TRANSMISSION OF NOXIOUS INFORMATION TO A BRAINSTEM PAIN-MODULATING CIRCUIT

By

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A DISSERTATION

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LIST OF ABBREVIATIONS

aCSF	Artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ArchT	archaerhodopsin
CeA	Central nucleus of the amygdala
CFA	Complete Freund's adjuvant
CGRP	Calcitonin gene-related peptide
ChR2	Channelrhodopsin-2
DMH	Dorsal medial nucleus of the hypothalamus
EKG	Electrocardiograph
EMG	Electromyograph
FG	Fluoro-Gold
GABA	γ-Aminobutyric acid
KF	Kölliker-Fuse nucleus
NK1	Neurokinin 1
NMDA	N-methyl-D-aspartate
NSAIDs	Nonsteroidal anti-inflammatory drugs
NTS	Nucleus tractus solitarius
PAG	Periaqueductal gray
PB	Parabrachial nucleus

RVL	Rostral ventrolateral reticular nucleus
RVM	Rostral ventromedial medulla
SAP	Saporin
SCP	Superior cerebellar peduncle
SNL	Spinal nerve ligation
SP	Substance P
vF	von Frey fibers

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ABSTRACT

Pain is a prevalent condition with high costs to the individual and society, and profound physical, psychological, and socioeconomic impacts.[142] An important contributor to both normal and clinically significant persistent pain is an intrinsic pain-modulating system, which regulates nociceptive processing via projections from the brainstem to the dorsal horn. The output of this modulating system, the rostral ventromedial medulla (RVM) can facilitate or suppress nociceptive processing at the level of the dorsal horn. This bidirectional control is mediated by two physiologically defined RVM cell classes, “ON-cells” and “OFF-cells,” that respectively facilitate and inhibit dorsal horn nociceptive transmission. A defining characteristic of these pain-modulating neurons is a change in their activity in response to noxious stimulation: ON-cells are activated, leading to a “burst” of activity associated with behavioral responses to noxious inputs, whilst OFF-cell firing is suppressed, producing a “pause” in any ongoing activity. This nociception-related response of pain-facilitating ON-cells and pain-inhibiting OFF-cells has been implicated in pathological pain states. Surprisingly, the pathway through which noxious inputs reach RVM has not yet been defined. The parabrachial complex (PB) is a major nociceptive relay that has direct and indirect anatomical connections with RVM. The first aim of this thesis was to examine whether PB conveys nociceptive information to RVM. The second aim was to explore how the interaction between PB and RVM varies as acute inflammation develops into a persistent pain state.

Under basal conditions, blocking PB contralateral to the stimulation significantly attenuated the ON-cell burst and OFF-cell pause. Inhibition of the PB terminals in RVM mimicked this effect. Together these data indicate that PB is a major relay through which nociceptive information from the contralateral side of the body reaches the RVM to influence activity of pain-modulating neurons, and that PB conveys these pain-related signals to RVM at least in part through a direct projection.

In acute and persistent inflammation, RVM pain-modulating neurons are “sensitized,” responding to normally innocuous stimuli, which increases behavioral responsiveness and presumably limits further damage. In acute inflammation, blocking PB contralateral to the site of inflammation suppressed the nociception-related activity of both ON- and OFF-cells, as under basal conditions. In persistent inflammation however, the role of contralateral PB in conveying nociceptive information to RVM was greatly reduced, and PB *ipsilateral* to the inflammation became the major relay of nociceptive information to RVM. However, lesions of contralateral PB prior to inflammation prevented recruitment of the ipsilateral PB, and RVM ON- and OFF-cells failed to develop sensitivity to subthreshold stimuli despite the persistent injury. These results indicate that ipsilateral and contralateral PB input have distinct roles in adapting to a prolonged injury and make distinct contributions to sensitization of RVM in persistent pain.

Collectively, the data in the current thesis establish a functional link between PB and RVM. My findings further detail the process through which inputs from PB sensitize and alter RVM ON- and OFF-cell activity during the transition from acute to persistent pain, shifting the balance between these two cell classes at different time points as inflammation develops. The dynamic interaction between PB and RVM is likely adaptive, while maintaining enhanced responsiveness in the injured area, while limiting the development of more widespread, maladaptive chronic pain. This work thus provides a greater understanding of the interaction between the pain-transmitting system and the pain-modulating system, and could aid in developing novel treatments with fewer side effects for clinical pathological pain.

CHAPTER 1

INTRODUCTION

1.1 OVERVIEW

Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, serving as a vital survival mechanism for an organism to rapidly learn and alter its behaviors. The magnitude of pain sensation evoked by a given stimulus can be influenced by genetic background, stimulus history, and behavioral state. Nociception, the neural system encoding and transmitting noxious or potentially harmful stimuli, is under tight regulation, and can be either inhibited or facilitated, depending on the emotional state and behavioral priorities of the organism. One example of inhibition of pain and nociception (analgesia) in prioritization of survival is that of an alerted prey escaping from its immediate predatory threat despite an injury. This phenomenon is described as "stress-induced analgesia." On the opposite end of the spectrum, pain can be enhanced (hyperalgesia) in instances of illness or prolonged stress, sometimes independent of physical injury. The organism in such cases is in a hypersensitive state such that even innocuous touch is perceived as painful. This hypersensitivity can be adaptive: it prevents excess physical activity, which allows the body to rest and recover, as well as memory formation and learning of behaviors to prevent further damage. This bidirectional modulation of pain is made possible by a complex neural network involving many levels of the nervous system, including ascending nociceptive input from the periphery and the descending influence of higher circuits. Many of these inputs eventually converge at a brainstem structure called the rostral ventromedial medulla (RVM).

The RVM is the output node of a pain-modulating system that processes various inputs and modulates nociception via its projections to the dorsal horn of the spinal cord. It receives information from higher structures such as the hypothalamus and amygdala, providing a circuit through which cognitive and emotional factors can influence pain. The RVM also responds to peripheral noxious stimulation, changing its activity in such a way that responses to subsequent inputs are facilitated.[87; 99] This response thus contributes to sensitivity to nociception, and

may play a role in pathological pain. *Surprisingly, the pathway through which noxious inputs drive changes in RVM activity has not yet been defined.*

This thesis addresses this gap in our knowledge, by examining the circuit through which noxious information is relayed to the pain-modulating neurons of the RVM, and the plasticity of this circuitry in states of persistent inflammation. In this work, I identified the parabrachial complex (PB), an important nociceptive, emotional, and autonomic processing and relay center, as a major input to the RVM neurons, at least in part through its direct projection to the RVM. Furthermore, the influence of PB on RVM neuronal activity is dynamic, varying as acute inflammation persists over a period of days, and undergoing plasticity in persistent pain, as neurons become sensitized to additional nociceptive inputs. I conclude that PB is a major nociceptive input to the RVM pain-modulating neurons. These data also demonstrate an important role in RVM plasticity in persistent inflammation. This work thus furthers our understanding of the fundamental mechanisms of brainstem nociceptive modulation, and may ultimately make it possible for therapies to target this important pain-modulating circuit to treat pathological pain.

1.2 ROSTRAL VENTROMEDIAL MEDULLA AND THE MODULATION OF NOCICEPTION

Current pharmacological treatments for chronic pain have limited efficacy and undesirable side effects, particularly when used long-term.[142] Relief of acute pain is relatively straightforward, ranging from over-the-counter pain relievers, such as nonsteroidal anti-inflammatory drugs (NSAIDs), to prescription drugs, such as opioids, which can provide powerful pain control. Management for chronic pain conditions, on the other hand, is much more complicated. Many chronic pain conditions are without apparent underlying physical injury, or develop after the primary injury has recovered, making targeted treatments or surgical interventions difficult. Pharmacological therapies used in acute pain are typically less effective in chronic pain conditions. Opioids, one of the most powerful class of analgesics, become less

effective in managing pain symptoms when used long-term, and the accompanying side effects, such as constipation, respiratory depression, and dependence, become more severe. Other centrally acting drugs, such as antidepressants (e.g. serotonin-norepinephrine reuptake inhibitors) and anticonvulsants (e.g. $GABA_A$ receptor modulators), have shown benefits in treating some pain conditions, but these are also counterbalanced by undesirable side effects. These clinical caveats highlight the complexity underlying the pathologies of the pain-related neural circuits in chronic pain conditions.

There is now increasing evidence that pathological pain states are, at least in part modulated, by the brainstem intrinsic pain-modulatory system. This descending modulatory system is known to mediate top-down regulation of nociceptive processing, transmitting cortical and limbic influences to the dorsal horn. RVM, the key output node in this modulating system, controls the perception of pain and the detection of potentially damaging stimulation. Its pain-inhibiting output is crucial for morphine-induced analgesia,[3; 77] and its pain-facilitating output has been implicated in contributing to various chronic pain conditions.[75; 81]

1.2.1. Anatomy of a major descending pain-modulating pathway

Painful stimuli are transmitted to the central nervous system via peripheral C- and Aδ-fibers that synapse onto spinal dorsal horn neurons. The majority of the axons from these secondary neurons cross the midline at the level of spinal cord, and project to supraspinal targets. Conversely, supraspinal structures project diffusely throughout the spinal cord, where they control the activity of dorsal horn neurons and modulate nociceptive transmission (Figure 1). Extensive work over the past 30 years has gone into characterizing the anatomy and physiology of this descending pain modulatory system. Stimulation of specific brainstem structures, such as periaqueductal gray (PAG), inhibits dorsal horn neurons.[5; 6] However, PAG does not produce analgesia directly, but rather through the RVM. The RVM, primarily encompassing the area of the raphe magus and surrounding reticular region, has been established as the major

output node in descending modulation of nociception. Its relevance in modulating nociception was originally based on analgesia achieved by pharmacologically or electrically stimulating the region.[5; 47; 208] The RVM sends diffuse bilateral projections to the spinal dorsal horn at multiple levels where it modulates the activity of nociceptive dorsal horn neurons.[114-116]

Based on the early stimulation studies, the PAG-RVM circuit was classically viewed as part of a descending pain-inhibitory pathway.[5; 57; 125; 190; 208] However, studies later revealed that RVM modulates nociception in a bidirectional manner.[55 ; 81; 156; 157] While stimulation of RVM produces a net inhibition of nociception and behavioral analgesia, RVM neurons also facilitate nociception and hyperalgesia in some conditions.[104; 122; 143]

1.2.2. Physiology of the rostral ventromedial medulla

The facilitatory and inhibitory effects of the RVM are mediated by the output to the spinal dorsal horn from two classes of neurons, ON-cells and OFF-cells.[76] These pain-modulating neurons receive nociceptive input, and are characterized by their nociception-related responses to noxious stimuli: ON-cells increase firing, creating a "burst" of activity, while OFF-cells abruptly "pause" firing in a reciprocal manner(Figure 2).[58] There is a third class of neurons in the RVM are called “NEUTRAL cells,” which do not alter their activity in response to noxious stimuli. Although they also project to the superficial dorsal horn, like ON- and OFF-cells, their role in pain modulation, if any, remains undetermined.[75]

The ON-cells are responsible for descending facilitation of nociception. They exhibit a "burst" of firing at the withdrawal, and increase nociception and sensitivity to subsequent stimulation. ON-cell activity mediates the pain-facilitatory component of RVM output. Increased ON-cell firing is associated with animal models of inflammatory and neuropathic pain.[54; 81; 104; 143; 206] Selective activation of ON-cells (e.g., with focal application of a low dose of the neuropeptide neurotensin) is sufficient to cause hyperalgesia.[143] Furthermore, it has been shown that ON-cells are the only cell-type in the RVM that expresses μ-opioid

receptors, and administration of morphine directly suppresses ON-cell activity, contributing to an analgesic effect.[3; 56; 80]

The OFF-cells, on the other hand, are responsible for descending inhibition of nociception. In the presence of noxious stimuli, OFF-cells cease any ongoing activity prior to the withdrawal. This "pause" of OFF-cell firing has been shown to be necessary for behavioral withdrawal. Administration of morphine indirectly increases the ongoing firing of OFF-cells and abolishes the nociception-related pause, which leads to analgesia.[56] This effect is mediates by its descending protection to the dorsal horn, and increased OFF-cell ongoing activity is associated with analgesia.[56; 60] Furthermore, complete elimination of the OFF-cell pause, with improgan or morphine, will produce analgesia independent of the changes in ON-cell activity.[77; 78] This indicates that OFF-cell activity is a major determining factor for behavioral sensitivity of the animal.

ON- and OFF-cell activity is usually antiphase-synchronized under unstimulated conditions, having alternating periods of silence and activity.[4] This means during the ON-cell active phase, the OFF-cells are silent. The nociception-related ON-cell burst and OFF-cell pause are also antiphase-synchronized in response to noxious stimulations.[59] Thus, their output modulates the nociceptive transmission at the level of spinal dorsal horn in a parallel fashion, where the withdrawal threshold for the organism is determined by their ongoing and nociception-related activity collectively.[73; 75] However, it is possible in some conditions that expression of behavioral hypersensitivity is independently mediated by an increase in ON-cell firing or a decrease in OFF-cell pause threshold.[35]

NEUTRAL-cells are physiologically different from ON- and OFF-cells, as their activity does not alter in response to nociceptive inputs or withdrawal behaviors. Their exact function, if any, in pain-modulation is unknown. κ-opioid receptors are found on both NEUTRAL- and OFF-cells, and activation of these receptors suppresses hyperalgesia associated with inflammation.[71;

176; 199] A few studies have also demonstrated a subset of NEUTRAL- and ON-cells in the RVM express serotonin,[158, Gau, 2013 #526; 199] and serotonin outputs from the RVM region have been suggested to modulate nociception in dorsal horn neurons.[1; 69; 158] However, NEUTRAL-cells have not been shown to modulate nociception independently. Furthermore, some studies have found conflicting evidence that blocking serotonin with ondansetron has no anti-nociceptive effect in animal models of neuropathy and patients with neuropathic pain.[152; 186] It is thus unclear whether NEUTRAL-cells have any role in pain-modulation.

1.2.3. Inputs that drive ON- and OFF-cell activity

Activity of ON- and OFF-cells can be influenced by "top-down" inputs from higher structures, such as dorsal medial hypothalamus (DMH) and central nucleus of amygdala (CeA), which can alter the output modulatory "tone" in response to stress and fear.[81; 122; 126; 127] However, the characteristic ON-cell "burst" and OFF-cell "pause" are mainly driven by ascending nociceptive inputs. The rapid change of ON- and OFF-cell activity in response to a noxious stimulus has an important physiological value, because this acute, pro-nociceptive shift in RVM output functions as a positive feedback loop, promoting responses to subsequent stimuli that are potentially damaging.[86; 99] Furthermore, it is now recognized that activation of the ON-cell dominant pro-nociceptive positive feedback loop in response to injury or inflammation contributes to pathological pain states.[35; 68; 156]

Recent reports suggest RVM OFF-cell output is an important inhibitory drive in suppressing pain-related behaviors in persistent injury. Blocking RVM potentiates hyperalgesia in models of persistent inflammation, suggesting RVM output is dominated by OFF-cell inhibitory ongoing activity.[35; 43] However during persistent inflammation, these OFF-cells also respond to previously innocuous stimulation, suggesting that sensitization of OFF-cells and nociceptive input are important in maintaining behavioral hypersensitivity during a prolonged injury.[26; 35] Thus, it is crucial for us to understand how ON- and OFF-cells are activated by noxious inputs.

Despite over 30 years of research on descending modulation, the ascending pathways and brain relays through which noxious inputs drive changes of RVM ON- and OFF-cells have not been defined. *The first goal of this thesis is to identify the pathways through which noxious stimuli gain access to this RVM pain-modulating circuitry.*

There are a number of possible routes through which nociceptive information could reach RVM. Direct inputs to the RVM have been identified from the medullary dorsal horn,[179] but spinoreticular projections to RVM have been considered sparse.[30] Indirect pathways through higher structures such as the insula and amygdala are also a possibility.[11; 23; 96; 127] However, the ON-cell burst and OFF-cell pause are observed in decerebrated animals, indicating that diencephalic and telencephalic structures are not required for noxious information to reach RVM neurons.[34] Another possible candidate is the parabrachial complex, which is the focus of this thesis.

1.3. PARABRACHIAL COMPLEX AS MAJOR INPUT TO THE RVM

1.3.1. Anatomy of the parabrachial complex

The parabrachial complex (PB) is a functionally and anatomically complex structure involved in a wide range of homeostatic functions.[62; 139; 140] It encompasses the area surrounding the superior cerebellar peduncle (SCP) in the pontine midbrain. PB is an elongated structure has three major divisions: the medial PB , the lateral PB , and the Kölliker-Fuse nucleus (KF), which is a ventrolateral extension at the ventral tip of the SCP. These major divisions are further divided into different subnuclei based on cytoarchitecture.[62] Afferent connections to PB correlate closely with the major divisions, which hint at their general functions. The medial PB primarily receives projections from the gustatory portion of the nucleus of the solitary tract (NTS),[146] which is consistent with its functions in taste and salt homeostasis by relaying taste information to the thalamus and cortical areas.[62; 160; 168]

In contrast with the lateral and medial PB, the lateral PB and KF receive common inputs. Innervation from the NTS, makes them a major relay center for cardiovascular, respiratory, and other homeostatic information.[27; 28; 40; 42; 62; 72; 168; 173] Both regions send efferent projections to higher structures, such as amygdala and hypothalamus, as well as other brainstem structures, such as PAG, RVM and adrenergic regions.[16; 62; 173] These anatomical findings support the idea that PB and KF are important structures for homeostatic function (e.g. thermoregulation, cardiovascular regulation) as well as nociception.[33; 52; 107; 132; 139; 140; 195] Additionally, KF provides efferent projections to ventrolateral medulla and NTS, and has been implicated in having important functional roles in modulation of respiration.[16; 44; 50; 62; 100; 173; 198] Thus to avoid functional overlaps, the current thesis will focus mainly on lateral PB.

1.3.2. The role of parabrachial complex in nociception transmission and pain modulation

PB also receives, processes, and relays nociceptive signals.[63] It is the primary supraspinal target of nociceptive transmission neurons with cell bodies in the contralateral lamina I and lamina III of the dorsal horn. PB also receives sparse projection from the ipsilateral dorsal horn, but the functional role of these ipsilateral projections has not been investigated.[2; 20; 40; 53; 183] Many of the PB projecting neurons in Lamina I/III express the NK1 receptor (NK1R), a receptor for substance P.[46; 183] Substance P (SP) is a neuropeptide that is released in spinal dorsal horn by primary afferents upon a noxious stimulation.[49; 109] Together, these data suggest that dorsal horn neurons, spinoparabrachial pathway, and PB are part of an important axis in nociceptive signal transmission. Indeed, nociceptive neurons have been identified in the PB, primarily in lateral PB and KF.[11; 13; 85; 92; 131]

Functional studies have linked PB to major pain-related functions. It has been implicated in emotional and autonomic aspects of pain,[10; 70; 163] as well as in modulation of nociception.[33; 107; 132] PB projects to the midbrain PAG, part of the brainstem pain-

modulating system.[63] A direct projection from the PB to the RVM has also been identified, but this has not been investigated in any detail.[8; 84] Studies using SP-Saporin (SP-SAP) to lesion NK1R-positive spinal neurons demonstrated impaired serotonergic descending modulation, presumably through RVM, on dorsal horn nociceptive neurons in both healthy animals, and in animals with persistent inflammation.[103; 180] However, these studies suffer a major limitation of not testing PB directly. Specifically, lesion of all NK1R-positive neurons may affect other non-spinoparabrachial pathways,[121] as well as the intraspinal circuits themselves. Thus, these data do not demonstrate a functional connection between PB and RVM. *Aim 1* of this thesis is to determine the contribution of PB to the activity of RVM ON- and OFF-cells using a combination of pharmacological tools and in *vivo* single-cell recording, and to delineate the functional role of the direct projection from PB to RVM using optogenetic methods.

1.4 Plasticity of the pain modulation network in acute and persistent pain

The PB input to RVM may also play an important role in the transition from acute to persistent pain. Ablation of NK1R-positive neurons in the superficial dorsal horn, a major nociceptive input to PB, resulted in the inability to develop and maintain persistent hyperalgesia in models of inflammatory injury.[103; 145; 164; 180] When dorsal horn neurons were recorded in these animals, they lacked the increased responsiveness, or sensitization, to noxious stimulation that is normally associated with inflammatory injury.[180] Khasabov et al. demonstrated that NK1R-positive neurons can influence the activity of dorsal horn nociceptive neurons through engaging a descending modulatory pathway.[103] However, direct evidence for the PB-to-RVM connection in modulating acute and persistent pain is still lacking. *Aim 2* of this thesis is to explore the mechanistic timeline of how input to RVM from PB influences ON- and OFF-cell activity in acute and persistent pain.

1.4.1. Descending modulation in acute and persistent pain states

The responsiveness of dorsal horn nociceptive neurons is modulated by the activity of RVM ON- and OFF-cells, and can be altered during various pain states. As mentioned previously, RVM neuronal activity is mainly classified by two categories: the ongoing activity, and the nociception-related activity. The ongoing firing is measured as the average firing rate in a period without any stimulation. It is the output of ON- and OFF-cells at the onset of stimulation that plays a role in setting the behavioral threshold to withdraw, such that an increase in ON- and decrease in OFF-cell ongoing firing leads to hyperalgesia, and a decrease in ON- and increase in OFF-cell firing leads to hypoalgesia.[73]

The functional implication of nociception-related activity is less straightforward. ON-cell nociception-related “burst” is typically measured as the total number of spikes within the "burst" period, although other parameters have also been used (e.g. peak firing rate). The magnitude of the ON-cell response is positively correlated with the magnitude of the behavioral response.[99] On the other hand, the OFF-cell “pause” can be quantified as the duration of the pause. The duration of the pause could be interpreted as the period over which the animal remains vigilant for subsequent stimuli, because the absence of OFF-cell activity is associated with lower withdrawal threshold.[73] Furthermore, the threshold at which OFF-cell pauses directly correlates with the threshold of withdrawal. A decreased threshold for OFF-cell pause can also manifest as hyperalgesia in persistent inflammation.[35] This enhanced sensitivity to stimuli in persistent inflammation, due to a decreased OFF-cell response threshold, is the same behavioral phenotype seen in acute inflammation, but from increased ON-cell ongoing firing.[104; 143] These two mechanisms, changes in *either* ON-cell or OFF-cell firing, can thus contribute independently to behavioral hypersensitivity in acute and persistent inflammation.

1.4.1.1. Acute inflammatory pain

RVM plays an important role in acute pain. Increased ON-cell ongoing activity has been associated with hyperalgesia in a number of acute inflammatory pain models. Local injection of pro-inflammatory agents, such as Complete Freund's Adjuvant (CFA) or capsaicin, results hyperalgesia with increased ON-cell and decreased OFF-cell ongoing activity, and injection of lidocaine in RVM reduces hyperalgesia.[35; 104] This suggests that the increased RVM ON-cell ongoing activity mediates hyperalgesia in acute inflammation. Similarly, visceral acute inflammation induced by application of capsaicin increases ON-cell ongoing activity, and decreases the threshold for nociception-evoked hindpaw withdrawal.[172] NMDA receptors are upregulated in RVM neurons during CFA-induced acute inflammation. These receptors are presumably expressed on ON-cells, because activation of NMDA receptors potentiates hyperalgesia.[66; 182] Taken together, these results demonstrate a clear influence of descending facilitation of nociception mediated by ON-cells in acute inflammatory pain.

PB is also involved in acute pain. Lesion of NK1R-positive neurons in superficial dorsal horn suppresses development of hyperalgesia after acute unilateral injection of capsaicin at the hindpaw,[120] presumably due to the lack of descending pain facilitatory output from RVM.[103] However, it is unknown whether the connection between PB and RVM is responsible for driving ON-cell activity during acute inflammation.

1.4.1.2. Persistent inflammatory pain

Despite the similar behavioral hypersensitivity, persistent pain is not simply a continuation of acute pain. Animals injected with CFA display acute, but transient spontaneous pain-related behaviors, while their hypersensitivity to external stimuli persists.[64; 110; 148; 178] The timeline of this change in behaviors correlates with the functional and biochemical plasticity seen in RVM neurons.[65; 66; 161] After the first day of inflammation, RVM neurons begin to upregulate AMPA receptors. Activating these receptors enhances descending inhibition and

attenuates hyperalgesia.[65] RVM κ- and μ-opioid receptor-mediated descending inhibition is also enhanced during persistent inflammation, and lasts more than 2 weeks.[176] Functionally, blocking RVM during persistent inflammation potentiates hyperalgesia, which indicates that RVM increases descending inhibition from OFF-cell output as a form for compensatory adaption to persistent inflammation.[35; 43; 161] However, RVM still helps maintain behavioral hypersensitivity by decreasing ON- and OFF-cell response thresholds.[35] This plasticity which occurs in RVM during the transition from acute to persistent pain may be driven by sensory input, as it has a profound impact on the physiology of RVM neurons.[182]

The PB is a likely candidate input that contributes to RVM plasticity in persistent inflammation. NK1R-positive neurons can influence dorsal horn nociceptive neurons through engaging descending pain-modulatory pathways.[103] In models of persistent inflammatory pain, lesion of NK1R-positive neurons using SP-SAP attenuates the development of persistent pain, and decreases the excitability of dorsal horn nociceptive neurons.[145; 164; 180; 197] This suggests the failure of developing persistent pain in these NK1R-lesion animals may be in part due to the inability of the spinoparabrachial pathway to engage descending pain-modulation. One key component of the circuit may be the supraspinal interaction between PB and RVM. Previous experiments did not directly examine the interaction between PB and RVM, and thus can only infer the involvement of these areas in the sensitization process. *Aim 2* of this thesis project is to study the mechanisms through which PB drives RVM activity, allowing the maintenance behavioral hypersensitivity during the transition from acute to persistent pain.

1.5. SUMMARY

The sensation of pain is subjective and unique to each individual because the experience of pain depends not only on the input of noxious information (nociception) but the state of the body – an interaction between pain transmission and pain modulation. The transmission of nociceptive information through the spinal cord dorsal horn is subject to bidirectional control by

brainstem pain-modulation systems. The output of the best-studied modulatory system is through the RVM, which exerts bidirectional modulatory effects mediated by two sets of physiologically identified neurons: the “ON-cells” and the "OFF-cells.” The RVM ON- and OFF-cells respectively facilitate or inhibit the passage of noxious information from the periphery through the dorsal horn. However, the ascending pathway through which noxious inputs gain access to the RVM is still unknown. This work will examine the effects of input from PB, a major nociceptive relay center in the brainstem, upon RVM’s pain-modulating properties. *The overarching goal of this thesis is to address an important gap in our knowledge regarding the intrabrainstem circuitry which drives RVM activity in acute and persistent pain.*

1.5.1. Aim 1: ON- and OFF-cell responses to nociceptive input from PB

The focus of the first aim of this thesis is to determine the pathway through which noxious signals reach RVM pain-modulating neurons. If PB relays spinal nociceptive information from the spinal cord to RVM neurons, interfering with PB input, either by inhibiting the cell bodies or its terminals in RVM, should have effects similar to intrathecal lidocaine application, which attenuates RVM nociception-related activity, and alters ongoing activity. Because the spinoparabrachial projection is predominantly crossed, the RVM's response to block of PB contralateral to stimulated hindpaw is expected to be attenuated to a greater extent than that after blocking PB ipsilateral to the stimulus. If bilateral block of PB completely eliminates the OFF-cell pause and ON-cell burst, it would suggest that the spinoparabrachial pathway is the *sole* source of nociceptive input to the RVM.

Here in Aim 1, I measure the change in ON- and OFF-cell physiology in response to inactivation of the PB cell bodies using lidocaine or $GABA_A$ agonist (Chapter 2), or to inactivation of PB terminals in RVM using optogenetic methods (Chapter 3). *My hypothesis is PB is a major nociceptive input to RVM pain-modulating neurons, and interfering with PB inputs will attenuate nociception-related responses and ongoing activity of ON- and OFF-cells.*

1.5.2. Aim 2: influence from PB on RVM plasticity in acute and persistent pain

The spinoparabrachial pathway has been linked to central sensitization during persistent inflammation, presumably through engaging the RVM descending pathway. The focus of the second aim is to examine whether PB contributes to the sensitization of RVM pain-modulating neurons in acute and persistent pain. If PB continues to relay nociceptive information to RVM in acute and persistent inflammation, then PB contributes to RVM sensitization and plasticity in persistent pain. Since previous studies involving lesion of neurokinin 1 receptor (NK1R) - expressing neurons effectively ablated both the contralateral and ipsilateral side of spinoparabrachial tract, disregarding the potential difference between the two inputs, the current thesis will examine the influence of contralateral PB input (Chapter 4) and ipsilateral PB input (Chapter 5) on RVM neuronal response. *I hypothesize that contralateral and ipsilateral PB inputs to RVM drive and maintain ON- and OFF-cell sensitization in acute and persistent pain.*

1.5.3. Significance and Innovation

There is growing evidence that failure or abnormality in a brainstem pain-modulating system is an important factor in many chronic pain states. RVM, the output node of this system, modulates the sensitivity for the body, and its activity heavily influenced by inputs of pain-related information. Yet, we do not know how the pain-related information reaches the pain-modulating neurons in the RVM. The current thesis addresses this gap in our knowledge, delineating the pathways through which pain transmission systems link to pain modulation systems, and how such pain-related input drives sensitization of the pain modulation system in the transition from acute to persistent pain. This information is critical if we are to develop better approaches to treating chronic pain.

Aim 1 of this thesis defines PB as a source of noxious inputs to the RVM, which modulates ON- and OFF-cell activity and subsequent pain behaviors. The data in this thesis will therefore

be the first to delineate a specific spinal nociceptive transmission pathway as a direct driver for the nociceptive responses of identified RVM pain modulating neurons.

The experiments in *Aim 2* expand from the data in Aim 1 and prior knowledge of RVM physiology in persistent pain to examine the process through which PB and RVM contribute to sensitization of the pain modulation system during an injury. Thus these experiments provide a crucial mechanistic timeline describing how the interaction between the pain-transmission system and the pain-modulation systems can lead to pathological pain.

The tools used in this thesis are innovative, employing optogenetic methods to study the direct projection of PB to RVM. This approach provides a distinct advantage over global activation or inactivation techniques by isolating a single component of a complex circuit, which allows me to measure PB's influence on RVM cellular physiology and subsequent pain behaviors.

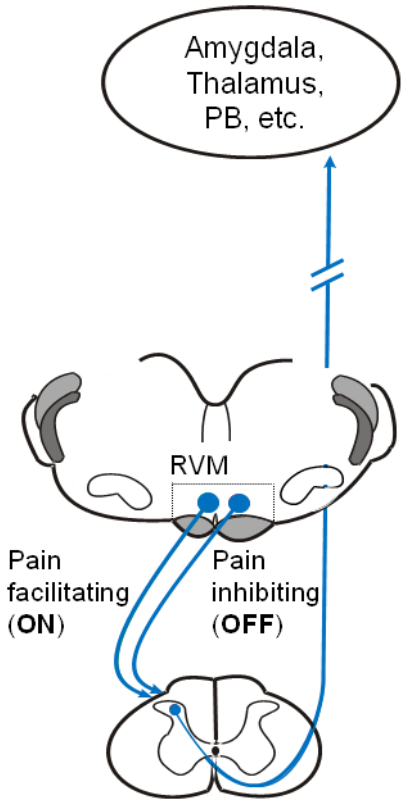

Figure 1 Modulation on dorsal horn nociceptive transmission by RVM efferents

The rostral ventromedial medulla (RVM) is the output of a brainstem pain-modulating network. RVM efferents to the dorsal horn can have facilitatory (ON-cells) or inhibitory (OFF-cells) effect on nociceptive signal transmission to various structures, such as amygdala, thalamus, and parabrachial complex (PB).

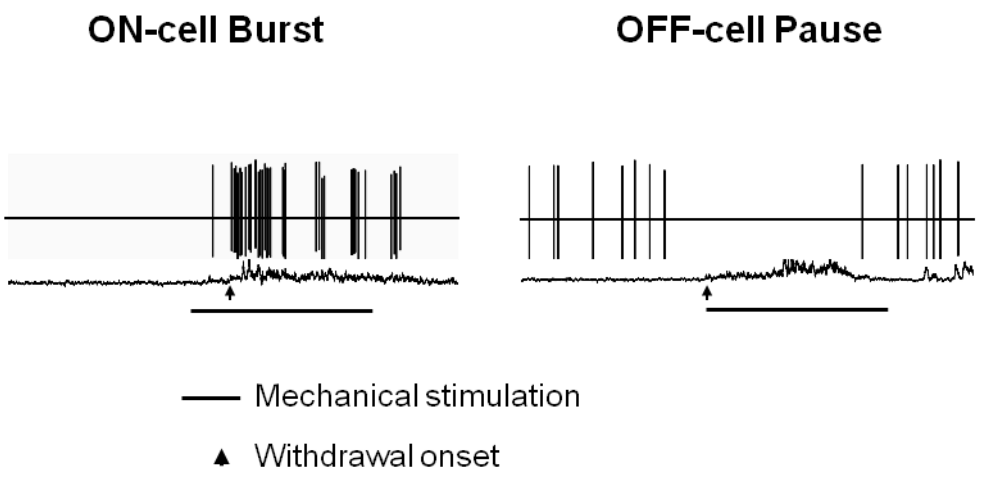

Figure 2 Temporal patterns of ON- and OFF-cell firing

Individual action potentials from the ON-cell (left) and OFF-cell (right) are shown during withdrawal from a noxious mechanical stimulus (8 s of 100 g mechanical stimulation). EMG recording from the hamstring muscle shows the moment of withdrawal.

CHAPTER 2

MANUSCRIPT #1

The parabrachial complex links pain transmission to descending pain modulation

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ZR, QC – electrophysiological experiments, data analysis, manuscript writing and review

SD – data analysis

JDC – experimental design

DT – immunohistochemical experiments

2.1. ABSTRACT

The rostral ventromedial medulla (RVM) has a well-documented role in pain modulation, and exerts anti-nociceptive and pro-nociceptive influences mediated by two distinct classes of neurons, OFF-cells and ON-cells. OFF-cells are defined by a sudden pause in firing in response to nociceptive inputs, whereas ON-cells are characterized by a "burst" of activity. Although these reflex-related changes in ON- and OFF-cell firing are critical to their pain-modulating function, the pathways mediating these responses have not been identified. The present experiments were designed to test the hypothesis that nociceptive input to the RVM is relayed through the parabrachial complex (PB). In electrophysiological studies, ON- and OFF-cells were recorded in the RVM of lightly anesthetized male rats before and after an infusion of lidocaine or muscimol into PB. The ON-cell burst and OFF-cell pause evoked by noxious heat or mechanical probing were substantially attenuated by inactivation of the lateral, but not medial, parabrachial area. Retrograde tracing studies showed that neurons projecting to the RVM were scattered throughout PB. Few of these neurons expressed calcitonin gene-related peptide (CGRP), suggesting that the RVM projection from PB is distinct from that to the amygdala.

These data show that a substantial component of "bottom-up" nociceptive drive to RVM pain-modulating neurons is relayed through the parabrachial complex. While the parabrachial complex is well-known as an important relay for ascending nociceptive information, its functional connection with the RVM allows the spinoparabrachial pathway to access descending control systems as part of a recurrent circuit.

2.2. INTRODUCTION

An important factor in both acute and chronic pain is an intrinsic pain-modulating system that regulates nociceptive processing via descending projections from the brainstem to the dorsal horn. This system contributes to abnormal pain in animal models; in patients, impaired descending control has been documented in many chronic pain syndromes.[35; 43; 75; 81; 113; 156; 157; 181; 191; 202] The output of this modulating system, via the rostral ventromedial medulla (RVM), can facilitate or suppress nociception via two physiologically-defined cell classes, "ON-cells" and "OFF-cells," that respectively facilitate and inhibit dorsal horn nociceptive transmission.[75; 76; 81] However, these modulatory neurons also *receive* noxious input: ON-cells are activated by noxious stimulation, giving a "burst" of activity, whilst OFF-cell firing is suppressed, producing a "pause" in any ongoing activity. This acute activation of pain-facilitating ON-cells and suppression of pain-inhibiting OFF-cells can function as a positive feedback loop, promoting responses to subsequent afferent inputs.[86; 99] Pain transmission and modulating systems thus constitute a recurrent circuit, with noxious stimulation as the primary "bottom-up" influence on pain-modulating outputs. Surprisingly, the pathway through which noxious inputs drive changes in RVM activity has not been defined. Not knowing how pain transmission is linked to pain modulation has severely limited our ability to define the function and plasticity of the RVM at the level of the synapse, cell membrane, and circuit.

One candidate with the potential to relay noxious information to the RVM is the parabrachial complex (PB). The PB comprises a functionally and anatomically diverse region involved in a range of homeostatic functions.[10; 61; 62; 102; 140] It is a major supraspinal target of nociceptive transmission neurons with cell bodies in the contralateral superficial dorsal horn (with sparse ipsilateral input). It also receives projections from deep dorsal horn.[12; 17; 53; 155] Nociceptive neurons have been identified in the PB, primarily in lateral PB (lPB) and the Kölliker-Fuse nucleus (KF).[11; 13; 23; 85] There is evidence that spinal neurokinin-1 (NK1)-

positive neurons, presumed to relay through PB, engage both descending inhibition and descending facilitation.[107; 180; 194]

A direct, but relatively sparse projection from the PB to the RVM region has been identified anatomically.[8; 84; 195] In addition, PB projects directly to the central nucleus of the amygdala and through a thalamic relay to the insula, implicating the PB in emotional and autonomic aspects of pain.[23; 62; 95; 203] It also projects to the midbrain periaqueductal gray.[63] Each of these parabrachial targets has outputs that converge, directly or indirectly, at the RVM.[97; 126; 127; 174] Despite these wide-reaching anatomical links, the functional influence of PB on RVM pain-modulating neurons has not been investigated.

The present experiments were designed to test the hypothesis that nociceptive input to pain-modulating neurons of the RVM is relayed through the PB. We also used immunohistochemical methods to determine whether the projection from PB to RVM includes CGRP neurons, since CGRP-expressing neurons comprise a significant proportion of the nociception-related projection from PB to the amygdala.[29; 41; 177] We found that blocking the lateral PB complex, but not the medial PB are, significantly attenuated nociceptive responses of RVM pain-modulating neurons, indicating that a substantial component of pain-related drive to RVM pain-modulating neurons is relayed through the lateral PB.

2.3. Materials and Methods

All experimental procedures were approved by the Institutional Animal Care and Use Committee at Oregon Health & Science University and followed the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain.

2.3.1. Electrophysiological experiments

2.3.1.1. Animal preparation

Male Sprague-Dawley rats were purchased from Charles River (240-340 g) and acclimated for at least one week in the vivarium with a 12-h light/dark cycle and food and water available *ad libitum*. Experiments were performed during the light phase.

On the day of the experiment, animals were deeply anesthetized (isoflurane) and a catheter inserted in the external jugular vein for subsequent infusion of the short-acting barbiturate methohexital. They were then transferred to a stereotactic frame. Small craniotomies were made to gain access to the RVM and PB, and the meninges were opened. Heart rate was monitored using EKG, and respiratory rate using a ventilation pressure transducer.[37] Body temperature was monitored and maintained at 36-37 °C with a heating pad. When preparatory surgeries were complete, rats were placed on a continuous methohexital infusion. The anesthetic plane was set at a depth that allowed a stable heat-evoked hindpaw withdrawal reflex, while preventing spontaneous movement. Animals were stabilized for at least 45 minutes at an anesthetic flow rate before beginning data collection.

2.3.1.2 Microinjections

Drugs were delivered to the PB using a glass microinjector (70 μm outer diameter) attached to a 1 μl Hamilton syringe using PE50 tubing. Lidocaine (4%), the $GABA_A$ receptor agonist muscimol (8 pmol), or artificial cerebrospinal fluid (aCSF, comprised of NaCl 128 mM, KCl 2.6 mM, $CaCl_2$ dihydrate 1.3 mM, $MgCl_2$ hexhydrate 0.9 mM, $NaHCO_3$ 20 mM, Na_2HPO_4 1.3 mM, with pH of 7.4) was injected in 200 nl over a period of 3 to 4 min. Fluorescent beads (FluoSpheres, Invitrogen, Eugene, OR) were included in the drug solutions in a 1:100 concentration to verify the injection site, and injectors were rinsed before being lowered into the brain. Injections were generally made into the right lateral PB (contralateral to stimulation of the

left hindpaw), but in some experiments, injections were made bilaterally. Stereotactic coordinates (relative to interaural zero, head level) were as follows: lateral PB (AP -1.2 mm with the injector directed caudally at 14°, ML ± 2.3 mm, DV +3; right medial parabrachial area (mPB) (off-target placement controls; AP -1.2 mm with the injector directed caudally at 14°, ML -1.4 mm, DV +3).

2.3.1.3. Extracellular recording

Extracellular single-unit recordings were made with stainless-steel microelectrodes (Microprobes, Gaithersburg, MD) with gold- and platinum-plated tips. Signals were amplified (10k) and bandpass filtered (400 Hz to 15 kHz) before analog-to-digital conversion at 32k samples/s.

An RVM neuron was isolated and classified as an ON-, OFF-, or NEUTRAL-cell using methods described previously.[4; 35] Both ON- and OFF-cells have whole body receptive fields, but inverse responses during nocifensive behaviors. That is, ON-cells exhibit a burst of action potentials beginning immediately before a nocifensive withdrawal or continue firing if already active. OFF-cells cease firing (if active), or remain silent (if inactive). NEUTRAL-cells show no response associated with nocifensive reflexes, as described previously.[4; 35] To further confirm cell classification, a 10 s noxious pinch was delivered to the hindpaw using a toothed forceps: ON-cells fire and OFF-cells cease firing throughout the stimulus. Activity of only one neuron was recorded in a typical experiment, but that of more than one neurons was recorded simultaneously in seven cases (of 94 total).

2.3.1.4. Experimental protocol

An ON-, OFF-, or NEUTRAL-cell was isolated. Lidocaine or muscimol was used to block PB and effects on spontaneous firing and changes in cell activity associated with nociceptive withdrawal reflexes were then determined.

Both noxious heat and von Frey fiber stimulation of the hindpaw were used. For heat-evoked withdrawal, the left hindpaw was placed on a platform overlying a feedback-controlled halogen bulb, with trials at approximately 5 min intervals. The paw surface temperature was increased at a rate of 1.2 °C/s from 35 °C to a maximum of 53 °C. The withdrawal temperature and latency were determined using a transducer attached to the paw. Withdrawals evoked by mechanical stimuli (von Frey filaments) were also tested in a separate set of experiments. Filaments (26, 60, and 100 g) were applied, in ascending order, to the interdigital webbing of the plantar surface of the left hindpaw for 8 s, with three trials of each force. A minimum of two testing sites was used in rotation. Individual trials were initiated at intervals of at least 30 s, with longer interstimulus intervals (up to 2 min) used when necessary to capture a period when the cell under study was inactive (ON-cell) or active (OFF-cell) so that a "burst" or "pause" could be quantified. Withdrawals were recorded as calf muscle electromyography (EMG), with the first positive inflection of the rectified EMG used as the onset of the response. If there was no withdrawal within the stimulus period (15 s for heat, 8 s for mechanical stimuli), this cut-off latency (i.e., 15 or 8 s) was assigned for assessment of response latencies.

For heat stimulation, lidocaine, muscimol, or aCSF was infused into the right lateral PB complex (contralateral to the stimulated paw), right medial parabrachial area, or both the left and right lateral PB following a baseline period with four trials. Additional trials were performed after the infusion was complete, for a total time of up to one hour. Trials were initiated at approximately 5 min intervals throughout, as noted above. For mechanical stimulation, lidocaine was infused into the right lateral PB nucleus (contralateral to the stimulated paw) following a baseline consisting two sets of the von Frey stimulus series. Two additional von Frey stimulus series were then delivered over the 10 to 15 min period after completing the microinjection. As noted above, individual trials were initiated at intervals of at least 30 s, with longer interstimulus intervals (up to 2 min) used when needed in an attempt capture a period

when the cell under study was inactive (ON-cell) or active (OFF-cell) so that a “burst” or “pause” could be quantified. If the ON-cell remained active or OFF-cell silent beyond those limits, no “burst” or “pause” data were obtained for that trial.

2.3.1.5.Data analysis

The extracellular recording signal, EMG, EKG, and respiratory transducer output were digitized and collected using Spike 2 software (Cambridge Electronics Design Ltd, Cambridge, UK). Each waveform was sorted using Spike2 template matching and cluster analysis, and verified on an individual spike basis.

A number of cell parameters, defined as previously described,[4; 26; 123] were used to characterize reflex-related changes in RVM cell activity. In experiments testing heat-evoked withdrawals, we analyzed the number of spikes per ON-cell burst, the latency from burst onset to hindpaw withdrawal, and the peak firing rate during the burst. For OFF-cells, we analyzed the duration of the OFF-cell pause and the latency from the pause to hindpaw withdrawal. In experiments testing mechanically-evoked withdrawals, we analyzed spontaneous activity (firing rate in the 30 s period before stimulus sets), number of spikes per ON-cell burst, and the duration of the OFF-cell pause.

Data were compared between average baseline and post-drug times appropriate for each agent. For lidocaine, the testing protocol was completed within 15 min of the injection. For muscimol, which has a longer duration of action than lidocaine, the mean of heat trials at 10, 15 and 20 min post-injection was used as the post-drug response.

Latencies were analyzed using a paired t-test or Wilcoxon’s signed ranks test to compare post-block responses to baseline (the latter when the distribution of responses was truncated because some responses were at the cut-off value. Because cell parameters for RVM neurons are typically highly skewed, some were analyzed using paired *t*-test of log-transformed data,

again comparing post-block responses to baseline. For all tests, $p < 0.05$ was considered significant. Data are reported as mean and SEM or, for log-transformed data, geometric mean with 95% confidence limits.

2.3.1.6. Injection and recording sites

At the completion of the recording, an electrolytic lesion was made in the RVM to localize the recording site. Rats were overdosed with methohexital and transcardially perfused. The brains were removed and sectioned on a Leica CM3050 S cryostat (60 μm sections). The RVM lesion and fluorescent beads marking the injection site in the PB region were photographed with an Optronics Microfire camera attached to an Olympus BX51 microscope. If beads or lesion were not found, the data were not analyzed further. Injection sites were plotted on hand-drawn sections in Adobe Illustrator using landmarks defined by Paxinos and Watson.[150] For "on-target" injections, if injection sites were not located within the lateral parabrachial complex, the data were not analyzed further. The RVM was defined as the nucleus raphe magnus and adjacent reticular formation medial to the lateral boundary of the pyramids at the level of the facial nucleus.

2.3.2. Anatomical study of projections from PB to RVM and overlap with CGRP expression

2.3.2.1. Animal preparation

Two adult male Wistar rats, anesthetized with 3% isoflurane in 100% O_2, were injected with fluorogold (FG, 4%, 30nl, Fluorochrome, LLC) into RVM (stereotactic coordinates: AP -3.0 mm, ML 0 mm, DV -9.2 mm relative to lambda with incisor bar at -4 mm). Rats were treated with antibiotic (40,000 units/kg penicillin G, s.c.), analgesic (0.05 mg/kg buprenorphine, s.c.) and saline (3 ml, s.c.). After 7 days, the rats were deeply anesthetized with pentobarbital (80 mg/kg, i.p.) and transcardially perfused with 200 ml isotonic saline followed by 200 ml paraformaldehyde (4% in PBS). The brains were post-fixed in 4% paraformaldehyde for 1 h and

equilibrated overnight in PBS with 30% sucrose and 0.01% sodium azide. Serial coronal sections (30 µm) were cut with a freezing-stage microtome, collected sequentially in 6 sets and stored in PBS with 0.01% sodium azide at 4 °C.

2.3.2.2. Immunohistochemical procedures

Sections containing PB were pre-incubated in an antibody dilution solution (ADS: 500 ml PBS, 0.3% Triton-X 100, 1.25 g carrageenan, 100 mg NaN_3, 5 ml normal donkey serum) for 3 h and incubated overnight at room temperature with the primary antibodies for CGRP (1:2000, Mouse-anti-CGRP, Ab81887, Abcam) and FG (1:70,000, Rabbit-anti-Fluoro-Gold, 52-9600, Fluorochrome, LLC). After two washes in PBS containing 0.3% Triton-X 100 (TPBS, 20mM), the tissue was incubated for 1 h in ADS containing the secondary antibody for CGRP (1:500, Alexa Fluor594-donkey-anti-Mouse, Invitrogen). After two washes in TPBS, the tissue was incubated for 1 h in ADS containing the secondary antibody for FG (1:500, AlexaFluor488-donkey-anti-Rabbit, Invitrogen). After fluorescent labeling, the tissue was washed in PBS and mounted onto coated slides, air dried and coverslipped with anti-fade mounting medium (Pro-Long Gold, Invitrogen).

2.3.2.3. Tissue analysis

The neuroanatomical designations of the injection sites in RVM and those of FG- and CGRP-labeled neurons in the PB are based on the stereotaxic rat brain atlas of Paxinos and Watson.[150] Photomicrographs of brain sections and labeled neurons were taken with a camera attached to an Olympus BX51 fluorescence microscope. The photomicrographs were assembled into a plate using Adobe Photoshop to adjust contrast and brightness without altering the original colors.

2.4. RESULTS

2.4.1. Effect of blocking lateral PB on ON- and OFF-cell firing and withdrawal evoked by noxious heat and von Frey hindpaw stimulation

Activity of RVM ON-, OFF-, and NEUTRAL-cells was recorded before and during blockade of the contralateral lateral PB complex. We successfully completed the entire protocol for a total of 39 ON-cells, 40 OFF-cells, and 7 NEUTRAL-cells in 79 animals before and after block of the lateral PB. We also recorded 8 ON-cells and 7 OFF-cells before and after block of the medial PB area (15 animals). PB injection sites are plotted in Figure 3.

Blocking the lateral PB substantially attenuated the ON-cell burst and OFF-cell pause evoked by either thermal or mechanical noxious stimulation. Figure 4 shows representative examples of an OFF-cell and ON-cell response during heat-evoked withdrawal before and after lateral PB block. The duration of the OFF-cell pause was substantially reduced, as was the magnitude of the ON-cell burst.

Group effects are summarized in Figure 5 for thermally evoked responses. As shown in Figure 5a, blocking the lateral PB contralateral to a noxious heat-evoked paw withdrawal using either lidocaine or muscimol significantly attenuated the associated ON-cell burst (lidocaine: $t_9 = 2.48$, $p = 0.035$, n = 11 with one cell active at stimulus onset, precluding quantification of the burst; muscimol: $t_8 = 2.76$, $p = 0.025$, n = 10, with one cell active). Infusion of aCSF into lateral PB had no effect on the ON-cell response ($t_4 = 0.25$, $p = 0.81$, n = 10, with five cells active at stimulus onset). The OFF-cell pause was also significantly reduced during block of lateral PB using either lidocaine or muscimol (Figure 5b, lidocaine: $t_{10} = 2.52$, $p = 0.03$, n = 11; muscimol: $t_7 = 3.16$, $p = 0.016$, n = 8). Again, vehicle infusion was without effect (aCSF: $t_6 = 1.37$, $p = 0.22$, n = 8 with one cell silent at stimulus onset, precluding quantification of pause duration). The fact that both lidocaine and muscimol were effective suggests that the effect of the block was on lateral PB neurons, and not simply fibers of passage.

As Figure 4and 5 illustrate, the RVM response during heat-evoked withdrawal was not *eliminated* following PB block. For ON-cells, although the evoked response was reduced substantially, it was not eliminated completely (i.e., to 0 spikes associated with paw withdrawal). Moreover, although the OFF-cell pause was substantially reduced with lateral PB block, it remained significantly longer than the mean interspike interval in the 5 s period immediately prior to onset of the noxious stimulus. Thus, for example, although the reflex-related pause was reduced from 6.75 s [95%CI: 2.69, 16.75] in baseline to 2.2 s [95% CI: 1.21, 4.01] during muscimol block of PB, the pause remained significantly longer than the interspike interval immediately prior to heat onset for those trials (0.19 s [95% CI: 0.08, 0.43] $t_7 = 6.9$, $p = 0.0002$, *t*-test for correlated means, n = 8). This confirms that the OFF-cell pause was not *entirely* eliminated with lateral PB block.

Similar effects of blocking lateral PB were seen when ON- and OFF-cell responses associated with mechanically evoked withdrawals were examined. Figure 6 shows representative examples of an OFF-cell and ON-cell response during withdrawal evoked by von Frey probes before and after lateral PB block. The duration of the OFF-cell pause was substantially reduced. The ON-cell burst was eliminated in this case. These effects are quantified in Figure 7, which shows group data for ON- and OFF-cell responses during withdrawal evoked by 26, 60, or 100 g von Frey filaments in baseline and during block of the lateral PB contralateral to the stimulus using lidocaine. Both the ON-cell burst (Figure 7a) and OFF-cell pause (Figure 7b) were significantly attenuated, but again generally not completely eliminated, during lateral PB block.

In addition to the significant reduction in response magnitude, the *latency* of the residual response during peripheral stimulation was increased. Thus, both the pause and burst associated with heat-evoked withdrawal occurred at a longer latency during lidocaine block of PB (Figure 8a). Vehicle (aCSF) injection had no effect on the latency of either the pause ($t_6 =$

1.21, $p = 0.34$, n = 8 with one silent cell) or burst ($t_7 = 0.88$, $p = 0.40$, n = 10 with two active cells). Responses during intense mechanical stimuli (60 and 100 g) also occurred at a longer latency (Figure 8b and c).

The relationship between the neuronal response and the behavioral withdrawal was not altered, with no overall change in the latency from the onset of the residual neuronal response to withdrawal during lateral PB block. For example, the latency from withdrawal-related pause to the withdrawal itself was 0.70 ± 0.12 s in baseline, and 1.22 ± 0.70 s during block ($t_{10} = 0.71$, $p = 0.49$, n = 11). The latency from the withdrawal-related burst to the withdrawal itself was 0.77 ± 0.20 s in baseline, and 2.15 ± 0.89 s during block ($t_9 = 1.69$, $p = 0.12$, n = 11 with one cell showing no burst during block). Therefore, given the increase in neuronal response latency during inactivation of lateral PB, it should not be surprising that behavioral response latency was also increased. Figure 9a shows that latencies of heat-evoked responses were increased during lidocaine block of PB, but not following injection of aCSF. For mechanical stimuli (Figure 9b), the change in latency of the behavioral response was significant only in the noxious range (60 and 100 g), which parallels the change in neuronal response latency described above (Figure 8). The lack of change in both neuronal and behavioral responses with the 26 g stimulus is probably related to the fact that the proportion of cut-off values was relatively high with this filament. (Even in baseline, half of the 22 animals tested showed no response within the 8 s cut-off time at this stimulus intensity, Figure 9b).

In addition to the attenuation of withdrawal-related changes in firing, PB block altered the spontaneous discharges of both ON- and OFF-cells. As shown in Figure 10, lidocaine block of lateral PB resulted in a significant increase in the spontaneous firing of OFF-cells and a decrease in the ongoing firing of ON-cells. NEUTRAL-cell firing was unaffected by parabrachial blockade.

2.4.2. Bilateral lateral PB block does not completely eliminate ON- and OFF-cell reflex-related activity

As described above, the ON-cell burst and OFF-cell pause were largely attenuated by unilateral blockade of the lateral PB contralateral to the noxious stimulus, but they were rarely fully abolished. To determine whether the burst and pause could be eliminated *completely* with block of lateral PB bilaterally, we recorded from 4 ON-cells and 5 OFF-cells before and after bilateral lidocaine or muscimol injections (ipsilateral and contralateral to the noxious stimulus). In neither case was the response entirely abolished with the ON-cells showing at least one reflex-related spike in all cases, and the OFF-cells similarly exhibiting a pause that was at least three times the mean interspike interval in the 5-s period immediately before the stimulus onset. Thus, the average number of evoked spikes in the ON-cell burst was 16.8 [range: 3.8 – 50.0] in baseline and 6.3 [range: 0.67 – 16] during bilateral PB block. The duration of the OFF-cell pause was reduced from 55.4 s [range: 11.0 to 136.1] in baseline to 13.9 s [range: 3.4 – 34.1] during PB block.

2.4.3. Off-target placement control injections

Anatomical studies show a direct projection from the dorsal horn to the lateral PB, and Fos expression evoked by noxious hindpaw stimulation is mainly restricted to lateral PB.[85] The medial PB area therefore provided a reasonable off-target placement control site. We tested the effect of blocking the medial PB and adjacent tegmentum on the responses of ON-cells and OFF-cells during heat-evoked withdrawal. Neither the ON-cell burst nor the OFF-cell pause were significantly reduced following infusion of muscimol in medial controls sites (Figure 11). Paw withdrawal latency was also unaffected (baseline: 2.6 ± 0.4s, block: 2.7 ± 0.5s; $t_{14} = 0.29$, $p = 0.77$, n = 15).

2.4.4. CGRP-expressing neurons in PB do not project to RVM

The neuropeptide CGRP has been proposed to have important roles in a number of pain syndromes. CGRP-immunoreactive (ir) neurons in PB are thought to contribute to the affective component of a nociceptive experience through a projection to the central nucleus of amygdala.[41; 177] However, it is unclear whether CGRP-positive neurons also project directly to the RVM. To address this question, we injected the retrograde tracer FG in RVM (Figure 12). The tracer injection extended about 1 mm rostral and caudal from the focus of the injection, covering a large portion of the raphe magnus and dorsal parapyramidal area. FG-ir neurons in PB demonstrate projections to RVM arising from the Kölliker-Fuse nucleus, the lateral PB, including the dorsal, crescent, and central lateral nuclei, and more sparsely, from medial PB (Figure 12).

Immunostaining for CGRP revealed a population of neurons extending from 0.24 anterior to 0.36 posterior to the interaural line (Figure 12), confirming previous observations.[105; 177; 203] However, only one CGRP-ir neuron in PB was found to be double-labeled for FG (Figure 12). Indeed, the population of neurons projecting to RVM seemed to be segregated from the CGRP-ir neurons in PB. The dense plexus of CGRP-ir fibers in PB prevented an accurate analysis of the number of CGRP-ir neurons in the region. Nevertheless, the distribution of FG-ir neurons did not overlap with that of CGRP in PB, as shown in the example in Figure 12.

2.5. DISCUSSION

The RVM is the output node of an intrinsic brain pain-modulating circuit that can facilitate or inhibit pain depending on stimulus history (e.g., persistent inflammation) as well as “top-down” cognitive and emotional factors.[75; 76; 81] The interactions between the *inputs* to the RVM and its *output* form a recurrent positive and negative feedback network that are important for acute and persistent pathological pain. Analysis of the *output* of the RVM using selective

pharmacological manipulation has shown that the facilitatory and inhibitory outputs of the RVM are represented by ON-cells and OFF-cells.[75; 76; 81]

The *inputs* to the RVM are less well understood. "Top-down" inputs, e.g., from the amygdala, periaqueductal gray, and hypothalamus, have been implicated in modulation of pain during stress, fear, and immune activation,[75; 79; 81; 122; 127] although the relevant circuitry is just beginning to be elucidated. The primary "bottom-up" input to RVM is nociceptive sensory information: pain-facilitating ON-cells are activated by noxious stimuli, whereas the pain-inhibiting OFF-cells cease firing. These changes in firing are abrupt, and time-locked to the behavioral withdrawal from the stimulus,[59] forming a positive feedback loop. Despite the well-known fact that RVM ON- and OFF-cells respond to noxious somatic stimuli, the specific pathways through which nociception-related information reaches RVM pain-modulating neurons in acute and chronic pain states had not previously been identified.

The present experiments provide strong evidence that a significant share of the acute nociceptive input to ON- and OFF-cells is relayed through PB, specifically lateral PB. Infusion of lidocaine into the lateral PB in normal animals substantially attenuated the ON-cell burst and OFF-cell pause evoked by either thermal or mechanical noxious stimulation contralateral to the PB injection site. Similar attenuation of the burst and pause with muscimol indicated that the effect of lidocaine was not due to inactivation of fibers of passage, e.g., to nucleus cuneiformis, a known input to RVM.[7; 205] The reduction in the nociceptive responses of RVM ON- and OFF-cells was unlikely to be due to spread of lidocaine or muscimol to adjacent structures, since inactivation of medially adjacent control sites in the medial PB area did not significantly attenuate either the burst or pause. These data therefore show that the lateral PB is a major relay for nociceptive inputs triggering ON- and OFF-cell responses to acute noxious stimulation. Inactivation of lateral PB also led to significant changes in the spontaneous firing of RVM ON- and OFF-cells, with an increase in OFF-cell discharge and a decrease in ON-cell discharge.

This change implies that information relayed through lateral PB also contributes to the ongoing "tone" of RVM ON- and OFF-cell output, a conclusion consistent with the fact that blocking ascending transmission from the lumbar spinal cord also alters spontaneous firing of these neurons.[74]

The firing of RVM NEUTRAL-cells was unaffected by PB block, confirming the distinct properties of these neurons as a separate RVM cell class. However, the sample of NEUTRAL-cells here was relatively small, and we did not record from raphe pallidus, a subregion of the RVM and known target of PB efferents, which has a well-documented role in thermoregulation.[138]

2.5.1. Parabrachial complex as a major relay for supraspinal nociceptive transmission

PB comprises a functionally and anatomically diverse region involved in a wide range of homeostatic functions.[10; 61; 62; 102; 140] PB also has an important role in receiving, processing, and relaying nociceptive signals.[63] It is a major supraspinal target of nociceptive transmission neurons with cell bodies in the contralateral superficial dorsal horn (with sparse ipsilateral input). It also receives projections from deep dorsal horn.[12; 17; 53; 155] Nociceptive neurons have been identified predominantly in the lateral PB.[11; 13; 23; 85] Functionally, there is evidence that spinal NK1-positive neurons, considered to constitute the primary nociceptive input to PB, leads to engagement of both descending inhibition and facilitation.[107; 180] However, whether the PB *itself* engages RVM pain-modulating neurons had not been tested. The present study provides direct evidence that RVM pain-modulating neurons receive nociceptive input relayed through lateral PB, which could subsequently modulate nociceptive transmission and nocifensive behaviors.

The present experiments do not define the circuitry linking PB to the RVM. Although we confirmed a direct anatomical connection from the PB to the RVM,[8; 84; 195] we cannot conclude that this projection conveys nociceptive information to RVM ON- and OFF-cells, since

although RVM-projecting neurons are distributed in both medial and lateral PB, only inactivation of lateral PB interfered with nociceptive responses in the RVM. Moreover, these projections could potentially contribute to one of the other roles shared by PB and RVM (e.g., thermoregulation, cardiovascular regulation[27; 28; 42; 90; 137; 140; 170; 171]). Further, there are a number of indirect connections that could mediate the PB input to RVM. Among the parabrachial targets that have outputs converging directly or indirectly at the RVM are the central nucleus of the amygdala, midbrain periaqueductal gray, and insula (through a thalamic relay).[23; 39; 62; 95; 97; 126; 127; 203] In either case, our finding that RVM-projecting neurons in the PB are scattered across nuclei of the PB complex and do not generally express CGRP suggests that RVM-projecting neurons in PB are distinct from the CGRP-containing population that constitutes a substantial proportion of the PB projection to the central nucleus of the amygdala.[41; 70; 177] Additional studies will be needed to isolate direct and indirect influences on the RVM from PB, and to define underlying synaptic mechanisms.

Although the reduction in both the ON-cell burst and OFF-cell pause during PB block was substantial, it was not complete. This could be due to a less than total elimination of lateral PB activity with either lidocaine or muscimol.[38] In addition, the parabrachial complex is a large elongated structure, and RVM-projecting neurons are distributed along the entire rostrocaudal axis. The blocking agents are unlikely to have spread throughout the entire complex. However, using a larger volume to influence the entire lateral PB would have unavoidably suppressed activity in surrounding structures. Another possibility is that nociceptive information is conveyed through PB ipsilateral to the noxious stimulus.[11] However, even *bilateral* lateral PB block was not sufficient to eliminate the burst and pause completely. This raises the possibility that additional pathways, such as a direct input from dorsal horn,[179] or other relays, such as the periaqueductal gray, also carry nociceptive information to the RVM.

2.5.2. Functional role of the PB relay to RVM pain-modulating neurons.

The ongoing “tone” of ON- and OFF-cell firing shifted during PB block, with increased ON-cell firing and decreased OFF-cell firing, and the ON-cell burst and OFF-cell pause were delayed, not eliminated. The withdrawal reflex latency increased in parallel with the delay in the burst and pause. This modest increase in withdrawal latency is consistent with the known subtle influence of ongoing RVM ON- and OFF-cell activity on withdrawal threshold under basal conditions,[73; 74] and with the fact that elimination of the majority of dorsal horn neurons projecting to PB using a toxin selective for neurons expressing the NK1 receptor similarly has no or limited effects on nociceptive behaviors under basal conditions.[103; 145; 164; 180]

The PB input to RVM may nevertheless play an important role in hyperalgesia and persistent pain, in which both structures have been implicated. Hyperalgesia and sensitization of dorsal horn neurons in models of injury, inflammation, and neuropathy either fails to develop or is significantly attenuated following elimination of NK1-expressing neurons, the major nociceptive input to the parabrachial complex.[103; 145; 164; 180] Similarly, interfering with ON-cell activation in acute inflammatory states attenuates behavioral hypersensitivity.[35; 104; 201] Moreover, there is abundant indirect evidence that ON-cells contribute to hyperalgesia in neuropathy, opioid-induced hyperalgesia, and other persistent pain states.[25; 32; 51; 156; 164; 184; 187; 193] A better understanding of the interactions between the PB and RVM in conditions of injury and inflammation should therefore provide critical insights into the plasticity of pain-modulation in persistent pain.[26; 35; 43; 162]

2.5.3. Conclusion

Despite over 30 years of research on descending control, the pathway(s) through which noxious inputs drive changes in brainstem pain-modulating activity had not previously been defined. The present experiments have identified PB as a major relay through which noxious stimulation, the primary “bottom-up” influence on pain-modulating circuits, reaches the RVM to

influence activity of identified pain-modulating neurons, the ON- and OFF-cells. Thus the present study fills an important gap, revealing a novel *functional* link between an ascending nociceptive transmission pathway (PB) and the primary output node (RVM) of a descending pain-modulating circuit. Additional research will be needed to determine whether the influence of PB on RVM is mediated by direct connections, and how this circuit is modified in persistent pain states.

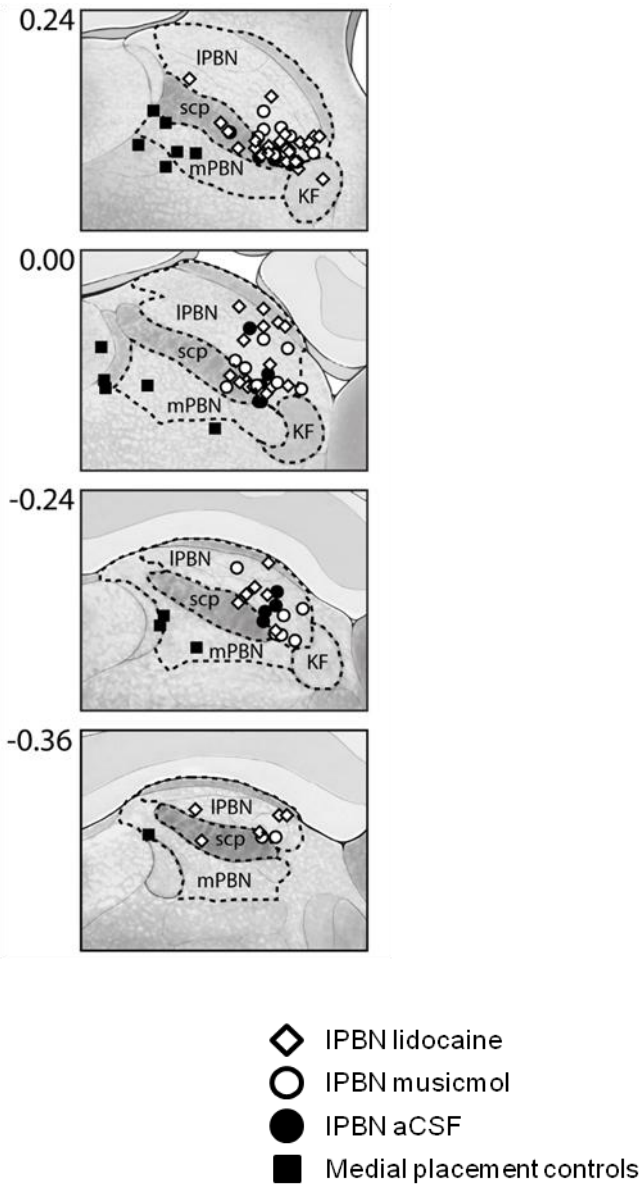

Figure 3 Locations of microinjection sites in the lateral PB complex and medial PB area.

Injections were distributed among sections at +0.24 to -0.36 relative to the interaural line. KF: Kölliker-Fuse, lPB: lateral parabrachial complex, mPB: medial parabrachial area, scp: superior cerebellar peduncle.

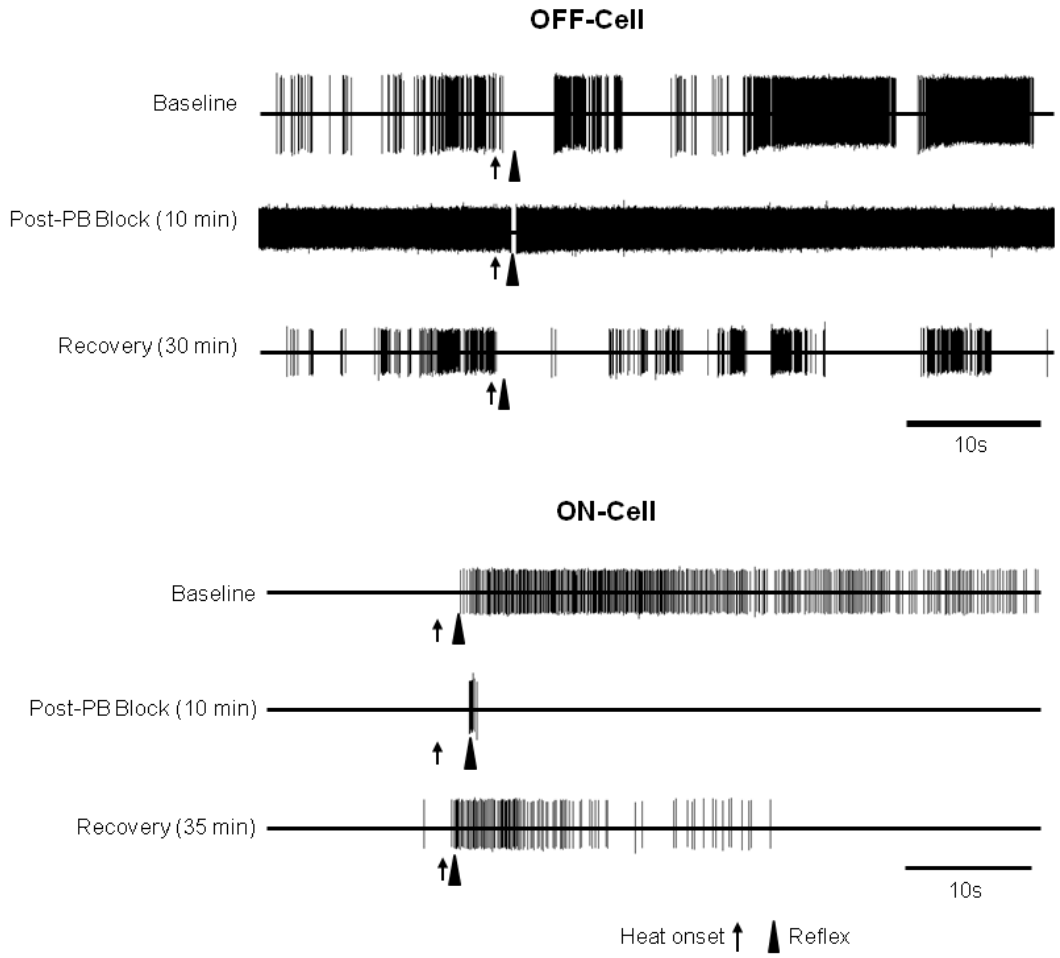

Figure 4 Examples of the effect of lateral PB block on noxious stimulus-evoked, reflex-related activity of ON- and OFF-cells

Representative examples show OFF- and ON-cell activity during withdrawal from noxious heat stimulus at baseline compared to during lidocaine block of lateral PB contralateral to the stimulus, as well as subsequent recovery. In both cases, reflex-related changes in firing were substantially reduced, although not entirely eliminated. Increased spontaneous firing of the OFF-cell is also evident.

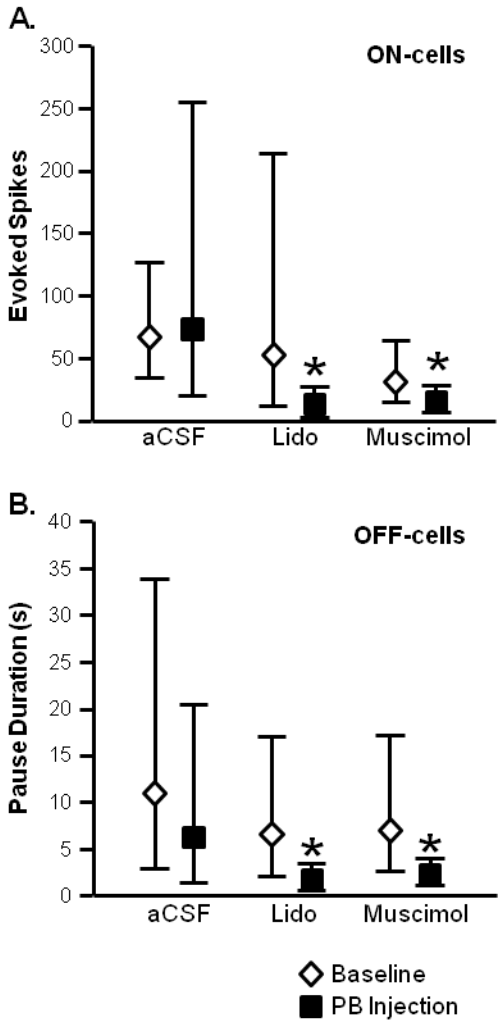

Figure 5 Inactivation of lateral PB interfered with the ON-cell burst and OFF-cell pause in response to heat stimulation.

A. ON-cells. Effect of lateral PB microinjection of aCSF (200 nl), lidocaine (4%, 200 nl), or muscimol (8 pmol in 200 nl) on the ON-cell burst (measured as total evoked spikes). **B**. OFF-cells. Effect of lateral PB microinjection of aCSF, lidocaine, or muscimol on OFF-cell pause (measured as pause duration). (PB injections were contralateral to the peripheral stimulus. Reported as geometric mean with 95% confidence limits, *$p < 0.05$ compared to baseline, *t*-test for correlated means, n = 8 to 11 cells per class/treatment.)

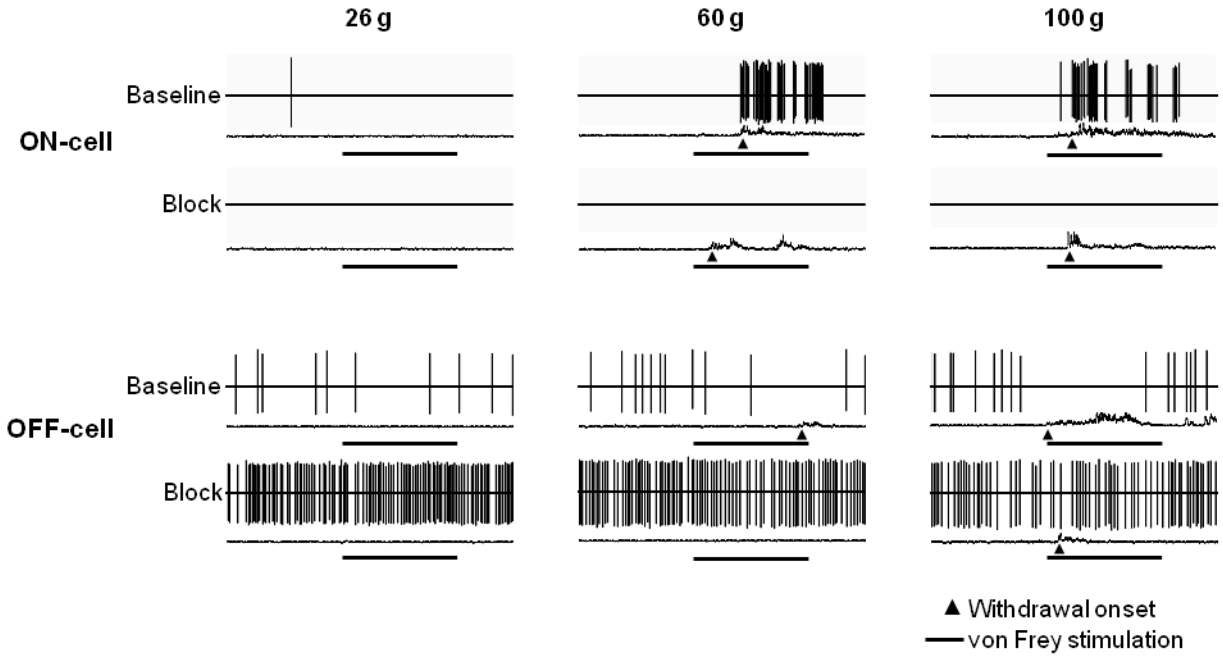

Figure 6 Examples of the effect of lateral PB block on mechanically evoked, reflex-related activity of ON- and OFF-cells.

Representative examples show ON- and OFF-cell activity and associated EMG during trials using 26, 60, and 100 g von Frey probes both in baseline and during PB block. A baseline (top-trace) and block trial (lower trace) are shown for each force. Reflex-related changes in firing were substantially reduced in both cases, and even eliminated for the ON-cell. Period of von Frey fiber application (8 s) is shown below each trace, with arrowhead indicating behavioral responses, which occurred in response to 60 and 100 g stimuli in baseline.

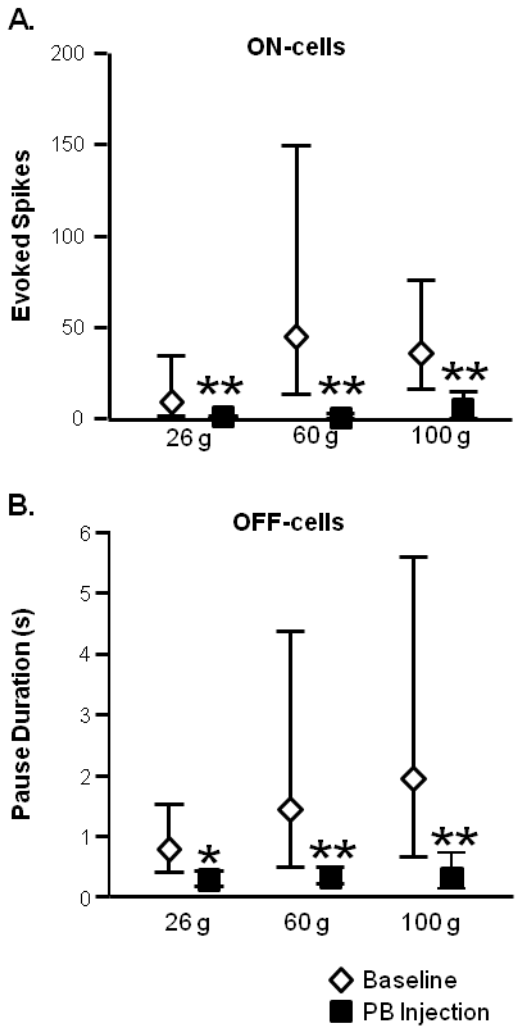

Figure 7 Inactivation of lateral PB interfered with the ON-cell burst and OFF-cell pause in response to mechanical stimulation.

A. ON-cells. Effect of lidocaine (4%, 200 nl) microinjected into the lateral PB on activity triggered during application of von Frey probes (26, 60 and 100 g, measured as total evoked spikes, n = 8). **B**. OFF-cells. Effect of lidocaine (4%, 200 nl) microinjected into the lateral PB on OFF-cell pause (measured as pause duration, n = 13). (PB injections were contralateral to the peripheral stimulus. Reported as geometric mean with 95% confidence limits, *t*-test for correlated means, *$p < 0.05$, **$p < 0.01$ compared to baseline.)

Statistical analysis.

ON-cells (n = 8): 26 g: $t_7 = 3.80$, $p = 0.0067$; 60 g: $t_5 = 6.99$, $p = 0.0009$ (two cells active at stimulus onset; 100 g: $t_7 = 3.77$, $p = 0.0069$.

OFF-cells (n = 13): 26 g: $t_{11} = 2.67$, $p = 0.022$ (one cell silent at stimulus onset); 60 g: $t_{11} = 3.17$, $p = 0.009$ (one cell silent at stimulus onset); 100 g: $t_{11} = 3.81$, $p = 0.0029$ (one cell silent at stimulus onset).

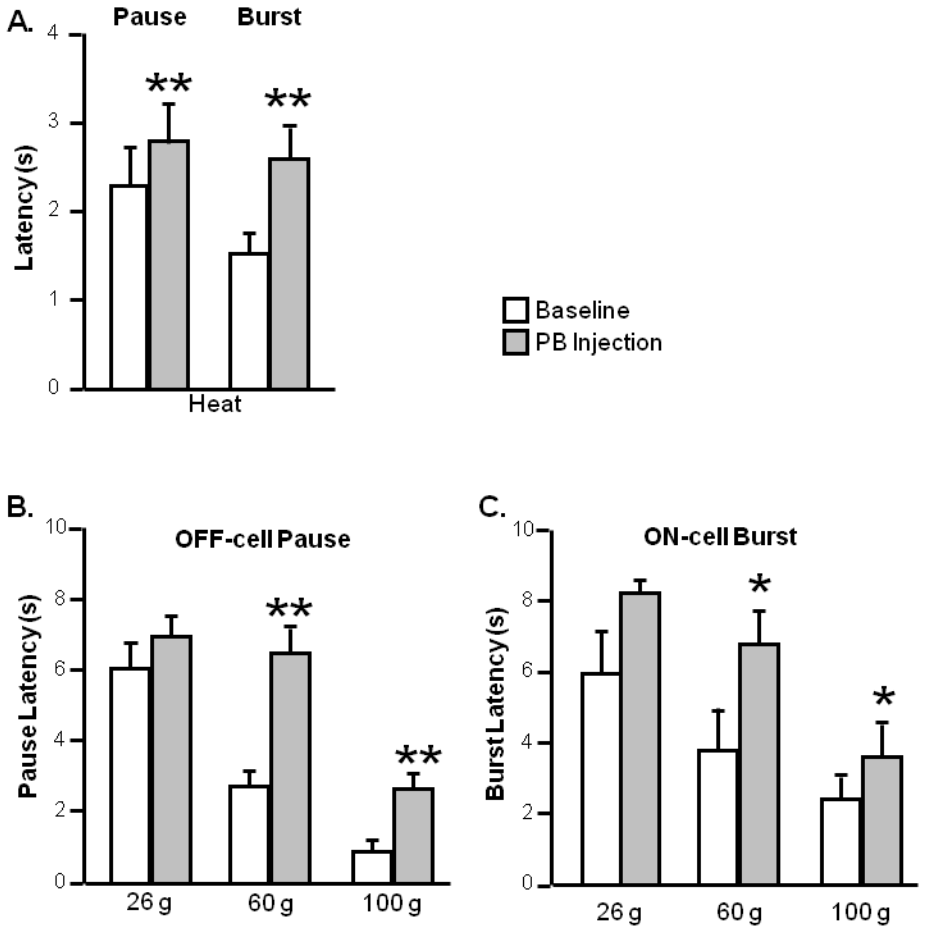

Figure 8 The latencies of the residual ON-cell burst and OFF-cell pause were increased during inactivation of lateral PB

Effect of lateral PB block (lidocaine) on the latencies of the ON-cell burst and OFF-cell pause during noxious heat (A) and stimulation with VF probes (26, 60 and 100 g, B and C). (PB injections were contralateral to the peripheral stimulus. Mean + SEM, *$p < 0.05$, **$p < 0.01$ compared to baseline using t-test for correlated means or Wilcoxon's signed ranks test, 8 to 13 cells/group)
Statistical analysis:
Heat. Pause: $t10 = 3.93$, $p = 0.0028$, $n = 11$, Burst: $t10 = 3.84$, $p = 0.0033$, $n = 11$.
Mechanical stimulation, pause. 26 g: $W = 11$, $p = 0.19$, $n = 13$; 60 g: $W = 78$, $p = 0.0005$, $n = 13$ (with one cell silent); 100 g: $W = 74$, $p = 0.0015$, $n = 13$ (with one cell silent).
Mechanical stimulation, burst. 26 g: $W = 10$, $p = 0.12$, $n = 8$; 60 g: $W = 21$, $p = 0.031$, $n = 8$ (two cells active); 100 g: $W = 24$, $p = 0.047$, $n = 8$

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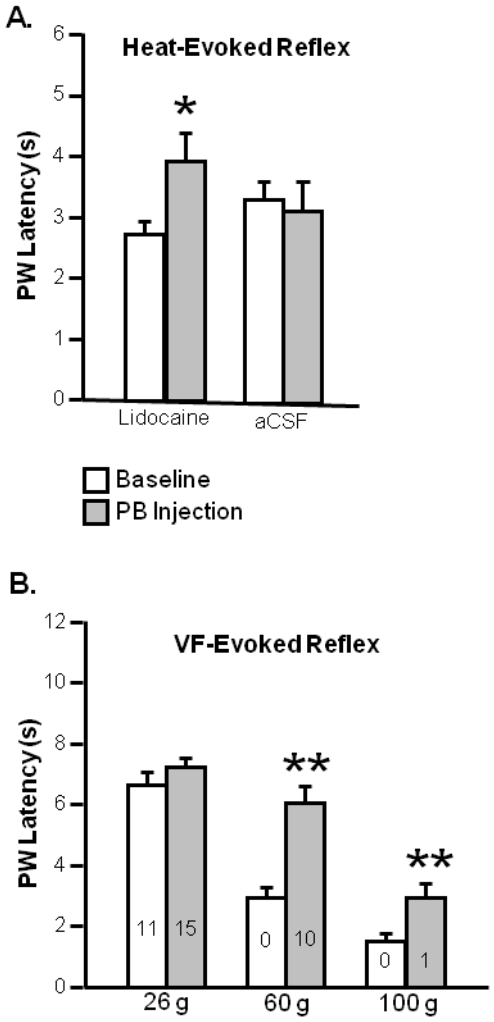

Figure 9 Modest behavioral hypoalgesia during inactivation of lateral PB

A. Effect of lidocaine or aCSF microinjected into the lateral PB on latency to heat-evoked withdrawal. Latency was significantly increased during lidocaine block of PB ($t_{25} = 2.17$, $p = 0.040$, $n = 26$), but unaffected by injection of aCSF ($t_{14} = 0.43$, $p = 0.68$, $n = 15$). B. Effect of lidocaine microinjected into the lateral PB on withdrawal evoked by VF probes (26, 60 and 100 g, $n = 22$, number of animals not responding within 8 s cut-off time shown within each bar). Latency was increased for stimuli in the noxious range (60 g: $W = 243$, $p < 0.0001$, $n = 22$; 100 g: $W = 203$, $p = 0.0004$, $n = 22$) but not for the 26 g stimulus ($W = 31$, $p = 0.13$, $n = 22$). PB injections were contralateral to the peripheral stimulus. (Data shown as mean + SEM, *$p < 0.05$, **$p < 0.01$ compared to baseline using t-test for correlated means or Wilcoxon's signed

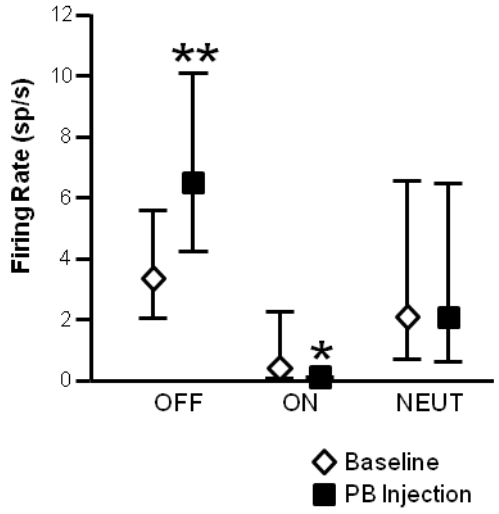

Figure 10 Effect of lidocaine (4%, 200 nl) microinjected into the lateral PB on spontaneous firing of OFF-cells, ON-cells, and NEUTRAL-cells in RVM

OFF-cell firing was significantly increased (t_{12} = 3.77, p = 0.0027, n = 13), while ON-cell firing was reduced (t_7 = 3.03, p = 0.0019, n = 8). NEUTRAL-cells did not respond to lateral PB block (t_5 = 0.98, p = 0.37, n = 6). (PB injection was unilateral. Geometric mean with 95% confidence limits, *p < 0.05, **p < 0.01 compared to pre-block baseline using t-test for correlated means)

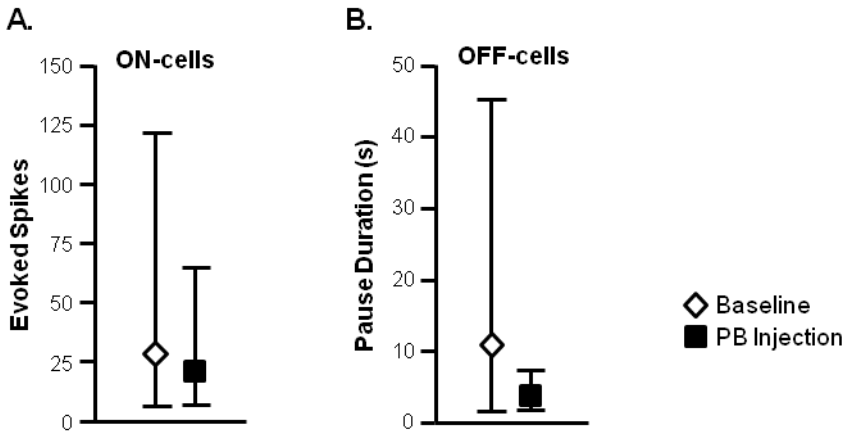

Figure 11 Medial PB (mPB) does not contribute to the ON-cell burst and OFF-cell pause.

A. Muscimol (8 pmol in 200 nl) microinjected into the mPB and adjacent tegmentum had no effect on heat-evoked activity of ON-cells (t_7 = 1.26, p = 0.25, n = 8). **B**. Muscimol (8 pmol in 200 nl) microinjected into the mPB and adjacent tegmentum had no effect on the heat-related OFF-cell pause (t_5 = 1.34, p = 0.24, n = 7 with one neuron silent at heat onset). (PB injections were contralateral to the peripheral stimulus. Reported as geometric mean with 95% confidence limits.)

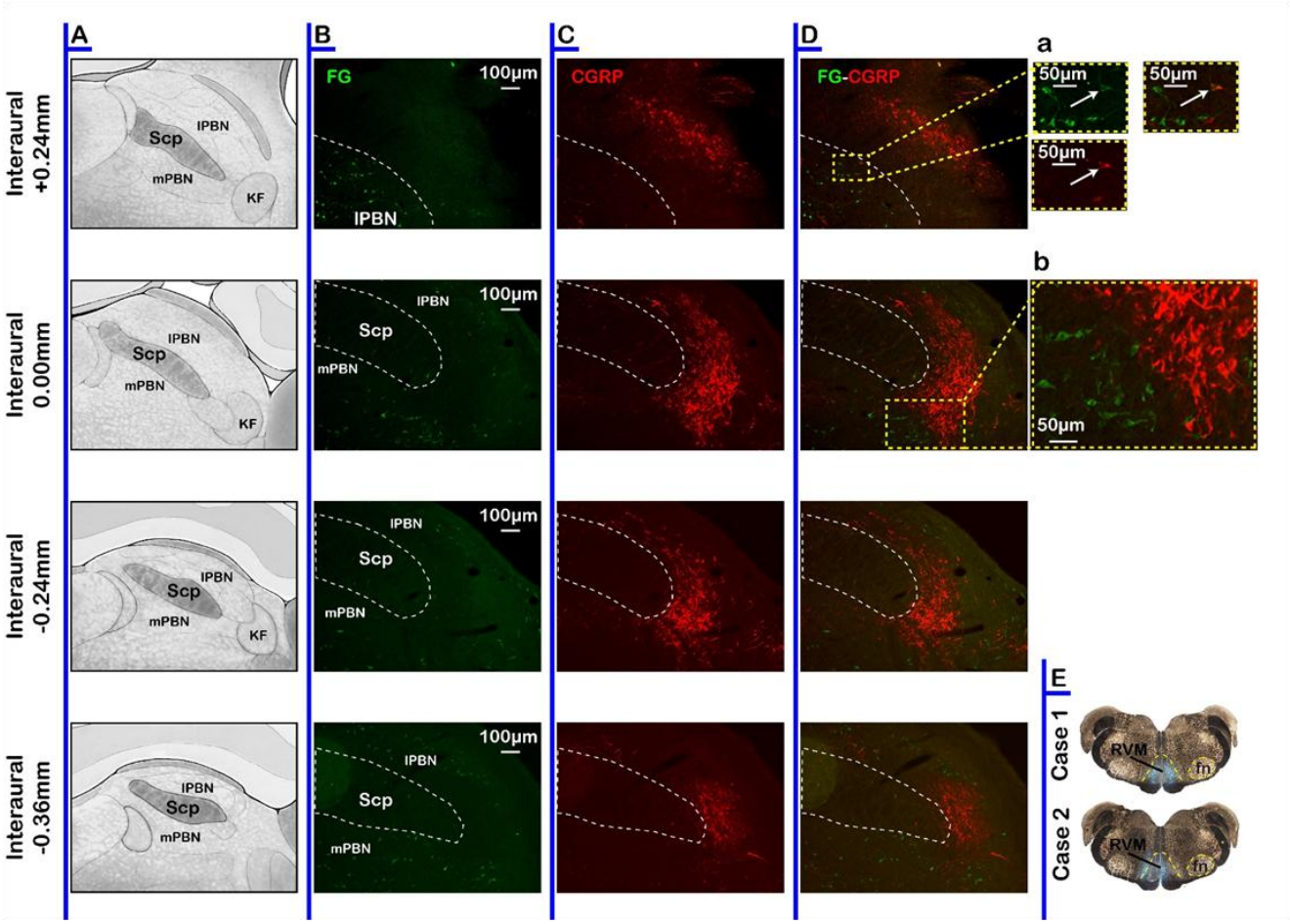

Figure 12 CGRP-ir neurons in PB do not project to the RVM.

A Schematic representation of the PB. Immunohistochemical label for FG (**B**, green), CGRP (**C**, red) and overlap (**D**) in the PB. **Da**. Inset showing higher magnification view of the only CGRP/FG double labeled neuron found in PB. **Db**. Inset showing higher magnification view illustrates segregation of CGRP-ir neurons from RVM-projecting neurons.

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CHAPTER 3

MANUSCRIPT #2

Optogenetic evidence for a direct circuit linking the parabrachial complex with pain-modulating neurons of the rostral ventromedial medulla

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QC – experimental design, electrophysiological experiments, data analysis, manuscript writing and review

ZR – experimental design

YZ – surgical preparation

3.1 ABSTRACT

The parabrachial complex (PB) is an anatomically diverse structure that has been considered to play an important role in ascending nociceptive relay. It has also been implicated to participate in regulating pain-transmission in spinal dorsal horn by engaging the intrinsic pain-modulating system. The output of this modulating system, the rostral ventromedial medulla (RVM), can facilitate or suppress nociceptive transmission at the dorsal horn by the respective action of two distinct classes of neurons, “ON-cells” and “OFF-cells”. Both classes are known to respond to noxious inputs: ON-cells are activated, leading to a “burst” of activity associated with behavioral responses to noxious stimulation, whilst OFF-cell firing is suppressed, producing a “pause” in any ongoing activity. Thus, these reflex-related changes in ON- and OFF-cell firing are critical to their pain-modulating function, and our laboratory recently identified a substantial share of nociceptive information is relayed through the lateral region of PB. A direct projection from PB was anatomically identified in RVM, but its functional roles were not studied. The present experiments were designed to test the hypothesis that nociceptive input to the RVM is relayed through this direction projection from PB. In electrophysiological studies, the reflex-related activity of ON- and OFF-cells was significantly attenuated, and produced behavioral antinociception with archaerhodopsin-induced PB terminals inhibition. Furthermore, both excitatory inputs and inhibitory inputs were revealed when PB terminals were globally excited using channelrhodopsin2, providing a number of possible functional implications for the PB-to-RVM circuit in acute and chronic pain.

These data show that a substantial component of the relevant nociceptive drive to RVM pain-modulating neurons is relayed through PB via its direction projection. Reflex-related changes in firing of these neurons thus depend on a short intrabrainstem loop conveying information related to noxious stimuli. Furthermore, the variation of PB inputs also suggests possible plasticity transitioning from acute to chronic pain. While the PB is well known as an

important relay for ascending nociceptive information, the connection with the RVM allows the spinoparabrachial pathway to access descending control systems as part of a feedback circuit. The current study thus provides concrete evidence for PB's capability to influence activity of RVM pain-modulating neurons in both excitatory and inhibitory manners via its direct projection.

3.2. INTRODUCTION

The parabrachial complex (PB) is a functionally and anatomically complex structure involved in a wide range of functions,[10; 31; 52; 62; 102; 117; 136; 139; 163; 168] including having an important role in receiving, processing, and relaying nociceptive signals.[63] Nociceptive neurons have been identified in the PB, with the highest density in the lateral region.[23; 85] A subset of nociceptive PB neurons, those expressing CGRP, are implicated in recruitment of amygdala circuits implicated in the affective dimension of pain.[144] PB can also engage descending pain-modulating systems to enhance or suppress nociceptive processing at the level of the dorsal horn.[107; 180] Our laboratory recently reported that the lateral PB is a major relay of nociceptive information to identified pain-modulating neurons of the rostral ventromedial medulla (RVM), which is the output node of the best-characterized brainstem pain-modulating system.[166]

The PB influences the RVM's bidirectional facilitatory and inhibitory control of spinal nociceptive transmission by modulating the output of two physiologically distinct classes of RVM neurons, the pronociceptive ON-cells and the antinociceptive "OFF-cells".[75; 76] Additionally, the PB also relays noxious stimulation to RVM ON- and OFF-cells, contributing to the reflex-related ON-cell “burst” (activity associated with behavioral responses), and OFF-cell "pause" (cessation of any ongoing activity). By means of this influence over the pain-facilitating ON-cells and pain-inhibiting OFF-cells in RVM, PB could be the primary driver for a positive or negative feedback loop, and contributing to normal and pathological pain.[43; 76; 156] However, due to the structural and functional complexity of PB efferent projections, it is unclear through which pathways the PB could influence the activity of RVM pain-modulating neurons.

The present experiments were designed to examine the functional roles of this PB-to-RVM direct projection in a brainstem pain-modulating system. We use optogenetic methods to test the hypothesis nociceptive information is relayed to RVM through this direct connection from

PB, thus completing a short intra-brainstem circuit that conveys spinal nociceptive information to a pain-modulating circuit. Furthermore, we tested whether this direct projection from PB to RVM influences the activity of all functional classes of RVM neurons, and whether each cell class receives a distinct influence, facilitatory or inhibitory, from PB.

3.3. MATERIALS AND METHODS

All experimental procedures were approved by the Institutional Animal Care and Use Committee at Oregon Health & Science University and followed the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain. Since we have identified lateral, not medial, PB relays nociceptive information to RVM in our earlier report,[166] all PB injections in the current study were done in the lateral PB.

3.3.1. Optogenetic animal preparation

Rats (90-110 g) deeply anesthetized (isoflurane) throughout the procedure. The rat was placed in a stereotaxic apparatus, and maintained on a circulating warm-water pad to prevent hypothermia. A mixture of bupivacaine HCl injection solution (5 mg/mL, Hospira, Inc., Lake Forest, IL) and adrenalin (1:100,000. JHP Pharmaceuticals, LLC, Rochester, MI) was administered subcutaneously at the incision. After the application of povidone iodine, an incision was made along the midline on top of the head, a craniotomy was performed approximately 1-3 mm lateral to the sagittal suture and 0.5-2.5 mm rostral to the lambdoid suture, and the dura was removed to allow the placement of a microinjection cannula with a 14 ºC angle to the coronal plane into the lateral parabrachial nucleus (AP: -1.2 mm, ML: ±2.3 mm, DV: +3 mm from interaural). A Picospritzer® (Parker Hannifin, Hollis, NH) was used to deliver 200 nL of a solution containing AAV9-hSyn-ChR2-eGFP or AAV9-CAG-ArchT-eYFP (Penn Vector Core, University of Pennsylvania). The microinjection cannula was left at the injection site for 10 min before retraction to minimize back-flow. The craniotomy site was filled with gelfoam, and the incision site closed with sutures. Rats received lidocaine ointment, penicillin G

(80 kU/mL, i.m.) and carprofen (5 mg/mL, s.c., Pfizer, New York) after the procedure, and were returned to the home cage for 4 to 5 weeks to allow expression of ChR2 and ArchT. An example and a plot of expression sites is shown in Figure 13.

3.3.2. Extracellular recordings

Four to 5 weeks after vector injection in the PB, the animals were deeply anesthetized (isoflurane) and a catheter inserted in the external jugular vein for subsequent infusion of the short-acting barbiturate methohexital. They were then transferred to a stereotactic frame. Small craniotomies were made to gain access to the RVM and PB, and the meninges were opened. Heart rate was monitored using EKG. Body temperature was monitored and maintained at 36-37 °C with a heating pad and a space heater.

When preparatory surgeries were complete, rats were placed on a continuous methohexital infusion. The anesthetic plane was set at a depth that allowed a stable heat-evoked hindpaw withdrawal reflex, while preventing spontaneous movement.

Extracellular single-unit recordings were made using an optoelectrode, constructed by pairing a stainless-steel microelectrode (Microprobes, Gaithersburg, MD) with gold- and platinum-plated tips with an optical fiber (200 µm diameter, ThorLabs). Signals were amplified (10k) and bandpass filtered (400 Hz to 15 kHz) before analog-to-digital conversion at 32k samples/s.

3.3.2.1. Validating opsin functions in PB

A subset of ChR2- or ArchT-injected animals were used to record PB neurons, and to validate the activation function with ChR2, and inhibition function with ArchT. A neuron in lateral PB was isolated, and classified as an nociception responsive neurons based on its increased firing rate during a noxious heat stimulus applied to the plantar surface of the hindpaw using a Peltier device (Yale Instrument). For the ArchT validation, a light source with mean wavelength

of 565 nm (range 540-575nm) and power of 0.5mW was used (ThorLab). An example of ArchT-induced inhibition in a PB neuron is shown in Figure 14a. For ChR2 validation, a light source with mean wavelength of 470 nm (range: 465-475, 1.1 mW power) was used, and an example of ChR2-induced activation of a PB neuron shown in Figure 14b.

3.3.2.2. Recording RVM neurons in response to PB terminal manipulations

In recording experiments, an RVM neuron was isolated and classified as an ON-, OFF-, or NEUTRAL-cell[4; 35; 59] using a noxious heat stimulus applied to the plantar surface of the hindpaw using a Peltier device (Yale Instrument). The surface temperature was held at 35 °C, and increased at a rate of 1.2 °C/s from 35 °C to a maximum of 53 °C. Withdrawals were recorded as hamstring muscle electromyographic response (EMG), with the first positive inflection of the rectified EMG used as the onset of the response. ON-cells exhibit a burst of action potentials beginning immediately before a nocifensive withdrawal or continue firing if already active. OFF-cells cease firing (if active), or remain silent (if inactive). To further confirm cell classification, a 10 s noxious pinch was delivered to the hindpaw using a toothed forceps: ON-cells fire and OFF-cells cease firing throughout the stimulus. All remaining neurons (i.e., those not showing a withdrawal-related burst or pause) were classified as NEUTRAL-cells. To avoid misclassifying ON-cells as NEUTRAL-cells,[4] a bolus of methohexital anesthetic was given to the point that withdrawal reflex was abolished. Firing of NEUTRAL-cell is unaltered during this maneuver, whereas spontaneously active ON-cells slow or stop. After confirmation of cell type, animals were re-stabilized at a constant anesthetic flow rate before beginning data collection. One neuron per animal was recorded from in a typical experiment, but two or three neurons were sometime recorded from a single animal.

Once a neuron was isolated and characterized, we determined responses to optogenetic manipulations of cell bodies in PB and PB terminals in RVM. Three trials with light-induced inhibition of PB terminals in RVM were tested first, followed by three additional trials with light-

induced inhibition of cell bodies in. Each light trial (terminal or cell body stimulation) consisted of approximately 60 s of total light stimulation, with a heat stimulus delivered in the second half of the light train No-light/heat-only trials were performed before every light stimulation, to provide a control heat-evoked response. Because the densest spinoparabrachial projection ascends to the contralateral PB,[12; 17; 20; 53; 155; 183] the heat stimulus was always delivered to the paw contralateral to the PB opsin expression.

For the ChR2 experiments, only terminal stimulations were performed. Each light trial consisted of approximately 45 s of total light stimulation, with a heat trial in the last 30 s of the light train. To screen RVM neuron responses to pulses of different widths and frequencies, six different stimulation protocols were used: continuous light, 50 ms at 10 Hz, 20 ms at 10 Hz, 10 ms at 10 Hz, 20 ms at 20 Hz, and 20 ms at 40 Hz, delivered in that order. Each light protocol was tested once. To control for any possible antidromic activation of PB cell bodies by terminal stimulation, 4% lidocaine (200 nl) was microinjected in PB in some experiments. Following the injection, two additional terminal stimulations were performed in these animals (continuous light, and 50 ms pulse width at 10 Hz).

3.3.3. Histology

At the completion of the electrophysiological experiment, recording sites were marked with an electrolytic lesion. Animals were then overdosed with methohexital and perfused transcardially with saline followed by 10% formalin. The brains were removed, blocked, and sectioned on a Leica CM3050 S cryostat (40 μm sections). Recording sites marked with an electrolytic lesion at the conclusion of the experiment, as well as viral expression in PB cell bodies and in terminals around the recording sites in the RVM were plotted on hand-drawn sections in Adobe Illustrator using landmarks defined by Paxinos and Watson.[150] Only data from "on-target" reporter expression and recording site were analyzed (Figure 15) The RVM

was defined as the nucleus raphe magnus and adjacent reticular formation at the level of the facial nucleus (-1.04 to -2.6 mm IA, ±0.6 mm lateral, and 9-10 mm ventral to the brain surface).

3.3.4. Analysis

The microelectrode signal, EMG, and EKG output were digitized and collected using Spike 2 software (Cambridge Electronics Design Ltd, Cambridge, UK). Each waveform was sorted using Spike2 template matching and cluster analysis, verified on an individual spike basis.

We characterized reflex-related changes in OFF-cell activity by the duration of the reflex-related pause. ON-cell activity was quantified as the total number of spikes in each reflex-related burst (which may last for many seconds or tens of minutes). In ChR2 experiments in which a reflex-related burst could not be defined because the neuron under study showed light-related activity, the number of spikes in the 3 s period beginning 0.5 s before the paw withdrawal reflex was used instead of total evoked spikes.

Spontaneous activity was defined as the firing rate in the 10 s period before each stimulus (heat or light) stimulus or stimulus train (light). To assess the effect of each specific light-stimulation protocol, a ratio of firing during the light stimulus to that prior to stimulus onset was obtained. To be considered an effect, firing was changed by more than 50%. A change of less than +/-50%, or if the cell was silent, a change less than 1Hz were considered no difference.

We also measured the latency of the cell response to light pulses in ChR2 experiments. To calculate the response latency for those cells with excitatory inputs, a peri-stimulus time histogram (0.5 ms bins) was constructed to include the 10 s prior to, and 10 s at the beginning of each light stimulation trial. A mean and standard deviation of the control firing rate was calculated for each pre-light period, and the trial-specific response latency defined as the time at which the light-induced firing rate exceeded a threshold of 1.95 standard deviations above the pre-stimulation mean. The average response latency over all light-pulse trials was calculated

for each light-evoked response. To calculate response latency for cells with inhibitory responses to optogenetic activation of PB afferents, similar means and standard deviation calculations were done, except that inter-spike intervals were used rather than firing rate.

Paw withdrawal latencies were analyzed using a paired Wilcoxon's signed rank test, because distribution of responses was truncated when some responses were at the cut-off value. Because cell parameters for these neurons are typically highly skewed, these parameters were analyzed using paired t-test, or one-way ANOVA with repeated measures and post hoc Dunn's multiple comparison test of log-transformed data. The ratio of activity during light to that immediately prior to light onset was analyzed using Wilcoxon's signed rank tests. For all tests, $p < 0.05$ was considered significant.

3.4. RESULTS

3.4.1. Expression of ArchT or ChR2 on neurons and terminals projecting from lateral parabrachial complex to RVM

Our goal in this study was to determine how direct PB input to RVM affects the activity of identified RVM neurons *in vivo*. ArchT or ChR2 viral vectors were injected in the right PB 4 to 5 weeks prior to the electrophysiological experiment.

Reporter was found to be expressed along the PB projecting fibers and terminals. Positive fibers were found regions consistent with previous studies using anterograde tracing methods.[89; 173] For the current study, we confirmed that PB fibers project abundantly throughout the RVM area(raphe magnus n., raphe pallidus n., gigantocellular reticular n.), with the span from the facial nerve (-1.32 mm IA) to the caudal end of the facial nucleus (-3.12 mm IA). Besides the RVM, opsin-positive fibers of similar density were also found in rostral ventrolateral reticular nucleus (RVL) and the facial nucleus. In addition, there were also opsin-

positive fibers in other reticular nuclei (Gi, IRt and PCRt), solitary nucleus abducens nucleus and the dorsal part of medial longitudinal fasciculus, but with much less density.

3.4.2. Light evoked inhibition of PB terminals in RVM attenuates noxious evoked changes in RVM ON- and OFF-cell firing in ArchT-injected animals

We recorded RVM ON- and OFF-cell responses evoked by heat stimulation of the hindpaw in animals expressing ArchT in PB cells and projection targets. 12 ON-cells and 14 OFF-cells were recorded in 18 animals. Trials were done with and without light-induced inhibition of PB terminals in RVM and cell bodies in PB.

Light-evoked inhibition of cell bodies in PB attenuated the ON-cell burst and OFF-cell pause evoked by noxious thermal stimulation, consistent with previous work using microinjection of the $GABA_A$ agonist muscimol in PB,[166] and confirming PB as a relay of nociceptive information to RVM ON- and OFF-cells. As shown in the examples of Figure 16a, light-evoked inhibition of PB terminals in RVM also significantly attenuated the ON-cell burst and OFF-cell pause, similar to the effects of suppressing PB activity at the cell body. The effects of light-evoked suppression of activity in PB and PB terminals in RVM are summarized in Figure 16b-c. These observations indicate that a direct projection from PB to the RVM conveys nociceptive information to RVM pain-modulating neurons.

Light-evoked inhibition of activity in PB terminals in RVM also altered the spontaneous discharges of both ON- and OFF-cells (Figure 17), with a decrease in ON-cell firing and increase in OFF-cell firing. However, inhibition of PB activity at the cell body did not produce significant changes in ON- and OFF-cells spontaneous activity. In parallel with the light-induced changes in ON- and OFF-cell firing, the heat-evoked paw withdrawal latency in these animals was moderately increased by inhibition of PB-terminals in RVM($W = -236.0$, $p = 0.0008$, $n = 25$), while inhibition of cell bodies in PB did not significantly alter withdrawal behaviors ($W = -96.0$, $p = 0.1747$, $n = 25$)

3.4.3. RVM neurons can be activated or inhibited by optogenetic activation of PB terminals in RVM in ChR2-injected animals

Animals in which ChR2 viral vectors were injected in PB were used to assess the characteristics of the direct inputs from PB to RVM. Responses of RVM ON-, OFF- and NEUTRAL-cell to light-induced activation of PB terminals were. A range of light pulse widths and frequencies were tested. We also determined whether optogenetic activation of PB terminals in RVM altered heat-evoked neuronal and behavioral responses. We recorded 13 ON-cells, 14 OFF-cells, and 9 NEUTRAL-cells in 28 animals.

Light-induced activation of PB terminals resulted in significant *net* changes in spontaneous activity for both ON- and OFF-cells, but not for NEUTRAL cells (Figure 18a-c). When comparing RVM neuronal activities during the light-on periods to the pre-light periods, all stimulation protocols were able to alter spontaneous firing of both ON- and OFF-cells, whereas they had no *net* effect on NEUTRAL cells (Figure 18d-f)

When examining light-induced responses on each individual neuron, at least some neurons within each of the functionally defined RVM cell classes, the ON-cells, OFF-cells, and NEUTRAL-cells, responded to light-induced activation of PB terminals in RVM with all light-pulse protocols tested. For ON-cells, 9 of 13 cells recorded (69%) showed an increase in firing rate during the light pulse train, 1 exhibited a decrease in firing rate, and 3 showed no change in activity. Similarly, 10 of 14 (71%) OFF-cells displayed increased firing rate in response to light, 2 showed decreased spontaneous firing rate, and 2 showed no response. NEUTRAL cells were less responsive to light-induced activation of PB terminals than ON- and OFF-cells, with 6 of 9 cell recorded (67%) exhibiting no change in their spontaneous firing. However, 2 out of 9 NEUTRAL-cells showed an increase, and 1 showed a decrease in activity. These varied responses to PB terminal activation were not due to recruitment of other non-RVM PB targets

via antidromic impulses, as these ChR2-induced changes were preserved when lidocaine (4%, 200nl) was injected in PB to block conduction through the soma (data not shown).

For cells that exhibited an excitatory response, the latency of the response to light-induced terminal activation was determined for the different cell classes. The OFF-cells responded at an average latency of 2.2 ± 0.2 ms from onset of the light pulse, consistent with a single synaptic delay. ON-cells responded at a significantly longer latency to activation of PB terminals, 3.5 ± 0.5 ms (unpaired *t*-test, $t_{16} = 2.537$, $p = 0.022$). For the two NEUTRAL cells activated by terminal stimulation, the mean latency was 2.1 ms (1.5 and 2.6 ms for the two cells). For the few cells that exhibited an inhibitory response to terminal activation, the response occurred at a much longer latency. For the two OFF-cells inhibited by light, the response occurred with a delay of 139 ms, whereas that for the sole ON-cell was 228 ms, and for the NEUTRAL cell was 161 ms.

The duration of the OFF-cell pause was reduced during activation of PB terminals ($t_{(11)} = 6.881$, two silent cells, $p < 0.0001$), whereas the ON-cell burst (3s around the time of withdrawal) was not changed significantly ($t_{(12)} = 1.945$, $p = 0.0756$). Noxious heat-evoked paw withdrawal was delayed with activation of PB terminals ($W = -318.0$, $n = 28$, $p = 0.0003$), indicating an overall anti-nociceptive state for the animals.

3.5. DISCUSSION

3.5.1. PB modulates ON- and OFF-cell activity via its direct projection to RVM

Descending pain-modulating pathways regulate nociceptive processing at the level of the dorsal horn. The facilitatory and inhibitory outputs of RVM, mediated by ON-cells and OFF-cells, can be modulated by “top-down” inputs from areas including the amygdala, periaqueductal gray, and hypothalamus, and contribute to the effects of fear, stress, and immune activation on pain behavior.[75; 81; 122] “Bottom-up” nociceptive sensory inputs also play an important role

in modulating RVM functional outputs: noxious stimuli activate pain-facilitating ON-cells, giving rise to a “burst” of firing, while suppressing pain-inhibiting OFF-cells, leading to a “pause” of any ongoing activity. These sudden changes in firing begin immediately before the behavioral withdrawal from the stimulus, allowing the response to occur by removing OFF-cell-mediated descending inhibition and enhance the magnitude of the response.[59; 99] It is clear that RVM ON- and OFF-cells respond to noxious somatic stimuli, however the specific pathway or pathways through which nociception-related information reaches RVM pain-modulating neurons are only now beginning to be defined.

Our laboratory recently identified the lateral region of PB as a significant nociceptive relay to the RVM, showing that global inactivation of the PB area attenuated both the ON-cell burst and OFF-cell pause.[166] Although we also confirmed a direct anatomical connection from the PB to the RVM,[8; 84; 195] we could not conclude that this connection mediated the ON-cell burst and OFF-cell pause since there was no evidence that the projection was to ON- and OFF-cells rather than NEUTRAL-cells, since the direct connection could have been related to other functions of PB and RVM (e.g., thermogenesis[137; 138]), and since the PB could have been an intermediate relay between the dorsal horn and other sites that project to RVM such as the amygdala, periaqueductal gray or insula.[23; 62; 63; 72; 95; 119; 174; 203] To address this question, the present experiments used optogenetic methods to manipulate the terminals of PB neurons in the RVM, providing definitive evidence that the direct circuit linking PB to the RVM conveys nociceptive signals to the RVM. Thus, optogenetic inactivation of the PB terminals in the RVM substantially attenuated ON-cell burst and OFF-cell pause evoked by noxious demonstrating that this direct projection from PB contributes to the ON-cell burst and OFF-cell pause. As with global inactivation of the PB area using the $GABA_A$ receptor agonist muscimol,[166] neither the ON-cell "burst" or the OFF-cell "pause" were completely eliminated

using optogenetic inhibition of the PB terminals in RVM. This suggests that other direct pathways or relays also carry nociceptive information to the RVM.

Optogenetic inactivation of PB terminals was also sufficient to modulate the spontaneous discharge of RVM ON- and OFF-cells, with a decrease in ON-cell firing and increase in OFF-cell firing. This indicates that a direct input from PB to RVM can modulate the overall "tone" in RVM pain-modulating neurons, and suggests that sensory information relayed through the PB is an important factor in that tone, at least in lightly anesthetized animals.[74]

3.5.2. The PB supplies both excitatory and inhibitory inputs to the pain-modulating neurons in RVM

The experiments using optogenetic inactivation of PB terminals in animals expressing ArchT in PB demonstrate a net contribution of PB inputs to the ON-cell burst and OFF-cell pause. However, the inactivation approach can only identify inputs that are active under a given experimental condition. We therefore used optogenetic activation of PB terminals expressing ChR2 to identify potential "latent" inputs from PB to RVM. The majority of both ON- and OFF-cells surveyed were excited by activation of PB terminals. These observed effects were not due to antidromic activation of PB cell bodies, as lidocaine injection in PB did not alter the light-induced spontaneous discharge of in RVM neurons. These findings indicate that a net excitatory input to either or both cell classes exists, and has the potential to be engaged under different physiological or pathophysiological conditions.

We also found a small percentage of ON- and OFF-cells that were inhibited by activation of local PB terminals. The small number of neurons showing inhibition could have been due to a sampling bias, but the fact that activation of PB terminals produced a net hypoalgesia would be consistent with the effects of activating ON- and OFF-cells,.[58; 149; 153] which suggests that the predominant excitatory effect of terminal stimulation reflected the overall response of the population as a whole. The latencies of inhibitory responses were noticeably longer than those

of excitatory responses, suggesting that the former were due to circuit-level interactions. In any case, although the precise balance of active inhibitory and excitatory inputs to RVM ON- and OFF-cells under different physiological conditions remains unknown, and our experiments using ArchT indicate that the net influence of PB inputs on ON-cells was excitatory whereas that on OFF-cells was inhibitory under the conditions of these experiments, the responses to optogenetic activation of PB terminals make it clear that PB has the capacity to both activate and inhibit ON-cells and OFF-cells in RVM.

In vivo recordings do not allow us to determine whether PB terminals synapse directly onto the ON- and OFF-cells recorded. However, the short latency of the OFF-cell response to PB terminal activation is consistent with a single synaptic delay, which suggests that projections from PB form synaptic connections with RVM OFF-cells. The longer latency of the ON-cell response to PB terminal stimulation could be due to differential location of PB inputs on the ON-cells compared to the OFF-cells, or the intrinsic membrane property only allows slower depolarization in ON-cells. Further research should investigate the synaptic properties of this connection.

Lastly, the ChR2 studies also revealed an influence of PB on NEUTRAL-cells. Although the proportion of NEUTRAL-cells responding to activation of PB terminals in RVM was small, both activation and suppression of firing were seen. Since inactivation of PB terminals in the present studies and inactivation of PB itself had no effect on the firing of NEUTRAL-cells,[166] it would be interesting to know when and how parabrachial input to NEUTRAL-cells is engaged. It is likely that at least some of these NEUTRAL-cells serve other, non pain-related physiological functions that are shared between PB and RVM (e.g., thermoregulation).

3.5.3. Conclusion

Although it has been thought for some time that PB is an important relay through which nociceptive information engages descending control circuitry,[107; 180] it was only recently that

a functional link between PB and RVM was established.[166] The present study demonstrated that the anatomical connection from PB to RVM conveys nociceptive information relevant to pain-modulation. Under the conditions of these experiments, PB conveyed a net inhibitory input to OFF-cells and an excitatory input to ON-cells. However, excitatory inputs to OFF-cells and inhibitory inputs to ON-cells were also demonstrating, suggesting that PB could activate OFF-cells or inhibit ON-cells under different conditions, such as in persistent pain states. Given the importance of presumed spinoparabrachial dorsal horn neurons in pathological pain,[145; 164; 180] the interaction between PB and RVM is likely to be crucial in development of central sensitization and compensation during persistent inflammation or following nerve injury. Certainly it would be interesting to know whether these inputs are activated or unmasked in persistent pain, to either contribute to or limit expression of hyperalgesia.[25; 35; 43; 51; 156; 164; 185; 187; 194] It would also be useful to isolate the synaptic mechanisms linking specific PB input to RVM neurons, which could provide unprecedented insights into the factors underlying sensitization of brainstem circuitry in persistent pain.

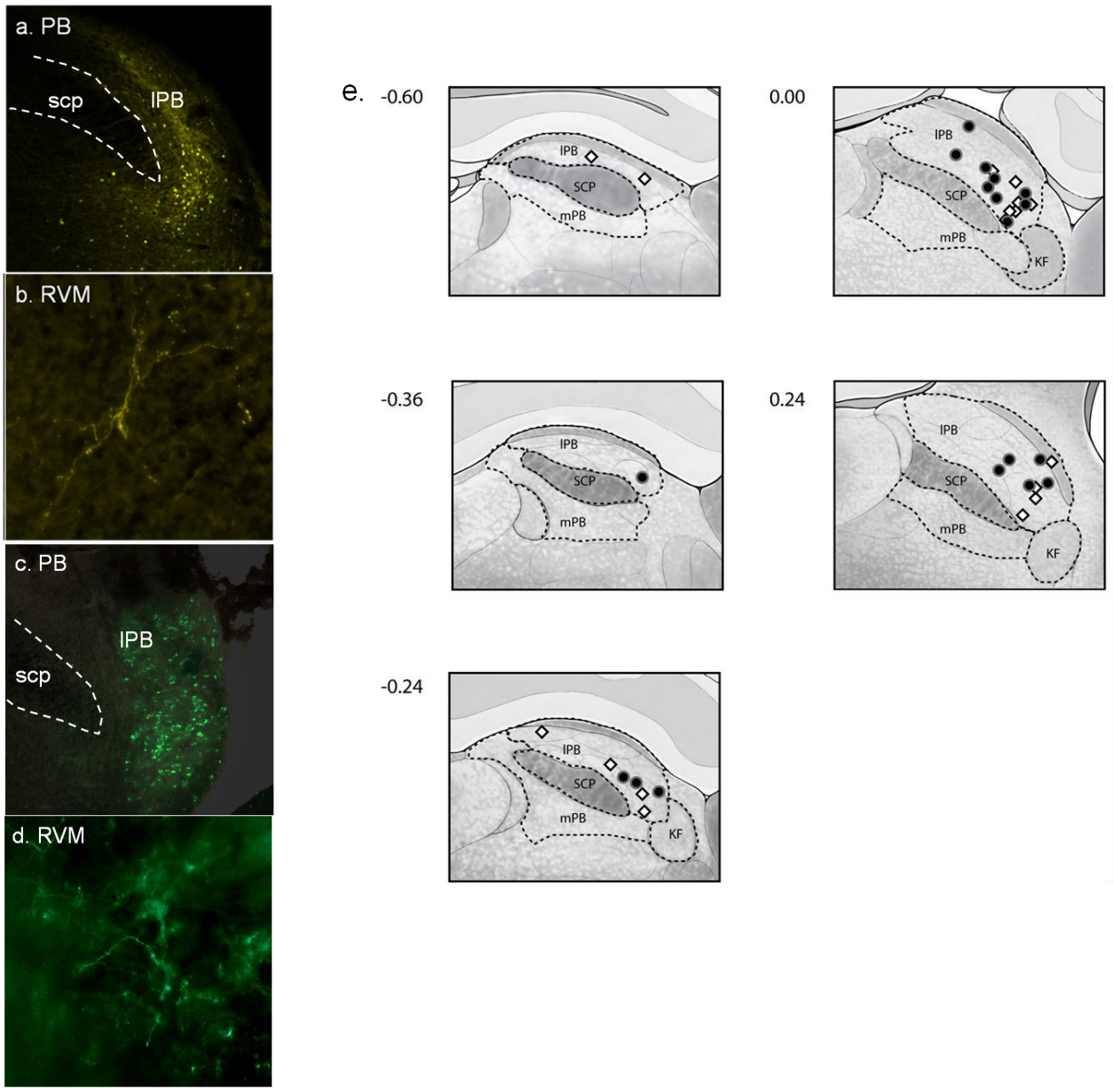

Figure 13 Locations of ArchT or ChR2 expression in the lateral PB complex

Examples of ChR2 expression in PB cell bodies **(a)** and PB projecting fibers in RVM**(b)**. Examples of ArchT expression PB cell bodies **(c)** and PB projecting fibers in RVM **(d)**. The expression sites were distributed among sections at +0.24 to -0.60 relative to the interaural line **(e)**. Injections inside the lateral PB (lPB) region were considered "on-target." White diamonds represent ChR2 expression sites, and black circles represent ArchT expression sites. KF: Kölliker-Fuse, lPB: lateral parabrachial complex, mPB: medial parabrachial area, SCP: superior cerebellar peduncle.

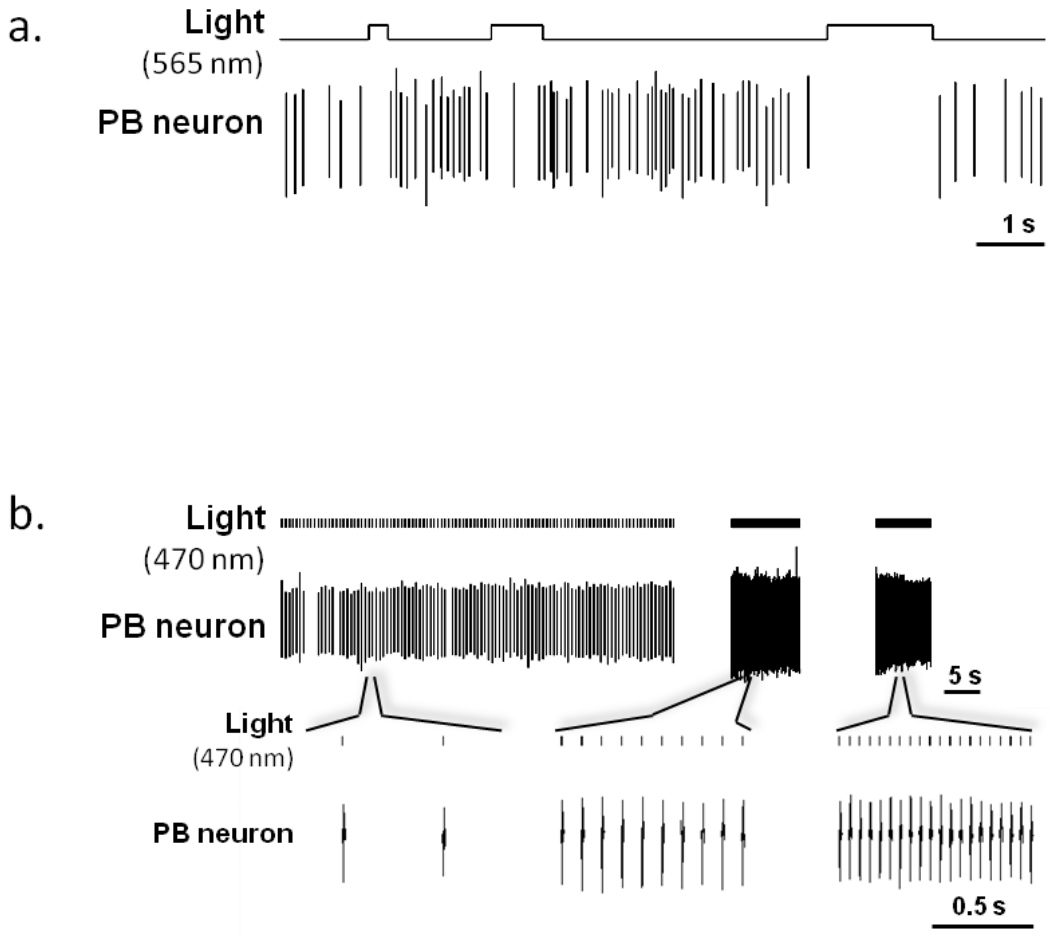

Figure 14 Validation of ArchT and ChR2 function in PB neurons.

a. Inhibition of PB neuronal activity by light-induced activation of ArchT opsins. **b.** Excitation of PB neuronal activity by light induced activation of ChR2 opsins. Note that firing of PB neurons followed light stimulation trains.

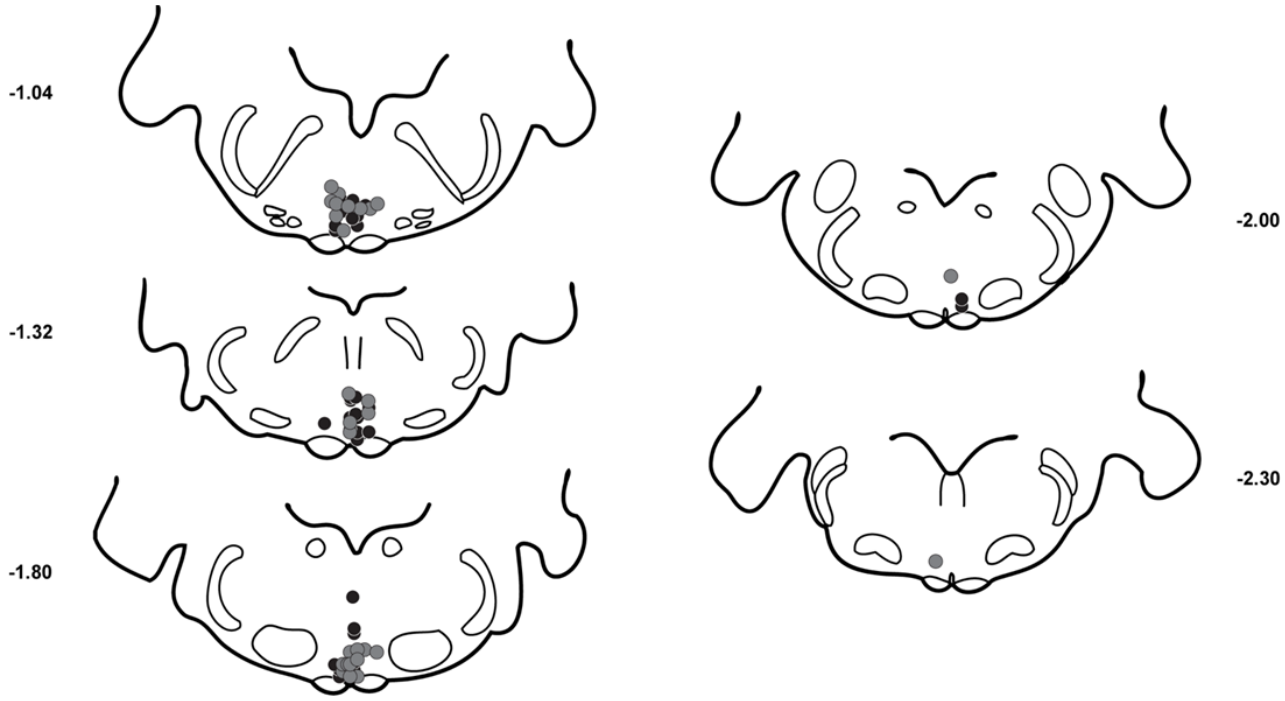

Figure 15 Locations of recording sites within the RVM.

Recording site for both ArchT (gray circles) and ChR2 (black circles) were distributed between at -1.04 mm and -2.30 mm relative to the interaural line, with majority of cells recorded between -1.04 mm to -1.80 mm.

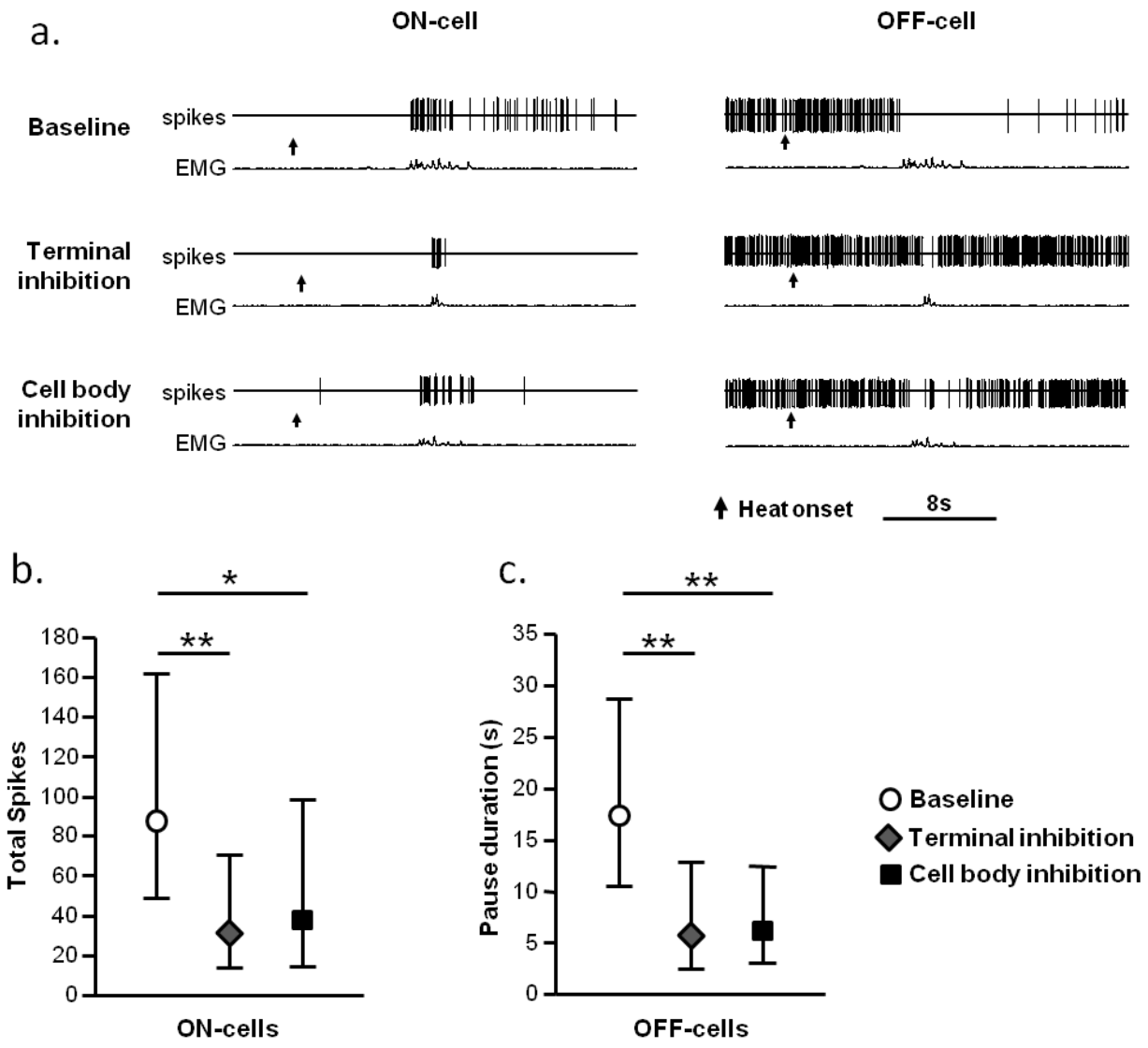

Figure 16 Effects of ArchT-induced inhibition of PB terminals and cell bodies on ON- and OFF-cell nociception-related activity.

a. Representative examples show ON- and OFF-cell activity during withdrawal from a noxious heat stimulus at baseline compared to during PB terminal or cell body inhibition. In both cases, nociception related changes in firing were attenuated. **b.** ON-cells. The effect of ArchT-induced inhibition of PB terminals and cell bodies on the ON-cell burst (measured as total evoked spikes). **c.** OFF-cells. The effect of ArchT-induced inhibition of PB terminals and cell bodies on the OFF-cell pause (measured as pause duration). (Reported as geometric mean with 95% confidence limits, *$p < 0.05$ and **$p < 0.01$ compared to baseline, one-way ANOVA with repeated measures and post hoc Dunn's multiple comparison test, n = 12 to 14 cells

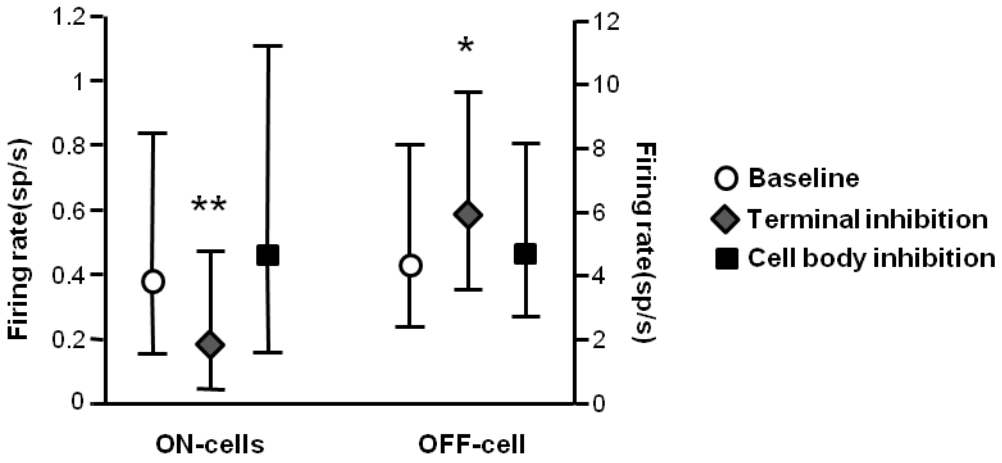

Figure 17 Effects of ArchT-induced inhibition of PB terminals and cell bodies on ongoing firing of ON- and OFF-cells in RVM.)

Terminal inhibition significantly decreased ON-cell ongoing firing, while increasing OFF-cell firing. Cell body inhibition had no significant impact on ongoing firing of both ON- and OFF-cells. (Reported as geometric mean with 95% confidence limits, *$p < 0.05$ and **$p < 0.01$ compared to baseline, one-way ANOVA with repeated measures and post hoc Dunn's multiple comparison test, n = 12 to 14 cells per class)

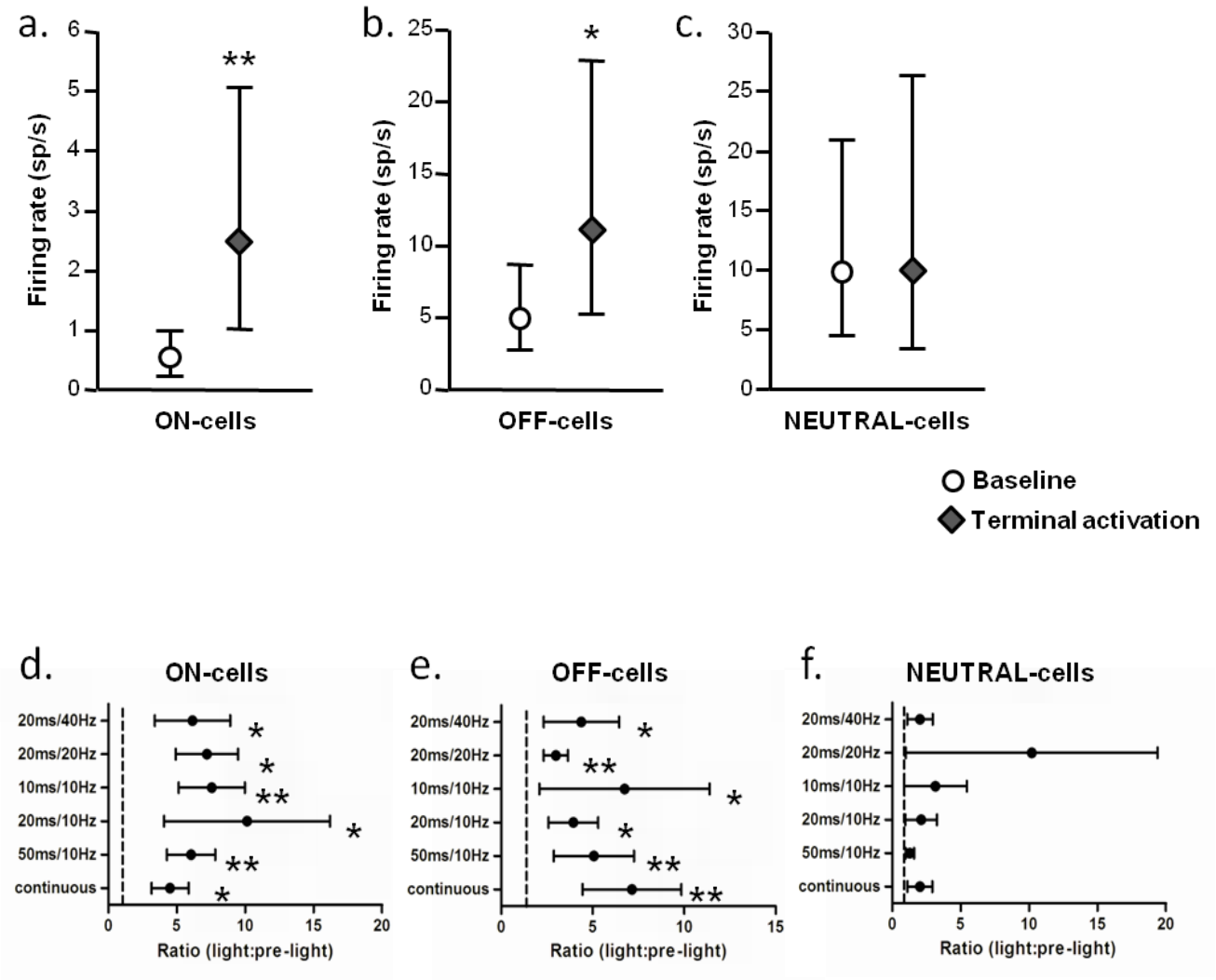

Figure 18 Effect of ChR2-induced activation of PB terminals on ongoing firing of ON-, OFF- and NEUTRAL-cells in RVM.

a-c Terminal activation significantly increased the net firing rate of both (a)ON- and (b)OFF-cells, but had no net effect on (c)NEUTRAL cells. (Reported as geometric mean with 95% confidence limits, *$p < 0.05$ and **$p < 0.01$ compared to baseline, paired t-test, n = 9-14 cells per class) **e-f.** Light vs. pre-light ratios for each light stimulation protocol. (d)ON- and (e)OFF-cells showed significant responses to all light stimulation trains, and (f)NEUTRAL-cells did not. (Reported as geometric mean with 95% confidence limits, *$p < 0.05$ and **$p < 0.01$ compared to the hypothetical value of 1 (no change), Wilcoxon's signed rank test, n = 12 to 14 cells per class)

CHAPTER 4

MANUSCRIPT #3

Plasticity in the link between pain-transmitting and pain-modulating systems in acute and persistent inflammation

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4.1. ABSTRACT

The parabrachial complex (PB) has recently been identified to be a major nociceptive relay for RVM pain-modulating neurons under basal conditions. The current study current study was designed to examine the role of PB in influencing ON- and OFF-cell activity during acute and persistent inflammation. Recording of RVM ON- and OFF-cells were perform in animals subjected to either acute or persistent inflammation. In animals with acute inflammation, PB remained as the major pronociceptive driver by exciting ON-cell and suppressing OFF-cell activity, similar to basal activity. However in persistent inflammation, PB no longer relays nociceptive information to ON-cells, and its influence on OFF-cell activity is reduced. Inactivation of PB during persistent inflammation also did not attenuate withdrawal latency in response to mechanical stimulations. These results thus provided evidence for a dynamic pain-modulating circuit between PB and RVM, and a neurophysiological basis for changes in nociceptive behavior during acute and persistent inflammation.

4.2. INTRODUCTION

The rostral ventromedial medulla (RVM), the output node of a major brainstem pain-modulating system, is now generally recognized to have an important role in persistent pain states, including inflammation and nerve injury.[43; 156; 187; 188] Molecular and cellular studies document plasticity in RVM circuits in persistent pain states, with changes in NMDA, AMPA, trkB, opioid, and neurokinin-1 receptor expression and function,[19; 65-67; 93; 106; 162; 175] local glial activation,[165] and even neuronal loss.[111] However, changes in RVM output may also reflect altered inputs. Although "top-down" inputs, from hypothalamus, amygdala, and the periaqueductal gray, have been shown to modulate nociception via the RVM,[75; 76; 81] plasticity in pain-transmission pathways has been extensively documented in persistent pain states, with sensitization of both primary afferents and central circuits,[94; 108; 154; 159] making it important to understand how nociceptive inputs reach RVM pain-modulating neurons under both basal conditions and following injury and inflammation.

The parabrachial complex (PB), especially its lateral region, was recently identified as a major pathway through which nociceptive signals gain access to the RVM.[166] PB receives a substantial projection from nociceptive neurons in the contralateral superficial dorsal horn. There is also a less dense input to PB from the ipsilateral superficial dorsal horn and from deeper laminae.[12; 17; 20; 53; 155] PB neurons show increased c-fos expression during inflammation or following peripheral nerve injury, as well as in response to acute noxious stimuli.[9; 15 ; 85; 98] Selective activation of a subset of PB neurons elicits aversive behaviors that may reflect aspects of a pain experience.[70] Although the contribution of PB itself to persistent pain has not been specifically tested, indirect evidence supports a role for this structure. A large proportion of superficial dorsal horn neurons projecting to the contralateral PB express the neurokinin-1 (NK1) receptor, and elimination of these neurons interferes with persistent hyperalgesia in animal models of inflammatory and neuropathic pain.[103; 145; 164;

180] The contribution of NK1-positive neurons to hyperalgesia is thought to be via engagement of a supraspinal loop through PB that leads to activation of a descending pro-nociceptive serotonergic pathway.

Functionally, the RVM modulates nociceptive transmission via two classes of neurons, pain-facilitating ON cells and pain inhibiting OFF-cells.[75] In acute inflammatory injury, the pronociceptive ON-cell ongoing discharge significantly increase and the antinociceptive OFF-cell firing is suppressed.[24; 35; 104] ON- and OFF-cell nociception-related response become sensitized, and exhibit a lower response threshold.[35] These changes form an ON-cell dominant, net pain-facilitatory loop, and are necessary for the expression of behavioral hyperalgesia. However when an injury persists, ON- and OFF-cell "tone" is restored to normal levels, although their response threshold decreases.[26; 35; 151] The net RVM output in persistent pain states is an OFF-cell-mediated antinociceptive influence that limits behavioral hypersensitivity.[35; 43]

Expanding upon our previous findings, the goal of the current study was to examine the contribution of PB to the activity of identified ON- and OFF-cells in acute (1 hour), as well as early (1 day) and late (5 or 6 days) phases of persistent inflammation induced by localized intraplantar injection of complete Freund's adjuvant (CFA) in one hindpaw. The time points were chosen based on reports that RVM physiology and its contribution to behavioral sensitivity fluctuate during acute injury and the first day following injury, but are then stable for at least ten days.[35; 65; 66; 118; 161; 182] We hypothesized that inputs from PB contribute to the sensitization and compensation process of RVM during the transition from acute to persistent inflammation.

4.3. MATERIALS AND METHODS

All experimental procedures were approved by the Institutional Animal Care and Use Committee at Oregon Health & Science University and followed the guidelines of the Committee

for Research and Ethical Issues of the International Association for the Study of Pain. Since we have identified lateral, not medial, PB relays nociceptive information to RVM in our earlier report,[166] all PB injections in the current study were done in the lateral PB.

4.3.1. Induction of Inflammation

Male Sprague-Dawley rats weighing between 250 and 400 g were used in the acute/persistent inflammation recording studies within the first hour (acute), 1 day or 5 to 6 days (persistent) after induction of inflammation.

For studies of acute inflammation, animals were anesthetized and maintained in a lightly anesthetized state. Complete Freund's Adjuvant (CFA, 0.1 ml, heat-killed Mycobacterium tuberculosis in mineral oil, 1 mg/ml, Sigma-Aldrich) was injected subcutaneously in the plantar surface of the left hindpaw, and neuronal activity and behavioral responses to probing with calibrate von Frey fibers were recorded as described below. In a subset of animals, paw thickness was measured at the base of the ankle before and 1 hour after hindpaw injection.

To induce persistent inflammation, rats were briefly anesthetized with isoflurane (4%, 4-5 min). CFA (0.1 ml) was injected subcutaneously into the plantar surface of the left hindpaw. Animals were anesthetized either 1 day or 5 to 6 days later for electrophysiological studies. These time points are consistent with the known duration of behavioral hypersensitivity following plantar CFA administration, which typically peaks at one day (24 h), but is maintained for 10 days or more.[35; 65; 66; 118; 161; 182]

4.3.2. Electrophysiological experiments

4.3.2.1. Animal preparation

On the day of the experiment, animals were deeply anesthetized using isoflurane (5% in oxygen) and a catheter inserted in the external jugular vein for subsequent infusion of the short-

acting barbiturate, methohexital. They were then transferred to a stereotactic frame. While animals were still deeply anesthetized, two small craniotomies were drilled for access to the RVM and the PB. Heart rate was monitored using EKG. Body temperature was monitored and maintained at 36-37 °C with a heating pad and a space heater.

After the surgical preparation, the rate of methohexital infusion was adjusted so that animals displayed no spontaneous movement or apparent distress, while allowing a stable heat-evoked hindpaw withdrawal reflex. Animals were stabilized for at least 45 minutes at a constant anesthetic flow rate before beginning data collection.

4.3.2.2. Electrophysiological recording

Extacellular single-unit recordings were made with stainless-steel microelectrode (Microprobes, Gaihersburg, MD) with gold- and platinum-plated tips. Signals were amplified (10k) sampled at 32k Hz, and bandpass filtered (400 Hz to 15 kHz).

RVM neurons were isolated and classified as ON- or OFF-cells as described previously using a Peltier device on the plantar surface of the hindpaw (Yale Instruments, a 35 °C holding temperature, increased at a rate of 1.2 °C/s from 35 °C to a maximum of 53 °C).[35; 58; 59] Both ON- and OFF-cells have whole body receptive fields, but inverse responses during nocifensive behaviors. ON-cells exhibit a “burst” of activity (if not already active), while OFF-cells exhibit a “pause” of any ongoing activity (if active), or remain silent (if inactive). To further confirm cell classification, a 10 s noxious pinch was delivered to the hindpaw using a toothed forceps: ON-cells burst and OFF-cells pause throughout the stimulus. Anesthetic depth and cell identification were assessed regularly throughout the experiment using heat stimulations. One neuron per animal was recorded from in a typical experiment, though two neurons could be simultaneously recorded on some occasions.

4.3.2.3. Experimental protocol

All studies used a within-subject design. For experiments testing PB's role in naive animals, an ON- or OFF-cell was isolated and characterized. Baseline mechanical sensitivity was tested using von Frey fibers (26, 60, and 100 g) applied in ascending order to the interdigital webbing of the right and left hindpaw for a period of 8 seconds. Withdrawal latencies were determined from hamstring EMG. Three trials were performed with each force, and a minimum of two testing sites were used in rotation. Individual trials were initiated at intervals of at least 30 seconds, with longer interstimulus intervals (up to 4 min) used when necessary to capture a period when the cell under study was active (OFF-cell) or inactive (ON-cell). Muscimol (200 nl, 8 pmol) mixed with florescent beads (1:100) was microinjected over 4 min into the right lateral PB, since previous work has shown that the lateral, and not medial, PB area is an important relay to RVM.[166] Four minutes later, mechanical sensitivity testing was repeated as in baseline.

For experiments involving acute inflammation, an ON- or OFF-cell was isolated and baseline parameters determined as above using 26, 60 and 100 g von Frey fibers. CFA was then injected in the plantar surface of left hindpaw. The animal was then left undisturbed for 10 min. Mechanical testing of the CFA-treated and untreated hindpaws was performed between 15 - 50 min following CFA injection. Muscimol was injected in the right PB as above 60 min post-CFA. Mechanical sensitivity was assessed between15 - 45 minutes after muscimol injection as described above.

For experiments involving persistent inflammation, CFA was injected into the left hindpaw 1 day or 5-6 days prior to the electrophysiological experiment. Following isolation and characterization of an RVM neuron as above, baseline mechanical sensitivity and withdrawal latencies were determined for the two hindpaws with von Frey fibers (4, 8, 15, 26, 60, and 100

g) as above. Muscimol was then microinjected into the right PB. Mechanical sensitivity testing was then repeated with the two hindpaws starting 4 minutes after completion of the injection.

4.3.3. Histology

Upon the conclusion of the recording experiment, an electrolytic lesion was made in the RVM to localize the recording site. Rats were overdosed with methohexital and transcardially perfused with saline followed by formalin. The brains were removed and sectioned on a Leica CM3050 S cryostat (60 μm sections). The RVM lesion and fluorescent beads marking the injection site in the PB were photographed with an Optronics Microfire camera attached to an Olympus BX51 microscope. If beads or lesion were not found, the data were not analyzed further. Injection sites were plotted on hand-drawn sketches of the sections in Adobe Illustrator using landmarks defined by Paxinos and Watson.[150] For "on-target" injections, if injection sites were not located within the lateral parabrachial complex, the data were not analyzed further. PB injection sites are plotted in Figure 19. The RVM was defined as the nucleus raphe magnus and adjacent reticular formation medial to the lateral boundary of the pyramids at the level of the facial nucleus.

4.3.4. Analysis

The microelectrode recording, EMG, and EKG output were digitized and collected using Spike 2 software (Cambridge Electronics Design Ltd, Cambridge, UK). Each waveform was sorted using Spike2 template matching and cluster analysis, verified on an individual spike basis.

In all experiments, data were compared between average pre- and post-PB block. Nociception-related activity was defined as the total number of spikes in each ON-cell burst, and the duration of the OFF-cell pause. Although trials using von Frey fibers were generally not initiated until ON-cells were inactive or OFF-cells active, some ON-cells became active or OFF-

cells became inactive for a prolonged period of time (>5 min). For those cases where ON-cells were continuously active, spike count during the 8 s von Frey stimulation period was used. For OFF-cells that were inactive, a pause duration of 8 s was assigned.

Ongoing activity was defined as the average firing frequency over 30 s prior to heat trials or during the 30 s immediately before beginning the von Frey stimulation protocol. In defining the direction of change of ongoing firing, at least 50% change from baseline would be considered an increase or decrease in activity following a PB-block.

Because cell parameters are typically highly skewed, they were analyzed using paired *t*-test of log-transformed data. Paw withdrawal latencies were analyzed using repeated-measures ANOVA followed by Sidak's post-hoc test to compare behavioral responses to the full range of stimulus forces tested. For all tests, $p < 0.05$ was considered significant. Withdrawal latencies are reported as mean + SEM, cell parameters are reported as geometric mean with 95% confidence limits, or before-after line graphs.

4.4. RESULTS

RVM ON- and OFF-cell responses during mechanical stimulation of the hindpaw were studied before and after blockade of the contralateral PB using the $GABA_A$ receptor agonist muscimol. This was done in the following conditions: untreated naïve animals, the first hour following plantar injection of CFA, 1 day later, and 5 to 6 days later. These studies focused on responses evoked by mechanical stimulation, because substantial mechanical hyperalgesia is robust in lightly anesthetized rats following plantar injection of CFA[35]. A total of 53 ON-cells, and 48 OFF-cells were recorded in 92 animals.

Injection of CFA into the plantar surface of the hindpaw produced localized inflammation that was evident within the first hour, with an increase in paw thickness from 4.92 $\pm$ 0.38 mm prior to the injection, to 9.2 $\pm$ 0.5 mm by 1 h post-injection, and 10.5 ± 0.6 mm by the fifth day. The

threshold for withdrawal of the CFA-treated paw was reduced significantly compared to the untreated paw at each of the three time points examined, as summarized in Figure 20. In the experiment performed during acute inflammation, we used 26, 60 and 100 g fibers. Prior to CFA injection, only 2 of 24 animals responded to a 26 g fiber, although all responded to 60 and 100 g. Following injection of CFA, all animals responded to the 26 g fiber. For more prolonged inflammation (1 and 5/6 days post-CFA), we used 4, 8, 15, 26, 60 and 100 g fibers. In all cases, withdrawal of the CFA-treated paw was elicited at 15 g or less. By comparison, the threshold for the untreated paw was 60 g in most cases (15 of 18 animals tested at 1 day post-CFA, and 21 of 23 animals at 5/6 days post-CFA).

4.4.1. Effect of blocking PB on RVM activity in naïve animals

We first considered the effect of blocking PB on the ON-cell burst and OFF-cell pause associated with stimulation of the contralateral hindpaw in naïve animals. Both the burst and pause were significantly reduced during PB block, as shown in the examples in Figure 21a and summarized in Figure 21b-c. Blocking PB *ipsilateral* to the stimulated paw in a separate group of animals had no effect on reflex-related firing of either the ON- or OFF-cell (data not shown). The ongoing firing exhibited by both ON- and OFF-cells was also altered Figure 21d. *All* of the ON-cells tested displayed decreased ongoing firing during PB block, and *all* OFF-cells showed increased firing.

4.4.2. Effect of blocking PB on RVM activity in acute inflammation (within 1 h after injection of CFA)

In the second set of experiments, we tested the effect of blocking PB contralateral to the acutely inflamed paw on RVM cell activity. As shown in the examples in Figure 22a, and summarized in Figure 22b-e, blocking PB in animals subjected to acute inflammation significantly attenuated reflex-related changes in the firing of ON- and OFF-cells associated with stimulation of the CFA-treated paw. The effect of PB block was therefore comparable to that

seen in naïve animals, although the effect on the ON-cell burst was less robust, with the response to the strongest stimulus used (100 g) not changed significantly. As in naïve animals, the ON-cell burst and OFF-cell pause associated with stimulation of the untreated paw were unchanged by the PB block (which was ipsilateral to untreated paw). Finally, the ongoing firing rate of ON- and OFF cells was also altered, with a decrease in ON-cell firing and an increase in OFF-cell firing, an effect similar to what was seen in naive animals (Figure 22f). All OFF-cells recorded showed an increase in ongoing activity compared to pre-block baseline, and 9 of 10 ON-cells with ongoing firing in baseline showed a decrease in ongoing activity during PB block.

4.4.3. Effect of blocking PB on RVM activity in early inflammation (1 day after injection of CFA)

In this set of experiments, animals were treated with CFA one day prior to the recording session.

Blocking PB at this time point failed to alter the evoked responses of either ON- or OFF-cells (Examples shown in Figure 23a). Summary data are shown in Figure 23b-c, with the total response summed across the innocuous (4, 8 and15 g) and noxious (60 and 100 g) ranges for stimulation of the CFA-treated and untreated paws. (Again, block was contralateral to the CFA-treated paw but ipsilateral to the untreated paw).

In these animals, the ongoing firing of both ON- and OFF-cells exhibited considerable variability in response to PB blockade. In contrast to the typical effect of PB block in naïve animals, OFF-cell firing was *not* significantly changed following PB block after 1 day of inflammation, and only 3 of 10 OFF-cells sampled showed an increase in firing rate; the remaining seven exhibited decreased or no change in firing. Although ON-cells overall showed a statistically significant decrease in ongoing activity compared to pre-block baseline, 9 of the 14 cells with ongoing activity showed a decrease in firing, and 2 cells showed an *increase* in activity (three cells without ongoing activity remained silent during PB block). The data is summarized in Figure 23d.

4.4.4. Effect of blocking PB on RVM activity in persistent inflammation (5 to 6 days after CFA injection)

In the final set of experiments, animals were treated with CFA 5-6 days prior to the recording session.

ON- and OFF-cells showed differential responses to PB block at this time point. For ON-cells, similar to what was seen 1 day post-CFA, blocking PB at 5-6 days post-CFA failed to alter the ON-cell burst associated with stimulation of either the inflamed (contralateral to PB block) or uninflamed (ipsilateral to PB block) paw (Figure 24a). However, the OFF-cell pause associated with both innocuous and noxious stimulation of the inflamed paw was significantly attenuated. Moreover, PB block also led to a reduction of the pause associated with noxious stimulation of the noninflamed paw (ipsilateral to the PB block, Figure 24b).

At the fifth day post-CFA, the ongoing firing of both ON- and OFF-cells was not altered in a uniform way by PB block. Eight out of 13 ON-cells showed either decreased or unchanged activity with PB block, and five were increased. OFF-cells showed an even greater variety of responses, with 5 out of 10 OFF-cells increasing their ongoing firing, and 5 decreasing or remaining unchanged. The overall activity of OFF-cells was thus unaffected by muscimol injection in the PB contralateral to the inflamed paw during the late phase of persistent inflammation (Figure 24c).

4.4.5. Effects of blocking PB on nociceptive withdrawal

In naïve animals, blocking the PB resulted in a small but statistically significant increase in the latency to withdraw the contralateral paw from intense (60 and 100 g) stimulation (Figure 25a), consistent with our previous report.[166] During acute inflammation (within the first hour following injection of CFA), blocking PB contralateral to the inflamed paw had an anti-hyperalgesic effect, significantly increasing the latency to respond to the 26 g fiber (Figure 25b).

However, the response to noxious stimuli (60 and 100 g) was unaffected, as was the responses to stimulation of the untreated paw (ipsilateral to the PB block, Figure 25c).

In contrast with the anti-hyperalgesic effect of PB block in the first hour following CFA injection, blocking PB contralateral to the inflamed paw at 1 or 5/6 days post-injection did not reverse hypersensitivity of the inflamed paw (Figure 26a-d). Moreover, PB block resulted in hyperalgesia (i.e., a *decreased* latency to respond to application of the 60 g fiber to the uninflamed paw (ipsilateral to the PB block), suggesting that any influence of PB had a net anti-nociceptive effect once inflammation was fully developed.

4.5. DISCUSSION

The lateral PB was recently identified as a major "bottom-up" driver of RVM ON- and OFF-cells, relaying information related to acute noxious stimulation to RVM neurons. The goal of the present study was to determine whether PB nociceptive input to RVM alters in persistent inflammation. The present experiments showed that PB is an important relay to RVM ON- and OFF-cells in acute inflammation as it is under basal conditions, but that its role as a driver of ON- and OFF-cell activity is diminished as inflammation develops and persists over a period of days. We also show that PB itself is not required for tactile allodynia in persistent inflammation.

4.5.1. PB as a relay to RVM in persistent inflammation

The current study is a first step towards delineating the functional dynamics of the PB as a nociceptive relay to RVM during different phases of inflammation. We focused on the PB contralateral to inflammation, since the major ascending projection from the superficial dorsal horn to PB ascends to the contralateral PB.[12; 17; 20; 53; 155]

In the naive condition, blocking PB contralateral to the stimulation site significantly attenuated the nociception-related ON-cell burst and OFF-cell pause evoked by noxious mechanical stimulation (60 and 100 g von Frey probes). PB block also resulted in a decrease in

the ongoing discharge of the ON-cells and an increase in that of OFF-cells. PB continues to function as an important relay to RVM in the early phase of developing inflammation, since PB block interfered with RVM responses to a subnoxious stimulus (26 g von Frey fiber applied to the inflamed paw) as well as those evoked by noxious stimuli, and also modified ongoing activity. These observations confirm the role of PB as a major relay of acute nociceptive information to RVM ON- and OFF-cells,[166] and demonstrate that the importance of PB as an input to RVM is maintained in acute inflammation. The latter finding suggests that PB could play a role in triggering molecular and cellular plasticity in RVM in inflammatory states. This idea is consistent with previous reports that spinal neurons expressing the NK1 receptor are essential for development behavioral hypersensitivity in persistent inflammation, since many of these neurons project to the parabrachial complex.[103; 145; 164; 180]

As inflammation persisted, the influence of the PB on RVM became less important. On the first day after injury, as acute inflammation transitioned into a persistent event, the ON-cell burst and OFF-cell pause were independent of PB, whether evoked by a noxious (60 or 100 g) or innocuous (4 to 15 g) stimulus. The ON-cell burst remained independent of PB at 5 to 6 days after CFA, although the OFF-cell pause was again reduced, although not eliminated, during PB block at this time point.

The contribution of PB to the ongoing firing of the ON and OFF-cells was also attenuated as inflammation developed. Although ON-cell discharge was still reduced significantly by PB block at 1 day post-CFA, implying that the nociceptive “tone” from RVM is still in part set by PB inputs during the early phase of inflammation, neither cell class showed an overall change in ongoing firing during PB block at 5 to 6 days post-CFA. Interestingly, some ON-cells showed increased ongoing discharge during PB block and some OFF-cells showed decreased activity in animals subjected to persistent inflammation. This is in contrast with the effect of PB block in naïve

animals and in acute inflammation, where PB block consistently led to a decrease in ON-cell firing and increase in OFF-cell firing.

Taken as a whole, these findings suggest that a distinct pathway or pathways is activated or unmasked to convey nociceptive information to RVM ON- and OFF-cells as inflammation is established. One possibility is that alternative pathways, such as a direct input from dorsal horn, or other relays, such as dorsal PAG or gracile nucleus/thalamus, convey relevant information to the RVM.[18; 179] Another possibility is that distinct pain-related cell populations or circuits exist within PB and differentially engage RVM ON- and OFF-cells under different conditions. Consistent with the latter suggestion, both pro-nociceptive and antinociceptive roles have been documented for PB in different experimental contexts,[33; 70; 98; 107; 124; 132; 144] suggesting that, as in RVM, there are multiple, functionally distinct pain-related cell populations or circuits within the PB. Plasticity within PB could change how circuits in this region process nociceptive information, and consequently alter its outputs, as has been documented in other PB projection targets such as the amygdala.[13; 163; 169] The net functional influence of PB on RVM pain-modulating neurons could thus depend on a balance among different PB outputs that shifts during inflammation.

Plasticity within RVM itself during prolonged inflammation could also alter the responses of ON- and OFF-cells to any input from PB. Molecular and cellular studies document plasticity in RVM circuits in persistent pain states, with changes in NMDA, AMPA, trkB, opioid, and neurokinin-1 receptor expression and function[19; 65-67; 93; 106; 162; 175], local glial activation,[165] and even neuronal loss.[111] The synaptic mechanisms of plasticity in RVM have not been established, in part because functionally relevant inputs to RVM ON- and OFF-cells have not been isolated in mechanistic studies. Nevertheless, there is evidence for opioidergic presynaptic plasticity in the region.[19; 175] Analysis of identified synapses will help

define the relationship between PB and RVM, and how this changes in acute and persistent inflammation.

4.5.2. Role of PB in nociceptive responsiveness during persistent inflammation

In these experiments, animals exhibited robust mechanical allodynia in the CFA-treated paw, compared to either pre-injection baseline or the untreated paw. Inactivation of PB in naïve animals resulted in a small, but statistically significant increase in the latency for withdrawal of the treated paw, but not the untreated, paw from noxious mechanical stimuli. In the first hour following injection of CFA, PB block substantially reversed mechanical hypersensitivity, without altering the responses to noxious stimuli. By contrast, inactivation of PB did not attenuate allodynia or noxious-evoked withdrawals as inflammation persisted (1 to 6 days after injection). Indeed, PB block unmasked moderate hypersensitivity in the untreated paw to the inflammation in animals subjected to persistent inflammation.

These observations confirm the modest hypoalgesia previously reported during inactivation of PB in naïve animals,[166] and indicate that PB contributes to the enhanced behavioral responsiveness in acute inflammation. By contrast, these data show that tactile allodynia in persistent inflammation does *not* require the contralateral PB, and in fact, PB may serve to limit hypersensitivity in the untreated paw. This suggests that lesions of dorsal horn NK1 expressing neurons should not interfere with maintenance of ongoing hyperalgesia, or that the effects of such lesions.[103; 145; 164; 180] are due to interactions within the spinal cord and not to loss of spinoparabrachial projections. Nevertheless, these experiments were focused on evoked pain, and we cannot rule out a possible contribution of PB to ongoing pain during persistent inflammation[70; 141; 144; 163], since the PB projection to the amygdala is distinct from that to the RVM.[166]

4.5.3. Pain-modulating neurons in RVM and PB are parts of a dynamic feedback loop throughout the time course of inflammation.

Descending control from the RVM during inflammation is dynamic: while RVM contributes to pain facilitation during acute injury and inflammation, the region can have a net inhibitory or compensatory role in persistent pain.[24; 35; 43; 104; 165; 172; 176; 201] A similar pattern was seen here for PB, with a net pro-nociceptive effect only in acute inflammation, but if anything, an anti-hyperalgesic influence as inflammation persisted. The parallel effects of blocking PB and RVM in acute and persistent inflammation is consistent with the idea that effects of PB on evoked pain are mediated by the RVM, and with recent evidence that PB is a major relay of nociceptive information to the RVM.[107; 166; 180]

In the first hour after local CFA administration, our data suggest that PB contributes to a pro-nociceptive positive feedback loop by contributing to the increased firing of ON-cells and suppression of OFF-cell activity. PB also has a role in the nociception-related changes in firing of both ON- and OFF-cells. Since the ongoing firing of these neurons impacts nociceptive tone, these changes in RVM cell activity promote hyperalgesia. Thus, blocking PB had a net anti-hyperalgesic effect.

By the first day of inflammation, although PB contributed to maintain ON-cell output, its pronociceptive influence was diminishing, as it no longer relayed nociceptive signals to either ON- or OFF-cells, nor suppressed anti-nociceptive tone mediated by OFF-cells. This loss of influence on the ON-cell burst, and the removal of suppression on OFF-cell anti-nociceptive tone may represent the early phase of a compensatory process that limits development of chronic pain.[176; 182] The lack of behavioral effect of blocking PB could be due to the fact that mechanical stimuli were delivered during ON-cells' inactive phases (or OFF-cells' active phases) whenever possible in order to capture their nociception-related activity. As a consequence, any contribution of ON-cell pronociceptive tone[73] would not be measurable.

By the fifth day of inflammation, the influence of PB on ON- and OFF-cell tone was completely removed, as blocking PB had no net effect on the ongoing discharge of either cell class or on withdrawal latency. The lack of an influence of PB at this point was not likely due to resolution of CFA-induced inflammation, as the inflammatory effect of CFA typically lasts for 7 days or more,[35; 93; 110] and both inflammation and hyperalgesia were maintained. The continued failure of PB to influence the RVM is consistent with the idea of RVM limiting development of chronic pain,[35; 182] with reduced pro-nociceptive output (ON-cells) while the anti-nociceptive influence of the OFF-cells curbs sensitized dorsal horn transmission, preventing spread of hypersensitivity to the contralateral, untreated paw.

4.6. CONCLUSION

PB is a major relay of nociceptive input to RVM pain-modulating neurons in naïve animals and in acute inflammation, but not during persistent inflammation. PB contributes to mechanical allodynia during acute inflammation, but not in persistent inflammation.

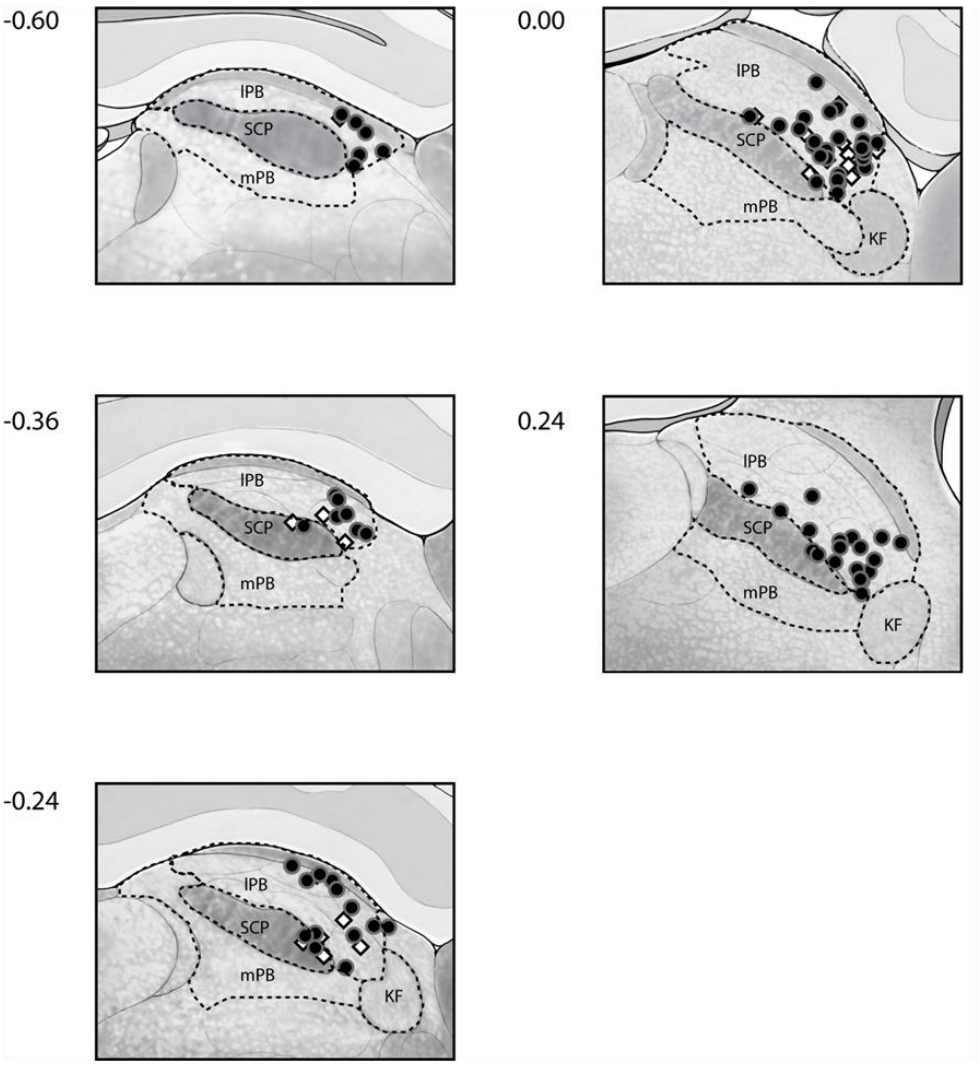

Figure 19 Locations of microinjection sites in the lateral PB complex.

Parabrachial injections were distributed among sections at +0.24 to -0.60 mm relative to the interaural line. Injections inside the lateral PB (lPB) region were considered "on-target." White diamonds represent naive untreated animals. Black circles represent animals with CFA induced inflammation. KF: Kölliker-Fuse, lPB: lateral parabrachial complex, mPB: medial parabrachial area, scp: superior cerebellar peduncle.

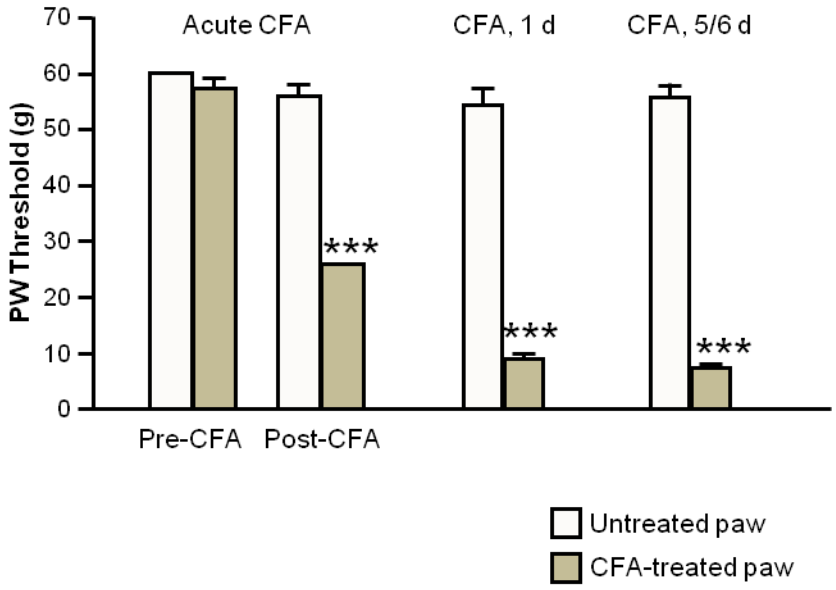

Figure 20 Effect of intraplantar CFA on withdrawal threshold measured acutely (within the first hour following injection), 1 day post-injection, and 5 or 6 days post-injection.

The contralateral, untreated paw is shown for comparison. Shaded gray represents "noxious stimuli." Two-way repeated-measures ANOVA followed by Sidak's post-hoc test to compare CFA-treated and untreated paw. ***$p < 0.0001$ compared to untreated paw.

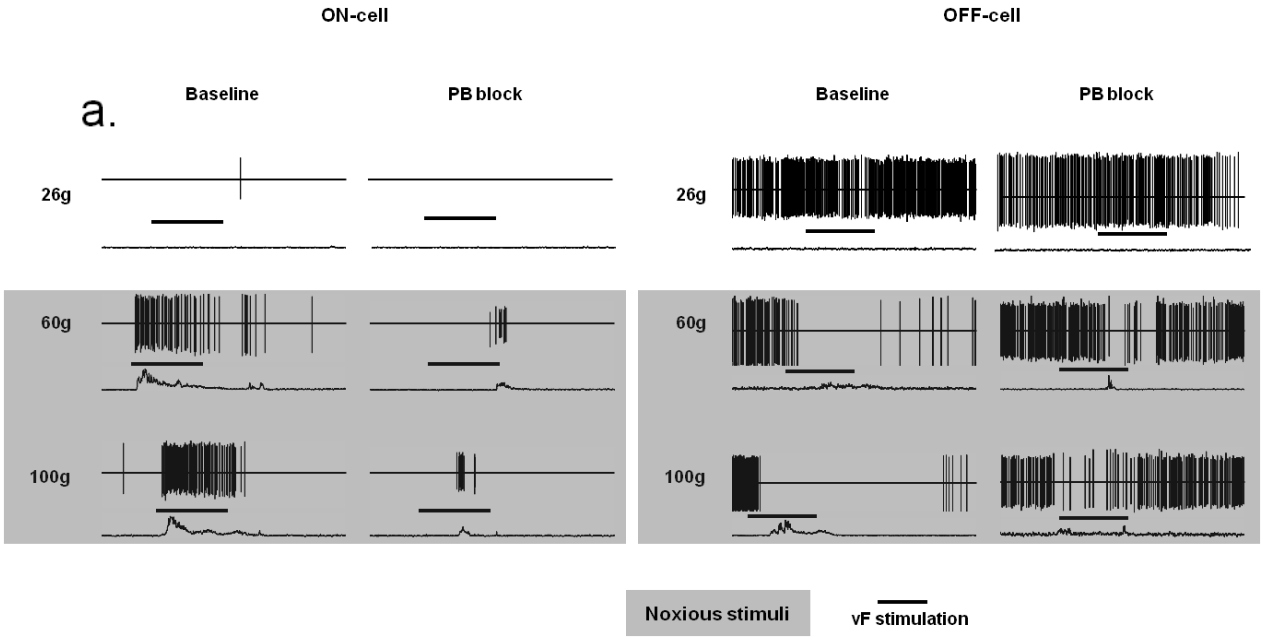

Figure 21 Inactivation of lateral PB interfered with the ON-cell burst and OFF-cell pause in response to mechanical stimulation in naïve animals.

a. Representative examples show ON- and OFF-cell activity during withdrawal from von Frey probes (26, 60 and 100 g) stimulus at baseline compared to during muscimol block of lateral PB contralateral to the stimulus. In both cases, nociception-related changes in firing were substantially reduced.

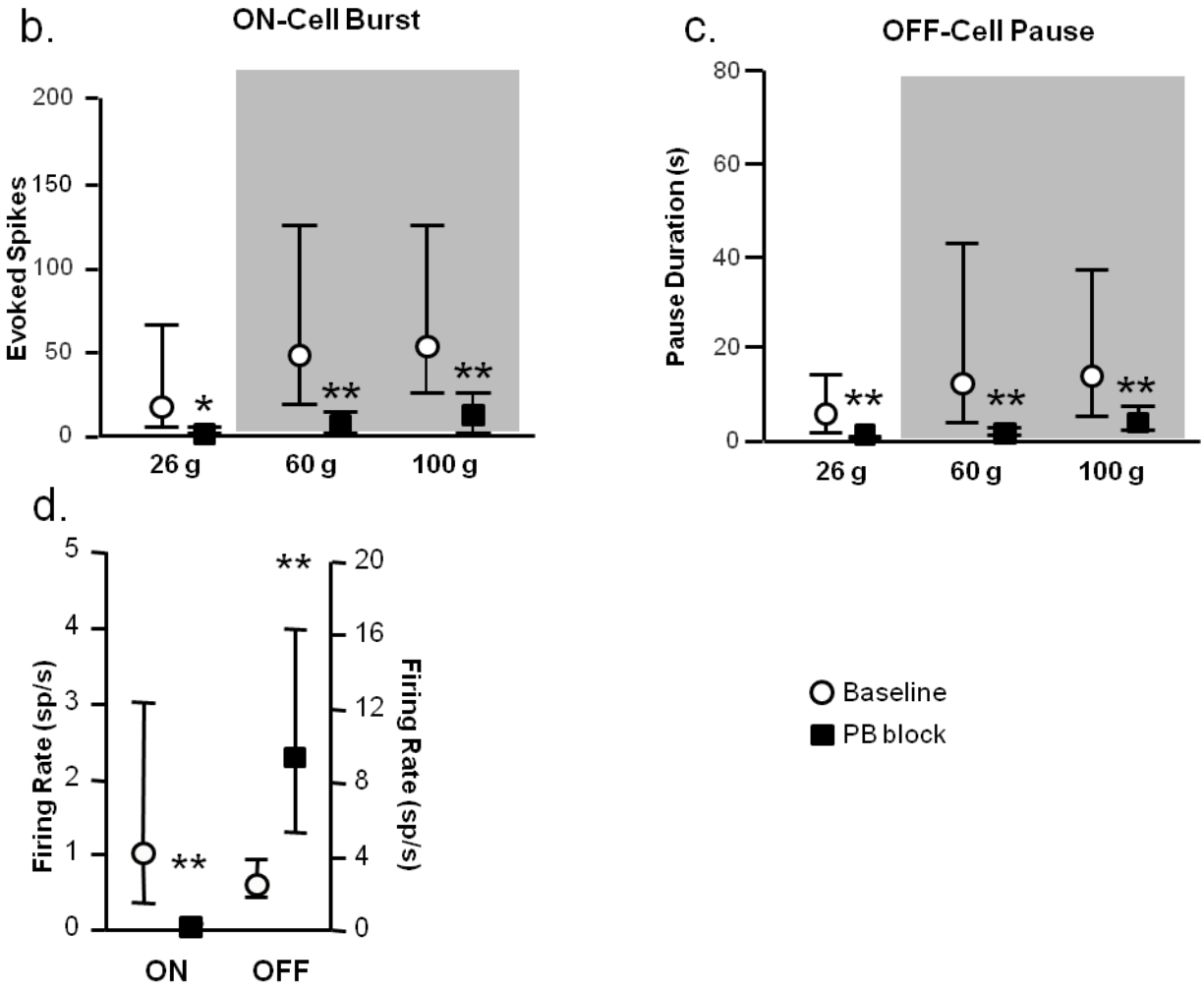

Fig. 21b-d

b. ON-cells. Effect of muscimol microinjected into the lateral PB on activity triggered during application of von Frey probes (measured as total evoked spikes, n = 14). **c**. OFF-cells. Effect of muscimol microinjected into the lateral PB on OFF-cell pause (measured as pause duration, n = 14). **d.** Ongoing firing of ON-cells was reduced and that of OFF-cells was increased. (PB injections were contralateral to the peripheral stimulus. Shaded gray represents "noxious stimuli" (60-100 g). Reported as geometric mean with 95% confidence limits, *t*-test for correlated means, *$p < 0.05$, **$p < 0.01$ compared to baseline.)

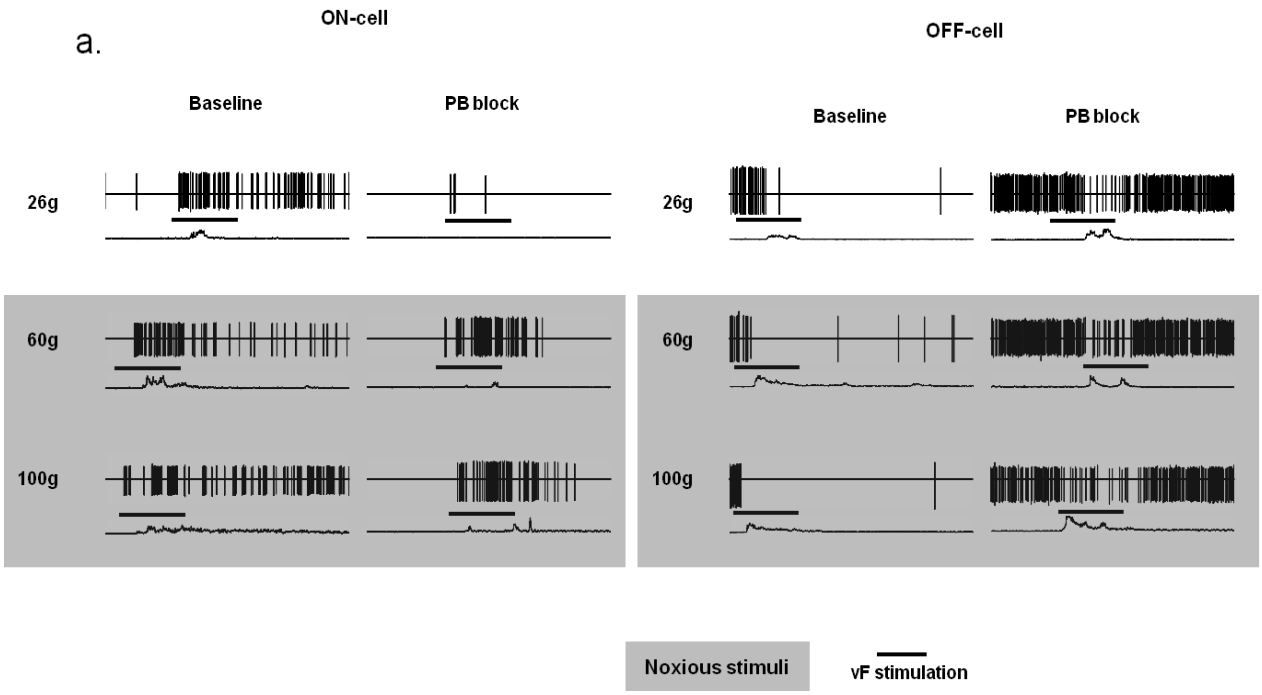

Figure 22 Inactivation of lateral PB interfered with the ON-cell burst and OFF-cell pause evoked during mechanical stimulation in acute inflammation.

a. Representative examples show nociception-related ON- and OFF-cell activity during acute inflammation before and after muscimol block of lateral PB contralateral to the inflammation. Shaded gray represents “noxious stimuli” (60-100 g). Unshaded area represents "subnoxious stimuli" (26 g).

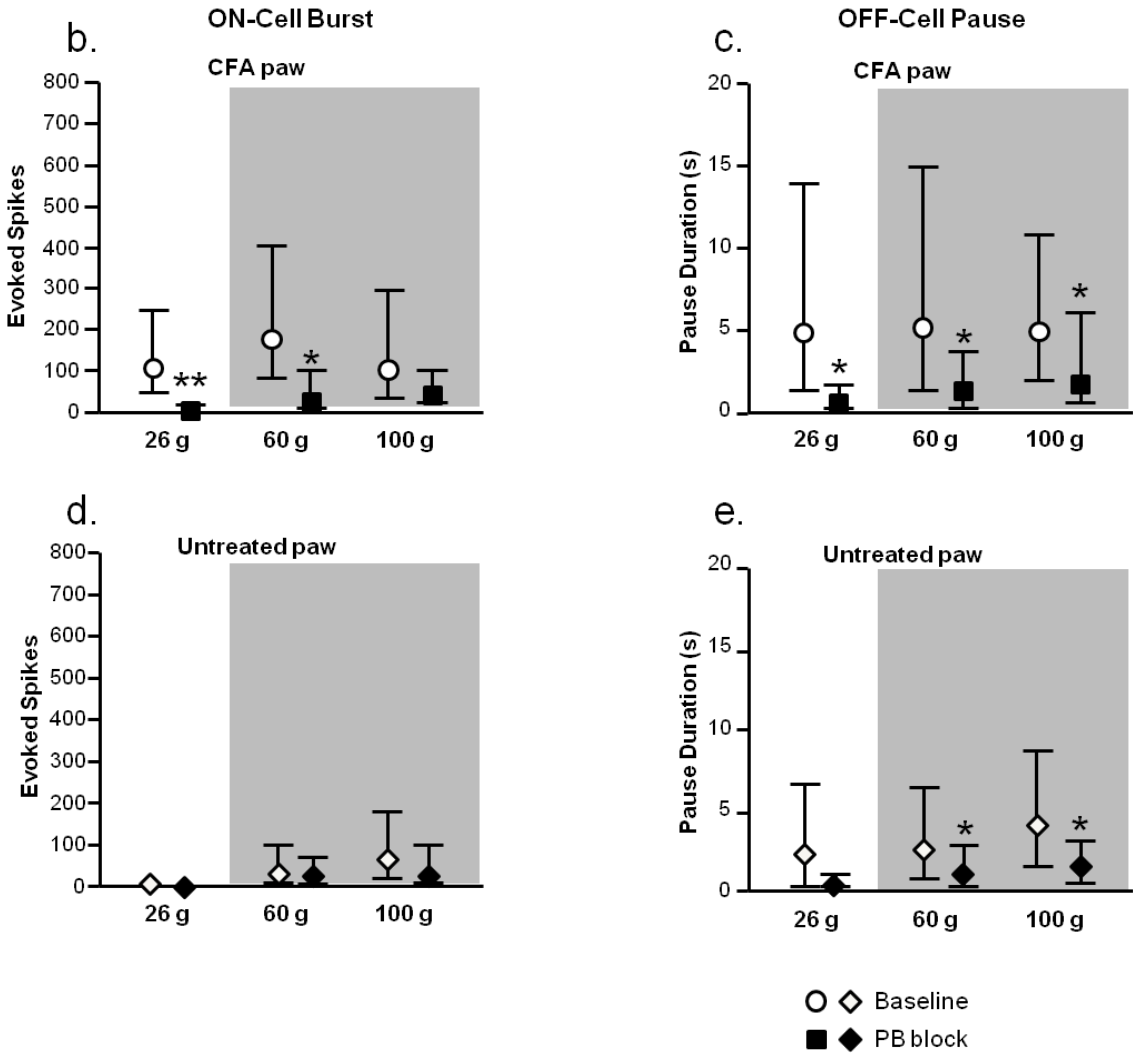

Fig. 22b-e

b. ON-cells. Effect of muscimol microinjected into the lateral PB on activity triggered during application of von Frey probes (26, 60 and 100 g, measured as total evoked spikes, n = 12 cells). **c**. OFF-cells. Effect of muscimol microinjected into the lateral PB on OFF-cell pause (measured as pause duration, n = 13 cells). Blocking PB had no effect on the nociception-evoked activity of (**d**) ON- and (**e**)OFF-cells when the ipsilateral hindpaw was stimulated. (PB injections were contralateral to the peripheral stimulus Shaded gray represents “noxious stimuli” (60-100 g). Unshaded area represents "subnoxious stimuli" (26 g). Reported as geometric mean with 95% confidence limits, *t*-test for correlated means, *$p < 0.05$, **$p < 0.01$ compared to baseline.)

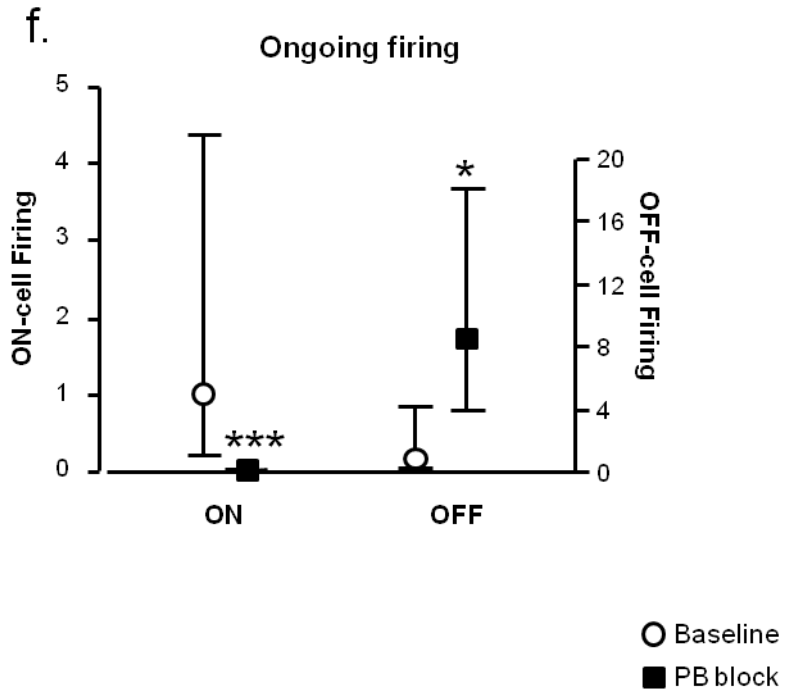

Fig. 22f

f. Ongoing firing of ON-cells was reduced (left) and that of OFF-cells was increased (right). (PB injections were contralateral to the peripheral stimulus. Reported as geometric mean with 95% confidence limits, *t*-test for correlated means, **p* < 0.05, ****p* < 0.001 compared to baseline.)

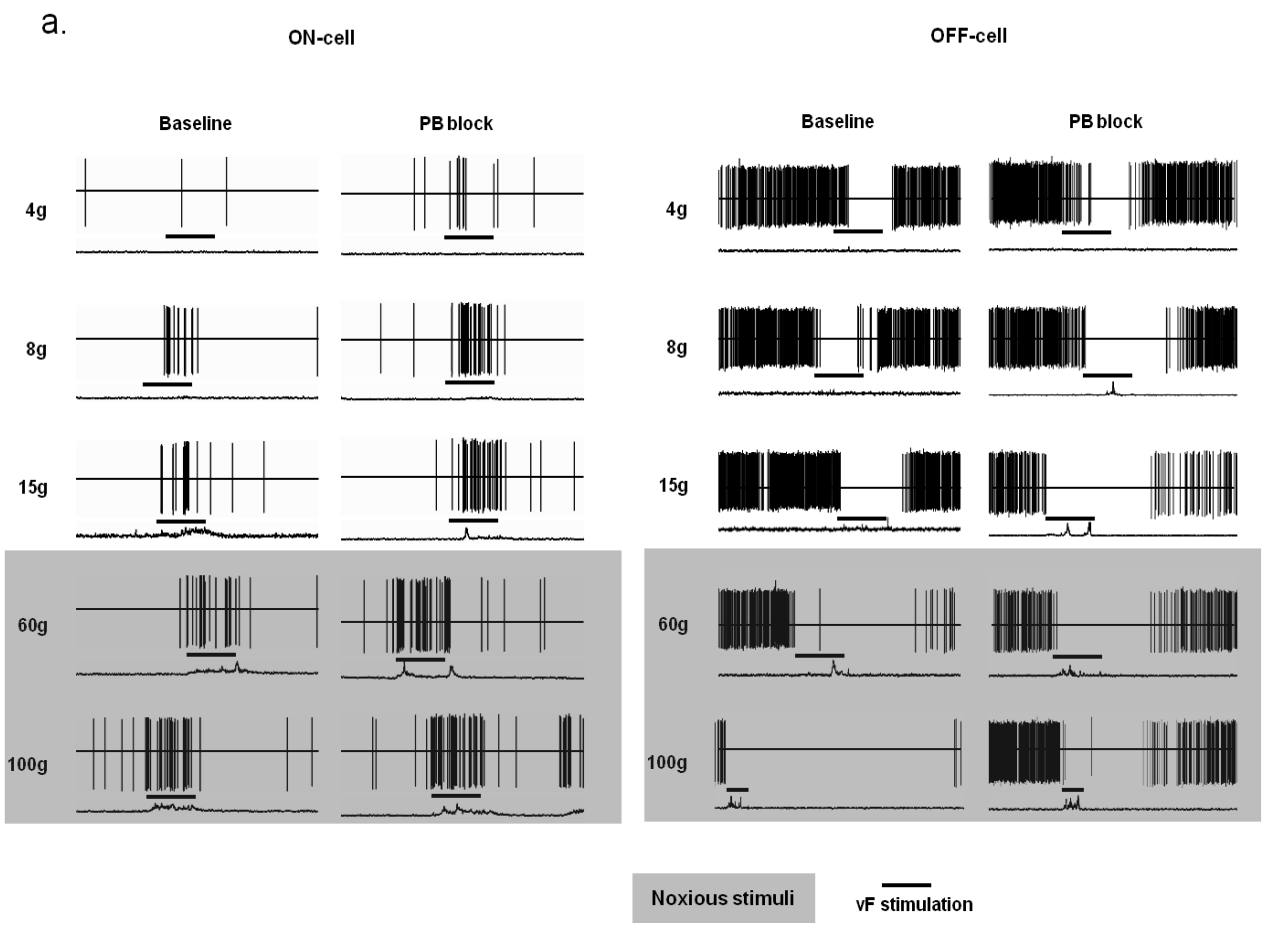

Figure 23 Inactivation of lateral PB had no effect on the ON-cell burst and OFF-cell pause evoked during mechanical stimulation 1 day post-CFA

a. Representative examples show nociception-related ON- and OFF-cell activity during the first day of inflammation, before and after muscimol block of lateral PB contralateral to the inflammation. Shaded gray represents “noxious stimuli” (60-100 g).

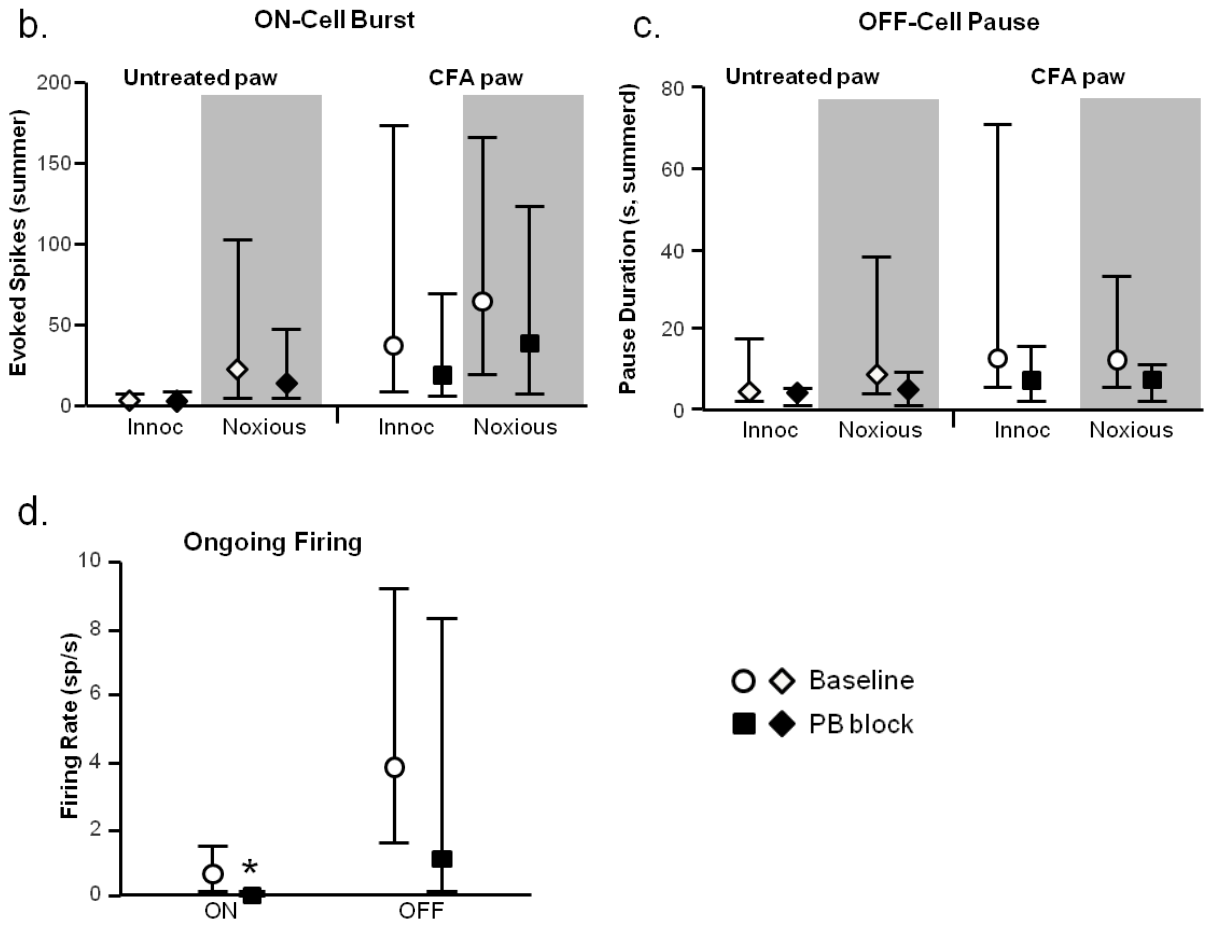

Fig. 23b-d

b. ON-cells. Effect of muscimol microinjected into the lateral PB on activity triggered during application of von Frey probes (innocuous: summed activity evoked by stimulation with 4, 8, and 15 g probes; noxious: summed activity evoked by stimulation with 60 and 100 g probes, n = 14 cells). **c**. OFF-cells. Effect of muscimol microinjected into the lateral PB on OFF-cell pause (measured as summed pause duration evoked by innocuous and noxious probes, n = 10 cells). **d.** Net ongoing firing of ON-cells was reduced (left), while OFF-cell firing was unchanged (right). (PB injections were contralateral to the peripheral stimulus/inflammation. Shaded gray represents “noxious stimuli” (60-100 g). Unshaded area represents "innocuous stimuli" (4-15 g). Reported as geometric mean with 95% confidence limits, *t*-test for correlated means, *$p < 0.05$, compared to baseline.)

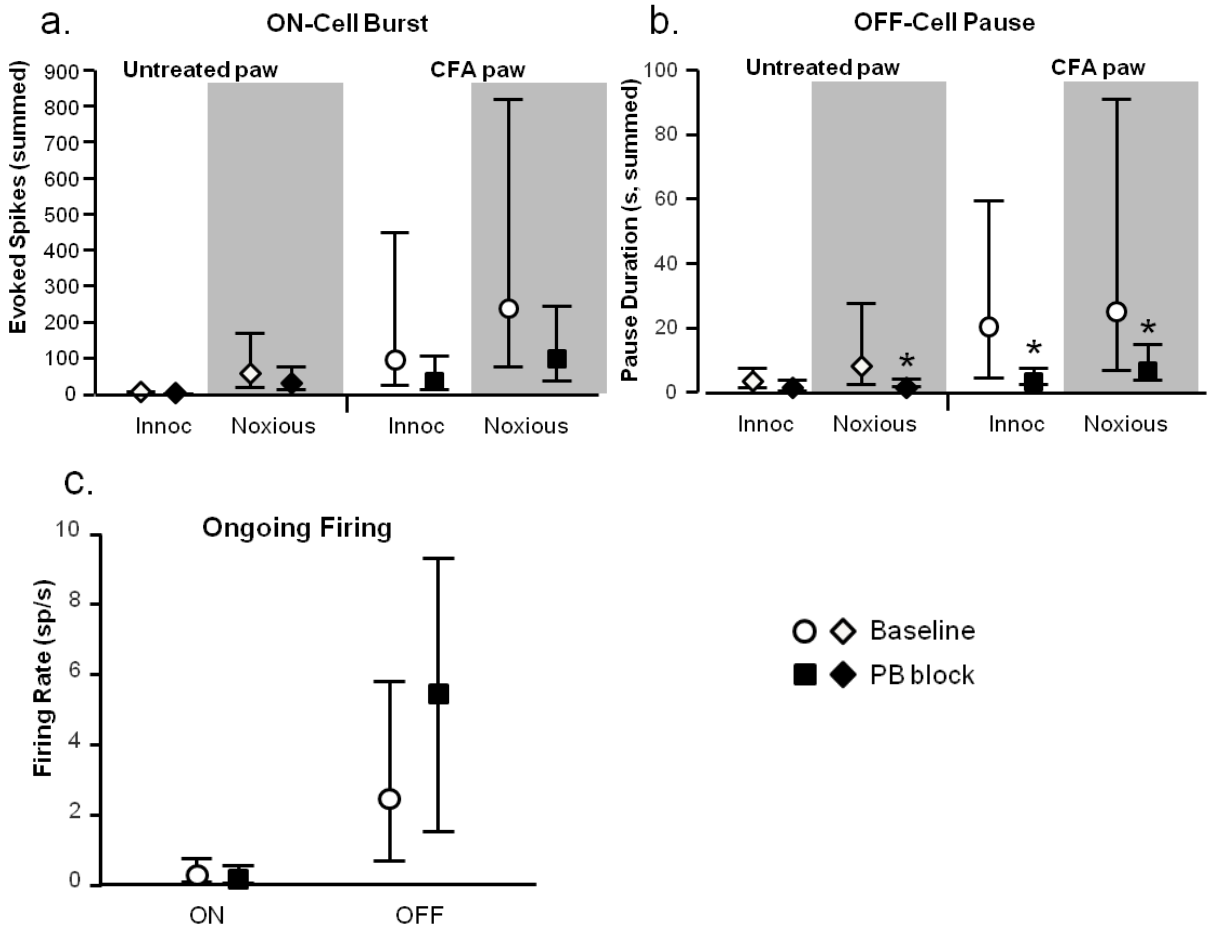

Figure 24 Inactivation of lateral PB had no effect on the ON-cell burst and OFF-cell pause evoked during mechanical stimulation 5/6 day post-CFA.

a. ON-cells. Effect of muscimol microinjected into the lateral PB on activity triggered during application of von Frey probes (innocuous: summed activity evoked by stimulation with 4, 8, and 15 g probes; noxious: summed activity evoked by stimulation with 60 and 100 g probes, n = 13 cells). **b**. OFF-cells. Effect of muscimol microinjected into the lateral PB on OFF-cell pause (measured as summed pause duration evoked by innocuous and noxious probes, n = 11 cells). **c.** Ongoing firing of ON-cells (left) and OFF-cells (right). (PB injections were contralateral to the peripheral stimulus. Shaded gray represents “noxious stimuli” (60-100 g). Unshaded area represents "innocuous stimuli" (4-15 g). Reported as geometric mean with 95% confidence limits, *t*-test for correlated means, *$p < 0.05$ compared to baseline.)

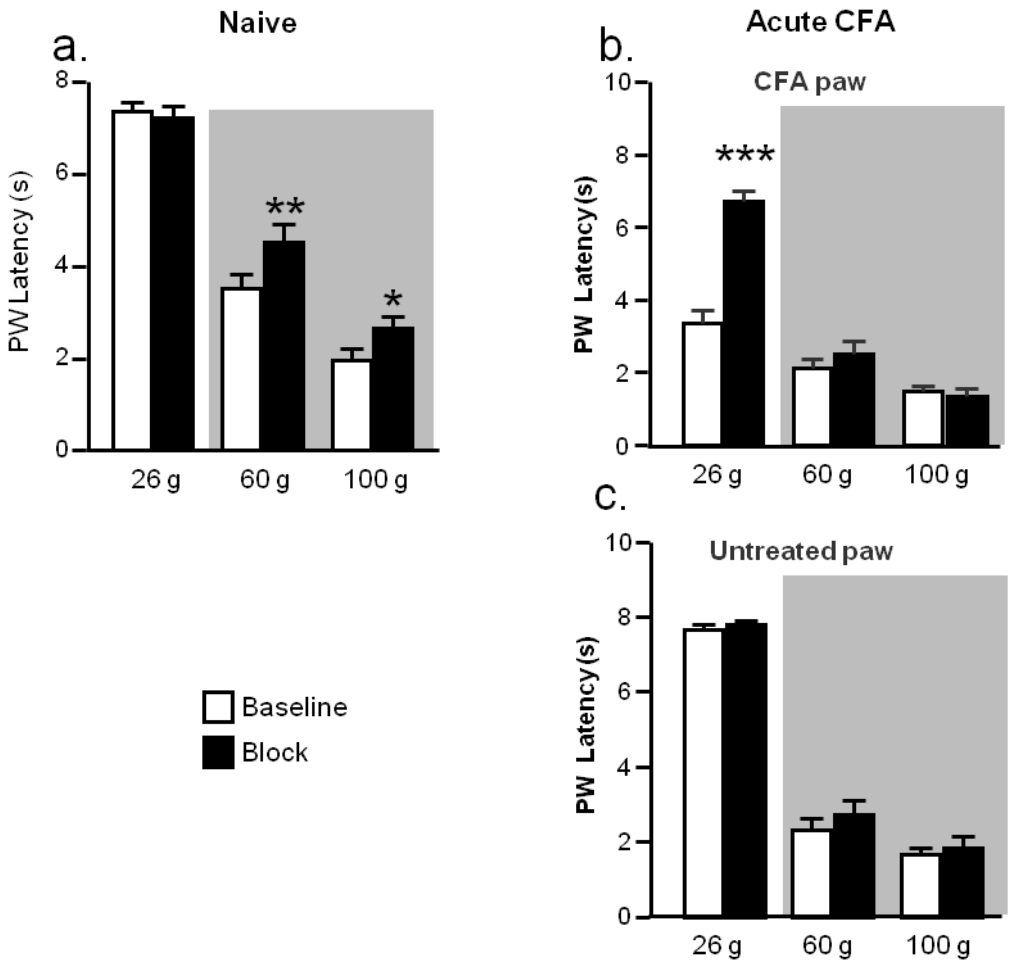

Figure 25 Effect of blocking PB on the withdrawal evoked by von Frey probes in naïve animals and during acute inflammation produced by plantar injection of CFA.

a. Naïve animals. PB injections were contralateral to the peripheral stimulus. **b**. Acute inflammation, with testing within the first hour following injection of CFA contralateral to the PB block. Two-way ANOVA with repeated measures on force and block, followed by Sidak's test for multiple comparisons showed a significant effect of force, a significant effect of block, and a significant interaction for the naïve animals and for the CFA-treated paw in acute inflammation. **c.** In acute inflammation, the untreated paw (contralateral to CFA paw) showed a significant effect of force, but no effect of block. Shaded gray represents "noxious stimuli" (60-100 g). Unshaded area represents "subnoxious stimulus" (26 g). 26 g does not belong to either categories. Reported as mean + SEM, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to baseline.

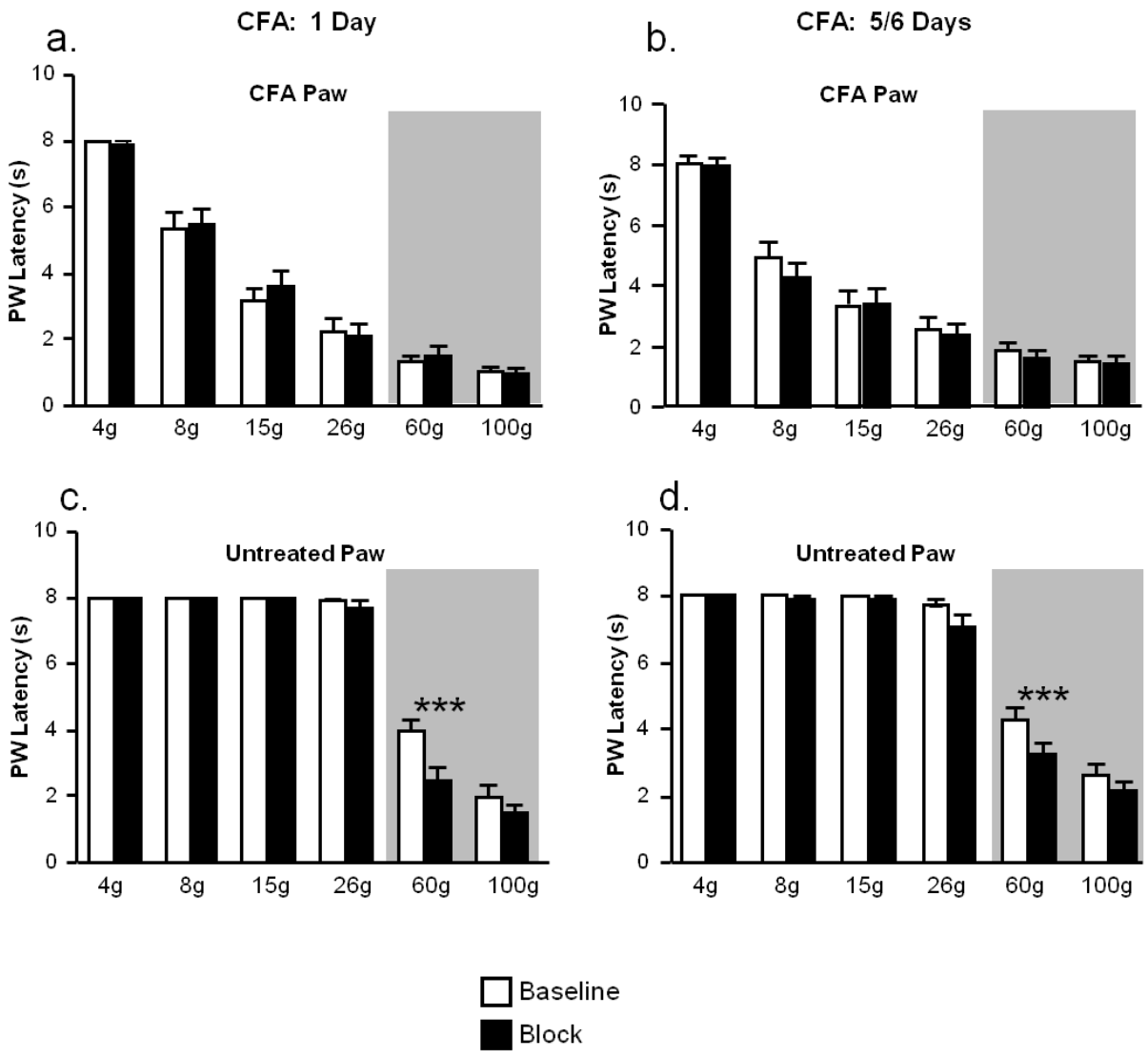

Figure 26 Effect of blocking PB on the withdrawal evoked by von Frey probes 1 and 5/6 days after CFA injection

a. 1 day post-CFA, with CFA-treated paw top, and untreated paw bottom. **b.** 5/6 days post-CFA. Two-way ANOVA with repeated measures on force and block, followed by Sidak's test for multiple comparisons showed a significant effect of force, an effect of block, and a force-by block interaction at both 1 and 5/6 days post CFA. Shaded gray represents "noxious stimuli" (60-100 g). Unshaded area represents "innocuous stimuli" (4-15 g). 26 g does not belong to either category. Reported as mean + SEM, ***$p < 0.001$ compared to baseline..

CHAPTER 5

MANUSCRIPT #4

Recruitment of the ipsilateral parabrachial input to the rostral ventromedial medulla in persistent inflammation maintains sensitization in a brainstem pain-modulating circuit

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5.1 ABSTRACT

Sensitization of the descending pain-modulating circuit is considered a major contributor to many chronic pain syndromes. The output node of this circuit, the rostral ventromedial medulla (RVM), can facilitate or suppress nociceptive transmission at the dorsal horn by the respective action of two distinct classes of neurons, pain-facilitating “ON-cells” and pain-inhibiting “OFF-cells.” A defining characteristic of these neurons is their responses to nociceptive input: ON-cells have a “burst” of activity, and OFF-cells have a sudden pause in firing, which allows for reflexive withdrawal. In persistent inflammation, these ON- and OFF-cells become sensitized, with significantly lower threshold and reduced latency for these nociception-related changes in firing. The parabrachial complex (PB) was recently identified as a major nociceptive relay that drives RVM neuronal activity. The PB conveys mostly contralateral, and some ipsilateral, nociceptive information to various structures, and has long been implicated to be crucial in maintaining central sensitization and behavioral hypersensitivity in persistent inflammation. However, the functional significance of the ipsilateral PB input to the RVM, and the difference between the two PB pathways, due to the laterality in respect to a local inflammation, were not defined. By examining the influence of ipsilateral PB on RVM neurons in normal and persistently inflamed animals, the current data demonstrated the recruitment of input to the RVM from the PB ipsilateral to the site of inflammation. Furthermore, this recruitment of ipsilateral PB input requires the presence of an intact contralateral PB. Taken together, the present study revealed a recruitment of a nociceptive relay to a sensitized pain-modulating circuit in persistent inflammation. This provides important insights to our understanding of the circuit responsible for central sensitization in some chronic pain syndromes.

5.2. INTRODUCTION

The sensitization of the descending pain-modulating system is thought to be a major contributor to many clinically significant chronic pain syndromes.[81; 200] The output node of this pain-modulating circuit, the rostral ventromedial medulla (RVM), comprises two classes of functionally distinct neurons, the pain-facilitating ON-cells and the pain-inhibiting OFF-cells.[55] These pain-modulating neurons enable the system to facilitate or suppress nociception through their projections to the spinal dorsal horn, and have been shown to undergo structural, molecular, and pharmacological plasticity in acute and persistent pain.[24; 65; 66; 93; 161; 165; 176 ; 182] Such plasticity within the RVM can be driven by "top-down" cognitive and emotional factors[122; 126; 127], as well as "bottom-up" nociceptive sensory history, such as neuropathy or inflammation.[26; 35; 76; 81] These ON- and OFF-cells also respond acutely to nociceptive signals, with ON-cells displaying a "burst" of activity, and OFF-cells producing a "pause" in any ongoing activity, thus allowing nocifensive withdrawal in response to noxious stimulation. Both response thresholds and latencies of these RVM neurons are significantly decreased in persistent pain states, and the nociception-related burst and pauses are enhanced.[26; 35; 143] However, the circuitry and mechanisms that drive these changes in neuronal response properties have not been identified.

It was recently found that the parabrachial complex (PB) relays nociceptive information to ON- and OFF-cells for the contralateral side of the body under basal conditions.[166] PB is an anatomically and functionally diverse mesopontine structure that receives, processes, and relays nociceptive signals from the spinal dorsal horn.[22; 63] It receives the majority of its nociceptive input from the contralateral dorsal horn, with sparse ipsilateral input.[12; 17; 20; 53; 155; 183] Although the functional difference between the contralateral and ipsilateral input to PB has not been explicitly demonstrated, they are thought to play a crucial role during central sensitization in persistent pain. Bilateral ablation of NK1 receptor-positive neurons in the spinal

dorsal horn, which presumably disrupts the majority of nociceptive input to both PBs, led to the inability to develop and maintain behavioral hypersensitivity in animal models of inflammatory and neuropathic injury.[103; 145; 164] Furthermore, the mechanism that the spinoparabrachial pathway and the PB drive central sensitization in persistent pain appears to be through recruiting the RVM.[180] However, we recently demonstrated that PB contralateral to the site of inflammation has a limited contribution to the sensitization of RVM, as it does not influence the net spontaneous activity and has a diminished role in conveying nociceptive information to ON- and OFF-cell. Furthermore, contralateral PB does not contribute to hyperalgesia in persistent inflammation (Chapter 4). This raises the possibility that an alternative pathway has been recruited during persistent inflammation, through which nociceptive signals are allowed to reach the sensitized ON- and OFF-cells, thus maintaining behavioral hypersensitivity.

Previous evidence consistently points to the spinoparabrachial pathway as a crucial component for the maintenance of spinal sensitization and behavioral hypersensitivity in persistent pain states.[103; 145; 164; 180] However, blocking the PB contralateral to the inflamed hindpaw, the presumed major nociceptive relay, had little impact on RVM neuronal activity and withdrawal behaviors (Chapter 4). This strongly suggests that the ipsilateral PB is a major contributor to central sensitization in persistent inflammation. Although the projection from the spinal dorsal horn to the ipsilateral PB is sparse compared to its contralateral projection, its involvement in persistent pain has been implied by reports of *c-fos* expression in ipsilateral as well as contralateral PB during a localized inflammation.[9; 15; 85] Thus we hypothesized that the PB ipsilateral to the inflammation is recruited to relay nociceptive information to RVM ON- and OFF-cells, helping to maintain behavioral hypersensitivity of the inflamed hindpaw in persistent inflammation. Further, since previous findings involving ablation of bilateral spinoparabrachial pathways used a neurotoxin approach that did not differentiate the functional role of PB ipsilateral and contralateral to inflammation,[103; 145; 164; 180] we

decided to explore the mechanism through which both PB inputs may contribute RVM sensitization during persistent inflammation. The current series of experiments thus provide important insights into how interactions between a pain-transmission system and a pain-modulating system contribute to central sensitization and maintain behavioral hypersensitivity in chronic pain.

5.3. MATERIALS AND METHODS

All experimental procedures were approved by the Institutional Animal Care and Use Committee at Oregon Health & Science University and followed the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain. Since we have established that lateral PB, but not medial PB, relays nociceptive information to RVM in previous studies,[166] all PB injections in the current study were done in the lateral PB.

5.3.1. Animal model preparation

Male Sprague-Dawley rats were purchased from Charles River and acclimated for at least 3 days in the vivarium with a 12 hr light/dark cycle and food and water available *ad libitum*. All preparations and experiments were performed during the light phase.

5.3.1.1. Persistent inflammation

For experiments involving persistent inflammation, naive or PB-lesioned male Sprague-Dawley rats weighing between 250 and 350 g were briefly anesthetized with isoflurane (4%, 4-5 minutes). Complete Freund's Adjuvant (CFA, 0.1 ml) was injected subcutaneously into the plantar surface of the left hindpaw, after which the rat was returned to its home cage for 5-6 days until the electrophysiological experiment. These time points were chosen based on previous studies from the Heinricher laboratory, and were consistent with the known duration of behavioral hypersensitivity following plantar CFA administration. Inflammation induced by CFA

typically peaks at one day (24 h), but is maintained for two weeks or more, and with the time-course of changes in RVM activity after CFA injection.[35; 65; 66; 118; 161; 182]

5.3.1.2. Neurotoxin (ibotenic acid) injections

For a subset of experiments involving chronic lesion, ibotenic acid was injected into the contralateral PB 3-4 days with respect to CFA injections into the hindpaw to induce persistent inflammation (CFA). Rats (250-300 g) were induced with isoflurane and placed in a stereotaxic frame. A small incision was made along the midline, a craniotomy made, and the dura removed to allow for the placement of a glass microinjector into the right PB (AP: -1.2 mm, ML: -2.3 mm, DV: +3 mm from Interaural at a 14º angle to the coronal plane). Ibotenic acid (200 nL, 10ug/uL in aCSF) mixed with blue fluorescent beads (FluoSpheres, Invitrogen, Eugene, OR) at 100:1 concentration was delivered using Picospritzer (Parker Hannifin, Hollis, NH). Following retraction of the microinjector, the craniotomy site was filled with gelfoam, and the incision closed. After the procedure, the rat then received lidocaine ointment, penicillin G (80 kU/mL, i.m.) and carprofen (5 mg/mL, s.c., Pfizer, New York), before return to the home cage for recovery. The apoptotic lesion induced by ibotenic acid and its anatomical placement were assessed histologically using Nissl stain.

5.3.2. Electrophysiological experiments

5.3.2.1. Animal preparation

On the day of the experiment, animals were deeply anesthetized using isoflurane, and a catheter inserted in the external jugular vein for subsequent infusion of the short-acting barbiturate methohexital. They were then transferred to a stereotactic frame. While the animals were still deeply anesthetized, two small craniotomies were drilled for access to the RVM and the PB. Heart rate was monitored using EKG. Body temperature was monitored and maintained at 36-37 °C with a heating pad and a space heater.

After competition the surgical preparation, the rate of methohexital infusion was adjusted so that animals displayed no spontaneous movements, and exhibited a stable heat-evoked hindpaw withdrawal reflex. Animals were stabilized for at least 45 minutes at a constant anesthetic flow rate before beginning data collection.

5.3.2.2. Electrophysiological recording

Extacellular single-unit recordings were made with stainless-steel microelectrodes (Microprobes, Gaihersburg, MD) with gold- and platinum-plated tips. Signals were amplified (10k), sampled at 32k Hz, and bandpass filtered (400 Hz to 15 kHz).

RVM neurons were isolated and classified as ON- or OFF-cells as described previously.[58; 59] Heat stimuli were delivered to both hindpaws using a Peltier device (Yale Instrument) to identify the cells and to test the anesthetic depth in the first 10 minutes of recording, prior to initiating von Frey trials. Both ON- and OFF-cells have whole body receptive field, but inverse responses during nocifensive behaviors. ON-cells exhibit a “burst” of activity (if not already active), while OFF-cells exhibit a “pause” of any ongoing activity (if active), or remain silent (if inactive). To further confirm cell classification, a 10 s noxious pinch was delivered to the hindpaw using a toothed forceps: ON-cells burst and OFF-cells pause throughout the stimulus. One neuron per animal was recorded from in a typical experiment, though two were neurons recorded simultaneously on some occasions.

5.3.2.3. PB block

During electrophysiological recording, the $GABA_A$ receptor agonist muscimol (1ng/200nl in aCSF) was delivered to the left PB using a glass microinjector (70 μm outer diameter, attached to a 1 μl Hamilton syringe via PE50 tubing). Green fluorescent beads (FluoSpheres, Invitrogen, Eugene, OR) were included in the drug solutions in a 1:100 concentration for *post hoc* verification the injection site. Injectors were rinsed and lowered into the brain using stereotactic

coordinates for the left lateral PB (AP -1.2 mm with the injector directed caudally at 14°, ML +2.3 mm, DV +3, relative to interaural zero, level head). Injections were made over a period of 4 minutes.

5.3.2.4. Experimental protocol

All studies used a within-subject design.

For experiments testing the influence of PB on the RVM when the ipsilateral hindpaw was stimulated in naive animals, at least one ON- or OFF-cell was isolated and characterized. Baseline mechanical sensitivity using von Frey fibers was then tested. von Frey fibers (26, 60, and 100 g) were applied in ascending order to the interdigital webbing of the left hindpaw for a period of 8 seconds. The withdrawal latencies were determined using hamstring EMG. Three trials were performed with each force, and a minimum of two testing sites was used in rotation. Individual trials were initiated at intervals of at least 30 seconds, with longer interstimulus intervals (up to 5 minutes) used when necessary to capture a period when the cell under study was inactive (ON-cell) or active (OFF-cell). Muscimol was then injected in the left PB, and mechanical sensitivity testing repeated as in baseline.

In electrophysiological studies involving persistent inflammation, animals with the left hindpaw injected 5-6 days prior were used. A subset of these animals had undergone lesion of the right PB (contralateral to inflammation) prior to induction of persistent inflammation as described above. Following isolation and characterization of an RVM neuron(s), baseline mechanical sensitivity and withdrawal latencies were determined for the inflamed and untreated hindpaws with von Frey fibers (4, 8, 15, 26, 60, and 100 g). Muscimol was then microinjected into the left PB as in experiments in naïve animals. Mechanical sensitivity testing was then repeated for both hindpaws.

5.3.3. Histology

At the conclusion of the recording, an electrolytic lesion was made in the RVM to localize the recording site. Rats were overdosed with methohexital and transcardially perfused. The brains were removed, and sectioned on a Leica CM3050 S cryostat (40 μm sections). The electrolytic lesion in the RVM and fluorescent beads marking the muscimol injection site in the PB were photographed with an Optronics Microfire camera attached to an Olympus BX51 microscope. If beads or electrolytic lesion were not found, the data were not analyzed further. For brains that received ibotenic acid injections, Nissl stain was performed on sections to verify the excitotoxic effect on PB cell-bodies. Cell body lesion of the PB by ibotenic acid was verified and photographed as described.

Muscimol-injection and ibotenic acid-induced lesion sites were plotted on hand-drawn sections in Adobe Illustrator using landmarks defined by Paxinos and Watson.[150] For "on-target" injections and lesions, if muscimol-injection or ibotenic acid-induced lesion sites were not located within the PB, the data were not analyzed further. The RVM was defined as the nucleus raphe magnus and adjacent reticular formation medial to the lateral boundary of the pyramids at the level of the facial nucleus.

5.3.4. Analysis

The extracellular recording signal, EMG, and EKG output were digitized and collected using Spike 2 software (Cambridge Electronics Design Ltd, Cambridge, UK). Each waveform was sorted using Spike2 template matching and cluster analysis, and verified on an individual spike basis.

In all experiments, data were compared between baseline and during block of PB. Nociception-evoked activity was measured using the number of spikes per ON-cell burst, and the duration of the OFF-cell pause. Total spikes per burst and pause duration were grouped

and summed according to stimulus intensity: innocuous stimuli were defined as 4-15 g of mechanical stimulation, and noxious stimuli were defined as 60-100 g of stimulations. Although trials using von Frey fibers typically did not take place until ON-cells were inactive, or OFF-cells were active, there were incidences where ON-cells were continuously active, or OFF-cells completely inactive. For those cases, the "burst" or the "pause" in activity is undefined, and hence cannot be obtained. Instead, the average spike counts during the von Frey stimulation in baseline and during PB block were used. Similarly, when an OFF-cell became completely inactive post-PB block (spontaneous activity = 0 spike/s), the pause duration for those trials would be maximized to full stimulus duration. Spontaneous activity for pre-PB block for all experiments was defined as the average firing frequency during the 30 s prior to the baseline heat trials. The PB-block spontaneous activity was defined as the firing frequency in the 30 s prior to the beginning of the PB-block von Frey series, at 4 minutes after the PB inject was completed.

Paw withdrawal latencies were analyzed using a paired Wilcoxon's signed ranks test. Because cell parameters for these neurons are typically highly skewed, these parameters were analyzed using paired t-test of log-transformed data. For all tests, $p < 0.05$ was considered significant.

5.4. RESULTS

5.4.1. PB ipsilateral to the stimulation site is not a relay to RVM in naïve animals

We showed recently that the PB is a major relay of nociceptive information from the contralateral side of the body to the RVM,[166] which is in keeping with the dense spinoparabrachial projection from the superficial dorsal horn to the contralateral PB. However, a sparse ipsilateral spinoparabrachial projection has been noted, although not described in any detail.[12; 17; 20; 53; 155; 183] We therefore blocked PB ipsilateral to the stimulation site in naïve animals to determine whether the ipsilateral PB conveyed nociceptive information to pain-

modulating neurons in the RVM under basal conditions. Muscimol injection sites in the PB area for naïve animals are shown in Figure 27.

Blocking PB ipsilateral to the stimulated paw had no effect on either the ON-cell burst or OFF-cell pause evoked by 26, 60, or 100 g von Frey fibers (Figure 28a-b), indicating that PB does not convey important nociceptive information from the ipsilateral side of the body in normal conditions. In contrast with the absence of an effect on evoked responses, the spontaneous firing of both ON- and OFF-cells was altered during PB block (Figure 28c), consistent with our earlier report.[166] The PB block slightly but significantly enhanced the response to stimulation of the ipsilateral hindpaw with a marginally noxious (26 g) von Frey fiber (Figure 29).

5.4.2. Recruitment of ipsilateral PB in persistent inflammation

NK1-expressing spinoparabrachial neurons have been shown to play an important role in heightened behavioral responsiveness in inflammation,[103; 145; 164; 180] yet inactivation of PB contralateral to the inflammation site fails to alter the stimulus-related changes in RVM firing or behavioral hyperalgesia (Chapter 4). The present experiments therefore explored a possible role for the PB *ipsilateral* to the site of inflammation in sensitization of RVM pain-modulating neurons during persistent inflammation. CFA was injected in the right hindpaw 5 days prior to the recording study. The activity of an RVM neuron and withdrawal latencies of both the inflamed and untreated paw were recorded before and after inactivation of the left PB (ipsilateral to the inflamed paw). Injection sites in the PB area are shown in Figure 30.

Blocking the ipsilateral PB significantly reduced the OFF-cell pause and ON-cell burst associated with von Frey stimulation of both the CFA-treated and untreated hindpaw in the innocuous (4 to 15 g) as well as noxious (60 and 100 g) ranges (Figure 31a-b). This indicates that the ipsilateral PB relays nociceptive signals, and contributes to the sensitized responses of RVM ON- and OFF-cells to both noxious and innocuous stimuli during inflammation. Ipsilateral

PB block also significantly altered the spontaneous firing of both ON- and OFF-cells in these animals, with a decrease in ON-cell firing and increase in OFF-cell firing (Figure 31c).

These changes in RVM cell activity following block of PB ipsilateral to the site of inflammation were accompanied by significant changes in nociceptive withdrawals of both the inflamed and untreated paw. The latency for withdrawal of the inflamed paw was increased throughout the subnoxious range, demonstrating a net anti-hyperalgesic effect of removing the ipsilateral PB (Figure 32a). For the untreated paw, where withdrawal was generally only elicited in the noxious range, blocking the ipsilateral PB produced hypoalgesia (Figure 32b).

5.4.3. Sensitization of ipsilateral PB in persistent inflammation requires contralateral PB

Given the observation that the PB ipsilateral and contralateral to a site of acute noxious stimulation or prolonged inflammation have differential influences on RVM pain-modulating neurons, the next set of experiments was designed to examine the process by which the PB ipsilateral to the site of injury is recruited to influence the RVM in persistent inflammation. Since the contralateral PB is a major relay to the RVM in normal animals and early in inflammation,[166] we hypothesized that the *contralateral* PB is required for the recruitment of *ipsilateral* PB during persistent inflammation. To test this, animals were subjected to an excitotoxic lesion of the *right* PB prior to induction of inflammation in the *left* hindpaw. Recording experiments took place 5 days after CFA injection.

The sites of ibotenic acid injection and examples of excitotoxic effects are plotted and shown in Figure 33. As in experiments focused on acute block of PB using the $GABA_A$ agonist muscimol, the focus of these experiments was on the lateral PB, as this region is functionally most relevant to RVM pain-modulating circuits, and has a denser concentration of RVM-projecting neurons.[11; 13; 23; 85; 166]

In contralateral-lesioned animals, blocking PB ipsilateral to the inflamed paw (the left PB) had no effect on the ON-cell burst or OFF-cell pause evoked by stimulation or either the inflamed or untreated paw, irrespective of stimulus intensity (Figure 34a-b). However, the spontaneous discharges of both ON- and OFF-cells were altered (Figure 34c), as in naïve animals and animals subjected to persistent inflammation without PB lesion.

Finally, development of mechanical allodynia was eliminated by lesion of the PB contralateral to the inflammation site (Figure 35a-b). Two-way ANOVA with repeated measures on force and side, followed by Sidak's test for multiple comparisons on paw withdrawal latency showed a significant effect of force ($F_{(5,80)} = 117.4$, $p < 0.0001$), but no effect on side ($F_{(1,16)} = 2.85$, $p = 0.11$), comparing between the CFA-treated and untreated hindpaw in baseline. In these animals, acute block of PB ipsilateral to the inflammation unmasked significant hyperalgesia in the CFA-treated paw ($F_{(1,16)} = 13.9$, $p = 0.002$), with no change in the untreated paw ($F_{(1,16)} = 0.14$, $p = 0.71$).

5.5. DISCUSSION

5.5.1. Recruitment of a novel nociceptive pathway as a means of RVM sensitization during persistent inflammation

Sensitization of the descending pain-modulating system that controls spinal pain transmission is thought to be a key factor in persistent pain after injury. The output node of the intrinsic pain-modulating circuit, the RVM, is known to undergo adaptive plasticity during persistent inflammation.[35; 65-67; 93; 106; 162; 175] Both ON- and OFF-cells become sensitized to previously innocuous stimuli, which contribute to behavioral hyperalgesia. At the same time, a "rebalancing" of ON- and OFF-cell output exerts a net antinociceptive influence, which can limit hypersensitivity.[35] We recently demonstrated a dynamic process by which the PB, a major nociceptive relay, contributes to RVM plasticity during persistent inflammation (Chapter 4). Under basal conditions, nociceptive information is relayed to the RVM via the PB

contralateral to the stimulus site.[166] The importance of the contralateral PB is maintained in the initial stages of inflammation, and is part of a pain-facilitating positive-feedback loop. However, as inflammation persists, PB contralateral to the inflammation site no longer provides a major input to RVM pain-modulating neurons, suggesting an adaptation of the pathway involving contralateral PB (Chapter 4). The present study describes an alternative pathway to RVM in persistent inflammation by showing that the PB ipsilateral to the site of injury contributes to the evoked responses of RVM ON- and OFF-cells. These experiments also suggest that recruitment or unmasking of an input from the ipsilateral PB to the RVM is triggered by input from the contralateral PB.

The current results further delineate the role of the PB in central sensitization during persistent inflammation. Recruitment of the PB ipsilateral to the inflamed hindpaw can help provide the necessary stimulus-evoked nociceptive input to the RVM ON- and OFF-cells. Conveyance of innocuous nociceptive information via PB thus contributes to RVM sensitization during persistent inflammation. This observation provides direct evidence for the long-assumed involvement of PB in maintenance of persistent pain, which was based on the fact that ablation of NK1-receptor positive dorsal horn neurons, many of which project to the contralateral PB (with sparse ipsilateral projections),[183] diminishes behavioral hypersensitivity associated with localized inflammation of several days duration.[180; 197] However, NK1 receptor-expressing neurons are also likely to exert effects on local spinal circuits, and could recruit descending modulation via relays other than PB. By directly manipulating PB, the target of the spinoparabrachial pathway, and determining its influence on the activity of RVM pain-modulating output neurons, the present experiments provide strong evidence that PB *ipsilateral* to the sites of injury contributes to sensitization of brainstem pain-modulating circuitry in persistent inflammation.

The fact that stimulus-evoked activity of RVM ON- and OFF-cells was not affected when PB *ipsilateral* to the stimulus site was blocked in naïve animals might reinforce the idea that an anatomically less dense projection is functionally less important. However, blocking PB ipsilateral to an inflamed paw interfered with the ON-cell burst and OFF-cell pause evoked by stimulation of that paw, and also substantially attenuated mechanical hyperalgesia exhibited on that side. These data suggest that the behavioral anti-hyperalgesia produced by lesion of NK-1 positive, presumed parabrachial-projecting neurons[103; 145; 164; 180; 197] is due to loss of the ipsilateral spinoparabrachial pathway.

Blocking PB ipsilateral to the inflamed paw also interfered with the ON-cell burst and OFF-cell pause evoked by stimulation of the opposite, uninflamed paw and reduced the behavioral sensitivity to stimulation of that paw. The effect on the uninflamed paw is consistent with the effects of blocking the PB contralateral to the stimulation site on RVM cell activity and behavior in naïve animals. This result thus indicates that the basal function of PB ipsilateral to the inflamed paw is intact in persistent inflammation, relaying nociceptive input to the RVM as under control conditions.

Blocking PB results in significant changes in the spontaneous firing of RVM ON- and OFF-cells in normal animals, with a depression in ON-cell discharge and increase in OFF-cell discharge, irrespective of whether the left (present experiments) or right PB is blocked.[166] Blocking PB ipsilateral to a site of inflammation resulted in a similar change in the spontaneous firing of RVM ON- and OFF-cells. By contrast, blocking PB contralateral to an inflamed paw did not consistently result in decreased ON-cell activity and increased OFF-cell activity. This suggests that the stimulus-evoked and ongoing activity of RVM neurons might be under the influence of distinct neurons or circuits within PB.

The second major observation in the current experiments is that PB contralateral to an inflammatory insult is required for the recruitment of the ipsilateral PB input to RVM ON- and

OFF-cells. The fact that blocking ipsilateral PB in the absence of intact contralateral PB during persistent inflammation had no net effect on RVM activity reaffirms the idea of *plasticity occurring in either the ipsilateral PB or RVM itself*, since acute bilateral blockade of PB interferes with the ON-cell pause and OFF-cell burst.[166]

5.5.2. Physiological implications of ipsilateral PB pathway, and its input to the RVM in chronic pain

The PB is a major nociceptive relay center that receives a dense somatosensory input from the contralateral dorsal horn.[12; 17; 20; 53; 155; 183] It projects to a number of areas relevant to pain processing and modulation, including the amygdala, hypothalamus, insular cortex, and some noradrenergic structures (e.g. A1, A5, locus coeruleus/subcoeruleus) as well as RVM.[16; 62; 96; 166; 173; 177 ; 195] The PB also receives a much sparser and less well-documented input from ipsilateral dorsal horn.[12; 17; 20; 53; 155; 183] Although studies using *c-fos* as a marker of neuronal activation have shown some responsive neurons in the ipsilateral PB,[9; 15; 85] the functional relevance of the ipsilateral projection has been largely overlooked. Indeed, we have found that blocking PB ipsilateral to a noxious stimulus fails to significantly reduce the noxious-stimulus related changes in ON- and OFF-cell firing.[166] The modest hyperalgesia seen with block of PB ipsilateral to a subnoxious stimulation (26 g) most likely reflects the effect of engagement of other non-RVM targets.[11; 21; 70; 91; 100; 101; 107; 163; 198]

Although input from the ipsilateral PB pathway has limited functional impact on the activity of RVM pain-modulating neurons in normal animals, its influence on the RVM became significant in animals with persistent inflammation, during which the contralateral PB contribution has been shown to be diminished (Chapter 4). Together, these findings indicate that ipsilateral and contralateral PB pathway have distinct roles in adapting to a prolonged injury and contributing to the maintenance of behavioral hyperalgesia in persistent pain. This process supports a sensitized RVM response to peripheral input via the ipsilateral PB while damping a

pronociceptive positive feedback loop through the contralateral PB. This dynamic process is likely adaptive in focusing sensitization to the injured tissue, and limiting development of more widespread maladaptive chronic pain.

5.5.3. Conclusion

The present findings provide a circuit-level explanation for the observation that ablation of NK1-expressing dorsal horn neurons interferes with behavioral hyperalgesia in persistent inflammation.[103; 145; 164; 180; 197] Combined with the recent report that the influence of the contralateral PB is diminished in persistent inflammation (Chapter 4), the current results demonstrated the different roles of contralateral and ipsilateral PB in adaptation during persistent inflammation, where they may respectively help initiate and maintain sensitization of RVM ON- and OFF-cells, and play distinct roles in behavioral hyperalgesia.

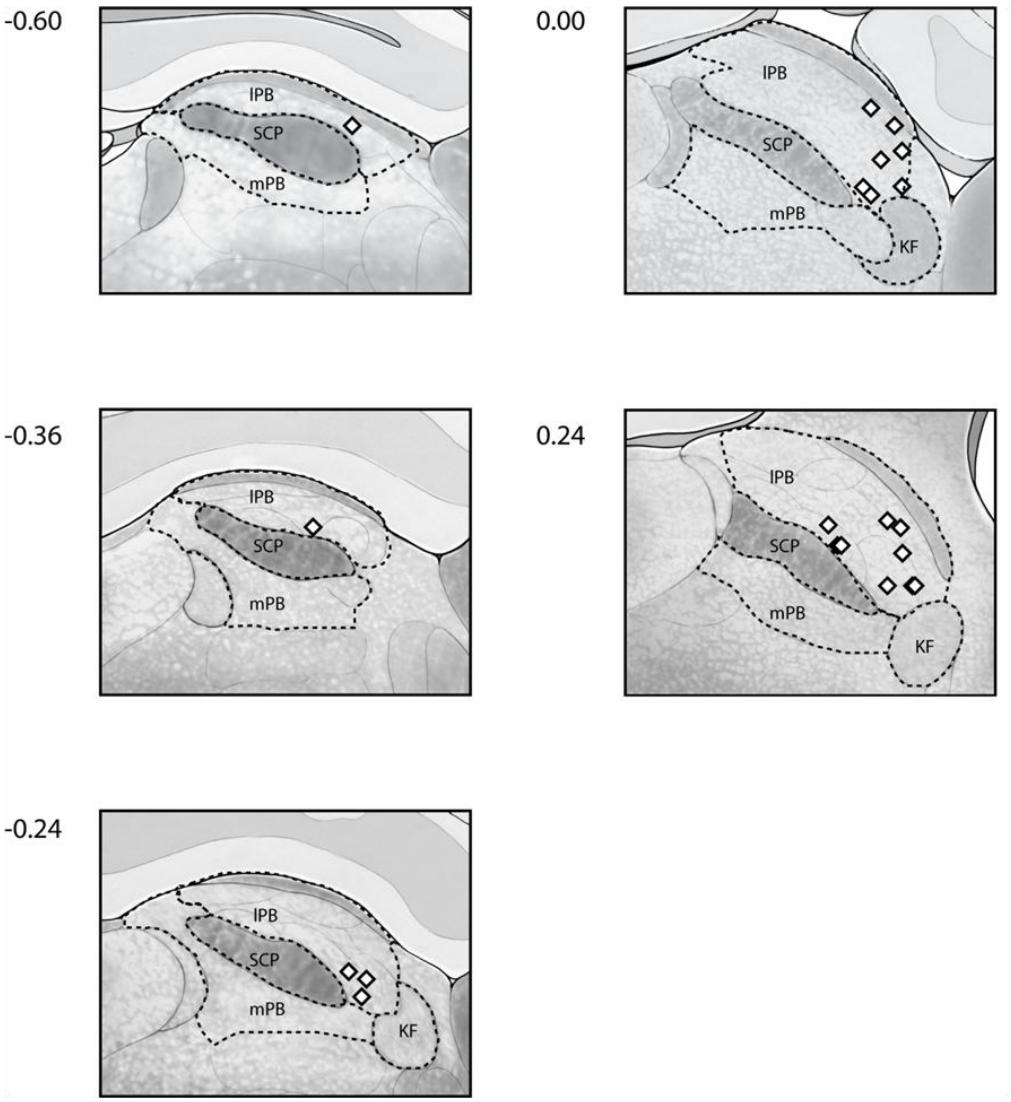

Figure 27 Locations of microinjection sites in the ipsilateral PB in naive, untreated animals

Parabrachial injections were distributed among sections at +0.24 to -0.60 mm relative to the interaural line. Injections inside the ipsilateral lateral PB (lPB) region were considered "on-target." White diamonds represent injection sites for naive untreated animals. KF: Kölliker-Fuse, lPB: lateral parabrachial complex, mPB: medial parabrachial area, scp: superior cerebellar peduncle.

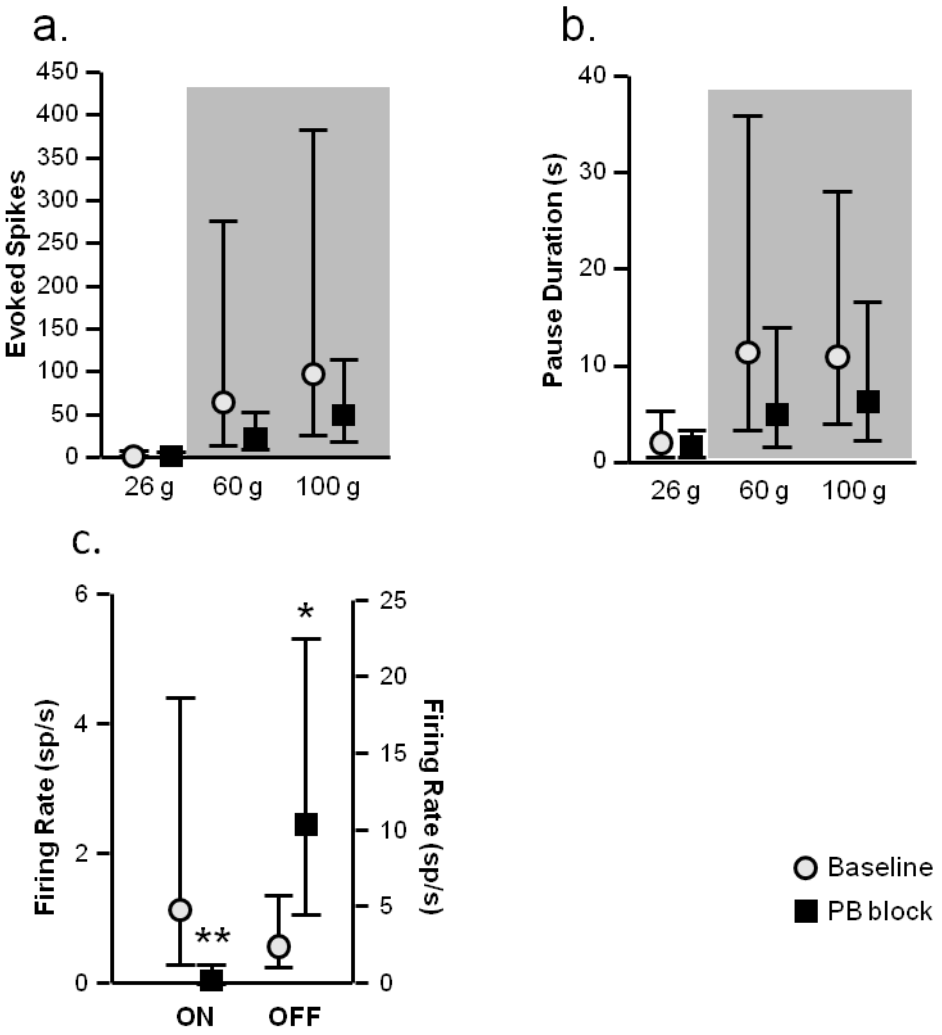

Figure 28 Inactivation of PB ipsilateral to the stimulated hindpaw did not interfere with the ON-cell and OFF-cell activity in response to mechanical stimulation.

a. ON-cells. Effect of muscimol (8 pmol, 200nl) microinjected into the left PB on activity triggered during application of von Frey probes (26, 60 and 100 g, measured as total evoked spike, n = `12). **b.** ON-cells. Effect of muscimol (8 pmol, 200nl) microinjected into the left PB on activity triggered during application of von Frey probes (measured as pause duration, n = `10). **c.** Effect of ipsilateral PB (left) inactivation on ongoing firing of ON-cells and OFF-cells in RVM. OFF-cell firing was significantly increased while ON-cell firing was reduced. Shaded gray represents "noxious stimuli" (60-100 g). (PB injection was unilateral, ipsilateral to stimulation. Data reported as geometric mean with 95% confidence limits, *$p <$ 0.05, **$p < 0.01$ compared to pre-block baseline using *t*-test for correlated means).

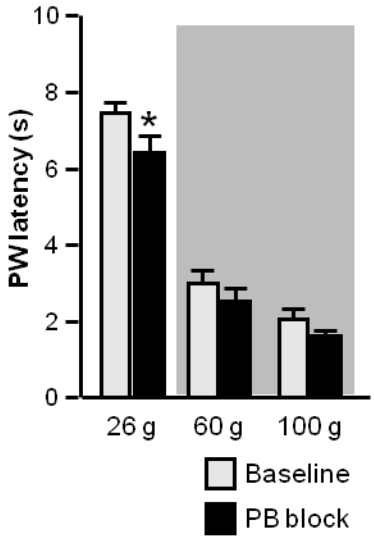

Figure 29 Behavioral effects on the hindpaw during inactivation of the ipsilateral PB.

There was modest decrease in hindpaw withdrawal latency to subnoxious stimulation (26 g) during inactivation of the ipsilateral PB. There were no significant changes in withdrawal latency to stimuli in the noxious range (shaded gray, 60-100 g). (Data shown as mean + SEM, *$p < 0.05$ compared to baseline using Wilcoxon's signed ranks test).

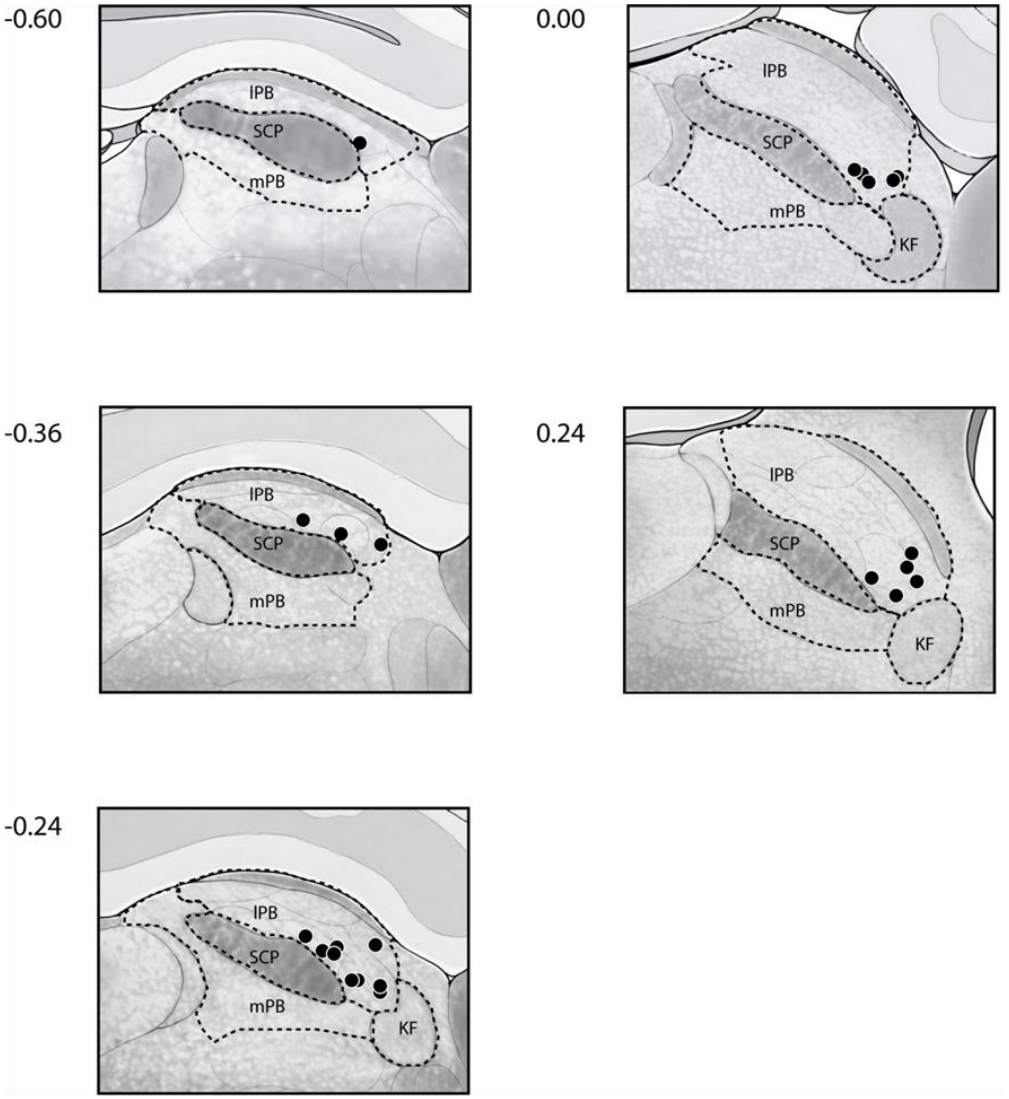

Figure 30 Locations of microinjection sites in the ipsilateral PB in CFA-treated animals.

Parabrachial injections were distributed among sections at +0.24 to -0.60 mm relative to the interaural line. Injections inside the ipsilateral lateral PB (lPB) region were considered "on-target." Black circles represent injection sites for CFA-treated animals. KF: Kölliker-Fuse, lPB: lateral parabrachial complex, mPB: medial parabrachial area, scp: superior cerebellar peduncle.

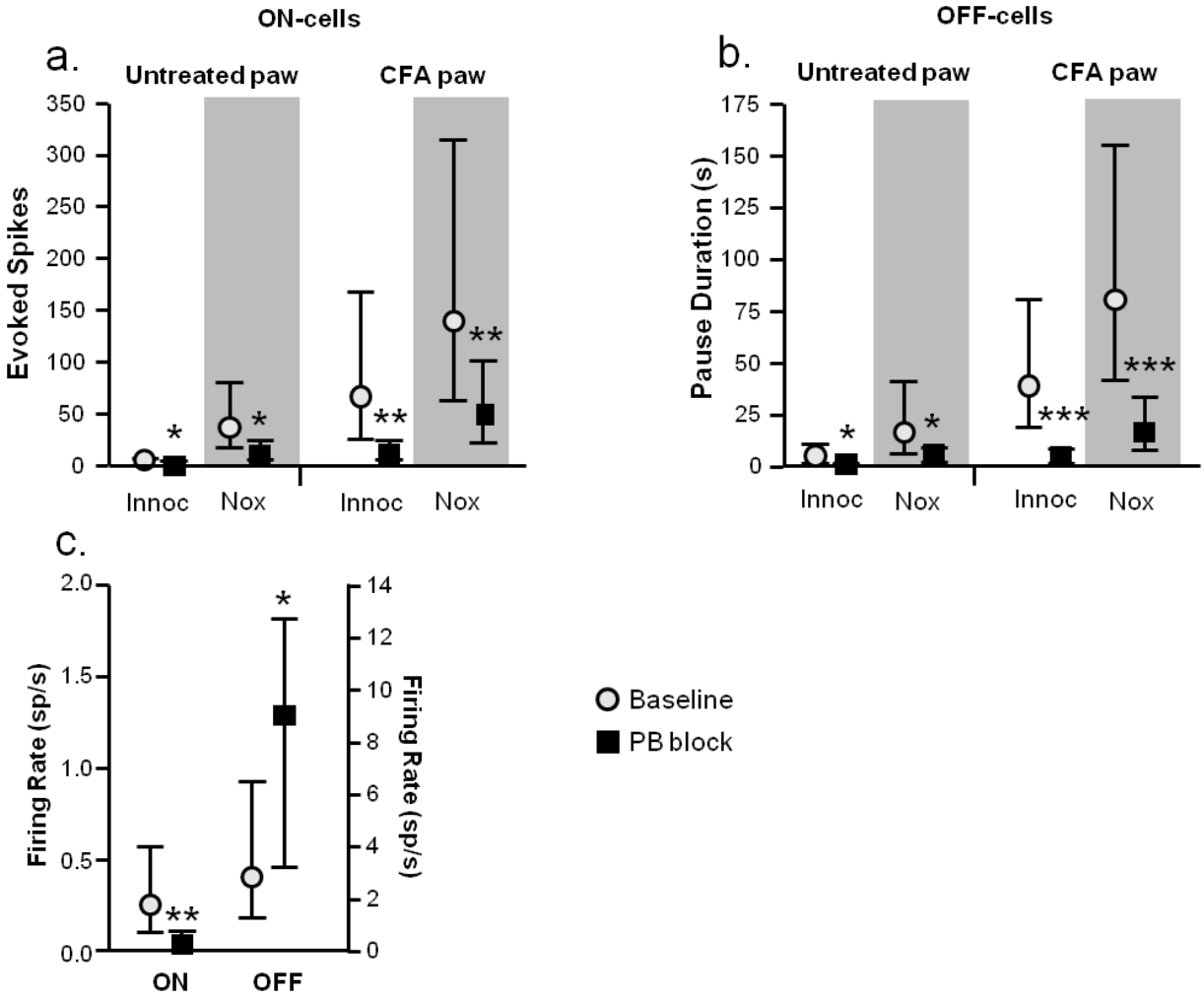

Figure 31 Inactivation of PB ipsilateral to inflammation interfered with the ON-cell burst and OFF-cell pause evoked during mechanical stimulation, as well as their ongoing activity in persistent inflammation.

a. ON-cells. Effect of muscimol microinjected into the ipsilateral PB on activity triggered during application of von Frey probes (26, 60 and 100 g, measured as total evoked spikes, n = 16 cells). **b**. OFF-cells. Effect of muscimol (8 pmol, 200 nl) microinjected into the ipsilateral PB on OFF-cell pause (measured as pause duration, n = 13 cells). **c.** Ongoing firing of ON-cells was reduced and that of OFF-cells was increased. (PB injections were ipsilateral to the inflammation. Shaded gray represents “noxious stimuli” (60-100 g). Unshaded area represents "innocuous stimuli" (4-15 g). 26 g does not belong to either categories. (Reported as geometric mean with 95% confidence limits, *t*-test for correlated means, **p* < 0.05, ***p* < 0.01, ****p* < 0.001 compared to baseline.)

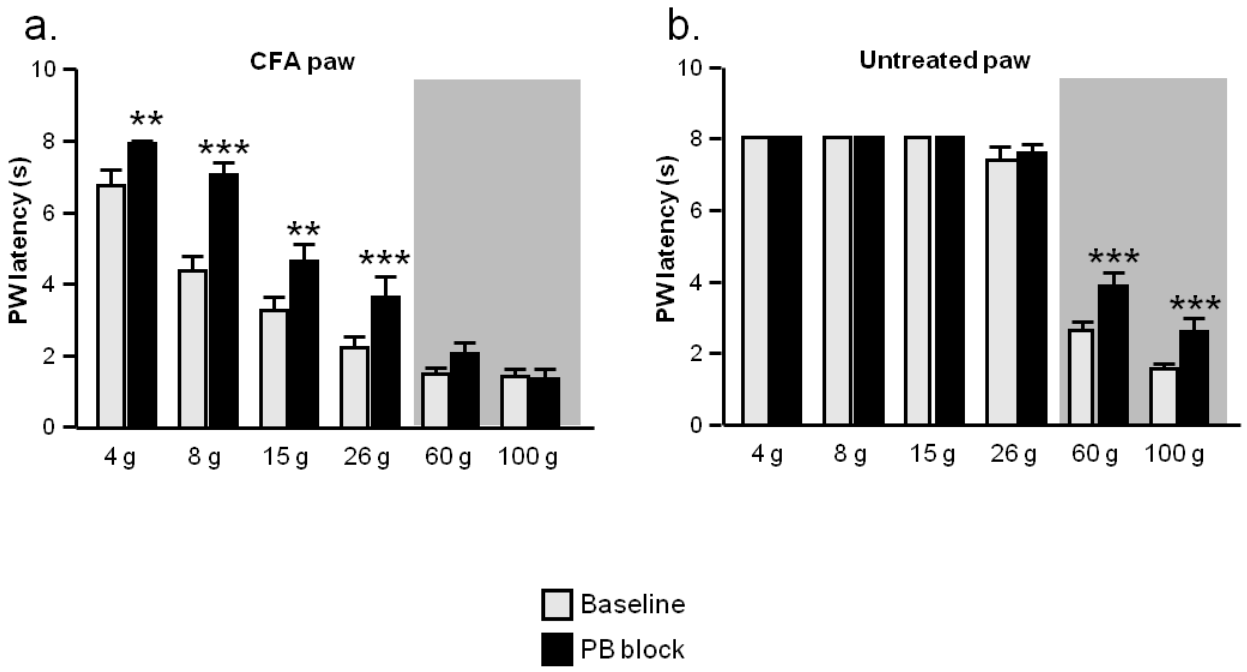

Figure 32 Effect of blocking ipsilateral PB on the withdrawal of the inflamed hindpaw evoked by von Frey probes 5/6 days after CFA injection.

5/6 days post-CFA treatment, blocking PB ipsilateral to the inflamed hindpaw significantly attenuate the withdrawal latency of **(a)** CFA-treated hindpaw, and **(b)** untreated hindpaw (contralateral to the block). Shaded gray represents “noxious stimuli” (60-100 g). Unshaded area represents "innocuous stimuli" (4-15 g). 26 g does not belong to either categories. Wilcoxon's sign ranked test, reported as mean + SEM, ***$p < 0.001$ compared to baseline.

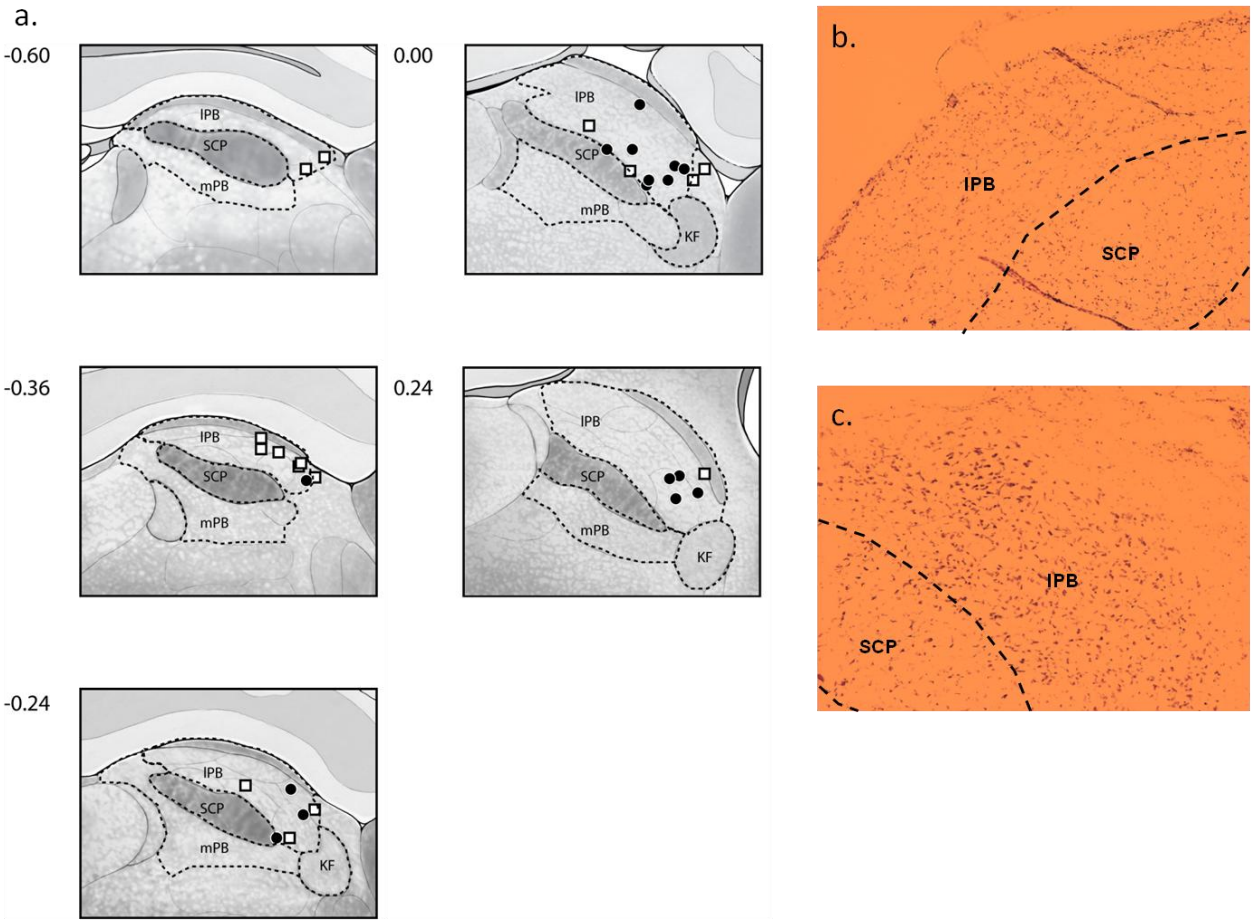

Figure 33 Locations of ibotenic acid microinjection in the contralateral PB prior to the induction of inflammation.

Prior to the induction of inflammation, the *right* PB was injected with the neurotoxin ibotenic acid to lesion the area. **a.** Ibotenic acid injections were distributed among sections at +0.24 to -0.60 mm relative to the interaural line. White squares represent ibotenic acid injection sites. Locations of muscimol injection in *left* PB during recording are also plotted in **(a)** (Black circles) **b-c.** Histological examples of ibotenic acid-induced lesion in PB (b) vs. control (c). Imagine shown are 5/6 days after inflammation. . KF: Kölliker-Fuse, lPB: lateral parabrachial complex, mPB: medial parabrachial area, scp: superior cerebellar

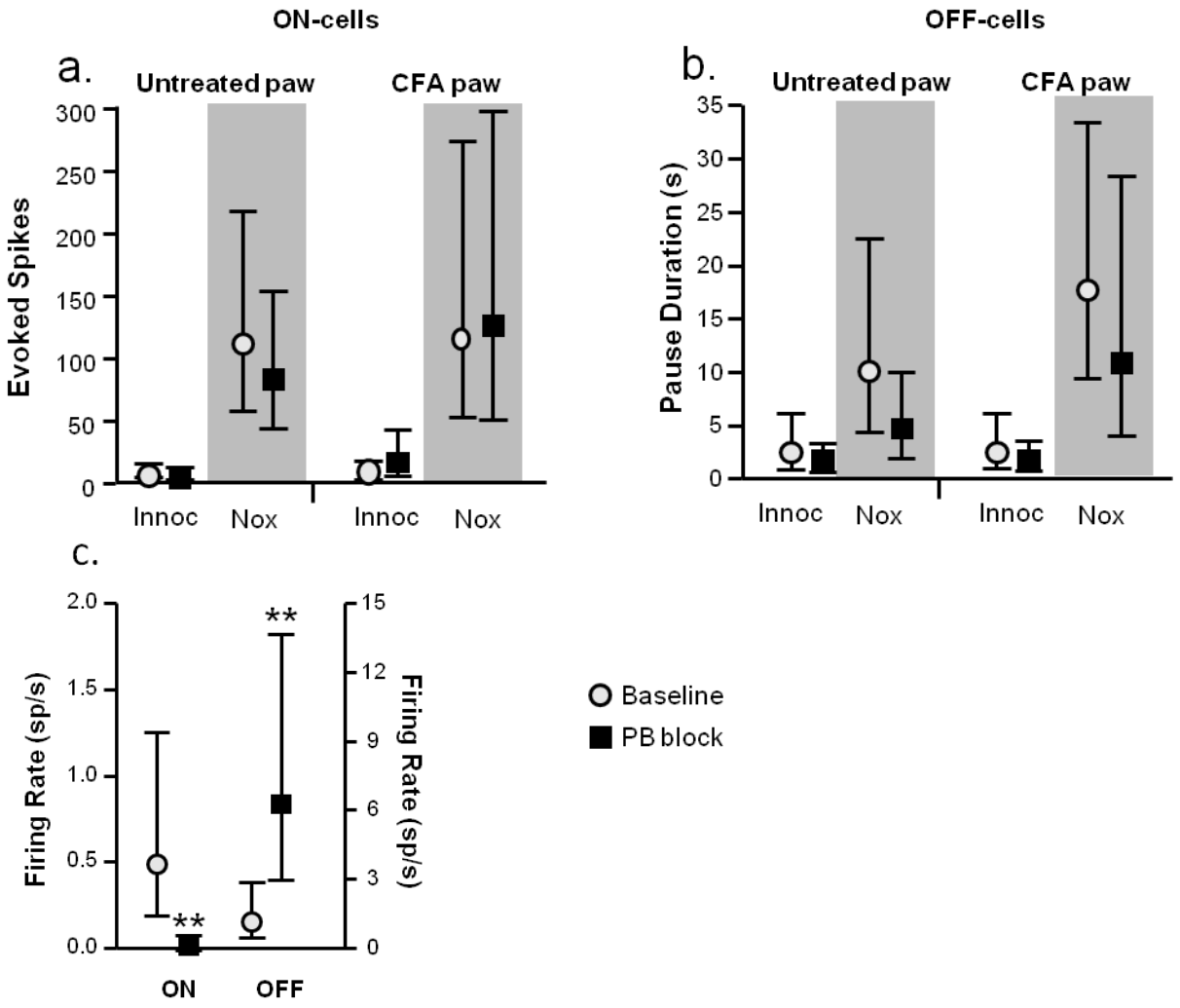

Figure 34 Effect of blocking PB ipsilateral to the inflamed hindpaw on ON- and OFF-cell activity in animals pre-treated with ibotenic acid.

Blocking PB ipsilateral to the inflammation did not attenuate **a.** ON-cell burst or **b.** OFF-cell pause in ibotenic acid-pretreated animals. **c.** Blocking ipsilateral PB significantly decreased ON-cell ongoing firing, and increased OFF-cell firing in ibotenic acid-pretreated animals. PB contralateral to the inflammation was pretreated with ibotenic acid, and PB ipsilateral to the inflammation was blocked during recording. Shaded gray represents “noxious stimuli” (60-100 g). Unshaded area represent "innocuous stimuli" (4-15 g) (Reported as geometric mean with 95% confidence limits, *t*-test for correlated means, $^{**}p < 0.01$ compared to baseline)

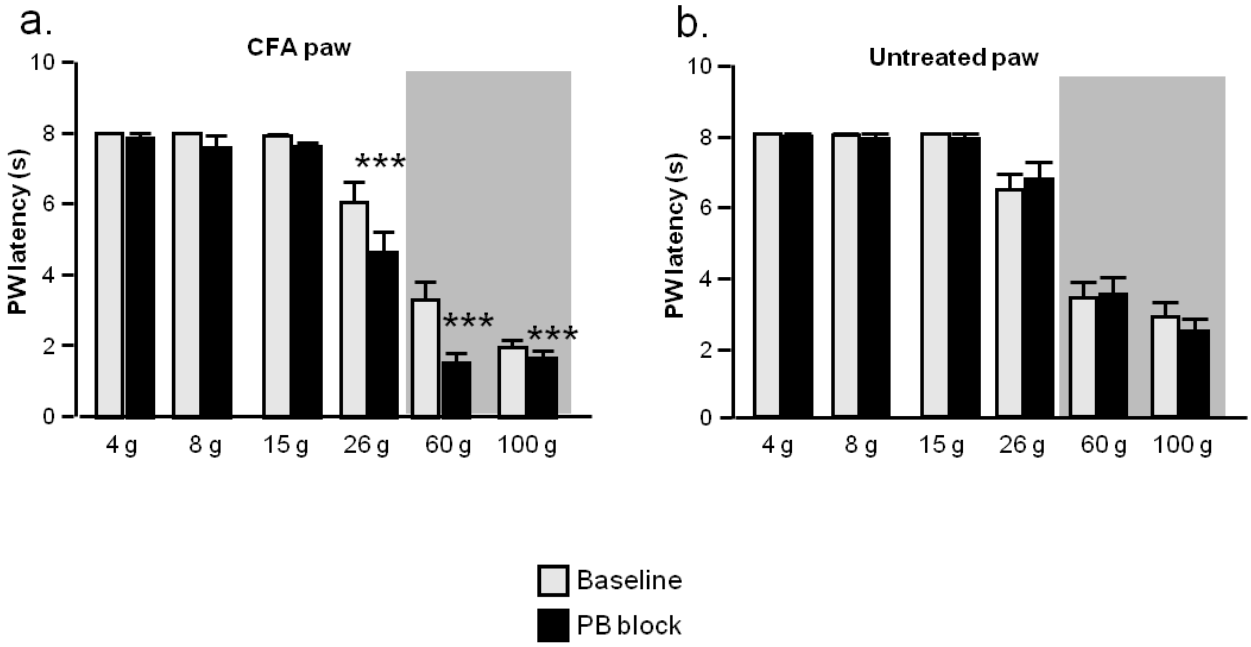

Figure 35 Effect of blocking ipsilateral PB on the withdrawal evoked by von Frey 5/6 days after CFA injection in ibotenic acid-pretreated animals

a. Blocking PB ipsilateral to inflammation reveal mild hyperalgesia on the inflamed hindpaw in the noxious range. **b.** Blocking PB ipsilateral to inflammation had no effect on the untreated hindpaw (contralateral to the block). Shaded gray represents “noxious stimuli” (60-100 g). Unshaded area represents "innocuous stimuli" (4-15 g). 26 g belongs to both categories. Wilcoxon's sign ranked test, reported as mean + SEM, ***$p < 0.001$ compared to baseline.

CHAPTER 6

DISCUSSION

6.1. KEY FINDINGS

- The PB carries nociceptive information from the contralateral side of the body to the RVM via a direct projection. This information contributes to both the nociception-related evoked responses of ON- and OFF-cells (the characteristic “burst” and “pause” that defines these cell classes) and to their ongoing activity (Figure 36a).

- Optogenetic manipulation reveals both excitatory and inhibitory components of the projection from the PB to the RVM to all RVM cell classes, NEUTRAL-cells as well as the pain-modulating ON- and OFF-cells.

- Activation of ON-cells and inhibition of OFF-cell firing during acute inflammation is mediated by PB contralateral to the inflammatory stimulus. However, this pro-nociceptive drive through the contralateral PB is diminished in persistent inflammation (Figure 36a).

- In persistent inflammation, nociceptive information is relayed to the RVM through the PB ipsilateral to the site of inflammation, rather than the contralateral PB as under basal conditions or in acute inflammation. This activation or unmasking of the ipsilateral PB input to the RVM requires the presence of an intact contralateral PB (Figure 36b).

- PB contributes to behavioral hyperalgesia associated with both acute and persistent inflammation. However, as with the input the RVM pain-modulating neurons, the role of PB depends on whether the block is ipsilateral or contralateral to the inflammation site. In acute inflammation, inactivation of contralateral PB attenuates hyperalgesia. By contrast, inactivation of ipsilateral PB attenuates hyperalgesia in persistent inflammation.

6.2. OVERVIEW

The RVM is the major output node of an intrinsic pain-modulating system that exerts bidirectional modulatory control via two physiologically defined classes of neurons: pain-

facilitating "ON-cells" and pain-inhibiting "OFF-cells." The output of these two classes of neurons modulates nociceptive transmission via projections to the dorsal horn. These neurons also respond to nociceptive signals, where ON-cells initiate a "burst" of firing, while OFF-cells "pause" any ongoing activity in response to an acute noxious input. These neurons can be also be “sensitized” following injury or inflammation, with the burst and pause evoked by normally innocuous stimuli, and this sensitization is believed to contribute to some forms of persistent pain. However, the pathway through which nociceptive signals reach the RVM had not been identified, leaving a gap in our knowledge of how peripheral inputs engage descending pain-modulating systems and how these inputs contribute to sensitization in persistent pain. The central goal of this thesis was to test the hypothesis that the PB complex is a relay of nociception-related information to the RVM.

PB is a known major relay of ascending nociceptive information, receiving a dense projection from the contralateral superficial dorsal horn as well as a sparser projection from the ipsilateral dorsal horn. The experiments described here demonstrated that the lateral PB is a major ascending relay of nociceptive input to RVM ON- and OFF-cells. Optogenetic experiments revealed that the influence of PB on RVM ON- and OFF-cells is mediated via a direct projection to RVM. Lastly, my experiments identified a dynamic process where PB contralateral and ipsilateral to a site of inflammation have interdependent roles that drive RVM sensitization and contribute to the initiation and maintenance of behavioral hypersensitivity. These findings demonstrate a functional link between an ascending nociceptive transmission relay (PB) and the primary output node (RVM) of a descending pain-modulating circuit under normal conditions, and during acute and persisting inflammation.

6.3. CIRCUIT MANIPULATION SHOWS THE INFLUENCE OF PB INPUT ON RVM NEURONAL FUNCTION

6.3.1. PB input to RVM under basal conditions (Chapter 2)

The defining characteristic of RVM ON- and OFF-cells is a reciprocal nocifensive reflex-related change in activity: ON-cells are activated ("burst") while OFF-cells abruptly stop firing ("pause").[58; 59] Nociceptive inputs to the RVM thus exert a major influence on the activity of ON- and OFF-cells. However, the source(s) of this primary sensory input was unclear. A diencephalic or telencephalic structure could be ruled out because ON-cell bursts and OFF-cell pauses are seen in animals following mid-collicular decerebration.[34] The PAG, which has dense projections to RVM, is another possible candidate, since there is a substantial direct spinal projection to the PAG. However, PAG lesion does not eliminate ON-cell bursts and OFF-cell pauses,[126] which indicates that the PAG is not a necessary relay for nociceptive input to RVM. Direct inputs to the RVM have been identified from the medullary dorsal horn, but these spinoreticular projections to RVM have been considered sparse, and the functional significance of this input has not been investigated.[30; 179] In this thesis, I considered another possible candidate, the parabrachial complex, specifically its lateral region, and showed it to be a major relay of nociceptive input to RVM pain-modulating neurons.

In Chapter 2, it was demonstrated that lateral PB is a primary relay for nociceptive to RVM ON- and OFF-cells under basal conditions, without ongoing injury or inflammation. The left and right PB each convey input from the contralateral side of the body, and have minimal role in relaying signals from the ipsilateral side, which is consistent with the dense spinoparabrachial projection from the contralateral superficial dorsal horn.[12; 17; 53; 155; 183] Studies using *c-fos* as a marker for neural activation also point to the contralateral PB as having a significant role in nociception, since the contralateral, but not ipsilateral, PB showed significant activation in response to acute noxious stimuli.[11; 13; 23; 85] Electrophysiological studies have identified nociresponsive neurons in PB with broad visceral and somatosensory receptive fields.[14; 130;

131]. Given the substantial reduction in ON-cell burst and OFF-cell pause during inactivation of PB demonstrated here, the share of acute nociceptive input to RVM that is relayed through PB is significant.

Inactivation of PB also led to changes in the ongoing firing of ON- and OFF-cells, which implies that information relayed through PB contributes to the ongoing "tone" of RVM ON- and OFF-cell pain-modulating output. ON-cell activity was reduced, whereas OFF-cell firing was increased. NEUTRAL-cell firing was unchanged during PB block, indicating that these neurons do not receive ongoing input from PB under the conditions of these experiments. It is likely that nociceptive, or at least somatosensory, information is the relevant factor in the effect of PB block on ongoing activity of ON- and OFF-cells, since similar changes in RVM activity are seen with spinal lidocaine administration, which would block ascending transmission of somatosensory inputs.[74] The ongoing balance between RVM ON- and OFF-cells is known to modulate the threshold and magnitude of nociceptive behavior,[73; 99] making it likely that the increased threshold for noxious-evoked withdrawal observed during PB block was due to the increase in OFF-cell firing and decrease in ON-cell firing.

6.3.1.1.The direct PB projection to RVM carries nociceptive information (Chapter 3)

In Chapter 2, PB was identified as an important nociceptive relay to RVM. However, the pathway linking PB to ON- and OFF-cells was not defined. PB could potentially influence RVM indirectly, through areas such as the amygdala, PAG, or insular cortex,[23; 62; 95; 126; 127; 174; 203] but as mentioned above (section x.3.1), these structures can be ruled out as the source of the nociception-related activity of ON- and OFF-cells. A sparse direct projection from PB to RVM had been identified in anatomical studies,[8; 84; 195] but it was unclear whether this direct connection is relevant to pain-modulation rather than one of the other functions shared by PB and the RVM (such as respiration, thermogenesis).

Optogenetic methods were used in this thesis to prove that the direct projection from PB to RVM can influence ON and OFF-cells. ArchT-induced inhibition of PB terminals in RVM mimicked the effect of pharmacological block of the PB area: the nociceptive responses of RVM pain-modulating neurons were reduced, while OFF-cells became more active and ON-cells less active. Because manipulating PB terminals in RVM would not influence pathways through other structures, these data indicate that the direct projection from PB to the RVM is sufficient to evoke the ON-cell burst and OFF-cell pause and modulate the ongoing "tone" of this system. Given that the projection from PB to RVM is not particularly dense, this finding cautions against the common assumption that the density of the connection between two structures is a measure of its functional importance.

Both excitatory and inhibitory inputs to all three classes of RVM neurons from PB were revealed using direct activation of RVM-projecting PB terminals expressing ChR2, although excitatory inputs were dominant and occurred at a short latency. By contrast, inactivation approaches (either microinjection of the $GABA_A$ agonist muscimol in lateral PB in Chapter 2 or ArchT-induced inhibition of PB terminals in RVM) identified only an inhibitory input to OFF-cells and an excitatory input to ON-cells, with no evident influence on NEUTRAL-cells. This indicates that only a subset of PB inputs to RVM neurons are active under the conditions of these experiments, raising the possibility that PB could either activate or inhibit any of the three RVM cell classes under different physiological or pathophysiological conditions. Given the lack of other evidence that NEUTRAL-cells play a role in pain-modulation, the input to this class brought to light by the optogenetic activation of PB terminals is likely to be important in some other function (e.g., respiration, thermogenesis).

Excitation of both OFF-cells and ON-cells in response to terminal stimulation using ChR2 occurred at a latency consistent with the time delay for monosynaptic input,[48] suggesting a direct excitatory synapse from PB with both cell classes. The fact that the latency of the ON-cell

response was, on average, longer than that of OFF-cells raises interesting questions as to whether there are differences the intrinsic membrane properties of the two classes or the relevant local circuit within the RVM. It is also possible that RVM neurons receive heterogeneous monosynaptic inputs from PB, which contribute to the difference in synaptic delays, a mechanism analogous to the ventrolateral PAG input to RVM.[147]

Inhibitory inputs to all three classes of RVM neurons appeared to have longer latency than excitatory inputs. Although only a few neurons that displayed inhibition could be found for each cell classes, it suggests that PB inputs can exert inhibition on RVM neurons via polysynaptic mechanisms.

6.3.2. PB is a relay in a positive-feedback loop during acute inflammation (Chapter 4)

Acute injury and inflammation typically lead to increased sensitivity of the injured body part, and this hyperalgesia is considered to be adaptive, leading to protective and recuperative behaviors. This behavioral hyperalgesia is linked to increased ON-cell activity by a considerable body of evidence. ON-cells enter a period of continuous activity in response to an acute inflammatory stimulus (e.g., topical mustard oil or capsaicin, localized injection of complete Freund's adjuvant), whereas activity of OFF-cells is suppressed.[24; 35; 104; 201] Both ON- and OFF-cells are sensitized to normally subthreshold inputs during acute inflammation, and can respond to light tactile stimuli that normally do not evoke a burst or pause.[26; 35] Under these conditions, with ON-cells dominating the output from the RVM, global inactivation of RVM reverses hyperalgesia, indicating that activation of ON-cells is critical for behavioral hyperalgesia.[35; 104] The concomitant suppression of OFF-cell activity may contribute to hyperalgesia in acute inflammation, but is not necessary, because selectively attenuating the increase in ON-cell activity without interfering with the depression of OFF-cell activity is sufficient to prevent hyperalgesia.[201]

In Chapter 4, it was shown that blocking PB contralateral to the inflamed site sensitization of ON- and OFF-cells, prevented the shift in ongoing activity so that ON-cell dominated the RVM output, and interfered with behavioral hyperalgesia in acute inflammation (first hour following intraplantar administration of CFA). Thus, continuing afferent input from the inflamed tissue forms the afferent limb of a positive feedback loop and is relayed to the RVM via the contralateral PB. The resulting activation of ON-cells has a global net pro-nociceptive influence that is sufficient to produce behavioral hyperalgesia in the inflamed tissue.[35; 188]

6.3.3. Adaptation of PB contralateral to inflamed site in persistent inflammation

In Chapters 2 and 4, there is supporting evidence for a significant role for PB in conveying input from the contralateral body to the RVM under basal conditions and in acute inflammation. In Chapter 5, it is shown that this influence diminished as inflammation persisted over a period of days.

As described in section 6.3.2., acute inflammation is associated with a shift in the ongoing activity of RVM ON- and OFF-cells so that ON-cell output dominates. The tactile threshold of both cell classes is also decreased, so that normally innocuous inputs evoke an ON-cell burst and OFF-cell pause. Animals display tactile allodynia maintained by a supraspinal positive feedback loop through the PB contralateral to the inflammation site and the RVM. This picture changes dramatically as inflammation persists over the next several days.[35] Both RVM cell classes remain sensitized to innocuous inputs, but the balance of ongoing activity between ON- and OFF-cells is restored. Tactile allodynia is maintained, but is independent of the RVM at these time points. Indeed, the net influence of RVM in persistent inflammation is anti-nociceptive.[35; 192] The restored balance between ON- and OFF-cell outputs has been considered a compensatory response that to some extent limits the full development and spread of hyperalgesia.[35; 43]

The role of the PB input to RVM also changes as inflammation persists. Unlike in naïve animals or during acute inflammation, blocking PB contralateral to the site of inflammation had no effect on either the ON-cell burst or OFF-cell pause when tested one day after CFA injection. By five days post-injection, block of contralateral PB reduced, but did not eliminate the OFF-cell pause, and the ON-cell burst was unaffected. Probably more important, contralateral PB block had mixed effects on the ongoing activity of ON- and OFF-cells, with some ON-cells showing *increased* activity with PB block and some OFF-cells showing *decreased* activity. As a result, contralateral PB block had no overall effect on ON- and OFF-cell output. These data suggest that additional influences from PB to RVM, not normally active under basal conditions or in acute inflammation, are recruited during persistent inflammation. These inputs have a net inhibitory influence on the activity of some ON-cells and a net facilitatory influence on the activity of some OFF-cells, which may contribute to the restoration of the normal balance in the ongoing activity of these two cell classes, "putting a brake" on the positive feedback loop triggered during acute inflammation, and allowing for increased descending inhibition as an adaption to a prolonged injury.[35; 43; 192]

6.3.4. Input to RVM from PB ipsilateral to an inflamed site is recruited in persistent inflammation

In Chapter 4, it is demonstrated that the contribution of PB contralateral to an inflammatory insult to nociception-related and ongoing activity of ON- and OFF-cells is diminished during persistent inflammation. Nevertheless, these neurons still exhibit the characteristic nociception-related burst and pause in response to noxious stimulation, and during innocuous stimulation of the inflamed limb. This finding suggested that a distinct pathway is activated or unmasked to convey nociceptive information to RVM pain-modulating neurons during persistent inflammation.

In Chapter 5, it is shown that PB *ipsilateral* to the inflamed paw conveys nociceptive information to RVM during persistent inflammation. Blocking the ipsilateral PB in animals in which CFA had been injected in one hindpaw 5 or 6 days prior to the recording study interfered

with ON-cell burst and OFF-cell pause evoked by noxious or normally innocuous mechanical stimulation of the inflamed paw. Blocking PB ipsilateral to an acute noxious stimulus had no effect on the RVM response to that stimulus. These data indicate that the PB ipsilateral to an inflamed site provides the necessary nociception-related input to RVM ON- and OFF-cells, and that this input is recruited or unmasked in persistent inflammation. This pathway is therefore involved in maintenance of a persistent inflammatory pain state.

These results add a new dimension to our understanding of spinoparabrachial pathways and their contribution to persistent or chronic pain. Elimination of spinal NK1R-expressing neurons using a selective toxin has been shown by a number of groups to interfere with persistent hyperalgesia in inflammatory and neuropathic pain models.[145; 164; 180; 197] The effect of the NK1R lesion was presumed to be mediated by the PB contralateral to the injury because the majority of dorsal horn NK1R-postive neurons send their axons to the PB on the contralateral side of the body.[12; 17; 53; 155; 183] However, the neurotoxin approach did not differentiate ipsilateral from contralateral targets of the spinoparabrachial pathway, nor could it confirm that PB itself was involved, NK1R-positive neurons are also involved in local spinal circuitry. The results of the experiments described in Chapter 5 suggest that the behavioral anti-hyperalgesia produced by lesion of NK1R-positive dorsal horn neurons is due to the loss of the ipsilateral spinoparabrachial pathway and its input to RVM.[145; 164; 180; 197]

6.3.4.1. Recruitment of ipsilateral PB input in persistent inflammation requires an intact contralateral PB

The recruitment of the ipsilateral PB input, and the sensitization of RVM in persistent inflammation were prevented when PB contralateral to the inflammation was lesioned prior to CFA injection. Behavioral hyperalgesia was also substantially attenuated. These data suggest that the *contralateral* PB triggers and/or maintains the engagement of the *ipsilateral* PB input to RVM in persistent inflammation. The observation that acute *ipsilateral* PB block had no effect

on RVM activity when the *contralateral* PB was lesioned in persistent inflammation is in contrast with the effect of acute *bilateral* block of PB under basal conditions, which interfered with the burst and pause. The current observation, that blocking PB ipsilateral to the inflamed site in the absence of an intact contralateral PB had no net influence on the nociception-related activity of ON- and OFF-cells, is therefore a direct result of the inability to recruit ipsilateral PB in the absence of contralateral PB.

6.3.5. Functional implications of the dynamic interaction between PB and RVM for acute and persistent pain

Despite the similarity in behavioral hypersensitivity between acute and persistent inflammation, considerable evidence, including some developed in pursuit of this thesis, indicates that hyperalgesia in persistent inflammation is not a mere continuation of the acute state, and that the underlying mechanisms vary over hours and days as inflammation develops and persists. Understanding these mechanisms is challenging because descending pain-modulating systems are part of a recurrent circuit, receiving inputs from the neural systems that they modulate. Further, the plasticity in descending control is driven by spinal cord circuits that are themselves sensitized and that in turn receive input from sensitized primary afferents. Teasing out the functions and connections of this dynamic circuit is therefore highly complex. The focus of this thesis was on how nociceptive information gains access to the output node of the descending limb of this circuit, the RVM.

RVM circuits undergo significant molecular and cellular plasticity in persistent pain states, with changes in NMDA, AMPA , trkB, opioid, and neurokinin-1 receptor expression and function,[65-67; 93; 106; 162; 175], local glial activation,[165] and even neuronal loss.[111] Nociceptive processing through PB is also known to be plastic,[13; 163; 169] it should therefore not be surprising that the interaction between PB and RVM is also altered during persistent inflammation. Although the net PB influence on RVM pain-modulating neurons under basal

conditions is to activate ON-cells and inhibit OFF-cell firing, optogenetic manipulation of PB terminals in RVM (Chapter 3) revealed that the direct projection from PB to RVM included excitatory and inhibitory influences on both ON- and OFF-cells. This raises the possibility that the net functional influence of PB on the RVM depends on a balance of excitatory and inhibitory inputs, and that this balance shifts during inflammation. This may be a key factor in switching RVM output from an ON-cell dominated pro-nociceptive influence in acute inflammation to an OFF-cell mediated anti-hyperalgesic and antinociceptive influence as inflammation is maintained.

The relative roles of the PB contralateral and ipsilateral to the inflammation site add an additional layer of complexity. Input from the contralateral PB contributes to the increased ON-cell activity in the first hour of inflammation, but its influence on RVM ON- and OFF-cell activity wanes by the first day of inflammation, and is mostly eliminated by the fifth day (Chapter 4). Meanwhile, PB ipsilateral to the inflammation develops a greater significance, although this recruitment of ipsilateral PB is triggered and possibly maintained by the contralateral PB (Chapter 5). Together, these findings indicate that the ipsilateral and contralateral PB have distinct roles in adapting to a prolonged injury or inflammation. This process supports a sensitized RVM response to peripheral input via the ipsilateral PB while damping a pronociceptive positive feedback loop through the contralateral PB. This process is likely adaptive in focusing sensitization to the injured tissue, and limiting development of more widespread, maladaptive chronic pain.

6.4. TECHNICAL CONSIDERATIONS

6.4.1. Anesthesia

The use of light anesthesia allows us to precisely study the physiology of an isolated neuron within an intact circuit, while measuring its behavior output. This lightly anesthetized model, in which a spinal reflex to noxious stimuli is used as the behavioral outcome, has been well

established.[4 ; 26; 35; 36; 75; 79] Moreover, ON-, OFF-, and NEUTRAL cells have been indentified in unanesthetized animals.[34; 82; 112; 128; 129] Therefore, although there may be quantitative differences between lightly anesthetized and awake behaving animals, the qualitative relationship between RVM activity and nociceptive sensitivity appears to be preserved in these lightly anesthetized animals.

The likely quantitative effects of anesthesia would be to suppress ON-cell excitability and increase OFF-cell firing, with no effect on NEUTRAL-cell firing.[112] Increasing anesthesia depth would also blunt ON- and OFF-cell nociception-related responses and depress the nocifensive behaviors.[99; 112] The lightly anesthetized preparation used here allows behavioral responses in animals at stimulus intensities comparable to those used in awake behaving animals.[26; 35] A report indicates that RVM neurons in unanesthetized animals may respond to innocuous stimulus in proportion to the intensity of the stimulation.[149; 167] However, a quantitative shift in ON- and OFF-cell responsiveness does not change the fundamental RVM physiology.

6.4.2. Optogenetic methods

The light stimulation protocol has a primary impact on the degree to which the circuit is inactivated (ArchT) or activated (ChR2). The level of channel expression and the field of illumination from the optoelectrode can also affect the neuronal response, and cause over- or under-stimulation of terminals, which would yield unreliable responses. Thus, our light stimulation protocol was optimized to ensure the reproducibility of the physiology by avoiding events like terminal fatigue from overstimulation. Another concern over the use of viral vectors is the over-expression of channels,[204; 207] which may change neuronal physiology. No-light controls were carried out in the infected animals to ensure the integrity of the circuit, and ON-cell bursts and OFF-cell pauses remained intact and comparable to naïve animals. Lastly, RVM neuron responses to terminal stimulation *in vivo* represent input to the RVM, but not necessarily

to the neuron under study. Whether an individual RVM neuron receives direct input from PB can only be addressed in slice recordings.

6.5. FUTURE DIRECTIONS AND IMPLICATIONS FOR HUMAN HEALTH

6.5.1. The role of PB direct projections to RVM in acute and persistent pain

The transition from acute to chronic pain is accompanied by functional, physiological, and pharmacological changes in the RVM.[81; 161] One of the most significant questions raised in this work is how the function of the direct projection to RVM from ipsilateral and contralateral PB evolves over the course of a persisting inflammation. The experiments employing ChR2-mediated activation of PB terminals in RVM revealed that PB has the capacity for bidirectional modulation of all three functional classes of RVM neuron, while the studies using global inactivation of PB suggested that a different balance of facilitating and inhibiting inputs to ON- and OFF-cells might be active at different stages of inflammation. Thus, a more in-depth investigation is warranted in to delineate functional changes in PB direct inputs to RVM during acute and persistent inflammation.

Excitatory ChR2 optogenetic approaches could be used to activate RVM terminals arising from contralateral and ipsilateral PB to test whether PB-RVM inputs are altered with persistent inflammation. Inhibitory ArchT opsins could then be used to silence RVM terminals arising from the ipsilateral and contralateral PB at different time points as inflammation develops and is maintained. I hypothesize that inactivation of terminals from PB contralateral to a site of persisting inflammation would have no net effect on RVM neuronal activity, while inhibiting terminals from ipsilateral PB would attenuate the ON-cell burst and OFF-cell pause.

6.5.2. Role of contralateral PB in initiation vs. maintenance of plasticity in the PB-RVM system

It would also be of interest to tease out the role of the contralateral PB in initiation vs. maintenance of RVM plasticity and behavioral hyperalgesia. PB contralateral to the

inflammation site relayed information to RVM in the first hour of developing inflammation, but whether this early input to RVM is sufficient to recruit or unmask a normally silent input from the PB ipsilateral to the inflammation site was not determined in these experiments, which used a permanent ablation approach to eliminate the contralateral PB. It would be useful to test the effects of a short-term block (e.g., expression of an inhibitory DREADD in contralateral PB with administration of CNO over a period of several hours) both early and late in development of inflammation to determine whether the contralateral PB is necessary to trigger recruitment of ipsilateral PB, required for maintenance of ipsilateral PB input, or both.

6.5.3. Synaptic characteristics of the PB-RVM circuit in persistent pain

Structural, molecular, and pharmacological alterations have been reported in the RVM in persistent pain states.[65; 66; 93; 161; 165; 176][111] Some of these changes likely reflect altered afferent input; others may be due to alterations in the intrinsic properties of RVM neurons and local circuits. Given the evidence presented here for dynamic interactions of PB input with RVM pain-modulating neurons, it would be useful to investigate the synaptic physiology of PB inputs to RVM, and determine how these are altered in persistent inflammation. This question could be pursued using *in vitro* patch recording from RVM neurons with optogenetic manipulation of PB terminals from PB contralateral and ipsilateral to a site of injury or inflammation. Although ON- and OFF-cells cannot at present be functionally identified *in vitro*, ON-cells could be labeled with Dermorphin-Alexa594 (DERM-A594),[133] while OFF- and NEUTRAL-cells can be inferred to comprise the non-labeled cells. This approach would allow one to determine if PB inputs synapse directly onto individual RVM ON- (DERM-A594-labeled) or OFF- (subset of unlabeled) cells, and whether local circuitry within the RVM mediates at least some of the PB-dependent responses during persistent inflammation.

6.5.4. Implications for human health

Current pharmacological treatments for chronic pain have limited efficacy and undesirable side effects, particularly when used long-term. [142] Understanding the central mechanisms underlying these pain conditions could provide novel targets for therapies that treat the underlying brain dysfunction, instead of symptoms. Given increasing evidence implicating descending modulatory systems in persistent pain,[43; 45; 189; 202] my data suggest that the connection from the PB to the RVM is potential target for clinical therapies that can block a pathological positive feedback loop or promote a shift to descending inhibition.

PB receives the majority of its nociceptive inputs from NK1R-positive spinal dorsal horn neurons. Knockout or ablation of these NK1R-positive neurons has been shown to attenuate behavioral hypersensitivity in preclinical animal models of persistent pain. However, over the past decades of clinical testing, NK1R antagonists have showed limited efficacy in achieving analgesia in clinical trials of a variety of clinical pain states. [83; 88] An obvious speculation for this discrepancy is the possible difference in physiology of NK1R between human and experimental animals. However, NK1R antagonists were not highly efficacious in animal models, which suggests that other factors are at work. A more likely explanation, based on the current data, is that NK1R-positive neurons are only involved during a narrow time window early in the development of inflammation, as shown here with PB driving RVM sensitization. Thus perhaps NK1R antagonists are best used as a prophylactic in events, such trauma and perioperative anesthesia, where they could block the spinoparabrachial inputs from feeding the supraspinal positive feedback loop, and prevent sensitization of descending pain-modulating pathways. Another potential therapeutic target for chronic pain is the PB itself. Stimulation of PB is reported to have antinociceptive effects,[33; 132] and direct manipulation of neurocircuits is effective or has shown promise in treating a number of diseases, such as Parkinson's

disease, depression and epilepsy.[134; 135 ; 196] Targeting PB using brain stimulation methods may be a non-pharmacological therapy to treat persistent pain.

6.6. CONCLUSION

In this thesis, I demonstrate a functional link between a pain-transmitting system (PB) and a pain-modulating system (RVM), and detail the process through which inputs from PB drive and sensitize RVM ON- and OFF-cell activity during the transition from acute to persistent injury. Input from the PB contralateral to the injury is part of a positive feedback loop, and sensitizes the pain-modulating neurons of RVM in acute inflammation. As the injury persists, input from the ipsilateral PB is recruited to relay nociceptive information to maintain RVM sensitization to innocuous stimuli. Understanding the interaction between the pain-transmitting system and the pain-modulating system will continue to elucidate the central mechanism of RVM sensitization, which may ultimately make it possible to manipulate this system to treat clinically relevant pathological pain with fewer side effects.

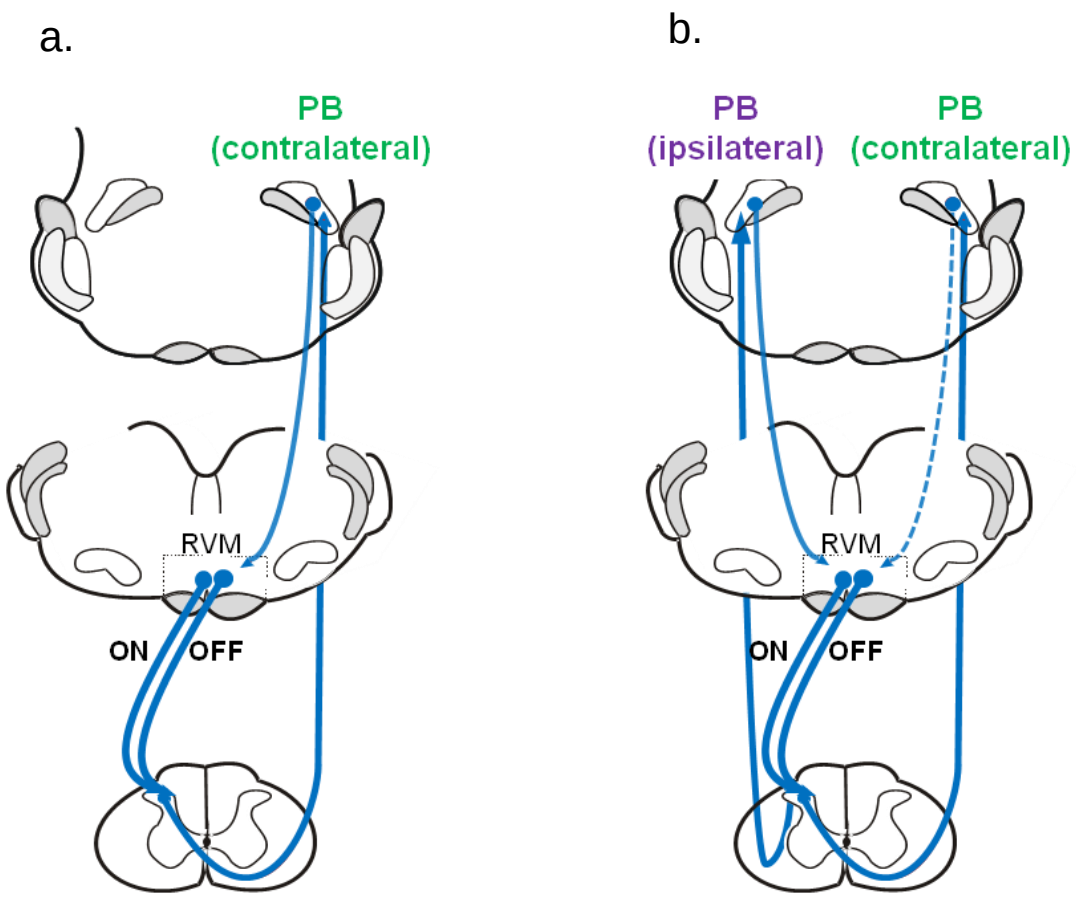

Figure 36 Changes of the PB-RVM circuit in different pain states

a. Under basal conditions and during acute inflammation, PB contralateral to the site of stimulation/ inflammation convey nociceptive information to RVM ON- and OFF-cells. **b.** During persistent inflammation, input from the contralateral PB to RVM diminishes, whereas input from the ipsilateral PB is recruited to convey nociceptive information to RVM ON- and OFF-cells.

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