either tracer or virus to be delivered into the desired site.

For fiber photometry experiments, we injected Vgat-ires-Cre and Vglut2-ires-Cre animals with 300nL of AAVDJ-EF1a-DIO-GCaMP6s, or C57BL/6 with 300nL of AAV8-hSyn-eGFP in the left vlPAG (Coordinates: -4.60mm AP, -0.7mm ML, -2.90mmDV) using a blunt 1µL syringe (Hamilton #80100). Immediately after, a fiber photometry implant was placed 200µm above the injection site (Coordinates: -4.60mm AP, -0.7mm ML, -2.70mmDV). The skull and implant were etched with a surgical scalp and secured using Metabond. Animals were allowed to recover for 4 weeks before behavioral testing.

Retrograde-tracing experiments were performed injecting 300-500nL of recombinant Cholera Toxin Subunit B (CTB) (Thermo Fischer) or Retrobeads conjugated to 488nm or 555nm fluorophores (Lumaflour) into the RVM (Coordinates: -5.85mm AP, 0.00mm ML, -5.85mm DV). For CTB tracing experiments we utilized a combination of (a) CTB 488 injected into the RVM of Vgat-ires-Cre animals crossed to the Ai9 (tdTomato, Vgat-Ai9) reporter to label Vgat+ neurons and, (b) CTB 555 injected into the RVM of GAD67-GFP reporter mice to label Gad67+ neurons, and allowed 7-10 before tissue collection. Lastly, we injected 300-500nL of Green Retrobeads IX (300nL) into the RVM (Coordinates: -5.85mm AP, 0.00mm ML, -5.85mm DV) of Vgat-Ai9, and
allowed 7-10 before tissue dissociation and fluorescence-activated cell sorting (FACS, see details below).

For the purpose of virally targeting RVM-projecting vlPAG neurons for tracing and optogenetic experiments, we injected 100-150nL of AAV2-Ef1a-DIO-eYFP or AAV2-Ef1a-DIO-ChR2-eYFP, in addition to AAV5-hSyn-mCherry (for injection site identification, 2:1 dilution), into the RVM (Coordinates: -5.85mm AP, 0.00mm ML, -6.00mm DV) of Vgat-ires-Cre and Vglut2-ires-Cre animals and allowed 4 weeks for optimal viral expression. For the purpose of achieving intersectional genetic expression of ChR2 in Vglut2 vlPAG that exclusively projected to the RVM, we bilaterally injected 200-300nL of AAV5-nEF-Con/Fon-hChR2(H134R)-eYFP-WPRE into the vlPAG of Vglut2 animals, followed by a midline injection of 400nL of CAV2-Flx-Flp into the RVM.

Using a similar approach as for viral injections, bilateral angled fiber optic cannulas were implanted at the vlPAG (-4.6mm AP, +/- 0.5mm ML 10° degrees, -2.55mm DV) 3-4 weeks after virus delivery for optogenetic manipulation of vlPAG-RVM projecting neurons in Vglut2-ires-Cre animals injected with AAV2-Ef1a-DIO-eYFP or AAV2-Ef1a-DIO-ChR2-eYFP.

2.3.2.2. Viral Constructs

Viral construct expressing AAVDJ-DIO-GCaMP6s (Titer: 8.0x10^{12}CG, Lot #: 4510) was acquired from the Stanford University Viral Core. Viral construct expressing AAV8-hSyn-eGFP (Titer: 8.0x10^{12}vg/mL, Lot #: AV4836F) was acquired from the University of North Carolina Viral Core. Viral constructs expressing AAV2rg-Ef1a-DIO-eYFP (1^{st} Titer: 2.8x10^{12}vg/mL, Lot #: 11-8-17; 2^{nd} Titer: 2x10^{13}vg/ml, Lot #: 3-13-19) or AAV2rg-Ef1a-DIO-ChR2(H134R)-eYFP (1^{st} Titer: 1.6x10^{13}vg/mL; 2^{nd} Titer: 5x10^{12}vg/ml, Lot #: 4-10-19) were acquired from the Washington
University School of Medicine Hope Center Viral Core. Viral construct expressing AAV5-hSyn-mCherry (Titer: $3.4 \times 10^{12}$ vg/mL, Lot #AV5043D) was acquired from the University of North Carolina Viral Core. All viruses were aliquoted into 2-5 µL aliquots and stored at -80°C until needed, after the initial thawing virus aliquots were kept at 4°C for a maximum of 3-5 days. Material transfer agreements have been acquired for each construct.

2.3.2.3. Fiber Photometry

Two LEDs were used to excite the genetically encoded calcium indicator (GECI), GCaMP6s, selectively in Vglut2+ and Vgat+ PAG neurons. Prior to recording, an optic fiber was attached to the implanted fiber using a ferrule sleeve (Doric, ZR_2.5). A 531-Hz sinusoidal LED light (Thorlabs, LED light: M470F3; LED driver DC4104) was bandpass filtered (470 +/- 20nm, Doric, FMC4) to excite GCaMP6s and evoke Ca$^{2+}$ dependent emission. A 211-Hz sinusoidal LED light (Thorlabs, LED light: M405FP1; Led driver: DC4104) was bandpass filtered (405 +/- 10nm, Doric, FMC4) to excite GCaMP6s and evoke Ca$^{2+}$-independent isosbestic control emission. GCaMP6s fluorescence traveled through the same optic fiber before being bandpass filtered (525 +/- 25nm, Doric, FMC4), transduced by a femtowatt silicon photoreceiver (Newport, 2151) and recorded by a real-time processor (TDT, RZ5P). The envelopes of the 531-Hz and 211-Hz signals were extracted in real-time by the TDT program Synapse at a sampling rate of 1017.25Hz. A photometry Analysis Custom MatLab script was developed to analyze fiber photometry data in context to mouse behavior. The isosbestic 405nm excitation control signal was subtracted from the 470nm excitation signal to remove movement artifacts from intracellular Ca$^{2+}$ dependent GCaMP6s fluorescence. Baseline drift was evident in the signal due to slow photobleaching artifacts, particularly during the first several minutes of each recording session. A double
exponential curve was fit to the raw trace and subtracted to correct for baseline drift. After baseline correction, the photometry trace was z-scored relative to the mean and standard deviation of the whole session. The post-processed fiber photometry signal was analyzed in the context of animal behavior. Control animals, expressing AAV5-hSyn-eYFP, were utilized to determine what the contribution of locomotor responses were to the transients observed.

For fiber photometry studies, mice were initially acclimated to their tethered state, using a rotary joint cable, for 7-10 days, in a home cage inside the same room behavior would take place. During the behavioral experiments mice spent 45-60min acclimating before any recording session in clear plexiglass boxes to limit their movement and facilitate the access to their hind paws for nociceptive stimulation. To assess the responses to mechanical stimuli, 4 recording sessions were performed in each animal, with the intensity of the stimuli increasing gradually between session (Order: 0.08g, 0.32g, 1.28g + brush stroke, and pin prick). Each recording session lasted 15 minutes and the animal was presented with 6-8 trials for each stimulus strength, alternating between left and right hindpaw. Animals were allowed to rest 5-10 minutes between sessions. The response to each stimulus, whether they withdrew from the stimulus or not, was recorded in addition to a timestamp of when the stimulus was applied. We also assayed heat (Hargreaves Test, 10-minute session) and cold stimulus (Cold Plantar Assay, 15-minute session). For these experiments, animals were placed in the same plexiglass boxes over a heated (30°C) glass plate for the Hargreaves test and in a ¾’ glass plate for the cold plantar assay; see below for more details on each test. The latency of response to each stimulus, or how long it took them to withdraw from the stimulus, was recorded in addition to a timestamp of when the stimulus was applied and or when they withdrawal occurred. Lastly, we performed the tail-pinch test. The response to a noxious pinch under light anesthesia (1% isoflurane), while the mouse was head-fixed in the stereotaxic
apparatus, was recorded during a 10-minute session. For this test we used a digital Randall Selitto apparatus to apply and record the force applied to different segments of the tail. The response to each stimulus, whether they withdrew from the stimulus or not, was recorded in addition to the magnitude of the force applied and a timestamp of when the stimulus was administered. All behavioral responses were recorded using two different cameras, one facing the animal in question and one from below. Manual timestamps of stimulus application and behavioral responses were corroborated by manual scoring the behavioral responses from the videos.

2.3.2.4. Tissue preparation and immunohistochemistry

Animals were anesthetized with a ketamine cocktail (43% Ketamine HCl 100mg/mL, 43% Xylazine 100mg/mL, 14% Acepromazine 10mg/mL), and allowed to rest until no withdrawal reflex could be observed from a tail pinch. Animals were then placed in a dissection pad, and their heart was exposed for transcardial perfusion of 20mL of PBS 1x, followed by 20mL of 4% paraformaldehyde solution. Brains were then dissected and fixed overnight (10-12hrs) in 4% paraformaldehyde at 4°C. Tissues were then transferred to a 30% sucrose PBS 1x solution for 24-48hrs, or until the tissue sank in the holding container. Brains were then dried and transferred to a tissue mold filled with OCT and let rest until solid in a -80°C freezer (30-45min). Tissue blocks were later sectioned in a cryostat (Leica CM1860) at a width of 45µm and stored as floating sections in a PBS1x 0.1% Na Azide at 4°C on Netwell Permeable Supports (15mm diameter) while covered to protect the samples from photo bleaching. To perform immunohistochemistry of floating sections, we first washed them with PBS1x. Tissues were then incubated for 1 hour at room temperature in blocking solution (2-3mL per well, 5% Normal Goat Serum, 0.3% Triton-X,
PBS1x). Tissue were washed once more and placed in blocking solution with primary antibody for 12-16 hours at 4°C (refer to methods table for antibody concentration). Afterwards, we washed the tissues 3 times in PBS1x for 10min each time. Tissues were then transferred to blocking solution with secondary antibody for 1 hour at room temperature (refer to methods table for antibody concentration). Nuclear or cell body markers like DAPI or Neurotrace were added at this stage. Finally, tissues were washed 3 times in PBS1x for 10min each time, to then be mounted in slides and finished using Vectashield Hardset.

2.3.2.5. Tissue dissociation and fluorescence activated cell sorting (FACS)

Dissociation of PAG neurons was performed using protocols modified from prior published work to improve neuronal survival 49–51. Animals were anesthetized with a ketamine cocktail, transcardially perfused with 20mL of cold aCSF (124 mM NaCl, 24 mM NaHCO₃, 12.5 mM glucose, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 2 mM CaCl₂, 1 mM MgCl₂, 5mM HEPES, pH 7.4, 300-310 mOsm) and decapitated for tissue dissection. The tissue was allowed to rest in cold oxygenated (95% O₂/5% CO₂) aCSF for 5-10min and then it was sliced coronally using a vibratome (Leica VT1000 S). Brain slices (400-μm thick sections) were collected and kept in cold oxygenated aCSF. Tissue slices were micro-dissected under a microscope (Leica S9i) using a 0.5mm biopsy punch (WPI, #504528). Tissue punches were incubated in activated HBSS+H and Papain solution (45U, Worthington) for 10-15min at room temperature. The tubes containing the tissue punches were then transferred to ice to inactivate the Papain, and mechanical trituration of tissue punches was performed using ~600, 300 and 150 μm fire-polished Pasteur pipettes. The resulting cell suspension was then centrifuged at 5k RPM for 5min to obtain a pellet, and cells
were re-suspended in fresh aCSF. This step was repeated twice to wash any remnants of Papain. Cells were ultimately resuspended for FACS sorting in cold oxygenated aCSF and kept on ice for the duration of the sorting. Cells were incubated with 7-AAD 7-Aminoactinomycin D (A1310, Thermo Fisher) to gate for live cells. We performed control experiments to set the appropriate gates for Ai9 (Tdtomato) expression. Negative control samples were obtained from the cortex of c57BL6/J animals, while positive controls were obtained from the striatum of Vgat Ai9.

2.3.2.6. Anatomical Characterization

For validation and anatomical analysis of tissue sections, images were acquired on an epifluorescence microscope (Leica DM6B). Anatomical characterization of PAG to RVM projections were done two-fold, either by using CTB as a retrograde tracer, or using a retrograde viral approach. For anatomical characterization of viral injections, sequential series of images (collected every 120µm) that covered both the PAG (AP -4.23mm to -5.19mm) and the RVM (AP -5.52mm to -6.84mm) were utilized for each animal. For the PAG, we manually superimposed its subdivisions onto the images acquired using an image analysis software. We used a mouse brain atlas to identify regions of interest. Segmentation of the PAG columns was done using DAPI images in an attempt to prevent any bias. We then manually quantified the number of cells located within each column of the PAG. In addition, immunohistochemistry was performed to co-stain for tyrosine hydroxylase (TH) and tryptophan hydroxylase 2 (TPH2). Each channel was annotated separately and the amount of co-labeling between these was annotated and quantified. Only staining that represented clear cell bodies with nuclear (DAPI) staining were selected for analysis. Cells that were on the edge of the drawn subdivisions of the PAG were only quantified if more than half of its volume were within a region. As for the RVM, we imaged both the signals for the
AAV2rg-Ef1a-DIO-eYFP, and also the control AAV5-hSyn-mCherry virus in order to localize our virus injection. This mCherry+ staining was manually mapped onto coronal sections of the RVM and represented as cartoons throughout the paper to portray an approximation of the volume and medulla nuclei occupied by our virus injection. To validate our results, tracer injections of CTB-488 or CTB-555 into the RVM of Vgat-Ai9 or Gad67-eGFP animals were performed, respectively. Representative images from both the rostral (-4.48mm) and caudal (-4.84mm) PAG were selected and the co-labeling between the Ai9 or GFP reporters and CTB was quantified.

2.3.2.7. Optogenetic stimulation

To perform optogenetic manipulations of vlPAG-RVM projecting neurons, we injected AAV2rg-Ef1a-DIO-CHR2-eYFP or AAV2rg-Ef1a-DIO-eYFP and waited 4-6 weeks to allow for maximal expression in PAG cell bodies. Bilateral fiber optics were implanted above the vlPAG of all animals 3-4 weeks after viral injection. Using a 473nm laser, we delivered 5-10mW per implant side to guarantee 10mW/mm² up to a depth of 300µm below the fiber optic implants, ensuring light delivery to our region of interest. Laser stimulation was initiated 10-15s before behavioral testing of a single animal and was ended after one trial of behavior for both paws was performed. Animals were allowed to recover 5-10 minutes between optogenetic stimuli. In addition, animals were allowed to rest 24-48 hours between experimental days. Stimulation parameters (20Hz, 10ms pulses) were modified from the literature and in-vivo vlPAG recordings experiments that include naïve and chronic pain conditions.53,54 A frequency response curve was generated using animals under persistent inflammatory pain (see below for details). We used unilateral wireless opto-electronic devices to drive vlPAG neurons that express ChR2 in glutamatergic PAG neurons that projected to the RVM (Figure S6A-6B).
2.3.2.8. Animal behavior

Throughout the study, animals with fiber optics implants were acclimated to their tethered state for 7-10 days before any behavioral experiment was performed. Tethered animals were allowed to behave freely in clean home cages with food pellets for 30-45 minutes every day. All behavioral experiments, unless noted differently, were performed in clear plexiglass enclosures (4.5’ x 4.5’ x 6.5’) in which animals spent 1-2 hours acclimating before the start of any behavioral assessment. For optogenetic experiments, animals were tested prior to the opto-stimulation (2-3 trials), during the light stimulation and in some cases, after the optical stimulation had ended. Animals were allowed to rest at least 24 hours between behavioral assessments.

2.3.2.9. Nociceptive assessment

2.3.2.9.1 Mechanical nociception – To measure mechanical withdrawal thresholds, I used von Frey hairs in conjunction with the Up and Down Method while resting on a metal grid to provide access to the animal’s hind paws. Testing was alternated between sides and measurements were acquired as averages of 3-5 replicates for each hindpaw using a wide spectrum of filaments (0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.28 and 2.56g). For photometry experiments 3 filaments (0.08, 0.32 and 1.28g) were selected and the percentage response to each one was calculated, 8 trials were performed per filament. To assess dynamic brush, we utilized a ‘000’ brush and ran it across the surface of the hindpaw from the toes to the heel. The percentage response to this stimulus was calculated; 6 trials were performed. Lastly, we used a 22G gauge spinal needle, to prick the hindpaw of our animals and the percentage response to this stimulus was calculated; 8 trials were performed.
2.3.2.9.2 Thermal nociception – To measure the withdrawal latency to a radiant heat stimulus we utilized a plantar analgesia meter (IITC), and we performed the Hargreaves test on animals resting on a heated glass plate (30°C). Baseline light intensity (12-15%) was determined around values that resulted in withdrawal latencies of 8-10 seconds to allow for detection of positive and negative magnitude changes. Paws were alternated and measurements were acquired as averages of 3-5 replicates for each hindpaw. For naïve antinociception assessment, we averaged the latencies for both paws. For CFA-induced heat hyperalgesia experiments (see below), we kept individual paw values separate to use the non-inflamed paw as an internal control.

2.3.2.9.3 Cold nociception – To measure withdrawal latencies to cold nociceptive stimulus, we used the Cold Plantar Assay on animals resting on a ¾” glass plate. Paws were alternated, and measurements were acquired as averages of 3-5 replicates for each hindpaw. To detect cold allodynia associated with neuropathic injury, we used the acetone test on animals with the spared nerve injury (SNI) while resting on a metal grid. A drop of acetone was applied to the hindpaw of animals and the time they spent in nociceptive behaviors (licking, biting, paw lifting, and guarding) were measure for 5 minutes after acetone application. Paws were alternated and an average of the time spent in nocifensive behaviors were measured during two trials on each hindpaw.

2.3.2.9.4 Noxious pinch – Anesthetized photometry recordings were performed in animals secured in a stereotaxic frame. Animals were induced with 2% isoflurane in a chamber and transferred to a stereotaxic unit in which their heads were fixed for safety and to reduce movement artifacts. Isoflurane percentage was the lowered to 1% and animals were allowed to rest for 15min before starting the behavioral testing. We used a digital Randall Selitto (IITC) to pinch the animal’s tail and recorded evoked calcium transients after 8 noxious pinch stimuli for 10 minutes. Pinch sites
throughout the tail were divided in 4 different 1cm sections starting from the most proximal section of the tail. Noxious stimulation was applied starting from the most distal site and continuing more proximal, repeating this twice. Maximal force applied and behavioral response to noxious pinching was recorded.

2.3.2.10 Anxiety-like behaviors

We used an elevated zero maze to measure anxiety like behaviors (Dimensions: height 20’, diameter 24’, walkthrough width 2’). Under low light conditions (50 lux) and constant 20Hz 10ms 473nm light was delivered during the duration of the 6-minute trial. Animals were started in an open arm of the maze and allowed to freely move between the closed and open arms. Using Anymaze’s video tracking software, we measured the time and distance spent in each of the zones to ascertain the anxiety level of our animals.

2.3.2.11 Real-time Place Preference/Aversion

We used a black plexiglass two-chamber apparatus to assess real-time place preference or aversion (Dimensions: 21’x10.5’x10.5’). Using Anymaze’s video tracking software, and a TTL output cable, we were able to confine 20Hz 10ms 473nm laser stimulation only in one side of the apparatus for a 15-minute trial. We measured the time and distance spent in each of the zones to ascertain the preference or aversion level of our animals in naïve and chronic pain conditions.

2.3.2.12 Pain Models

2.3.2.12.1 Persistent Inflammatory Pain – In order to model a state of persistent inflammation, a dose of 20µL of Complete Freund’s Adjuvant (CFA) was injected into the right hind paw of animals that expressed either ChR2 or EYFP in vIPAG neurons that projected to the RVM. The
procedure was done under anesthesia (2% isoflurane) to avoid tissue injury due to movement during administration. CFA was delivered using insulin syringes (29 G1/2). Behavioral assessment was performed 8hrs, 3- and 7-days post administration of CFA. Thermal hypersensitivity was assayed acutely using the Hargreaves test (6-8hrs), mechanical hyperalgesia was assayed at 3- and 7-days post-CFA injection using von Frey hairs (Up-Down method). Contralateral, uninjured, paw was used as a control within experimental groups.

2.3.2.12.2 Neuropathic Pain – In order to model a state of neuropathic injury, we performed the spared nerve injury (SNI) in the right hindpaw of animals that expressed either ChR2 or EYFP in vIPAG neurons that projected to the RVM. The procedure was done under anesthesia (2% isoflurane), it consisted in exposing the sciatic nerve, ligating the tibial and common peroneal branches and removing 1-2 centimeters of nerve tissue distal from the ligature. Behavioral assessment was performed 3-, 5-, 7- and 14-days after injury. Mechanical testing was performed using von Frey hairs (Up-Down method), targeting the sural distribution of the right hindpaw or its most lateral aspect, at 3- and 7-days following injury. Cold allodynia was assayed 5-days after SNI, using the acetone test. Lastly, real-time place preference was then assayed 14 days after neuropathic injury. Contralateral, uninjured, paw was used as a control within experimental groups.

2.3.3. Quantification and statistical analysis

Experimenters were blinded in all behavioral experiments, only to be unblinded during data analysis. Animals were randomly selected for surgeries involving viruses and injuries, using littersmate controls to account for variability between litters. Exclusion criteria for our study included failure to localize viral expression in our animals and/or off-site localization of the probes.
for stimulation or recording. In all of our behavioral assays, at least 3 replicates were collected from all animals and averaged for statistical analysis. The number of animals used in our experiments is indicated by “n” in each experiment. Statistics were performed using PRISM v8.0. All data sets were submitted to a normality test to determine if parametric analysis could be applied, if the data failed the D’Agostino Pearson test, such data sets would then be subjected to a non-parametric analysis. A paired t-Test was used for comparing paired observations, unless normality was not achieved, then we used a Wilcoxon matched pairs test. Two-way ANOVA was used for comparing between groups in terms of virus expression and time. Post-hoc tests were used to compare effects of variables. A value of p <0.05 was considered statistically significant for all statistical comparisons. All data was exported from PRISM GraphPad and handled in Adobe Illustrator for preparation of the manuscript.

2.3.4. Code availability – A similar version of the analysis code for fiber photometry experiments show in Figure 1, S1 and S2 is available online at: https://github.com/BruchasLab.
Table 2.1 List of Materials and Reagents

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**Experimental Models**

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2.4 Results

2.4.1 In-vivo assessment of vlPAG responses to acute nociceptive stimuli

In order to assess the role of different vlPAG neuronal populations in acute nociception, we expressed the genetically encoded calcium indicator, GCaMP6s, in vlPAG neurons to monitor in-vivo intracellular calcium transients as proxy for neuronal activation during a variety of acute nociceptive stimuli. A battery of acute sensory stimuli that included von Frey filaments, brush stroke, pinprick, tail pinch, heat and cold were applied to the hindpaw of animals while recording calcium responses in the vlPAG (Figure 2.1 A). Robust expression of AAVDJ-GCaMP6s in the vlPAG of glutamatergic (Vglut2+) and GABAergic (Vgat+) vlPAG neurons was achieved 4 weeks after viral delivery (Figure 2.1 B, C).

Mechanical stimulation of the hindpaw of Vglut2 Cre animals expressing AAVDJ-DIO-GCaMP6s in the vlPAG (Vglut2-GCaMP6s) revealed a stimulus magnitude dependent response that translated to a robust correlation coefficient between the percentage of times animals responded to a given stimulus and the transient’s area under the curve (AUC, $R^2=0.8413$) or peak amplitude (PA, $R^2=0.8195$) (Figure 2.2 A, B). Representative traces from a single Vglut2-GCamp6s animal undergoing the mechanical stimuli battery, each row represents a single trial.
Figure 2.2 In-vivo assessment of PAG evoked activity upon innocuous and noxious mechanical stimuli. (A) Evoked calcium transients from Vglut2+ vPAG neurons, color coded by the intensity of the stimulus from black (innocuous) to blue (noxious), arrow indicates hindpaw withdrawal. (B) Linear correlation analysis between the percentage response and the area under the curve (AUC) and peak amplitude (PA) of elicited transients (%Response/AUC R² = 0.8413; %Response/PA – R² = 0.8195). (C) Representative heatmap for a single Vglut2 animal undergoing the battery of mechanical stimuli, each row is an individual trial and represents 20s before and 20s after the stimulus application denoted by the white line. Color scale based on Z-score, 4.0 to -1.0. (D) Evoked calcium transients from Vgat+ vPAG neurons, color coded by the intensity of the stimulus from black (innocuous) to magenta (noxious), arrow indicates hindpaw withdrawal. (E) Linear correlation analysis between the percentage response and the area under the curve (AUC) and peak amplitude (PA) of elicited transients (%Response/AUC R² = 0.9158; %Response/PA – R² = 0.9168). (F) Representative heatmap for a single Vgat animal undergoing the battery of mechanical stimuli, each row is an individual trial and represents 20s before and 20s after the stimulus application denoted by the white line. Color scale based on Z-score, 4.0 to -1.0. (G) Behavioral responses to the battery of mechanical stimuli for both Vglut2 (blue) and Vgat (magenta) Cre animals. (H) AUC measurements for the initial 5s of the evoked transient upon mechanical stimulation. (I) PA measurements for the initial 5s of the evoked transient upon mechanical stimulation. (2way ANOVA Multiple Comparison analysis: Comparison vs 0.08g *p<0.05, **p<0.005, ***p<0.0005; vs 0.32g #p<0.05, ##p<0.005, ###p<0.0005; vs 0.32g ^p<0.05, ^^p<0.005, ^^^p<0.0005; vs Brush &p<0.05, &&p<0.005, &&&p<0.0005; Vglut2 n=6, Vgat n=6)
(Figure 2.2 C). Similarly, mechanical stimulation of the hindpaw of Vgat Cre animals expressing AAVDJ-DIO-GCaMP6s in the vlPAG (Vgat-GCaMP6s) revealed a stimulus magnitude dependent response that translated to a robust correlation coefficient when comparing the percentage of times animals responded to such stimulus and the transient’s area under the curve (AUC, $R^2=0.9158$) or peak amplitude (PA, $R^2=0.9168$) (Figure 2.2 D, E). Representative traces from a single Vgat-GCamp6s animal undergoing the stimuli gradient, each row represents a single trial (Figure 2.2 F). The percentage of times animals withdrew from the acute stimulus, the area under the curve and the peak amplitude of the evoked transients were quantified (Figure 2.2 G-I).

In addition, both Vglut2-GCaMP6s and Vgat-GCaMP6s animals displayed robust transients evoked by sensory stimuli such as noxious pinch (tail pinch), radiant heat (Hargreaves Test), and cold (Cold Plantar Assay) (Figure 2.3 A, B). The withdrawal latencies for the different stimuli, such as heat, cold and tail pinch, in addition to the area under the curve and the peak amplitude of the evoked transients were quantified (Figure 2.3 C-F).

To distinguish contributions of sensory input from the motor withdrawal component of evoked vlPAG transients upon acute stimulation, individual trials for a sub-threshold (0.32g) and a supra-threshold (1.28g) mechanical stimulus were sorted into two categories: withdrawal or non-withdrawal. Analysis of Vglut2-GCaMP6s animals displayed no significant differences at a sub-threshold mechanical stimulus, but a significant increase in the AUC of supra-threshold stimulus during withdrawal responses (Figure 2.4 A-C). Analysis of Vgat-GCaMP6s animals displayed no significant differences at a sub-threshold mechanical stimulus, but a significant increase in the PA of supra-threshold stimulus during withdrawal responses (Figure 2.4 D-E).
Figure 2.3 In-vivo assessment of PAG evoked activity upon tail pinch, heat and cold stimuli. (A) Evoked calcium transients from Vglut2+ (blue) and Vgat+ (magenta) vPAG neurons to noxious pinch, arrow indicates hindpaw withdrawal. (B) Evoked calcium transients from Vglut2+ and Vgat+ vPAG neurons to radiant heat and cold, arrow indicates hindpaw withdrawal. (C) Behavioral withdrawal latencies to heat and cold stimulus. (D) Behavioral tail-pinch withdrawal threshold. (E) Area under the curve (AUC) measurements for the initial 5s of the evoked transient upon noxious pinch, heat and cold stimulation. (F) Peak amplitude (PA) measurements for the initial 5s of the evoked transient upon noxious pinch, heat and cold stimulation. (n=6 Vglut2, n=6 Vgat)
Ramping thermal stimuli, in contrast to mechanical punctate stimulus, had potential different time lock options, in terms pairing the evoked transients to either the withdrawal response or the stimulus initiation, each resulting in substantially different results. Time locking the recordings to the behavioral withdrawal response of animals from a thermal stimulus (cold or heat), in both Vglut2-GCaMP6s and Vgat-GCaMP6s, reveals an abrupt and sharp increase in fluorescence that reflects neuronal activation (Figure 2.5 A, B). Meanwhile, time locking the recordings to the initiation of the thermal stimulus (cold or heat), in both Vglut2-GCaMP6s and Vgat-GCaMP6s, reveals a gradual increase in fluorescence that peaks around the time of behavioral withdrawal (Figure 2.5 A, B).
In order to account for confounding effects in our experiments, we performed a number of control experiments. Recording from animals which expressed solely AAV5-hSyn-eGFP in the vlPAG, revealed no autofluorescence artifacts as a response of behavioral withdrawal upon pin prick stimulation (Figure 2.6). In addition, we performed sham stimulations to evaluate any possible conditioned response of Vglut2-GCaMP6s or Vgat-GCaMP6s animals during or after the behavioral testing. For each of these experiments, we waited until the animals had been exposed to the stimuli for the determined testing periods (see methods section), and at the end we presented the sham stimuli. We time locked the recordings at the start of the sham stimuli, which lasted 10 seconds. For mechanical testing, we brought the von Frey filaments near the hindpaw of the animal but stopped short of touching its paw. For heat stimulus, we presented the radiant heat source in the line of sight of the animal without directing it to any part of his body. For cold testing, we brought an empty probe near the area of hindpaw of the animal but stopped short of touching the glass plate. Although no quantification was performed, data suggest that some level of prediction or conditioning takes places in both Vglut2-GCaMP6s or Vgat-GCaMP6s animals, specifically during mechanical
testing. Lastly, to provide validity of our recordings, histological analysis, together with a compilation of the individual trials that were performed during these experiments are shown for both Vglut2-GCaMP6s (Figure 2.8) or Vgat-GCaMP6s (Figure 2.9) animals.

**Figure 2.6 Auto-fluorescence control experiment.** Evoked calcium transients from wildtype animals (C57BL/6J) expressing a reporter vector, AAV5-hSyn-eGFP. Downward arrow indicates pinprick stimulus application. (n=3)

**Figure 2.7 In-vivo assessment of PAG anticipation evoked activity upon stimulus presentation.** (A) Evoked calcium transients from Vglut2+ (blue) and Vgat+ (magenta) viPAG neurons to the presentation of a sham innocuous (0.08g, upper panel) and noxious (pinprick, lower panel) mechanical stimulus, upward arrow indicates the start of a 10s sham stimulus presentation. (B) Evoked calcium transients from Vglut2+ (blue) and Vgat+ (magenta) viPAG neurons to the presentation of radiant heat stimulus (upper panel) and sham cold stimulus (lower panel), upward arrow indicates the start of a 10s sham stimulus presentation. (Vglut2 n=6, Vgat n=6)
Figure 2.8 Compilation of individual photometry trials for Vglut2 vIPAG neurons. Fiber placement map covering approximately 250μm on the vIPAG in the rostro-caudal axis, top left panel. Collection of individual trials for the whole set of nociceptive stimuli applied: 0.08, 0.32 and 1.28g vF filaments, brush stroke, pinprick, tail-pin, heat and cold. Heatmap represents 20s before and after stimulus application. Trials were sorted in descending fashion based on the average amplitude over the first 5s. (n=6)
Figure 2.9 **Compilation of individual photometry trials for Vgat vlPAG neurons.** Fiber placement map covering approximately 250μm on the vlPAG in the rostro-caudal axis, top left panel. Collection of individual trials for the whole set of noxious stimuli applied: 0.08, 0.32 and 1.28g vF filaments, brush stroke, pinprick, tail-pinches, heat and cold. Heatmap represents 20s before and after stimulus application. Trials were sorted in descending fashion based on the average amplitude over the first 5s. (n=6)
2.4.2 Anatomical characterization of vlPAG to RVM projections

In order to characterize the anatomy of descending PAG circuitry, we decided to use viral vectors to identify Vglut2+ and Vgat+ projections from the PAG to the RVM (Figure 2.10 A). To test the feasibility of such approach we injected AAV2rg-Ef1a-DIO-ChR2-eYFP into the RVM of Vglut2+ and Vgat+ animals. Cre-dependent viral tracing revealed a substantial number Vglut2+ vlPAG neurons, while not labeling any Vgat+ vlPAG neurons (Figure 2.10 B, C). To compare injections sites of AAV2rg-Ef1a-DIO-ChR2-eYFP between Vglut2 and Vgat animals, histological reconstructions were made based on the co-injection of a non-Cre dependent AAV5-hSyn-mCherry reporter. Coronal plates of the caudal medulla were utilized to map the spread of the virus injections for Vglut2 (Figure 2.11 A) and Vgat animals (Figure 2.11 B). In addition, tracing experiments revealed glutamatergic medulla inputs from the somatosensory cortex, the paraventricular hypothalamus (PVN), thalamus (Th), zona incerta (ZI) and parabrachial nucleus (PBn) (Figure 2.12 A). GABAergic medulla inputs were observed solely from the amygdala nucleus (Figure 2.12 B).

Figure 2.10 Retrograde tracing of vlPAG neurons that project to the Nucleus Raphe Magnus. (A) Cartoon representation of viral approach to target selectively Vglut2 and Vgat vlPAG neurons that project to the Nucleus Raphe Magnus (NRM). (B) Representative images of both vlPAG (upper and lower left panels) and RVM (upper and lower right panels) of Vglut2 Cre animals injected with 300nl AAV2rg-Ef1a-DIO-ChR2-eYFP in the NRM. (C) Representative images of both vlPAG (upper and lower left panels) and RVM (upper and lower right panels) of Vgat Cre animals injected with 50nl AAV2rg-Ef1a-DIO-ChR2-eYFP in the NRM.

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Figure 2.11 NRM Injection distribution for retrograde targeting of RVM-projecting vPAG neurons with AAV2rg-Ef1α-DIO-ChR2-eYFP. (A) Histologically reconstructed injections sites for AAV2rg-Ef1α-DIO-ChR2-eYFP in Vglut2 Cre animals (n=4). (B) Histologically reconstructed injections sites for AAV2rg-Ef1α-DIO-ChR2-eYFP in Vgat Cre animals (n=6).

Figure 2.12 Additional excitatory and inhibitory projections to the NRM. (A) Representative images from different nuclei that had positive labeling after injecting of AAV2rg-Ef1α-DIO-ChR2-eYFP in the NRM of Vglut2 Cre animals. (B) Representative images from different nuclei that had positive labeling after injecting of AAV2rg-Ef1α-DIO-ChR2-eYFP in the NRM of Vgat Cre animals.
Using AAV2rg-Ef1a-DIO-eYFP to quantify our previous findings, we confirmed that the vast majority of PAG cells that project to the RVM are of glutamatergic identity (Figure 2.13 A). Sagittal sections of the brainstem revealed a dense arrangement of eYFP+ neurons in all of the columns of the PAG (Figure 2.13 B). Quantification of eYFP+ PAG neurons in both Vglut2 and Vgat Cre animals injected with AAV2rg-Ef1a-DIO-eYFP in the RVM revealed that the majority of this circuit is composed of glutamatergic neurons arranged densely in the lateral and ventrolateral aspects of the PAG (Figure 2.13 C). Analysis of the arrangement of eYFP+ neurons in the PAG of Vglut2 animals injected with AAV2rg-Ef1a-DIO-eYFP (Vglut2-eYFP\textsuperscript{PAG-RVM}), revealed higher number of eYFP+ cells in the caudal PAG when compared to the rostral PAG.

![Figure 2.13 Anatomical characterization of vlPAG projections to the NRM.](image)

(A) Cartoon representation of viral approach to target Vglut2 and Vgat vlPAG neurons that project to the Nucleus Raphe Magnus (NRM). (B) Expression of AAV2rg-Ef1a-DIO-eYFP in Vglut2+ neurons in a sagittal cut of the brainstem, inset highlights distribution of cells within the PAG columns. (C) Quantification of eYFP+ neurons in the different PAG columns of Vglut2 and Vgat Cre animals injected with AAV2rg-Ef1a-DIO-eYFP in the NRM (2way ANOVA, Multiple Comparisons, **p<0.005, **** p<0.0001). (D) Quantification of eYFP+ neurons across the anterior-posterior axis, according to coordinates from the Paxino’s Mouse Brain atlas, of different PAG columns of Vglut2 Cre animals injected with AAV2rg-Ef1a-DIO-eYFP in the NRM. (Vglut2 n=8, Vgat n=4)
Figure 2.14 Distribution of vlPAG neurons that project to the NRM. (A) Histologically reconstructed representation of vlPAG neurons for Vglut2 Cre animals injected with AAV2rg-Ef1a-DIO-cYFP in the NRM. (B) Histologically reconstructed representation of vlPAG neurons for Vglut2 Cre animals injected with AAV2rg-Ef1a-DIO-cYFP in the NRM.
Figure 2.15 NRM Injection distribution for retrograde targeting of RVM-projecting sIPAG neurons with AAV2rg-Ef1a-DIO-eYFP. (A) Histologically reconstructed injections sites for AAV2rg-Ef1a-DIO-eYFP in Vglut2 Cre animals (n=8). (B) Histologically reconstructed injections sites for AAV2rg-Ef1a-DIO-eYFP in Vgat Cre animals (n=4). (C) Quantification of the frequency which RVM nuclei were targeted by NRM injections.
Histological reconstruction of the neuronal cell bodies located throughout the PAG of Vglut2 and Vgat animals recapitulates the arrangement and magnitude observed and reported previously (Figure 2.14 A, B). Reconstruction of the injection sites of AAV2rg-Ef1a-DIO-ChR2-eYFP in Vglut2 and Vgat animals, based on the co-injection of a non-Cre dependent AAV5-hSyn-mCherry reporter, reveal comparable spread and infection volume between the two groups (Figure 2.15 A, B). Quantification of the frequency with which RVM nuclei has mCherry positive cells reveals that the gigantocellular reticular nucleus (Gi), gigantocellular reticular nucleus, alpha (GiA), dorsal paragintatocellular nucleus (DPGi), lateral paragintatocellular nucleus (LPGi), medial longitudinal fasciculus (mlf), raphe magnus nucleus (RMg) and raphe obscurus nucleus (ROb) (Figure 2.15 C). Finally, immunohistochemical characterization of Vglut2-eYFP_PAG-RVM neurons expression for known ventral PAG neuronal markers, such as such as tyrosine hydroxylase (TH, dopamine expressing neurons) or tryptophan hydroxylase 2 (TPH2, serotonin expressing neurons), revealed that less than 1% of eYFP+ neurons co-express these markers (Figure 2.13 E, F).
2.4.3 Anatomical characterization of vlPAG GABAergic neurons

Additional tracing experiments were performed to validate our findings that only a small number of GABAergic vlPAG neurons project to the RVM. Histological analysis of tissue following anterograde labeling 3-4 weeks after injection of AAV5-hSyn-DIO-mCherry in the vlPAG on Vglut2 and Vgat Cre animals revealed the presence of terminal-like structures in the RVM of the former, but not in the latter (Figure 2.16 A). Injection of Alexa-555 conjugated cholera toxin subunit B (CTB 555) in the RVM of GAD67-eGFP reporter animals, which express GFP in GABAergic animals, revealed no co-labeling between GAD67-eGFP neurons and CTB 555 labelled neurons (Figure 2.16 C, D). Injection of Alexa-488 conjugated cholera toxin subunit B (CTB 488) in the RVM of Vgat animals crossed to Ai9 reporter animals (Vgat-Ai9), which express tdTomato in GABAergic animals, revealed no co-labeling between Vgat-Ai9 neurons and CTB 488 labelled neurons (Figure 2.16 F, G). Lastly, we attempted an unbiased approach to isolate retrogradely labelled Vgat Ai9 vlPAG neurons with green retro beads (Figure 2.17 A, B). We were able to robustly isolate tdTomato negative, tdTomato positive, and retro bead positive neurons from control animals, while not detecting any vlPAG double positive cells in Vgat Ai9 animals injected with retro beads into the RVM (Figure 2.17 C-F).
Figure 2.17 PAG to RVM anterograde and retrograde tracing. (A) Cartoon representation of viral approach to anterogradely and Cre-dependently express AAV5-hSyn-DIO-mCherry in Vglut2 and Vgat vlPAG neurons. (B) Representative images of vlPAG and RVM sections from Vlglut2 (upper and lower, left panels) and Vgat Cre animals (upper and lower, right panels). (C) Cartoon representation of tracing approach to retrogradely label vlPAG neurons that project to the RVM in GAD67-eGFP reporter animals with cholera toxin subunit B (CTB). (D) Quantification of GAD67-eGFP and CTB-555 co-labeling in the rostral (AP -4.48mm) and caudal (AP -4.84mm) PAG (n=5). (F) Cartoon representation of tracing approach to retrogradely label vlPAG neurons that project to the RVM in Vgat-Ai9 reporter animals with cholera toxin subunit B (CTB). (G) Quantification of Vgat-Ai9 and CTB-488 co-labeling in the rostral (AP -4.48mm) and caudal (AP -4.84mm) PAG (n=3).
2.4.4 Role of Vglut2\textsuperscript{PAG-RVM} neurons in nociception and anxiety

In order to determine the optimal stimulation parameters for Vglut2-ChR2\textsuperscript{PAG-RVM} neuron stimulation, we took an intersectional genetic approach to target RVM-projecting vlPAG cells.\textsuperscript{58,59} We injected Vglut2 animals with CAV2-FlxFlp in the RVM, and AAV-hSyn-Con/Fon-ChR2-eYFP bilateral into the vlPAG. The combination of these viruses allows us to express ChR2 exclusively on Vglut2 neurons that project to the RVM. After 6-8 weeks, we implanted wireless NFC-powered µLED devices to wirelessly drive the activity of ChR2 expressing cells in the left vlPAG (Figure 2.18 A).\textsuperscript{60} After inducing a persistent inflammatory state via bilateral delivery of CFA, we assayed withdrawal latencies from a radiant heat source at different frequencies (0, 5, 10 and 20Hz). Optogenetic stimulation (20Hz, 10ms) of Vglut2-ChR2\textsuperscript{PAG-RVM} neurons was sufficient to significantly increase the withdrawal latency in Vglut2 animals with 20Hz 10ms pulses of
473nm blue light, but not in control or Vglut2 Cre negative animals (Control n=3, Vglut2 ChR2 n=3, 2way ANOVA, p=0.0229; Figure 2.18 B).

Subsequently, we assessed the role of Vglut2<sup>PAG-RVM</sup> neurons in nociception and anxiety-like behaviors. We injected AAV2rg-Ef1a-DIO-ChR2-eYFP or AAV2rg-Ef1a-DIO-eYFP into the RVM of Vglut2-Cre animals and bilaterally implanted fiber optics to optically stimulate RVM-projecting glutamatergic vlPAG neurons using a 473nm laser and 20Hz 10ms pulses (Figure 2.19 A). As part of our nociceptive assessment, we measured cold, heat and mechanical withdrawal thresholds pre- and post-optical stimulation to determine the effects of neuronal activation in nociception (Figure 2.19 B).

Our results indicate that stimulation of Vglut2<sup>PAG-RVM</sup> neurons has no effect in cold withdrawal thresholds as measured by the cold plantar assay (eYFP n=13 Paired t test, p=0.2582; ChR2 n=12 Paired t test, p=0.1318, Figure 2.19 C). However, stimulation of Vglut2<sup>PAG-RVM</sup> neurons was sufficient to significantly increase paw withdrawal latencies to a radiant heat stimulus (eYFP n=12 Paired t test, p=0.7564; ChR2

Figure 2.19 Characterization of optogenetic stimulation parameters for Vglut2 vlPAG-RVM. (A) Cartoon representation of intersectional genetic viral approach to retrogradely target and optogenetically stimulate Vglut2 vlPAG neurons that project to the Nucleus Raphe Magnus (NRM). (B) Frequency-dependent stimulation curve (0, 5, 10 and 20Hz) during the Hargreaves test. Withdrawal thresholds were assessed in CFA-treated animals to detect stimulation-induced analgesia. Vglut2 negative control animals are represented by closed black circles, while Vglut2 positive CreON/FlpON ChR2 expressing animals are represented by blue open circles (n=3).
Figure 2.20 Stimulation of Vglut2 vIPAG-RVM neurons induces heat antinociception. (A) Cartoon representation of viral approach to retrogradely target and optogenetically stimulate Vglut2 vIPAG neurons that project to the Nucleus Raphe Magnus (NRM). (B) Cartoon representation of optogenetic stimulation parameters and nociceptive behavioral assessment for cold, heat and mechanical stimuli. Naïve withdrawal thresholds assessed with the (C) cold plantar assay, (D) Hargreaves test and (E) von Frey filaments for Vglut2 animals expressing either eYFP (black bars) or ChR2 (blue bars) in Vglut2vIPAG-RVM neurons, before and during 20Hz optogenetic stimulation.
**Figure 2.21 Stimulation of Vglut2 vIPAG-RVM neurons has no effect in anxiety-like behaviors.** (A) Cartoon representation of viral approach to retrogradely target and optogenetically stimulate Vglut2 vIPAG neurons that project to the Nucleus Raphe Magnus (NRM). (B) Cartoon representation of optogenetic stimulation parameters and the elevated zero maze, an assessment of anxiety-like behaviors. (C) Cartoon representation of optogenetic stimulation parameters and the real-time place preference/aversion paradigm. (D-G) Analysis of anxiety-like behaviors for Vglut2 animals expressing either eYFP (black bars) or ChR2 (blue bars) in Vglut2 vIPAG-RVM neurons. (H) Trace representation of animal behavior during the elevated zero maze. (I) Analysis of real-time place preference or aversion upon stimulation of Vglut2 vIPAG-RVM neurons expressing either eYFP (black bars) or ChR2 (blue bars). (J) Trace representation of animal behavior during real-time place preference or aversion assessment.
n=16 Paired t test, p=0.0265, Figure 2.19 D). Contrariwise, mechanical withdrawal thresholds were not affected by Vglut2\textsuperscript{PAG-RVM} neurons stimulation (eYFP n=12 Paired t test, p=0.1378; ChR2 n=14 Paired t test, p=0.1394, Figure 2.19 E).

Similarly, we interrogated the effects of stimulation of Vglut2\textsuperscript{PAG-RVM} neurons in anxiety-like behaviors and real-time place preference or aversion. Using an elevated zero maze, we stimulated Vglut2\textsuperscript{PAG-RVM} neurons for a total of 6 minutes, while the animal had free access to the closed and open arms of the maze (Figure 2.20 B). In brief, stimulation of Vglut2\textsuperscript{PAG-RVM} neurons had no effect in total distance (Paired t test, p=0.0905), average speed (Paired t test, p=0.0984), time spent in arms (2way ANOVA, open arm p=0.7511, closed arm p=0.7751) or distance travelled (2way ANOVA, open arm p=0.6366, closed arm p=0.1052) within the different arms (eYFP n=12 & ChR2 n=16, Figure 2.20 D-G). Similarly, optogenetic stimulation of Vglut2\textsuperscript{PAG-RVM} neurons had no effect in the real-time assessment of the preference or aversion of the stimulation (eYFP n=12 & ChR2 n=14, On zone p=0.6231, Off zone p=0.6231; Figure 2.20 I, J).

2.4.5 Role of stimulation of Vglut2\textsuperscript{PAG-RVM} neurons in persistent inflammatory pain

Expanding our assessment of the role of Vglut2\textsuperscript{PAG-RVM} neurons to a persistent inflammatory state, we administered 20µL of intraplantar CFA and tested the analgesic potential of stimulation in inflammation-induced heat and mechanical hyperalgesia (Figure 2.21 A-C). Our results reveal that 20Hz stimulation of Vglut2\textsuperscript{PAG-RVM} neurons can fully recover CFA-induced heat hyperalgesia 6-8 hours after induction (eYFP n=9, ChR2 n=10, 2way ANOVA, *p<0.05, **p<0.005, ***p<0.001). The analgesic effect of stimulating Vglut2\textsuperscript{PAG-RVM} neurons was gone 10 min after stopping the laser stimulation (Figure 2.21 D). No effect was observed
Figure 2.22 Stimulation of Vglut2/vIPAG-RVM neurons is sufficient to induce analgesia in the context of persistent inflammatory pain. (A) Cartoon representation of viral approach to retrogradely target and optogenetically stimulate Vglut2 vIPAG neurons that project to the Nucleus Raphe Magnus (NRM). (B) Cartoon representation of unilateral Complete Freund’s Adjuvant (CFA) injection. (C) Cartoon representation of optogenetic stimulation parameters and nocieptive behavioral assessment for heat and mechanical stimuli. (D) Ipsilateral and (E) contralateral assessment of the effects of optogenetic stimulation of Vglut2/vIPAG-RVM neurons in heat withdrawal thresholds assessed with the Hargreaves test, 6-8 hours after CFA injection. Measurements were collected before, during, and 10min after the end of optogenetic stimulation. (F) Ipsilateral and (G) contralateral assessment of the effects of optogenetic stimulation of Vglut2/vIPAG-RVM neurons in mechanical withdrawal thresholds assessed with von Frey filaments, 3 and 7 days after CFA injection. Measurements were collected before, during, and 10min after the end of optogenetic stimulation.
when stimulating Vglut2\textsuperscript{PAG-RVM} neurons that expressed eYFP in either paw, ipsilateral or contralateral to CFA injection (Figure 2.21 D, E). Assessment of mechanical hyperalgesia induced as a result of CFA administration revealed no effect of stimulation of Vglut2\textsuperscript{PAG-RVM} neurons, in either ipsilateral or contralateral paw 3 or 7 days after induction (Figure 2.21 F, G).

2.4.6 Role of stimulation of Vglut2\textsuperscript{PAG-RVM} neurons in neuropathic pain

Continuing our assessment of chronic pain states, we modeled neuropathic pain in our animals with the spared nerve injury (SNI) model (Figure 2.22 A-C). The SNI induces long lasting mechanical hypersensitivity and spontaneous pain that can be alleviated by analgesics.\textsuperscript{61,62} Our results reveal that 20Hz stimulation of Vglut2\textsuperscript{PAG-RVM} neurons has no effect in mechanical hypersensitivity induced by the SNI model in either control of ChR2 expressing animals, in either ipsilateral or contralateral paw 3 or 7 days after induction (Figure 2.22 C, D). Moreover, we repeated a real-time place preference paradigm to assess whether or not the spontaneous pain associated with neuropathic pain could be relieved and we observed no preferences at 14 days after injury when allowed to roam freely in the real-time apparatus for 15 minutes (Figure 2.22 E, F). Representative trace shown and highlights no differences in locomotor behavior (Figure 2.22 G).
Figure 2.23 Stimulation of Vglut2 vPAG-RVM neurons has no analgesic effect in the context of neuropathic pain. (A) Cartoon representation of viral approach to retrogradely target and optogenetically stimulate Vglut2 vPAG neurons that project to the Nucleus Raphe Magnus (NRM). (B) Cartoon representation of unilateral spared-nerve injury (SNI). (C) Cartoon representation of optogenetic stimulation parameters and nociceptive behavioral assessment for mechanical stimuli. (D) Ipsilateral and (E) contralateral assessment of the effects of optogenetic stimulation of Vglut2 vPAG-RVM neurons in mechanical withdrawal thresholds assessed with von Frey filaments, 3 and 7 days after SNI induction. Measurements were collected before and during optogenetic stimulation. (F) Cartoon representation of optogenetic stimulation parameters and the real-time place preference/aversion paradigm. (G) Analysis of real-time place preference or aversion upon stimulation of Vglut2 vPAG-RVM neurons expressing either eYFP (black bars) or ChR2 (blue bars). (H) Trace representation of animal behavior during real-time place preference or aversion assessment.
2.5 Discussion

During the last couple of decades, the periaqueductal gray (PAG) has been shown to be an important player in descending modulation of pain.\textsuperscript{6–8,19,25,63,64} The PAG has been described in terms of its role in several conditions of endogenous analgesia such as stress-induced analgesia, fear-induced analgesia, stimulation-produced analgesia (SPA), opioid-induced analgesia and even placebo analgesia.\textsuperscript{9,15,23,26,27,36,65} Its connection with the rostral ventromedial medulla (RVM) has been studied and has been characterized as an excitatory monosynaptic projection that engages downstream pathways and lead to antinociception or analgesia.\textsuperscript{22,66–68} However, the specifics of the mechanism by which the PAG can elicit SPA or any of the other form of analgesia, have long eluded us. The theory proposes that disinhibition of descending excitatory brainstem projections is necessary for analgesia to occur, but our efforts to understand the neuronal ensembles that might be mediating a scenario such as SPA, has only proven useful to reveal the heterogeneity that exist within PAG and RVM neurons.

Our current understanding of the role of PAG in nociception is based on two distinct types of cells ON and OFF cells. These were described and characterized by response to a noxious stimulus and subsequent withdrawal, while ON cells increase their firing rate right after the stimulus was applied, OFF cells decrease their firing rate.\textsuperscript{69} However, we do not fully understand how these functional properties arise, or what the significance of maladaptive changes in chronic pain conditions might mean for descending pain modulation. Although we have described the effects of many compounds and manipulations in both PAG and RVM, our knowledge of the components of such circuit remains limited. As a result, we used the GABA disinhibition model to gather a more thorough understanding of how the PAG is involved in nociceptive processing. Therefore, we have set out to expand the body of work surrounding PAG circuitry and its role in
descending pain modulation, specifically in terms of excitatory versus inhibitory neurotransmission at the level of the vIPAG to RVM projection.

Taking advantage of techniques that allow us to isolate specific subsets of PAG neurons, we decided to tease out the functional, anatomical and behavioral contribution of glutamatergic versus GABAergic vIPAG neurons. Initially we isolated glutamatergic (Vglut2+) and GABAergic (Vgat+) vIPAG neurons in an effort to interrogate any differences between how these two major populations were engaged during nociception. We expressed the genetically encoded calcium indicator, GCaMP6s, exclusively in Vglut2 or Vgat positive vIPAG neurons and recorded \textit{in-vivo} population-level calcium responses as a proxy for neuronal activity in response to a battery of sensory stimuli (Figure 2.1).

In order to assess the effects of acute sensory stimuli, we decided to test our animals using punctate mechanical stimuli. After designing a graded mechanical stimuli battery, ranging from innocuous to noxious (0.08, 0.32, 1.28g vF filaments, brush stroke and pin prick), we proceeded to assess the responses of Vglut2 and Vgat vIPAG neurons in real-time. It is important to report that our recording fiber optic was unilaterally located in the left vIPAG, an inherent limitation of our recording paradigm. It has been suggested that PAG circuitry might be lateralized, and data shows that PAG SPA results in stronger analgesia to the contralateral side in reference to the stimulation electrode.\textsuperscript{38,70} We decided to take this into account in our paradigm, thus we sorted the photometry data to interrogate any possible lateralization of evoked activity from either paw while recording unilaterally in the vIPAG. We recorded evoked responses elicited from both left and right hind paws and compared them against each other. Our results demonstrate that there are no differences and that a single side of the PAG faithfully represents vIPAG responses to sensory stimulation (data not shown). As a result, we averaged the evoked responses and discovered that
Vglut2 and Vgat vlPAG neurons exhibited similar responses to applied mechanical stimuli. Interestingly, the activity of vlPAG neurons protrayed a transient size that seemed correlate with the intensity of the stimulus (Figure 2.2 A, D). A linear regression analysis showed a correlation between the area under the curve and the peak amplitude of transients, and the percentage of times an animal responded on average to each stimulus (Figure 2.2 B, E).

Our findings are unexpected, but find support from early work that suggest that the magnitude of PAG electrical stimulation to achieve SPA is proportionate to the intensity of the stimulus applied. We believe that this all suggest that the PAG’s gain can be altered or modified to induced proportionate analgesia according to the situation. Thus, we suggest that the magnitude of sensory input is correlated with the magnitude of the response of PAG subpopulations. Our findings suggest that both Vglut2 and Vgat vlPAG neurons are engaged similarly during the process of acute nociception, and the magnitude with which they are recruited depends on the stimulus being felt.

It is important to highlight that it is not possible to differentiate if the similarities of these responses are due to a comparable but possibly divergent efferent roles of these two populations of vlPAG neurons, or due to incoming afferent signals that target them in a similar fashion. Regardless, our results provide the first piece of evidence that suggest that both glutamatergic and GABAergic vlPAG neurotransmission are engaged similarly during nociception. In addition, we have also shown that Vglut2 and Vgat vlPAG neurons exhibit similar evoked transients to noxious tail-pinche, heat and cold stimuli (Figure 2.3). Including the fact that a similar stimulus magnitude dependent effect was observed with heat stimulation, when increasing the intensity of the radiant heat source (data not shown).
Based on the close link between nociception and withdrawal reflexes, we decided to investigate what the contribution of withdrawal reflexes was on evoked vlPAG calcium transients. We screened and sorted our data to represent events in which a stimulus was applied but no behavioral responses were recorded, and in events in which the application of a stimulus coincided with the withdrawal. Our results revealed that both Vglut2 and Vgat vlPAG neurons exhibit evoked transients just as a result of the stimulus application and suggest that vlPAG responses in both Vglut2 and Vgat vlPAG neurons are independent of a behavioral withdrawal (Figure 2.4). This analysis was performed both at sub-threshold and supra-threshold intensities, exhibiting convincing evidence that even in conditions in which animals responded only 20-25% of the times a stimulus is applied, a transient could be detected. Moreover, systemic administration of morphine showed inhibition of the behavioral tail-pinchoff response but did not fully eliminate the evoked vlPAG transient in either Vglut2 or Vgat vlPAG neurons (data not shown). In brief, we believe that evoked vlPAG calcium transients in both glutamatergic and GABAergic neurons are elicited in large part due to engagement of both populationsvia afferent sensory pathways, but not by the behavioral withdrawal response to a noxious stimulus.

Up to this point, all the evidence presented has been discussed in the context of the application of a punctate stimulus, focal both in space and time. As a result, the analysis presented was time locked to the paw withdrawal, which in most cases coincides with the stimulus application. Although this approach seems to fit for mechanical stimuli, this analysis could be problematic for behavioral paradigms in which the stimulus is in the form of a “ramp” instead. Therefore, we decided to re-analyze the data from the Hargreaves test and cold plantar assay, to evaluate possible differences between time locking our analysis to the behavioral withdrawal and doing the same but to the initiation of the stimulus (Figure 2.5). Out data revealed that in contrast
to a sudden increase as portrayed initially, Vglut2 and Vgat vlPAG neurons exhibit a gradual increase in signal as the stimuli duration increases. This gradual ramping activity can be explained by the temperature transfer that leads to an increase or decrease of skin temperature preceding a withdrawal. Not to our surprise, the eventual plateau of this signal was reached around the time at which a behavioral withdrawal response was recorded. Ultimately, the details of when we time lock these behaviors for the purpose of data analysis will dictate what we can interpret from our data. Focusing on the withdrawal event, we are able to assess what seems as a more uniform population response but lose the real-time changes that precede a behavioral response but that lead to a sensory threshold.

Due to the complexity of our photometry experiments, a series of control experiments were performed as an attempt to account for several possible sources of variability. First, we expressed AAV5-hSyn-eGFP in vlPAG neurons as a control for any contribution of autofluorescence and/or movement artifacts. We performed our most noxious stimulus, pin prick, in a series of animals and were not able to observe any evoked responses (Figure 2.6), suggesting that the transients observed are related to neural activity and not an artifact of movement, for example. Second, we wanted to rule out that prolonged and repeated nociceptive stimulation of animals during behavioral testing might account for conditioned behaviors that could affect our analysis. Thus, we tested for the presence of conditioned responses from mechanical, cold and heat stimuli. This assessment was done via the presentation of a sham stimulus, after animals had exposed to several (6-8) trials of the same type. In the case of the mechanical stimulus, a von Frey filament was presented and approached but it never contacted the hindpaw of the animals. In the case of the cold stimulus, we presented the cold probe, but it never contacted the glass plate where the animal stood. Finally, in the case of the Hargreaves test, we initiated the radiant heat source near the visual field of the
animal while avoiding directing it to any part of the animal’s body. In brief, our results revealed what could represent a slight conditioned response in both Vglut2 and Vgat vlPAG neurons, being more profound for mechanical stimuli (Figure 2.7). However, these experiments were done as part of a series of control experiments and they do not represent an optimal way to measure any sort of conditioned response from the animals and/or vlPAG neurons. In future work, we plan to appropriately assess conditioned responses from vlPAG neurons using a classical cue-paired conditioning paradigm.

While expanding our understanding of how vlPAG neurons are involved in nociception is crucial to shed light into how descending modulation of pain works, our efforts would be incomplete without the context of a circuit. As a result, we used a novel viral approach to label vlPAG neurons that project to the Nucleus Raphe Magnus (NRM) of the caudal medulla. Again, we utilized Vglut2 and Vgat Cre animals to selectively express a retrogradely labeling vector (AAV2rd-Ef1a-DIO-ChR2-eYFP) in excitatory and inhibitory nuclei that projected to the RVM (Figure 2.10 A). Our results revealed that eYFP+ vlPAG neurons could only be observed in Vglut2 Cre, but not in the Vgat Cre animals (Figure 2.10 B, C). Moreover, our experimental approach revealed additional CNS nuclei that project to the NRM. Some of the sources of glutamatergic projections to the NRM include the somatosensory cortex, paraventricular hypothalamus, thalamus, zona incerta and parabrachial nucleus (Figure 2.12 A). Meanwhile, sources of GABAergic inputs to the NRM only included the amygdala (Figure 2.12 B).

Initial experiments were confirmed using a different viral vector for optimal visualization (AAV2rd-Ef1a-DIO-eYFP) and revealed that the significant majority of vlPAG neurons that project to the RVM are glutamatergic and are located in the lateral and ventrolateral columns of the PAG (Figure 2.13 A-C). In addition, characterization of the anterior-posterior distribution
RVM-projecting vlPAG cells, revealed an increase density of glutamatergic vlPAG cells in the caudal PAG (Figure 2.13 D). Histological reconstruction of vlPAG labelled neuron maps showcases how rostral PAG (-4.48mm) contains mostly vPAG labelled neurons, while as you travel to more caudal PAG areas (-4.96mm) the Vglut2+ neurons shift and now localize in the lateral/ventrolateral columns (Figure 2.14). Identification of target sites for our anatomy experiments reveals that among the most commonly infected nuclei you could find: the DPGi, Gi, GiA, LPGi, mlf, RMg, and ROb (Figure 2.15).

Moreover, immunohistochemical analysis of RVM-projecting vlPAG cells revealed low co-expression of two known markers of vPAG neurons, tyrosine hydroxylase (TH) and tryptophan hydroxylase 2 (TPH2) (Figure 2.16 A). Our experiments revealed that the population of glutamatergic RVM-projecting eYFP+ cells in the vlPAG did not co-express either TH or TPH2 (Figure 2.16 B). These findings support previous reports of dopaminergic vPAG neurons being a part of an ascending circuit that projects to the central amygdala (CeA) and the bed nucleus of the stria terminalis (BNST). However, the lack of co-expression of TPH2 suggests that these RVM-projecting vlPAG Vglut2 neurons could represent a subset of descending cells distinct from the serotonergic system. Considering that dorsal raphe serotonergic neurons are located in the caudal medial vPAG region, it can be appreciated how Vglut2 eYFP neurons avoid the medial caudal vPAG area, supporting the notion of a different subset of descending projections (Figure 2.14).

Anatomical findings were validated by multiple approaches that included anterograde and retrograde tracing. We injected AAV5-hSyn-DIO-mCherry into the vlPAG of Vglut2 and Vgat neurons and allowed 4-6 weeks for optimal expression in long-range projections. We observed projections (mCherry+, terminal-like structures) in the rostral ventromedial medulla of Vglut2
animals (Figure 2.16 A, B). In addition, we injected the retrograde tracer cholera toxin B (CTB) as part of a two-way approach to search for inhibitory vlPAG neurons that projected to the RVM. We used transgenic reporter mouse lines for glutamate decarboxylase 1 that expressed eGFP (GAD67-eGFP) and for the vesicular GABA transporter (Vgat Cre animals crossed to floxed tdTomato animals, Vgat-Ai9) together with RVM CTB injections, and our results confirmed that neither of those populations represent a viable candidate for significant inhibitory PAG to RVM projections (Figure 2.16 C-G). Finally, we retrogradely labeled Vgat-Ai9 vlPAG neurons after green retro beads injection into the RVM (Figure 2.17 A, B). Tissue dissociation in conjunction to fluorescence activated cell sorting (FACS) revealed no double positive neurons in the vlPAG (Figure 2.17 C-F). In brief, these results validated our conclusion that the majority of the vlPAG neurons that project to the RVM are of glutamatergic nature, we have not only used a robust viral approach to interrogate such statement, but we have used traditional and novel methods to go further and confirm that the absence of evidence in this case suggest that vlPAG GABAergic neurons comprise a subset of local inhibitory interneurons with minor descending output.16,50,74–77

As a next step, we decided to focus on studying the sufficiency of vlPAG glutamatergic neurons in the context of nociception and analgesia. But before we could perform any behavioral experiments, we decided to try and figure out the optimal parameters for stimulation of vlPAG neurons. It has been recorded in the literature that vlPAG neurons can fire in the range of 10-15Hz under naïve conditions and increase upwards of 20Hz in chronic pain conditions.53,54 Thus, we decided to gain access to RVM-projecting vlPAG glutamatergic neurons and express Channelrhodopsin2 (ChR2) to stimulate these cells and investigate their potential analgesic role in the setting of persistent inflammatory pain. We performed an intersectional genetic approach to express ChR2 dependent under the expression of two recombinases (Cre and Flp) in Vglut2 vlPAG
neurons that projected to the RVM.\textsuperscript{58,59,78} We expressed a retrogradely transported Cre-dependent version of Flipase (CAV2-FlxFlp) in the RVM, and in parallel expressed a Cre-dependent Flp-dependent ChR2 in the vlPAG of Vglut2 animals (Figure 2.18 A). This approach allowed us to manipulate the activity of solely glutamatergic RVM-projecting neurons. Next, we induced a persistent inflammatory state via intraplantar delivery of Complete Freund’s Adjuvant (CFA) and stimulated vlPAG neurons via wireless µLED implants.\textsuperscript{60} Our results show that stimulation of RVM-projecting Vglut2 vlPAG neurons is sufficient to induced analgesia under a state of thermal hypersensitivity in a frequency-dependent fashion, showing maximal efficacy at 20Hz 10ms pulses (Figure 2.18 B). Thus, we concluded that stimulation of vlPAG glutamatergic projection is sufficient to induce analgesia upon 473nm 20Hz 10ms optical stimulation.

Under the premise that stimulation of RVM-projecting vlPAG (Vglut2\textsuperscript{PAG-RVM}) neurons is sufficient to induce analgesia in the context of inflammation, we decided to evaluate the role of Vglut2\textsuperscript{PAG-RVM} neurons in naïve nociceptive thresholds. Using a novel adeno-associated vector we were able to express ChR2 in Vglut2\textsuperscript{PAG-RVM} neurons after 3-4 weeks and just one injection in the RVM of Vglut2 Cre animals (Figure 2.19 A). Animals were implanted with bilateral fiber optics over the vlPAG columns to optogenetically target Vglut2\textsuperscript{PAG-RVM} neurons, and animals were assayed for naïve cold, heat and mechanical sensory thresholds (Figure 2.19 B). Our results show that 20Hz 10ms pulses of 473nm light induced antinociception solely to heat stimuli, with no effects in cold or mechanical stimuli (Figure 2.19 C-E).

In contrast, previous work has shown that electrical stimulation of vlPAG neurons results in antinociception that is not limited to heat.\textsuperscript{11,33,34,36,38,79} This dichotomy could be due to the difference of stimulating a whole nucleus with electricity, which will exert its effects not only a large group of diverse neurons but also passing fibers, over using an approach like ours that limits
the stimulation to a small subset of neurons. Possible sources of passing fibers might originate in nuclei such as the hypothalamus, in which both the lateral and periventricular regions contain a substantial amount of excitatory (Vglut2+) neurons that are consistently retrogradely labelled from the RVM. In our case, we are not only stimulating neurons that are glutamatergic and reside in the vlPAG, but more specifically these neurons project to the RVM. We also have to take into consideration that we are stimulating the cell bodies of Vglut2\textsuperscript{PAG-RVM} neurons without special control over where any action potentials might be directed towards. Therefore, it is also worth mentioning that our results could potentially be mediated via collateral projections of Vglut2\textsuperscript{PAG-RVM} neurons. Glutamatergic PAG neurons have been shown to not only project to the RVM but send projections to areas such as the hypothalamus and the ventral tegmental area, both of which are involved in nociception. This concept is an important one, thus we are considering in our future work to map the collateral projections of these Vglut2\textsuperscript{PAG-RVM} neurons to understand this circuit better.

Additionally, stimulation of Vglut2\textsuperscript{PAG-RVM} neurons did not seem to induce any anxiety-like behaviors in the elevated zero maze or induce preference or aversion states using a real-time place preference/aversion paradigm (Figure 2.20 B-J). These results suggest that stimulation of Vglut2\textsuperscript{PAG-RVM} neurons modulates a single sensory modality and has no negative valence associated with it. Previous reports have shown that stimulation of vlPAG glutamatergic neurons both locally and projecting neurons to lateral aspects of the medulla have a role in anxiety and defensive behaviors.\textsuperscript{44,46} However this can be explained by differences in stimulating a large subset of glutamatergic neurons (via chemogenetics) that could potentially be projecting to multiple nuclei due to the lack of spatial resolution, versus a smaller subset that specifically projects to the NRM. Reports of chemogenetic activation of Vglut2 neurons in the PAG, have implicated these
101 cells in anxiety-like states measured by their behavior open field test. In addition, stimulation of Vglut2 vlPAG neurons that project to pre-motor center in the lateral aspect of the medulla have been shown to be implicated in freezing and defensive behaviors. Interestingly, we see none of these characteristics, suggesting that perhaps our small subset of ventromedial medulla projecting neurons are not implied in such behaviors due to their anatomical properties.

Consequently, we decided to expand our experiments to conditions of persistent or chronic pain models. We induced a persistent inflammatory state via unilateral intraplantar delivery of Complete Freund’s Adjuvant (CFA) and characterized an acute (6-8hr) window of thermal hyperalgesia, followed by up to 7 days of mechanical allodynia. Following optogenetic stimulation of Vglut2\textsuperscript{PAG-RVM} neurons we observed a short-lived (<10min) recovery of thermal thresholds in the CFA treated animals, but no changes in the mechanical allodynia assayed at 3- and 7-days post-CFA in our animals. In terms of neuropathic injury, stimulation of Vglut2\textsuperscript{PAG-RVM} neurons had no effect on the robust mechanical allodynia observed at either 3- or 7-days after the spared nerve injury (SNI). Lastly, we decided to interrogate if stimulation of Vglut2\textsuperscript{PAG-RVM} neurons could alleviate spontaneous behaviors associated with neuropathic injury in animal models. To our disappointment, the real-time place preference experiment revealed that even after injury, stimulation of Vglut2\textsuperscript{PAG-RVM} neurons induces no positive or negative valence state.

In summary, our result show that optogenetic stimulation of Vglut2\textsuperscript{PAG-RVM} neurons leads to an increase in withdrawal latencies to heat stimuli, and to recovery from CFA-induced heat hyperalgesia. We demonstrated that stimulation of Vglut2\textsuperscript{PAG-RVM} neurons has no effect on either cold or mechanical antinociception, anxiety-like behaviors, CFA-induced mechanical allodynia or nerve injury- associated mechanical alldynia. As a result, an open question remains: , Why are
the effects of Vglut2PAG-RVM neuron stimulation specific to heat nociception, and not other modalities?

One possible explanation relies on the observation that PAG stimulation exerts a modulatory effect upon a specific subset of spinal cord dorsal horn neurons. It has been shown that electrical stimulation of the PAG can increase the thresholds to heat stimuli, but in doing so it only alters the response of a subset of class II dorsal horn neurons that uniquely receive C-type fiber input. Moreover, it has been shown that this phenomenon has an anatomical component, targeting cells which are located both at superficial and deeper laminas of the dorsal horn, areas where a C-type exogenous pattern can elicit neuronal responses. In the context of persistent or chronic pain, it is important to note that every model is characterized by a specific pattern of nociceptor involvement. Inflammatory models such as CFA, are characterized by a primary hyperalgesia phase in which C-type fiber are sensitized (heat & mechanical allodynia present), followed by a secondary hyperalgesia phase in which is majorly A-type fiber driven (mechanical allodynia present). On the contrary, in a model of neuropathic injury such as SNI, both C-type and A-type fibers seem to be sensitized in unison. As a result, it seems likely that stimulation of Vglut2PAG-RVM neurons also preferentially modulates spinal cord dorsal horn neurons that receive C-fiber type input, and this could explain why we have a heat antinociceptive phenotype in naive states. We could hypothesize that the analgesic potential of Vglut2PAG-RVM neuron stimulation is limited to inflammatory states during the primary hyperalgesia phase due to its dependence on C-type fiber sensitization. Conversely, secondary hyperalgesia or neuropathic injury is mostly dependent on A-type fiber sensitization and to a minor extent C-fiber sensitization, which could explain the lack of effect of Vglut2PAG-RVM neuron optogenetic stimulation due to an overwhelming nociceptive information being transduced by sensitized A-fibers. Such a mechanism may rely on
survival circumstances where discriminatory sensory processing, proposed to be mediated by A-type fibers, is kept intact while nociceptive processing can be depressed.85

2.6 Conclusions

The role of the periaqueductal gray (PAG) in nociception has been extensively shown and characterized for decades, but it wasn’t until recently that we have been able to answer long-standing questions about physiological phenomena that couldn’t be addressed in animal models due to technical limitations. Our work has not only shown direct evidence of the involvement of a divergent set of neurons within the vlPAG in acute nociception but has characterized their role in a variety of conditions and modalities. We believe that our results extend past the concept of the GABA disinhibition hypothesis by suggesting that inhibitory and excitatory neuronal populations in the vlPAG co-participate to coordinate a diversity of behaviors related to acute nociception such as defensive behaviors, withdrawal and conditioning. Furthermore, our characterization of the anatomy of the vlPAG to RVM circuit, has shed light into the controversy regarding the identity of these projections. Lastly, and probably more importantly, our data supports the notion that PAG modulation can indeed modulate different sets of spinal cord dorsal horn neurons and lead to modality specific effects. As a result, we believe that our study does indeed extend our knowledge of what we knew about the PAG’s involvement in descending modulation of nociception. And further promotes the idea of the importance of re-visiting relevant circuits in important topics such as in pain, to attempt and learn more information that could potentially develop into druggable targets or novel approaches for the treatment of debilitating conditions such as chronic pain.
2.6 Acknowledgements

We would like to acknowledge the contribution of Dionett Bhatti for providing artistic support. In addition, we would like to recognize the support and help of all the members of the Gereau laboratory. Special thanks to Dr. Judy Golden for her support, mentorship and guidance throughout the duration of my training in the Gereau lab.
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