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EDUCATION

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Basic Science Judge (March 2010)
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RELATED EXPERIENCE

University of Maryland Dental School Summer Research Program
Student Researcher (May 2007 – August 2007)
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PUBLICATIONS

Chai, B. Guo, W. Wei, F. Dubner, R. Ren, K. Trigeminal-Rostral Ventromedial Medulla circuitry is involved in orofacial hyperalgesia contralateral to tissue injury. Mol Pain. 2012; 8: 78.

Guo, W. Wang, H. Gu, M. Zou, S. LaGraize, S. Chai, B. Ren, K. Dubner, R. Wei, F. Coordinated alterations in astroglial activity and descending facilitation of neuropathic pain. 3rd International Congress on Neuropathic Pain-NeuPSIG, 2010. 51-56.

Shimizu, K*.Chai, B*.LaGraize, S. Ren, K. Dubner, R. Microinjection of IL-1b into the trigeminal transition zone produces bilateral NMDA receptor-dependent orofacial hyperalgesia involving descending circuitry. Open Pain Journal, 2009, vol.2, 76-83.
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ABSTRACTS

Chai, B. Guo, W. Wei, F. Ren, K. Dubner, R. CFA-induced contralateral orofacial hyperalgesia requires descending RVM serotonergic input and activation of 5-HT_3 receptors in the contralateral spinal trigeminal nucleus. 2011. Annual meeting of the Society for Neuroscience. Washington, D.C

Chai, B. Wei, F. Ren, K. Dubner, R. Unilateral-induced contralateral orofacial hyperalgesia requires activation of trigeminal-RVM circuitry involving IL-1 and NK-1 receptors. 2010. Annual Meeting of the Society for Neuroscience. San Diego, CA

Chai, B. Shimizu, K. LaGraize, S. Ren, K. Dubner, R. Supraspinal circuitry mediates unilateral IL-1b-induced contralateral orofacial hyperalgesia. 2009. IADR/AADR Annual Conference. Miami, FL

Chai, B. Shimizu, K. Ren, K. Dubner, R. The effect of propentofylline on orofacial hyperalgesia. 2008. ADA Student Research Conference. Gaithersburg, MD

MEMBERSHIPS

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Abstract

Title of Dissertation: Trigeminal-rostral ventromedial medulla involvement in contralateral deep tissue orofacial hyperalgesia

Bryan Chai

Dissertation directed by: Ke Ren, MD, PhD
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In 2008, the National Institute of Dental and Craniofacial Research indicated that approximately 10 million Americans suffer from temporomandibular joint disorders (TMJD). Orofacial pain disorders not only impair the quality of life, but also seriously inhibit the health of the patient by impairing a person's ability to eat and drink. Reports have shown that patients with myofascial TMJD experience bilateral thermal hypersensitivity in the trigeminal region (Fernandez-de-las-Penas et al 2010). Our previous studies have shown that complete Freund's adjuvant (CFA)-induced masseter muscle inflammation and microinjection of the pro-inflammatory cytokine interleukin-1β (IL-1β) into the subnucleus interpolaris/subnucleus caudalis transition zone of the spinal trigeminal nucleus (Vi/Vc) induce contralateral orofacial hyperalgesia in rat models. Furthermore, ventral Vi/Vc second order neurons project to the rostral ventromedial medulla (RVM) (Sugiyo et al 2005), a critical site for descending pain modulation, and substance P (SP) and its neurokinin-1 (NK-1) tachykinin receptor in the RVM are involved in descending pain facilitation (LaGraize et al 2010). We hypothesize that the development of bilateral deep tissue orofacial hyperalgesia after unilateral inflammation

involves neuron-glial interactions in the ipsilateral Vi/Vc transition zone, the SP/NK-1 receptor signaling in the RVM, and subsequent activation of RVM 5-HT containing neurons terminating in the contralateral Vi/Vc transition zone. The results showed that 1) microinjection of the IL-1 receptor antagonist into the ipsilateral Vi/Vc attenuated the CFA-induced contralateral hyperalgesia, 2) lesions to the ipsilateral Vc did not prevent the development of contralateral hyperalgesia, 3) ibotenic acid lesion of RVM neurons prevented the development of IL-1β-induced contralateral hyperalgesia, 4) intra-RVM post-treatment injection of the NK-1 receptor antagonists attenuated CFA-induced bilateral hyperalgesia and IL-1β-induced bilateral hyperalgesia, 5) serotonin depletion in RVM neurons prior to intra-masseter CFA injection prevented the development of contralateral hyperalgesia, and 6) inhibition of 5-HT3 receptors in the contralateral Vi/Vc attenuated CFA-induced contralateral hyperalgesia. These results suggest that the development of CFA-induced contralateral orofacial hyperalgesia is mediated through descending facilitatory mechanisms involving the Vi/Vc-RVM circuitry.

Trigeminal-rostral ventromedial medulla involvement in contralateral deep tissue orofacial hyperalgesia

by
Bryan Y. Chai

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Abbreviations

5-HT - Serotonin

AMPAR - alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

BDNF - Brain-derived neurotrophic factor

CCI - chronic constriction-injury model

CCK - Cholecystokinin

CFA - complete Freund's adjuvant

CNS - central nervous system

Cox-2 - cyclooxygenase-2

GPCR - G-protein coupled receptor

IBO - ibotenic acid

IL-1β – interleukin-1beta

IL-1R – IL-1 receptor

IL-1ra - IL-1receptor antagonist

JNK - c-jun N-terminal kinase

MAPK - mitogen-activated protein kinases

NGCα - gigantocellular reticular nucleus pars alpha

NK1-R - neurokinin-1 receptor

NMDA - N-methyl-D-aspartate

NRM - nucleus raphe magnus

PAG - periaqueductal grey

PBN - parabrachial nucleus

PKC - protein kinase C

Po - posterior thalamic nuclear group

RNAi - RNA interference

RVM - rostral ventromedial medulla

Sm - submedius nucleus of the thalamus

STN - Spinal Trigeminal Nucleus

TMJD - temporomandibular joint and muscle disorders

TNF-α - tumor necrosis factor-α

Tph-2 - tryptophan hydroxylase-2

TRPV1 - Transient receptor potential vanilloid 1

Vc - subnucleus caudalis of the STN

Vi - subnucleus interpolaris of the STN

Vi/Vc - subnucleus interpolaris/subnucleus caudalis transition zone

Vo - subnucleus oralis of the STN

VPM - ventroposteromedial thalamic nucleus

Chapter 1: General Introduction

1.1 Pain

Pain is an unpleasant feeling or experience caused by an illness, injury, harmful, or potentially harmful stimulus to the body that is perceived through neurological processes and interpreted in the brain. In other words, pain is the transmission of neuronal stimulation to the brain and the emotional interpretation of what those signals mean. Pain can therefore be broken into three components: the sensory-discriminative, the affective-motivational, and the cognitive component (Johansen et al. 2001; Auvray et al. 2010).

The sensory-discriminative component is the neurological circuits and mechanisms responsible for the transmission of the noxious stimulus in the periphery to the brain. It is the individual neuronal responses to a noxious stimulus and determines the sensory characteristics of the pain such as quality, intensity, duration, and location. The neural processes leading to sensory pain transmission are referred to as nociception.

The affective-motivational component is the emotional aspect of pain each person undergoes when encountering a noxious stimulus. It is this emotional aspect that determines the anxiety or fear associated with a stimulus and pain. The affective-motivational component of pain has been shown to be associated with brain areas such as the prefrontal cortex, anterior cingulate cortex, and the amygdala (Apkarian et al. 2005; Maeoka et al. 2012; Iwata et al. 2011; Bourgeais et al. 2001). It is possible that the involvement of the limbic system is what leads to emotional modulation of pain such as

stress-induced hyperalgesia and analgesia (Martenson et al. 2009; Yilmaz et al. 2010).

The cognitive component is the avoidance learning that follows the primary reaction to pain. The memory and meaning that results from the initial noxious stimulus may alter future behavior when encountered with similar stimuli. Similar to the affective-motivational component, the cognitive component of pain has been associated with the anterior cingulate cortex and the prefrontal cortex (Johansen et al. 2001; Neugebauer et al. 2009). Together, these three components make up the behavioral output and symptoms of pain.

Acute pain is a natural physiological process that occurs in animals and humans alike. It is a protective mechanism to prevent permanent tissue injury from occurring or to prevent further harm from occurring to an existing injury. Without pain, a child would not know to remove their hand from a hot stove. Without pain, athletes would not provide necessary protection to injured joints or muscles. However, acute pain could persist and transition into chronic pain.

Chronic pain is pain that persists over an extended period of time and in many cases, the assault from the noxious stimulus is gone and the protective function of pain is no longer necessary. Chronic pain is therefore pathologic. Chronic pain occurs in disorders such as temporomandibular joint and muscle disorders (TMJD), trigeminal neuralgia, fibromyalgia, and diabetic neuropathy. In such pain disorders, it is common for people to experience hyperalgesia, an exaggerated pain response to a stimulus that is normally painful (stubbing your toe when it is already broken), and/or allodynia, a painful response that results from a stimulus that is normally not painful (touching your skin after a sun burn).

Orofacial pain disorders involving deep tissues, such as muscle and joint, are debilitating disorders that diminish the quality of life and seriously inhibit the health of the patient by impairing a person's ability to eat and drink. Trigeminal pain also spreads to wide orofacial areas and is a serious clinical problem. Reports have shown that patients with unilateral myofascial TMJD experience bilateral hyperalgesia (Fernandez-de-las-Penas et al. 2009; Fernandez-de-las-Penas et al. 2010). Similar reports have shown that chronic neck pain can result in bilateral mechanical-pain sensitivity of the trigeminal regions (La Touché et al. 2010). Despite the prevalence of orofacial persistent pain within the population, few treatments exist for patients due to the lack of understanding of the mechanisms involved. Through better understanding of the pathways and mechanisms involved in orofacial deep tissue pain, novel therapies and strategies may be developed for orofacial pain and other widespread pain conditions.

1.2 Animal models of persistent pain

Over the years, many preclinical models of persistent pain have been developed. In these models, long-lasting pain hypersensitivity develops, resembling some features of chronic inflammatory or neuropathic pain. Neuropathic pain is persistent pain that originated from an injury or disease process that affects the somatosensory system. Most neuropathic pain studies include nerve-ligation (Markus et al. 1984; Seltzer et al. 1990; Kim and Chung 1992) or chronic constriction-injury models (CCI). CCI uses loosely constrictive ligatures to induce behavioral signs in animals that closely mimic the clinical

symptoms seen in humans (Bennett and Xie 1988). The CCI model was initially shown to produce hyperalgesia from 2 days to 2 months after injury with the peak severity around 2 weeks (Bennett and Xie 1988). This temporal feature of the CCI model has made this a good model for testing persistent pain and plasticity (Mayer et al. 1999; Vanelderen et al. 2010). CCI of the infraorbital nerve has been used to test neuropathic pain conditions in the trigeminal system (Imamura et al. 1997; Benoist et al. 1999; Martin et al. 2010; Michot et al. 2012).

The study of inflammatory pain conditions has utilized many compounds to induce an inflammatory response. The formalin test in the hind paw has been used in many studies of analgesic and anti-nociceptive mechanisms in the spinal cord (Dubuisson and Dennis 1977; Watkins et al. 1997; Zhao et al. 2006; Sawynok and Reid 2011; Dang et al. 2011). It is thought that with formalin application one can observe the difference in peripheral and central mechanisms based upon the phasic hyperalgesic response that animals produce (Hunskaar and Hole 1987). Mustard oil and capsaicin are irritants that have also been used to induce hyperalgesia. Mustard oil has been used in the study of visceral pain (Ji et al. 2012; Chatter et al. 2012; Pereira et al. 2012), TMJ (Chang et al. 2012), headaches (Edelmayer et al. 2012), itch-behavior (Ji 2012; Spradley et al. 2012), and tooth-pulp inflammation (Narita et al. 2012). Capsaicin has been used to test transient receptor potential vanilloid 1 (TRPV1)-mediated hyperalgesia in the spinal and trigeminal systems (Park et al. 2011; O'Neill et al. 2012; Hitomi et al. 2012). However, both mustard oil and capsaicin are more acute in nature and have only been shown to induce hyperalgesia for brief periods of time (LaMotte et al. 1991; Woolf et al. 1994; Mansikka and Pertovaara 1997).

One of the most commonly used inflammatory agents is complete Freund's adjuvant (CFA). CFA is an antigen-in-oil emulsion that contains heat-killed *mycobacterium tuberculosis* to induce an immune response in the injected tissue. CFA is administered subcutaneously or intramuscularly and has been widely used for studying pain processing in the spinal and trigeminal systems. CFA has been shown to induce profound inflammatory hyperalgesia that lasts for 1-3 weeks after injection into the TMJ or masseter muscle (Ren 1999; Ambalavanar et al. 2005; Shimizu et al. 2009a).

Hyperalgesia has also been induced using specific molecular agents to activate receptors that are involved in the facilitation of hyperalgesia. Cholecystokinin (CCK) and neurokinin-1 receptor (NK1-R) activation in the rostral ventromedial medulla (RVM) and interleukin-1beta (IL-1β) application in the spinal trigeminal nucleus have been shown to induce hyperalgesia (Heinricher and Neubert 2004; Wei et al. 2008; Shimizu et al. 2009a; LaGraize et al. 2010).

1.3 Trigeminal system

The primary sensory afferents that innervate the face are provided by the fifth cranial nerve, the trigeminal nerve. The trigeminal nerve has three primary divisions that branch at the trigeminal ganglion: the ophthalmic (V1), maxillary (V2), and the mandibular (V3) divisions. The ophthalmic division provides sensory afferents to the superior third of the face including the forehead, eyebrows, eyelids, nose, cornea, conjunctiva, and lacrimal gland. The maxillary division provides sensory afferents to the

middle third of the face including the lower eyelids, nose, upper lip, skin overlying the zygoma, palate, and upper dentition. The mandibular division provides sensory afferents to the inferior third of the face including the skin overlying the temporalis, the muscles of mastication, lower lip, buccal mucosa, lower dentition, and tongue. The primary afferent neurons involved in nociception of all three project and synapse with neurons within the Spinal Trigeminal Nucleus (STN) that spans the brainstem from the caudal pons to the medulla and continuous with the upper cervical spinal cord. The STN can be subdivided into three subnuclei: the oralis (Vo), interpolaris (Vi), and caudalis (Vc).

The Vo is the most rostral subnucleus of the STN and is generally thought to participate in craniofacial reflexes (Capra and Dessem 1992; Sessle 2000). The Vo participation in craniofacial reflexes is also thought to be involved in processing nociceptive input from the oral cavity that also projects to the Vo as a possible component of an oral reflex (Dallel et al. 1990; Takemura et al. 2006). The Vo has also been shown to receive afferent input from the pulp of molar teeth in rats (Marfurt and Turner 1984). Noxious tooth pulp stimulation produces neuroplastic changes in the Vo (Sugimoto et al. 1998; Park et al. 2001). It has also been shown that mechanical overload on the dentition produces increased reactive oxygen species production in the Vo (Viggiano et al. 2010).

The Vi has been shown to be associated with sensory neuron projections from the vibrissa pad of rats (Furuta et al. 2010). The Vi- thalamic posterior nuclei pathway, also called the paralemniscus pathway, processes tactile information from the vibrissa pad (Chiaia et al. 1991). The Vi was found to have somatotopic organization in which the rostral-caudal representation of the vibrissa pad is similarly oriented in the Vi (Ma 1991).

Neurons that project to the Vi are also involved in processing noxious stimulation to the TMJ and the masseter muscle (Ohya 1992; Ohya et al. 1993; Ro and Capra 1999).

The Vc has been well established as a major component in the processing of orofacial pain (Amano et al. 1986; Hu 1990; Hu et al. 1992; Iwata et al. 1999; Nomura et al. 2002; Ro et al. 2007, Chun and Ro 2010). Research has shown that the Vc is involved in the development and maintenance of inflammatory pain, neuropathic pain, joint pain, and migraines (Villa et al. 2010; Zhu et al. 2011; Daigo et al. 2012; Miyamoto et al. 2012; Chun et al. 2012; Myren et al. 2012) The Vc also shows somatotopic differentiation in the termination of the primary afferents. In general, the primary afferents from the more anterior portions of the face terminate in the more rostral portion of the Vc. Similarly, the primary afferents from the more posterior portions of the face terminate in the caudal portions of the Vc (Shigenaga et al. 1986). Specifically with the masseter muscle, the Vc receives afferent projection from both the overlying skin and the underlying deep tissue (Wang et al. 2006). Extensive research has since implicated neuropeptides, ATP, excitatory amino acids such as glutamate, and many more neurotransmitters and neuromodulators as being involved in Vc nociception (Takemura et al. 2006; Chiang et al 2011). These various neurotransmitters are released from primary afferent neurons after noxious stimulation. The signal is then relayed to the thalamus and other higher centers of the brain.

The caudal Vi and the rostral Vc overlap at the obex level to form a subdivision of the STN called the Vi/Vc transition zone. The dorsal portion of the Vi/Vc transition zone mainly involves the rostral end of the Vc while the ventral portion involves the caudal Vi. Neuronal activation in the Vi/Vc can occur after corneal and ocular stimulation (Bereiter

et al. 1994; Okamoto et al. 2009; Chang et al. 2010) and orofacial stimulation (Sugimoto et al. 1994; Carstens et al. 1995; Hathaway et al. 1995, Zhou et al. 1999). The mandibular structures, masseter muscle, and TMJ are represented at the dorsomedial portion of the Vi/Vc transition zone (Capra 1987; Takemura et al. 1987; Pfaller and Arvidsson 1988; Shigenaga et al. 1988) and the orbital afferents are represented in the ventral Vi/Vc transition zone (Pozo and Cervero 1993; Hirata et al. 1999).

While the Vc has been shown to be involved in the sensory-discriminative component of pain, the Vi/Vc is unique in that it is involved in the sensory-discriminative component and the affective-motivational component of pain (Ren and Dubner, 2011). The Vi/Vc transition zone receives input from deep tissue masseter muscle afferents (Wang et al. 2006). Ventral Vi/Vc transition zone second order neurons then project to the nucleus submedius of the thalamus, the parabrachial nucleus (Ikeda et al. 2003, Yoshida et al. 1991), and the RVM (Sugiyo et al. 2005). The RVM has been shown to have reciprocal connections to the Vi/Vc transition zone bilaterally through an attenuation of masseter hyperalgesia after Vi/Vc or RVM lesion (Sugiyo et al. 2005). Orofacial hyperalgesia models show that bilateral activation of the ventral Vi/Vc transition zone occurs with deep tissue inflammation (Imbe et al. 1999). Similarly, it has been shown that orofacial tissue injury results in increased glia activation and cytokine release in the Vi/Vc transition zone (Guo et al. 2007).

1.4 Glia activation and pro-inflammatory cytokines

Neuronal-glial interaction is emerging as an important mechanism in chronic pain. The glia of the central nervous system (CNS), including microglia and astrocytes, have been shown to be involved in neuropathic and inflammatory pain (Ren and Dubner 2008; Milligan and Watkins 2009; Inoue and Tsuda 2009; Gosselin et al. 2010; Zhuo et al. 2011; Chiang et al. 2011). The development of neuropathic hyperalgesia can be prevented by glia inhibition (Ledeboer et al. 2005; Wei et al. 2008). Research has found that microglia and astrocyte activation differs in time after injury. Microglia activation has been shown to occur hours-days after injury while astrocyte activation occurs from days-weeks after injury (Tanga et al. 2004, Hald et al. 2009).

Microglia are considered the immune cells of the CNS and are thought to act as sensors for pathological conditions of the CNS (Kreutzberg 1996). An increase in microglia activation has been shown to occur in models of nerve injury (Eriksson et al. 1993; Coyle 1998). Astrocytes are a diversely functioning group of cells that regulate many aspects of neuronal function (Haydon 2001; Gosselin et al. 2010). Astrocyte activation has been shown to be upregulated in neuropathic pain, inflammatory pain, and cancer pain (Stuesse et al. 2001; Zhang et al. 2005; Guo et al. 2007). One downstream intracellular glial mechanism involved in the development of persistent pain is the activation of mitogen-activated protein kinases (MAPK). Research has shown that the p38 MAPK was activated in microglia following neuropathic pain (Jin et al. 2003; Tsuda et al. 2004) while inhibition of MAPK c-jun N-terminal kinase (JNK) in astrocytes was able to prevent the development of neuropathic hyperalgesia (Zhuang et al. 2006)

Another downstream mechanism of glia activation involves the upregulation and expression of proinflammatory cytokines. The inflammatory cytokines are among a group of chemical mediators that can be released by activated glia and have been implicated in persistent pain states (Watkins and Maier 2005). Interleukin-1, a prototype pro-inflammatory cytokine, is involved in a variety of diseases with pain components such as rheumatoid arthritis, inflammatory bowel disease, osteoarthritis, multiple sclerosis and neuropathy (Dinarello 2004; Kawasaki et al. 2008; Ren and Torres 2009). Although peripheral cytokines including IL-1β are upregulated as a part of the immediate inflammatory responses to injury (Watanabe et al. 2005), cytokines are also upregulated in the spinal cord and brain (Raghavendra et al. 2004).

IL-1β has been identified as a major inducer of cyclooxygenase-2 (Cox-2) in the CNS (Samad et al. 2001). IL-1β can directly activate nociceptors to generate action potentials and induce pain hypersensitivity (Binshtok et al. 2008). Intrathecally administered IL-1β produces enhanced dorsal horn nociceptive neuronal responses and behavioral hyperalgesia (Reeve et al. 2000; Falchi et al. 2001; Sung et al. 2005). Intracerebroventricular injection of IL-1β has been shown to produce hyperalgesia in animals (Oka et al. 1993; Watkins et al. 1994). Pretreatment with the IL-1receptor antagonist (IL-1ra) completely abolished centrally IL-1-induced hyperalgesia (Oka et al. 1993).

Glia activation participates in trigeminal pain processing. Microglia activation in the Vc is associated with neuropathic pain (Shibuta et al. 2012). The development and maintenance of orofacial pain involves increased cytokine expression and glia activation in the Vc (Daigo et al. 2012). An elevation of IL-6 level in the brainstem including the

spinal trigeminal nucleus is found after trigeminal nerve injury in the rat (Anderson and Rao 2001).

We have recently shown that glia-cytokine-neuronal interactions in the Vi/Vc transition zone play an important role in orofacial hyperalgesia (Guo et al. 2007). CFA-induced masseter muscle inflammation produced increased expression of IL-1β within the Vi/Vc transition zone. IL-1β activation of IL-1 receptor (IL-1R) was shown to produce phosphorylation of the N-methyl-D-aspartate (NMDA) receptor subunit GluNR1 (Guo et al. 2007). IL-1β microinjection into the Vi/Vc transition zone also produced orofacial hyperalgesia bilaterally (Shimizu et al. 2009a). Similarly, masseter inflammatory hyperalgesia was attenuated following glia inhibition and anti-inflammatory cytokine, IL-10 (Shimizu et al. 2009b).

1.5 Descending modulation in pain

The foundational concept that current mechanisms of descending modulation were built upon is what we know as the "gate hypothesis" proposed by Melzack and Wall in 1965. It was hypothesized that primary afferent fibers and the projection neurons at which they terminate receive integrated processes that modify nociceptive signals prior to their reaching supraspinal structures. Since the gate hypothesis, research has shown that fibers descending from the supraspinal structures modulate ascending nociceptive signals. The modulation has been shown to be inhibitory or facilitatory in nature (Fields 2000; Porreca et al. 2002).

Early research in descending modulation focused on the analgesic effects seen from descending inhibition (Oliveras et al. 1974; Akil et al. 1976). It was shown that direct stimulation and opioid injection into the periaqueductal grey (PAG) produced profound analgesia (Reynolds 1969; Mayer et al. 1971; Pert and Yaksh 1974; Jacquet and Lajtha 1976; Lewis and Gebhart 1977). Similar results were seen in the rostral ventromedial medulla (RVM) (Mayer and Liebeskind 1974; Proudfit and Anderson 1975). It has since been established that the PAG-RVM-spinal cord circuit is a key modulatory pathway involved in hyperalgesia and analgesia (Fields et al. 2006). The classical descending pathway includes sites within the forebrain such as the hypothalamus, amygdala, prefrontal cortex, insula, and sensory cortex, which project to the PAG. The RVM receives direct inputs from the PAG and projects to the spinal cord (Behbehani and Fields 1979; Mason 1999).

One mechanism of PAG involvement in analgesia was shown through mu opioid receptor activation-induced analgesia (Smith et al. 1988). It was further demonstrated that the opioid-induced disinhibition, or loss of inhibition, of GABAergic neurons in the PAG resulted in analgesia (Christie et al. 2000). It has also been suggested that motor cortex stimulation leads to inhibition of GABA interneurons in the PAG and subsequent disinhibition of PAG neurons involved in descending inhibition (Pagano et al. 2012). Cannabinoid receptor activation in the PAG has also been shown to participate in antinociception (Martin et al. 1995; Lichtman et al. 1996). Cannabinoid activation can enhance morphine antinociception (Wilson et al. 2008).

The RVM is a midline structure that is composed of the nucleus raphe magnus, the nucleus gigantocellularis pars alpha, and nucleus reticularis paragigantocellularis

lateralis (Zagon 1995). Neurons within the RVM have been classified as ON-cells, OFF-cells, and neutral cells based upon cell firing in relation to a tail flick seen after noxious stimulation (Fields et al. 1983). ON cells are defined as neurons that exhibit excitation after nociceptive stimulation. OFF cells are neurons that display inhibition in activity immediately prior to a nociceptive reflex (Fields et al. 1983). Neutral cells are considered the neurons of the RVM that are neither ON nor OFF cells (Barbaro et al. 1986). It is generally thought that OFF cells participate in the induction of descending inhibition while ON cells are involved in descending facilitation (Kaplan and Fields 1991, Heinricher et al. 1994). Morphine analgesia requires NMDA-mediated excitation of OFF cells while tail flick reflex activation of ON cells requires non-NMDA-mediated excitation (Heinricher et al. 2001).

1.6 Rostral ventromedial medulla and descending facilitation

There has been mounting evidence of RVM-mediated descending facilitation. Formalin-induced hyperalgesia and hyperalgesia from muscle inflammation are abolished with RVM lesions (Sugiyo et al. 2005, Wiertelak et al. 1997). The RVM is also necessary for the development of secondary hyperalgesia produced by mustard oil application (Urban and Gebhart 1999). Lidocaine microinjection into the RVM attenuated nerve ligation-induced allodynia (Pertovaara et al. 1996).

Within the RVM system, research has been conducted on various neurotransmitters and neuromodulators involved in the development and maintenance of

hyperalgesia (Heinricher et al. 2009). Earlier research had shown that neurotensin was involved in the facilitatory modulation of pain in the RVM (Urban and Gebhart 1997). Cholecystokinin microinjection in the RVM has also been shown to participate in descending facilitation of neuropathic pain (Kovelowski et al. 2000). Similarly, IL-1β and tumor necrosis factor-α (TNF-α) are involved in the enhancement of descending modulation when they are released and bind to their respective receptors in the RVM during a chronic constriction injury model (Wei et al. 2008; Roberts et al. 2009). NMDA receptor antagonist administration into the RVM attenuates neuropathic pain in rats (Wei and Pertovaara 1999). Furthermore, research has emphasized glutamate and GABA modulation by substance P and brain-derived neurotrophic factor (BDNF) release in the RVM (Guo et al. 2006; Budai et al. 2007; Pacharinsak et al. 2008; Zhang et al. 2009).

Despite considerable research involving RVM descending modulation in pain, there is very little research about descending modulation involvement in the trigeminal system and its role in secondary orofacial hyperalgesia (hyperalgesia at a site outside the zone of injury). Our research has shown that lesions of the RVM with ibotenic acid result in the loss of secondary orofacial hyperalgesia at the contralateral site (Shimizu et al. 2009a). This evidence parallels previous results that showed RVM involvement in spinal cord bilateral inflammatory hyperalgesia (Tillu et al. 2008).

1.7 Substance P and NK1-R in the RVM

Substance P is an eleven amino acid neuropeptide that preferentially binds NK1-R

which is a G-protein coupled receptor (GPCR) that activates a class of G-proteins called Gq. Gq activation leads to phospholipase C activation and activation of the inositol triphosphate signal transduction pathway. This results in increased intracellular calcium concentrations from endoplasmic reticulum release. Immunohistochemical localization shows moderate amounts of NK1-R in the RVM (Nakaya et al. 1994). Substance P has been shown to induce both analgesia (Hamity et al. 2010) and hyperalgesia (McCarson et al. 1994; Schafer et al. 1993). However, there is more recent evidence to suggest that substance P signaling in the RVM facilitates nociception (Cao et al. 1998; Rosen et al. 2004).

In the RVM, spinal cord projecting neurons express NK1-R (Khasabov et al. 2005; Pinto et al. 2008; LaGraize et al. 2012). NK1-R inhibition attenuates ON cell evoked excitatory responses and OFF cell evoked inhibitory responses from stimulation after prolonged inflammation (Khasabov et al. 2012). This activation of ON cells by NK1-R presumably leads to descending facilitation (Brink et al. 2012; Khasabov et al. 2012). Ablation of NK1-R containing RVM neurons reduces thermal and mechanical hypersensitivity in capsaicin and CFA–induced persistent pain models (Brink et al. 2009). Our laboratory has shown that NK1-R expression is increased in the RVM following hind paw inflammation. Further investigation reveals that substance P microinjection into the RVM induces thermal hyperalgesia in both left and right hind paws (Lagraize et al. 2012).

1.8 Descending serotonin system

Serotonin (5-HT) is a monoamine neurotransmitter that is synthesized from trypophan through hydroxylation by tryptophan hydroxylase-2 (Tph-2) in neurons. Seven families of 5-HT receptors are known, 5-HT(1-7), all of which are GPCRs, excluding the 5-HT3 receptor. The 5-HT3 receptor is a ligand gated non-specific cation channel that depolarizes the cell membrane when activated. Research has shown that 5-HT3 receptors are expressed ubiquitously throughout the peripheral and central nervous system of the rodent (Doucet et al. 2007).

Research has shown that the 5-HT system is involved in the descending facilitation of pain. Immunostaining for serotonin in the RVM shows that serotonin is primarily contained in neutral cells of the RVM (Potrebic et al. 1994). Intrathecal administration of a non-selective 5-HT receptor antagonist attenuated the facilitation of the tail flick reflex produced from RVM electrical stimulation (Zhuo and Gebhart 1991) and formalin-induced inflammation (Calejesan et al. 1998).

In the spinal cord, administration of the 5-HT3 receptor antagonist, ondansetron, inhibits evoked responses of dorsal horn neurons after noxious stimulation (Rahman et al. 2009) and intra-RVM CCK-induced mechanical and thermal hyperalgesia (Dogrul et al. 2009). Similarly, intrathecal administration of ondansetron attenuates formalin-induced hindpaw flinching and spinal ERK activation (Svensson et al. 2006). Interaction of facilitatory descending neurons in the RVM and spinal 5-HT3 receptors enhance the response of dorsal horn neurons to noxious stimulation (Suzuki et al. 2002). Ablation of serotonergic neurons attenuates mechanical and thermal hindpaw withdrawal in rats

under neuropathic pain conditions (Rahman et al. 2006). It was further shown that serotonergic neurons of the RVM are involved in descending facilitation through the depletion of endogenous 5-HT while maintaining the viability and function of serotonergic neurons (Wei et al. 2010).

These findings suggest that the 5-HT/5-HT3 receptor complex is involved in the descending facilitation of pain at the spinal cord. It is possible that this mechanism of descending facilitation in the spinal cord may also be seen in the trigeminal system.

1.9 Dissertation aims

Not much is known about the mechanisms involved in the development and maintenance of contralateral orofacial hyperalgesia. Possibilities of contralateral hyperalgesia were first seen with bilateral Fos expression in the Vi/Vc transition zone after unilateral orofacial noxious stimulation including intra-masseter CFA injection (Imbe et al. 1999; Strassman et al. 1993). The bilateral Fos expression was not seen in the Vc (Ikeda et al. 2003). We found that unilateral masseter injury resulted in bilateral hyperalgesia and allodynia (Shimizu et al. 2009b). We also found secondary hyperalgesia at the contralateral site after unilateral intra-Vi/Vc IL-1β microinjection. The Vi/Vc transition zone has been shown to have bilateral reciprocal connections to the RVM (Sugiyo et al. 2005). This suggests that IL-1β release in the Vi/Vc and the Vi/Vc-RVM circuit may be necessary for the development of bilateral hyperalgesia.

Substance P microinjection into the RVM induces thermal hyperalgesia in both

left and right hind paws (LaGraize et al. 2012). Attenuation of persistent pain after 5-HT depletion from descending serotonergic neurons of the RVM suggest that the 5-HT/5-HT3 receptor complex is involved in the descending facilitation of pain at the spinal cord (Wei et al. 2010).

Collectively, we hypothesize that the development of contralateral deep tissue orofacial hyperalgesia after unilateral inflammation involves neuron-glial interactions in the ipsilateral Vi/Vc transition zone that activates the substance P/NK1-R complex in the RVM and activates RVM 5-HT containing neurons terminating in the contralateral Vi/Vc transition zone.

In Specific Aim 1, we will test the hypothesis that ipsilateral IL-1β release in the Vi/Vc transition zone is necessary for the development of contralateral orofacial hyperalgesia after CFA-induced inflammation.

In Specific Aim 2, we will test the hypothesis that NK1-R-mediated activation of RVM neurons is necessary for the development of contralateral orofacial hyperalgesia after injury.

In Specific Aim 3, we will test the hypothesis that 5-HT expression in the RVM and activation of 5-HT3 receptors in the contralateral Vi/Vc transition zone facilitates the development of contralateral orofacial hyperalgesia.

These results will help us understand neural mechanisms involved in secondary hyperalgesia and contralateral orofacial hyperalgesia.

Chapter 2: General materials and methods

The following materials and methods described were used throughout all subsequent chapters. A detailed explanation of materials and methods specific to individual chapters will be explained in chapter sections "Specific materials and methods."

2.1 Animals

Male Sprague-Dawley rats (n=391 total) were used for all experiments. Cannulation experiments utilized rats weighing 250-300g (Harlan) and the RNAi experiments utilized rats weighing 200-250g (Harlan). Female rats were excluded to avoid complications of the estrous cycle. Rats were provided food and water *ad libitum* on a 12-hour light/dark schedule. The experiments were approved by the Institutional Animal Care and Use Committee at the University of Maryland Dental School.

2.2. Surgical preparation/cannulation

Rats were anesthetized using 50 mg/kg of pentobarbital sodium (i.p) and 2-3% isoflurane inhalation in a 30/70% oxygen/nitrogen gas mixture. Rats were placed in a stereotaxic device (Kopf Instruments, Model 900). A midline incision was made in the

scalp after debridement and sterilization of the surgical field with iodine wash. For administration of drugs via microinjection, guide cannulae (C315G, 26 gauge, Plastics One, Roanoke, VA) were implanted and cemented into the skull. A midline opening was made in the skull using a dental drill and a guide cannula was lowered into the ventral Vi/Vc transition zone and/or RVM by referring to the rat brain atlas (Paxinos and Watson 2005). The RVM is termed for the collective structures that consist of the midline nucleus raphe magnus (NRM) and the adjacent gigantocellular reticular nucleus pars alpha (NGCα). The guide cannula was then secured with cranioplastic cement. The wounds were cleaned with antiseptic solution and closed with 4-0 silk sutures. Animals were allowed to recover for 1 week before further experimentation.

Rats received unilateral intra-masseter muscle injections of the inflammatory agent, complete Freund's adjuvant (CFA, 0.05ml; 1:1 oil/saline) to produce hyperalgesia. IL-1β-induced hyperalgesic rats received a microinjection of recombinant rat IL-1β into the Vi/Vc through a 33-gauge injection cannula (C315I, Plastic One) inserted through the tip of the guide cannula. The injection cannula was connected to a 1-μl Hamilton syringe by polyethylene-10 tubing. All injections (0.5 μl) were performed by delivering drug or vehicle solutions slowly over a 2-minute period. Recombinant rat IL-1β was microinjected into the ipsilateral Vi/Vc transition zone.

The drugs used include: IL-1β and IL-1ra (PeproTech; Rocky Hill, NJ), L-733,060, RP67580, ibotenic acid, and Y-25130 (Tocris Bioscience, Ellisville, MO). IL-1ra was reconstituted in deionized water and injected at varying concentrations into the ipsilateral Vi/Vc. The Neurokinin-1 receptor antagonists L-733,060 and RP67580 were microinjected into the RVM. IL-1β and L-733,060 were reconstituted in deionized water

while RP67580 was reconstituted in DMSO. Ibotenic acid was reconstituted in deionized water and microinjected into the RVM as well. The 5-HT3 receptor antagonist, Y-25130, was reconstituted in deionized water. Vehicle and sham surgery control groups were performed for all experiments.

2.3 Behavioral Testing

Behavioral tests were conducted under blind conditions as described elsewhere (Ren 1999). A series of calibrated von Frey filaments were applied to the skin above the masseter muscle. Filaments were applied to the masseter muscle in increasing forces. Each von Frey filament was applied 5 times at intervals of a few seconds. A positive response was regarded as an active withdrawal of the head from the probing filament. The response frequencies [(number of responses/number of stimuli) X100%] to a range of von Frey filament forces were determined and a stimulus-response (S-R) curve plotted. After a non-linear regression analysis (GraphPad Prism), an EF_{50} value, defined as the von Frey filament force (g) that produces a 50% response frequency, was derived from the S-R curve. We used EF_{50} values as a measure of mechanical sensitivity. A leftward shift of the S-R curve, resulting in a reduction of EF_{50}, occurred after inflammation (Sugiyo et al. 2005). This shift of the curve suggests the presence of mechanical hyperalgesia and allodynia since there was an increase in response to suprathreshold stimuli and a decreased response threshold for nocifensive behavior.

2.4 Histology

Following behavioral testing, all rats were deeply anesthetized with pentobarbital sodium (100 mg/kg) and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. The brain and brain stem was removed and post-fixed in 4% formaldehyde for 24 hours before being transferred to 30% sucrose (w/v) for cryoprotection. 30 μm sections of the RVM or Vi/Vc were mounted on slides and stained with cresyl violet to confirm the accuracy of the cannula placement and injection site. Injections not located in the area of interest were excluded from experimental groups or used as an off target control (Figure 2.1; Figure 2.2).

2.5 Statistics

Data are presented as mean ± S.E.M. Statistical comparisons were made by two-way ANOVA and ANOVA with repeated measures and post hoc comparisons (Neuman-Keuls). $P<0.05$ was considered significant.

Figure 2.1: Photographs of histological confirmation of injection and lesion sites. The brain and brainstem of rats were removed after behavioral testing to confirm the site of injection. **A.** A representative photograph of the injection site in the Vi/Vc. **B.** The ibotenic acid lesion of the Vc resulted in necrotic tissue that was unable to be kept intact during the slice and staining procedure. **C.** A representative photograph of the injection site in the RVM. **D.** The ibotenic acid lesion of the RVM shows decreased cellular staining ventral to the injection site.

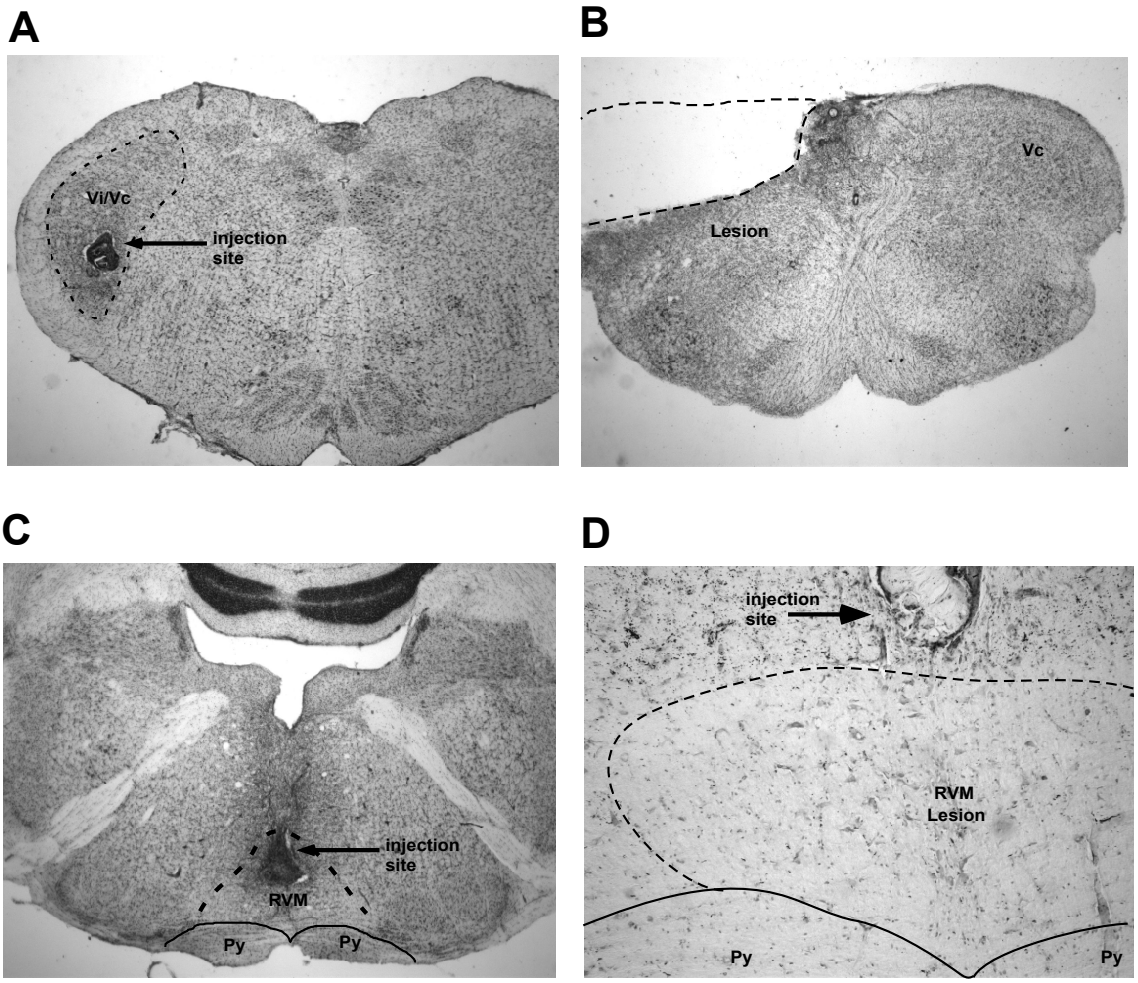
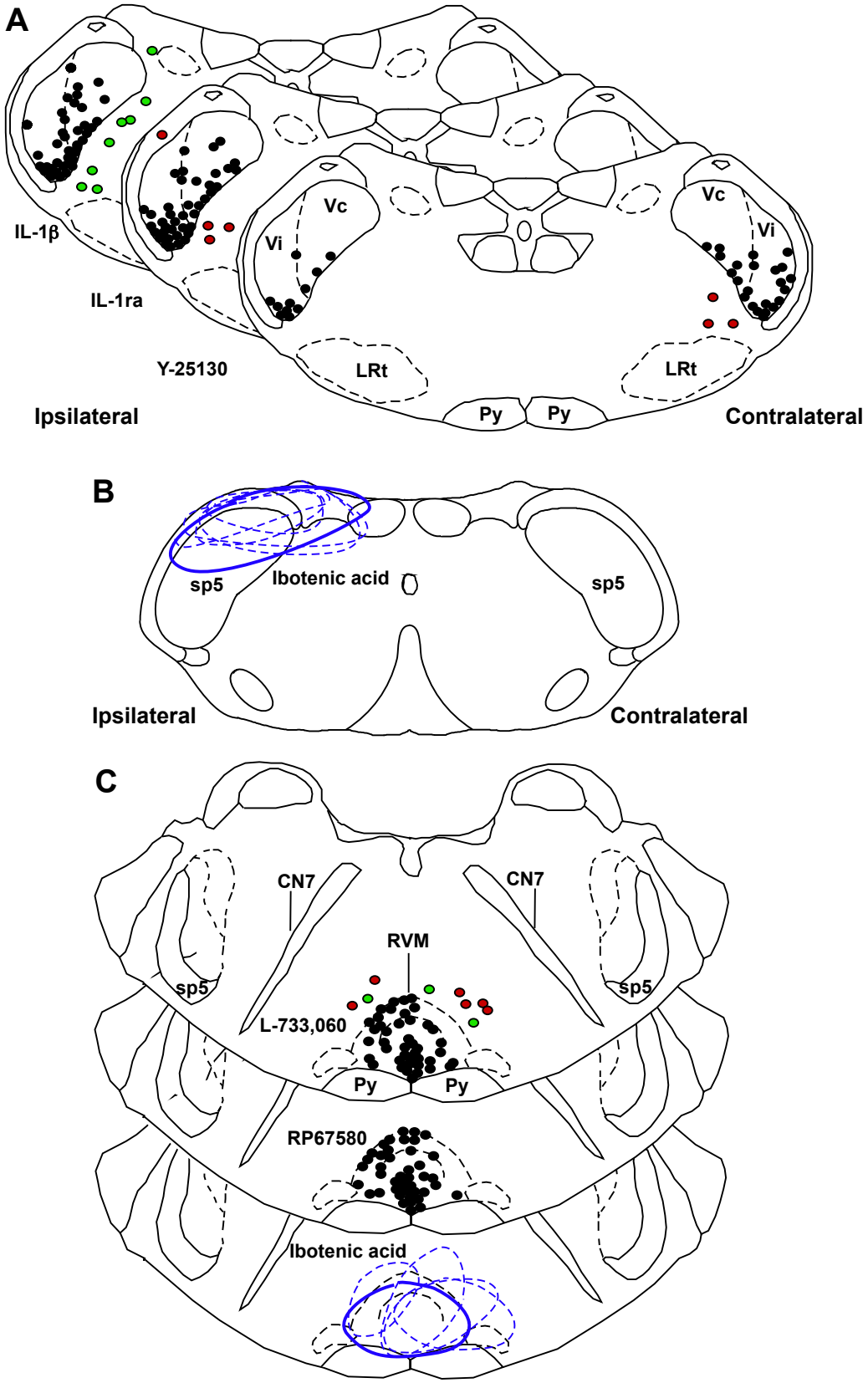


Figure 2.2: Map of histological confirmation of injection and lesion sites.

All injections and lesions were determined and mapped on Vi/Vc and RVM images. **A.** Reconstruction of all injections given into the Vi/Vc. The slices are categorized based upon the drug administered. **B.** Reconstruction of the ibotenic acid lesions in the Vc. The solid blue line of the ibotenic acid lesion represents the photograph shown in Figure 2.1B. **C.** Reconstruction of all injections given into the RVM. The solid blue line of the ibotenic acid lesion represents the photograph shown in Figure 2.1D. Black circles represent injection sites accurately placed within the area of interest. Green circles represent injections outside the area of interest that did produce an effect. Red circles represent injections outside the area of interest that did not produce an effect. Blue dotted lines represent the extent of the ibotenic acid lesions.


A
IL-1β
IL-1ra
Y-25130
Vc
Vi
LRt
Py
Py
Vc
Vi
LRt
Ipsilateral
Contralateral
B
sp5
Ibotenic acid
sp5
Ipsilateral
Contralateral
C
CN7
CN7
RVM
sp5
sp5
L-733,060
Py
Py
RP67580
Ibotenic acid

Chapter 3: IL-1β activation of the ipsilateral Vi/Vc in contralateral hyperalgesia

3.1 Introduction

Traditionally, orofacial pain research has focused on the activation of the subnucleus caudalis (Vc) of the spinal trigeminal nucleus (STN) (Dubner and Bennett 1983; Sessle 2000; Dubner and Ren 2004). Research has shown that trafficking and activation of the GluR2 and GluR3 subunits of the alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) in the Vc are involved in orofacial pain (Miyamoto et al. 2011; Miyamoto et al. 2012). Microglia and astrocyte activation in the Vc have also been shown to be involved in orofacial pain mechanisms (Okada-Ogawa et al. 2009; Shibuta et al. 2012). Research has shown that glia activation of MAPK signaling cascades as well as increased expression of pro-inflammatory cytokines in the Vc are involved in the orofacial hyperalgesia (Piao et al. 2006; Guo et al. 2007; Lee et al. 2010; Takahashi et al. 2011).

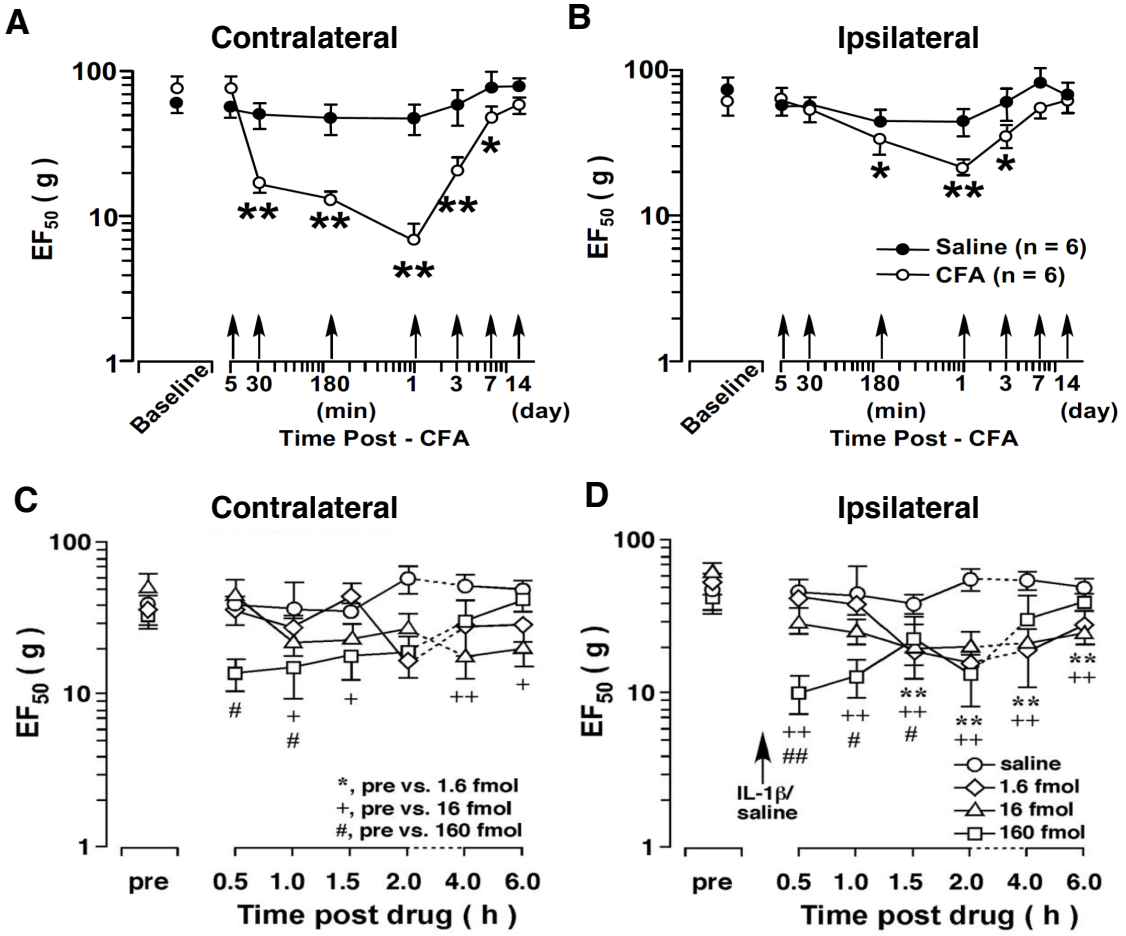
Despite the emphasis of orofacial pain research in the Vc, the Vi/Vc of the STN has been more recently shown to be involved in mechanisms of deep tissue trigeminal pain (Imbe et al. 1999; Zhou et al. 1999; Ro et al. 2003; Ren and Dubner 2011). Expression of Fos protein is observed in the Vi/Vc transition zone after various noxious stimuli applied to the TMJ and masseter muscle (Hathaway et al. 1995; Ro et al. 2003). Our previous studies showed that complete Freund's adjuvant (CFA)-induced masseter inflammation results in bilateral expression of Fos protein in the Vi/Vc transition zone

and ipsilateral expression of Fos protein in the Vc. The same study showed that CFA-induced inflammatory hyperalgesia was attenuated following ibotenic acid lesions in the Vi/Vc transition zone (Sugiyo et al. 2005).

Glia activation and increased expression of pro-inflammatory cytokines in the Vi/Vc transition zone have also been shown to participate in orofacial hyperalgesia (Guo et al. 2007). We have also shown that CFA-induced masseter inflammation leads to increased expression of the pro-inflammatory cytokine, IL-1β in astrocytes of the Vi/Vc transition zone. Furthermore, this increase in Vi/Vc IL-1β expression results in an IL-1 R-dependent upregulation of NMDA receptor activation (Guo et al. 2007). We have also found that CFA-induced masseter inflammation and intra-Vi/Vc microinjection of IL-1β induces bilateral orofacial hyperalgesia in rats (Figure 3.1). However, contralateral hyperalgesia is not seen with IL-1β microinjection into the Vc.

Thus, we hypothesize that CFA-induced masseter inflammation results in contralateral orofacial hyperalgesia via IL-1β activation of the ipsilateral Vi/Vc transition zone.

Figure 3.1: CFA masseter injection and IL-1β intra-Vi/Vc microinjection produces bilateral hyperalgesia. Rats injected with CFA into the masseter muscle resulted in bilateral hyperalgesia 3 hours to 3 days after CFA injection (**A,B**). Intra-Vi/Vc microinjection of IL-1β produced bilateral hyperalgesia 30 minutes to 1 hour after IL-1β was injected (**C, D**).



3.2 Specific methods

3.2.1 IL-1ra pre-treatment

IL-1ra (PeproTech; Rocky Hill, NJ) was reconstituted in deionized water and injected at varying concentrations into the ipsilateral Vi/Vc 7 days after cannula surgery. CFA (0.05ml; 1:1 oil/saline) was injected into the ipsilateral masseter muscle 15 minutes after the IL-1ra injection. Mechanical sensitivity was tested 1, 2, 3, 4, and 5 hours after CFA injection. Baseline mechanical sensitivity was tested before IL-1ra and CFA injection.

3.2.2 IL-1ra post-treatment

CFA (0.05ml; 1:1 oil/saline) was injected into the ipsilateral masseter muscle 7 days after cannula surgery. IL-1ra was injected at varying concentrations into the ipsilateral Vi/Vc 24 hours after CFA injection. Mechanical sensitivity was tested 0.5, 1, 2, 3, 4, and 5 hours after IL-1ra injection. Baseline mechanical sensitivity was tested before CFA injection and between CFA and IL-1ra injections.

3.2.3 Vc lesion

Vc lesions were made using 1-μg/0.5-μl of ibotenic acid (Tocris Bioscience) injected into the Vc unilaterally. Rats were anesthetized and a 1-μl Hamilton syringe was place between C1-C2 vertebrae and injected into the ipsilateral Vc over a 2-minute period. The rats were allowed to heal over 5-7 days. CFA was injected into the ipsilateral side and behavioral testing was performed after 24 hours.

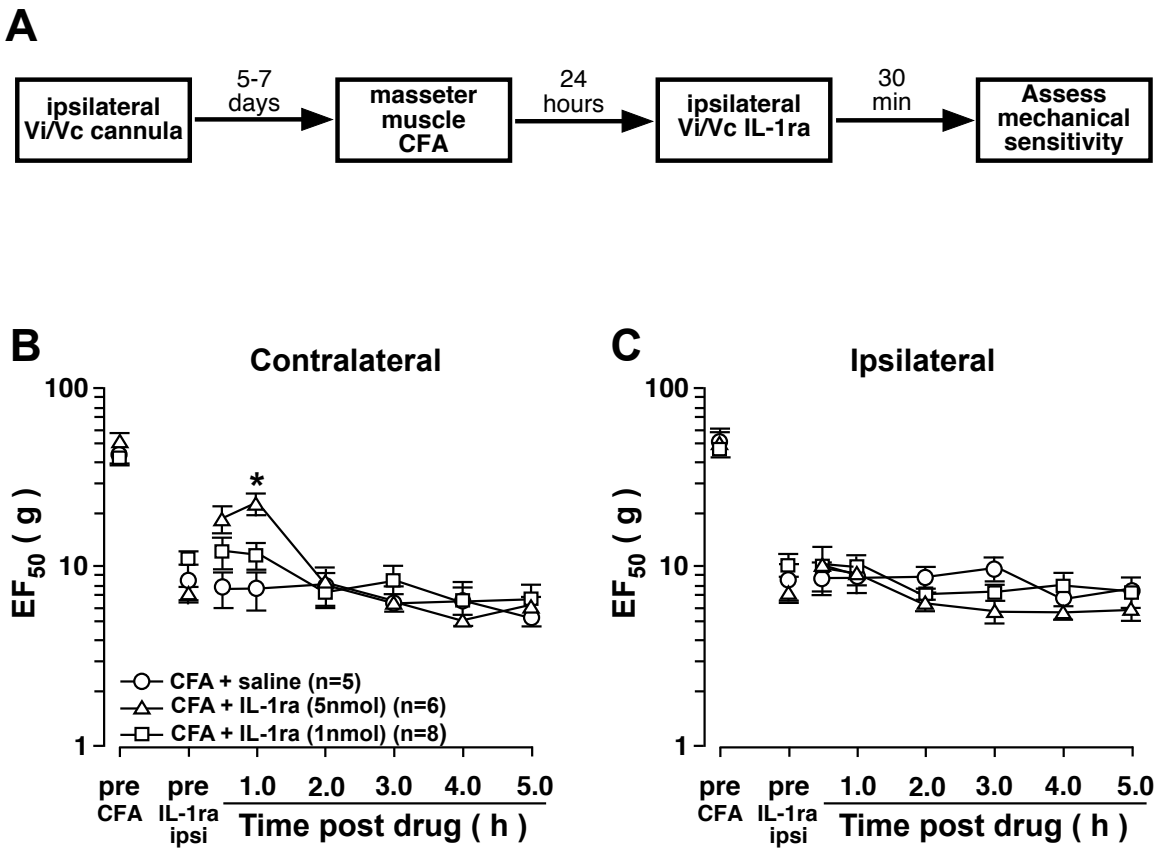
3.3 Results

3.3.1 IL-1R inhibition after CFA-induced hyperalgesia attenuates contralateral orofacial hyperalgesia

Our previous studies have shown that CFA injection into the masseter muscle and IL-1β injections into the Vi/Vc transition zone generate bilateral mechanical hyperalgesia as seen by the significant decreases in EF_{50} values (Shimizu et al. 2009a; Shimizu et al. 2009b). We have also shown that CFA-induced masseter muscle injection results in increased IL-1β expression in Vi/Vc astrocytes (Guo et al. 2007). To test whether activation of IL-1R in the Vi/Vc is involved in CFA-induced contralateral hyperalgesia, an IL-1R antagonist (IL-1ra) was injected.

In the post-treatment experiment, microinjection of IL-1ra into the ipsilateral Vi/Vc was done 24 hours after injection of CFA into the masseter muscle. Treatment of IL-1ra after CFA-induced hyperalgesia has been established to help determine the role of IL-1R activation in the maintenance of contralateral hyperalgesia. Mechanical sensitivity was measured 0.5-5 hours after IL-1ra microinjection (Figure 3.2A). IL-1ra (5 nmol, n=6) attenuated contralateral hyperalgesia 1 hour after microinjection as compared to saline-treated rats (Figure 3.2B), however, IL-1ra did not affect ipsilateral hyperalgesia (Figure 3.2C). Sham surgery control rats did not show signs of hyperalgesia. Similarly, saline control groups for CFA injections (saline+saline; saline+IL-1ra) did not produce hyperalgesia (results not shown). These results suggest that IL-1R activation in the ipsilateral Vi/Vc is involved in the maintenance of CFA-induced contralateral hyperalgesia.

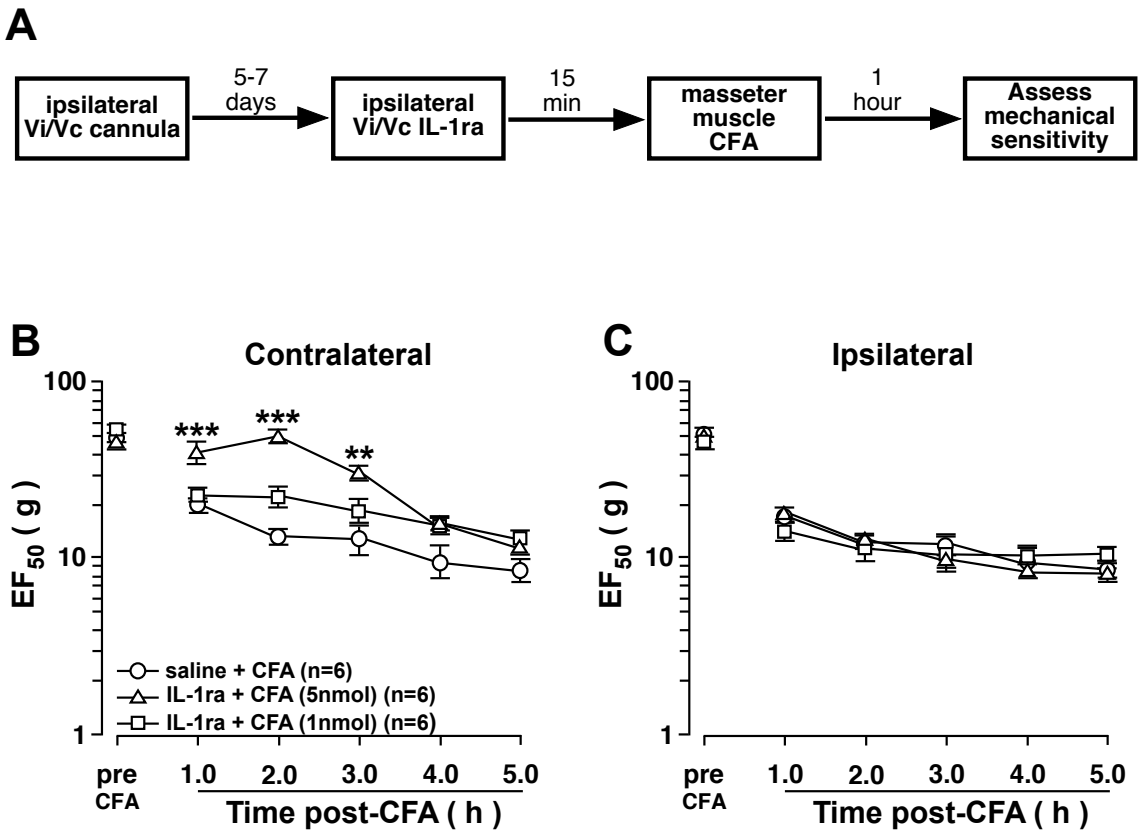
Figure 3.2: Inhibition of IL-1R in the ipsilateral Vi/Vc attenuates contralateral hyperalgesia. Contralateral orofacial hyperalgesia is attenuated by IL-1R inhibition in the ipsilateral Vi/Vc. **A.** Post-treatment experiment sequence: CFA (0.05 ml; 1:1 oil/saline) was injected unilaterally into the masseter muscle 24 hours prior to IL-1ra microinjection into the ipsilateral Vi/Vc (0.5ml). **B, C.** Post-treatment of IL-1ra attenuated CFA-induced contralateral hyperalgesia 1 hour after microinjection as compared to saline treated rats. IL-1ra did not attenuate ipsilateral hyperalgesia. *: IL-1ra (5 nmol) vs saline. *: $p<0.05$; (ANOVA with repeated measures and Student Neuman-Keuls post-hoc test).



3.3.2 IL-1R inhibition before CFA-induced hyperalgesia prevents the development of contralateral orofacial hyperalgesia

Treatment of IL-1ra before intra-masseter muscle CFA injection was performed to determine the role of IL-1R activation in the development of contralateral hyperalgesia. In the pre-treatment experiments, rats were injected with IL-1ra 15 minutes before CFA masseter muscle injection. Mechanical sensitivity was measured 1-5 hours after CFA injection (Figure 3.3A). IL-1ra (5 nmol, n=6) prevented the development of contralateral hyperalgesia 1-3 hours after CFA injection as compared to saline treated rats (Figure 3.3B). Pre-treatment of IL-1ra did not attenuate ipsilateral hyperalgesia (Figure 3.3C). Similar to the post-treatment experiment, sham surgery control and saline control groups for CFA injections (saline+saline; saline+IL-1ra) did not produce hyperalgesia (results not shown). These results suggest that IL-1R activation in the ipsilateral Vi/Vc is also involved in the development of contralateral hyperalgesia.

Figure 3.3: Inhibition of IL-1R in the ipsilateral Vi/Vc prevents the development of contralateral hyperalgesia. The development of contralateral orofacial hyperalgesia is prevented by IL-1R inhibition in the ipsilateral Vi/Vc. **A.** Pre-treatment experiment sequence: CFA (0.05 ml; 1:1 oil/saline) was injected unilaterally into the masseter muscle 15 min after IL-1ra microinjection into the ipsilateral Vi/Vc (0.5 ml). **B, C.** Pre-treatment of IL-1ra prevented the development of CFA-induced contralateral hyperalgesia 1-3 hours after CFA injection as compared to saline treated rats. IL-1ra did not attenuate ipsilateral hyperalgesia. *: IL-1ra (5 nmol) vs saline. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$ (ANOVA with repeated measures and Student Neuman-Keuls post-hoc test).

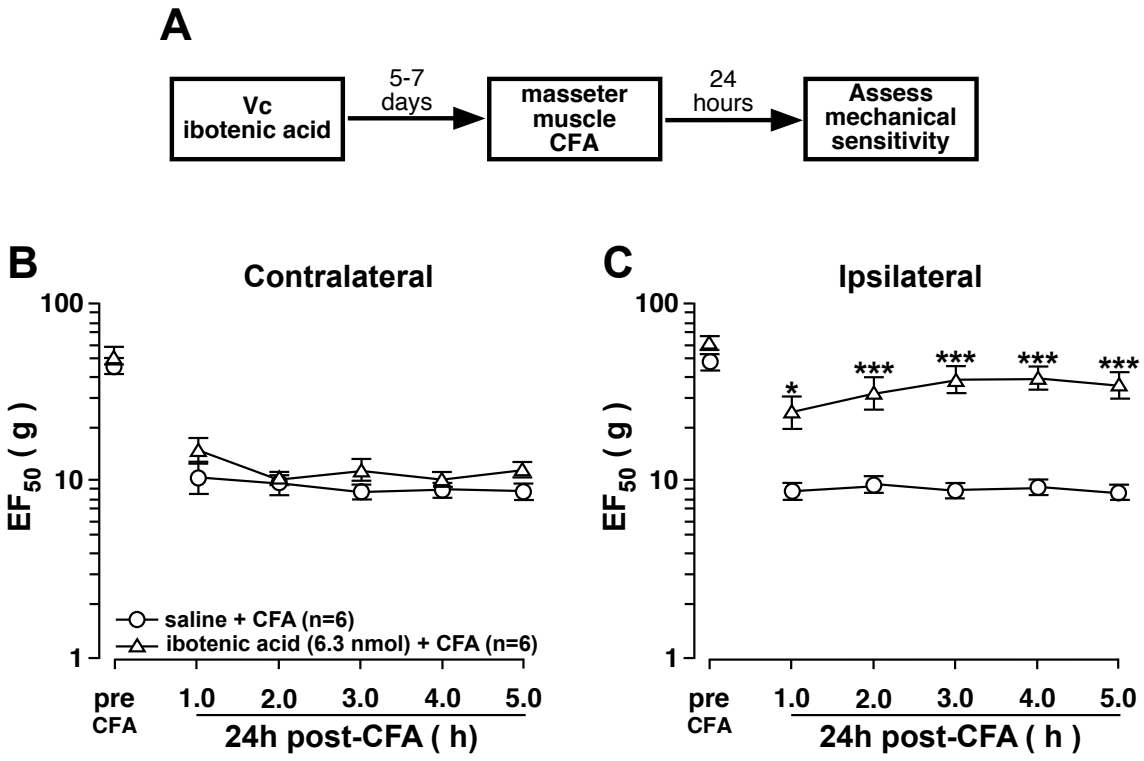


3.3.3 Ipsilateral Vc input is necessary for the development of CFA-induced ipsilateral orofacial hyperalgesia

The present data provide evidence that ipsilateral Vi/Vc was necessary for contralateral orofacial hyperalgesia. Specifically, our data showed that injection of IL-1ra into the Vi/Vc did not affect the ipsilateral hyperalgesia. Our previous studies have shown also that afferent nociceptive input into the Vc is critical for the development of ipsilateral hyperalgesia but does not affect contralateral hyperalgesia (Shimizu et al. 2009b). This suggests that the ipsilateral and contralateral hyperalgesia may be transmitted through different circuits in the trigeminal system.

To test this, a lesion was made in the ipsilateral Vc using 6.3 nmol of the excitotoxin, ibotenic acid. Ibotenic acid induces glutamate-mediated toxicity to neuron cell bodies through activation of glutamate receptors. An ipsilateral Vc lesion prior to CFA injection inhibited the development of ipsilateral hyperalgesia (Figure 3.4B) but did not inhibit contralateral hyperalgesia development (Figure 3.4A). This suggests that ipsilateral Vc input is primarily involved in the development of ipsilateral hyperalgesia but not for the development of hyperalgesia contralateral to the site of injury.

Figure 3.4: Vc activation is necessary for the development of ipsilateral hyperalgesia. Lesion of the ipsilateral Vc prior to CFA injection into the master muscle prevents the development of ipsilateral hyperalgesia. **A.** Ibotenic Acid (6.3 nmol) was administered to unilaterally to the Vc between vertebrae C1-C2, 7 days prior to ipsilateral CFA injection into the masseter muscle. **B.** Lesion of the ipsilateral Vc did not prevent the development of contralateral hyperalgesia. **C.** However, lesion of the ipsilateral Vc did prevent the development of ipsilateral hyperalgesia. *: Ibotenic Acid (6.3 nmol) vs. saline, $p<0.05$; **: $p<0.01$; ***: $p<0.001$ (ANOVA with repeated measures and Student Neuman-Keuls post-hoc test).



3.4 Discussion

In our present study, inhibition of IL-1R activation in the ipsilateral Vi/Vc before CFA treatment prevented the development of contralateral orofacial hyperalgesia. Similarly, inhibition of IL-1R activation in the ipsilateral Vi/Vc after CFA treatment resulted in an attenuation of the contralateral hyperalgesia. These results support the view that activation of IL-1R in the ipsilateral Vi/Vc is involved in the development and maintenance of deep tissue orofacial pain on the contralateral site.

Contralateral hyperalgesia has been seen with both unilateral CFA masseter muscle injection and unilateral intra-Vi/Vc IL-1β microinjection (Shimizu et al. 2009a). This contralateral effect also appears to be unique to the Vi/Vc transition zone. Previous research has shown that microinjection of IL-1β into the Vc does not produce contralateral hyperalgesia (Shimizu et al. 2009b). Similarly, rats injected with CFA into the hind paw do not develop contralateral hyperalgesia (LaGraize et al. 2010; Bai et al. 2010).

Previous studies also showed that masseter muscle inflammation produces increased IL-1β expression in Vi/Vc astroglia (Guo et al. 2007). This increased IL-1β expression was shown to produce IL-1R-dependent activation of the protein kinase-C signaling cascade. This resulted in GluNR1 subunit phosphorylation of the NMDA receptor and ultimately led to behavioral hyperalgesia (Guo et al. 2007).

An interesting observation is that ipsilateral Vi/Vc IL-1ra does not affect ipsilateral hyperalgesia. Conversely, lesions in the ipsilateral Vc (Figure 3.4) do not affect contralateral hyperalgesia but does prevent the development of ipsilateral

hyperalgesia. These results suggest that the contralateral hyperalgesia is predominantly mediated through the ipsilateral Vi/Vc. These results also suggest that the development of ipsilateral hyperalgesia is predominantly mediated through the ipsilateral Vc. One possible explanation is that ipsilateral hyperalgesia is dominated by hyperexcitability and central sensitization in the Vc driven by peripheral input from the site of injury. We have previously shown that the Vc receives afferent input from the overlying skin and deep tissue of the masseter muscle (Wang et al. 2006). Research has shown that the Vc neurons directly project to various brain structures including the parabrachial nucleus (PBN) (Cechetto et al. 1985; Feil and Herbert 1995), the ventroposteromedial thalamic nucleus (VPM) and posterior thalamic nuclear group (Po) (Guy et al. 2005). While the VPM is the classic thalamic structure involved in the trigeminothalamic pathway (Dubner and Bennett 1983), PBN has also been associated with nociception (Bourgeais et al. 2001). While we cannot rule out the involvement of ipsilateral Vi/Vc IL-1R activation in the development of ipsilateral hyperalgesia, it appears that it is not the predominant mechanism.

Lesions to the caudal Vc also suggest that ipsilateral Vc activation is not the predominant mechanism involved in the development of contralateral hyperalgesia. However, we cannot completely rule out the involvement of Vc. One potential pitfall of the Vc lesion experiment is the location and extent of the lesion within the Vc. The Vc is generally thought to descend from just caudal of the obex down to the level of C1-C2. This means that the ibotenic acid injection given at C1-C2 resulted in lesions to the caudal Vc and left the rostral Vc intact. Considering that masseter muscle afferents have been shown to project through the entirety of the medullary dorsal horn (Dessem et al.

2007), it is possible that the rostral Vc may play a part in the development of contralateral hyperalgesia.

Chapter 4: RVM involvement in contralateral hyperalgesia

4.1 Introduction

The rostral ventromedial medulla (RVM) is a midline site that is critical in the descending modulation of pain (Ren and Dubner 2002; Millan 2002). A critical pathway in descending modulation involves the periaqueductal grey (PAG)-RVM circuit. Neurons from areas such as the anterior cingulate cortex, amygdala, and hypothalamus descend and synapse in the PAG (Heinricher et al. 2009). Neurons of the PAG then descend and synapse in the RVM. The RVM sends projecting neurons to the spinal and medullary dorsal horn to modulate ascending sensory signals (Fields 2000). This descending modulation from the RVM can be facilitatory and inhibitory (Porreca et al. 2002; Ren and Dubner 2002). Descending facilitation from the RVM has been shown to be involved in formalin-induced hyperalgesia (Wiertelak et al. 1997), neuropathic hyperalgesia (Leong et al. 2011; Zapata et al. 2012), and deep tissue injury (Wei et al. 2010).

It has previously been shown that CFA-induced hyperalgesia was attenuated with ibotenic acid lesions in the RVM (Sugiyo et al 2005). Our previous studies have also shown that bilateral reciprocal connections exist from the Vi/Vc to the RVM (Sugiyo et al. 2005). This suggests that activation of RVM neurons may be necessary for the development of contralateral hyperalgesia after CFA-induced masseter muscle inflammation. It is possible that neuronal activation in the RVM produces a descending facilitatory signal to the contralateral side.

Thus, we hypothesize that neuronal activation in the RVM is necessary for the development of CFA-induced contralateral orofacial hyperalgesia.

4.2 Specific Methods

4.2.1 IL-1β-induced hyperalgesia

Following the "Surgical preparation/cannulation" section in Chapter 2, all rats received guide cannulae implanted in the RVM and the ipsilateral Vi/Vc. Rats were allowed to heal for 5-7 days before further manipulation.

In the Vc lesion experiment, the excitotoxin ibotenic acid was administered into the RVM to produce a lesion of the RVM cell bodies. Ibotenic acid was microinjected 10-15 minutes before IL-1β microinjection. IL-1β (PeproTech; Rocky Hill, NJ) was injected into the Vi/Vc and mechanical sensitivity was measured 0.5, 1, 1.5, 2, 3, 4, 4.5, and 5 hours after IL-1β injection. Baseline mechanical sensitivity was tested before ibotenic acid administration.

NK1-R antagonists, L-733,060 and RP67580 (Tocris Bioscience; Ellisville, MO), were administered to the RVM 15 minutes after IL-1β administration to the ipsilateral Vi/Vc. Mechanical sensitivity was tested 0.5, 1, 2, 3, 4, and 5 hours after L-733,060 and RP67580 injection. Baseline mechanical sensitivity was tested before IL-1β microinjection.

4.2.2 CFA-induced hyperalgesia

For CFA-induced hyperalgesia, a single cannula is implanted into the RVM as described in the "Surgical preparation/cannulation" section of Chapter 2. CFA (0.05ml; 1:1 oil/saline) is injected into the ipsilateral masseter muscle 5-7 days after cannula implantation. 24 hours after CFA injection, NK1-R antagonists L-733,060 and RP67580 were injected in to the RVM. Mechanical sensitivity was tested 0.5, 1, 2, 3, 4, and 5 hours after L-733,060 and RP67580 microinjection. Baseline mechanical sensitivity was tested before CFA injection and before L-733,060 and RP67580 microinjection.

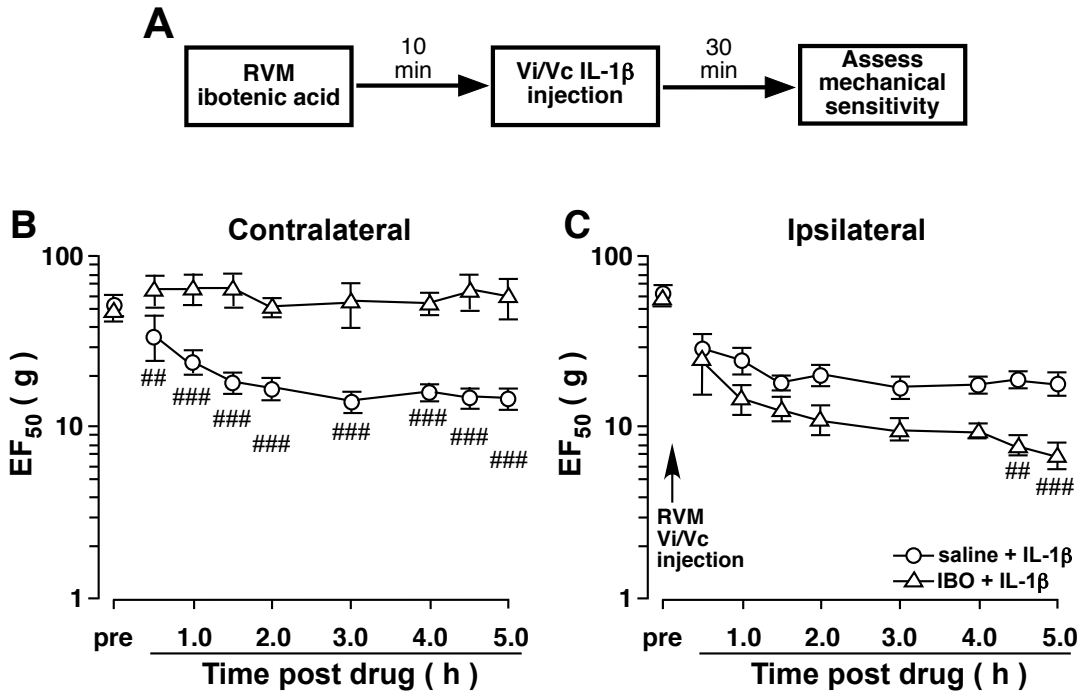
4.3 Results

4.3.1 Activation of RVM neurons is necessary for the development of contralateral orofacial hyperalgesia

The RVM is a midline structure that lacks laterality. It was previously shown that there are reciprocal facilitatory interactions between Vi/Vc and RVM pain modulatory circuitry (Sugiyo et al. 2005). The same study also showed that CFA-induced bilateral hyperalgesia was prevented with lesions to the RVM and the Vi/Vc. Thus, we tested the hypothesis that IL-1β-induced bilateral hyperalgesia was dependent on RVM and Vi/Vc interactions. To evaluate the role of RVM descending input in orofacial hyperalgesia, a soma-selective neurotoxin ibotenic acid was microinjected into the RVM to produce focal neural lesions and its effect on IL-1β-induced hyperalgesia was assessed. The lesions were localized in RVM, mainly in nucleus raphe magnus (NRM) and the

gigantocellular reticular nucleus pars alpha (NGCα). In rats receiving ibotenic acid injections, the IL-1β-induced hyperalgesia was abolished on the contralateral site as compared to saline-treated rats (Figure 4.1).

Figure 4.1: Intra-RVM ibotenic acid attenuated IL-1β-induced contralateral hyperalgesia. A. Ten-min before a unilateral injection of IL-1β into the Vi/Vc transition zone, ibotenic acid (IBO) (2 μg/0.2 μl, n=5) was microinjected into the RVM to produce excitotoxic neuronal lesions in RVM. Saline was injected as a vehicle control. The open circles indicate the injection sites for saline. **B**. Compared to saline-injected rats, RVM excitotoxic lesions prevented the development of contralateral hyperalgesia after injection of IL-1β into the Vi/Vc transition zone. **C**. Compared to saline control, there was a slight further decrease in EF50s on the ipsilateral site in the IBO-treated rats. ##, $p<0.01$, ###, $p<0.001$, saline vs. IBO (ANOVA with repeated measures and post-hoc test).



4.3.2 NK1-R activation in the RVM is necessary for the development of contralateral orofacial hyperalgesia

Research has shown that the neuropeptide substance P is involved in nociception and hyperalgesia in the spinal cord (Schafer et al. 1993; McCarson et al. 1994; Suzuki et al. 2002). Evidence also suggests that substance P is involved in descending facilitation of pain in the RVM (Budai et al. 2007; Brink et al. 2012). Immunohistochemical localization shows moderate amounts of the substance P receptor, neurokinin-1 receptor (NK1-R), in the RVM (Maeno et al. 1993; Nakaya et al. 1994; Saffroy et al. 2000). Furthermore, substance P activation of NK1-R in the RVM contributes to CFA-induced hyperalgesia and capsaicin induced hyperalgesia (Pacharinsak et al. 2008; Hamity et al. 2010; LaGraize et al. 2010).

NK1-R activation in the RVM has been shown to contribute to capsaicin-induced hyperalgesia (Pacharinsak et al. 2008). Similarly, it has been shown that NK1-R activation in the RVM is involved in mediating behavioral hyperalgesia after hind paw inflammation (LaGraize et al. 2010). To test whether NK1-R activation in the RVM was involved in orofacial contralateral hyperalgesia, two NK1-R antagonists, RP67580 and L-733,060 were microinjected into the RVM.

CFA was injected into the masseter muscle 24 hours before microinjection of NK1-R antagonist (Figure 4.2A). Both NK1-R antagonists attenuated CFA-induced bilateral hyperalgesia in a dose dependent manner. Attenuation of hyperalgesia was seen 0.5-5 hours post-injection with the highest dose of RP67580 (11.4 nmol; n=6) on the contralateral side (Figure 4.2B) and ipsilateral side (Figure 4.2C) when compared to vehicle control. Similar results were observed using the NK1-R antagonist L-733,060

(Figure 4.2D, E).

Inhibition of NK1-R activation in the RVM attenuated IL-1β (160 fmol) – induced bilateral orofacial hyperalgesia. Microinjection of RP67580 and L-733,060 into the RVM 15 minutes after IL-1β injection into the Vi/Vc (Figure 4.3A) resulted in a dose-dependent attenuation of bilateral hyperalgesia. Application of RP67580 (11.4 nmol; n=5) resulted in attenuation of hyperalgesia 0.5-4 hours post-injection on the contralateral side (Figure 4.3B) and ipsilateral side (Figure 4.3C). Similar results were observed using NK1-R antagonist L-733,060 (Figure 4.3D, E). Microinjection of vehicle controls into the RVM did not attenuate hyperalgesia. CFA injection control and sham surgery controls did not produce hyperalgesia. These results suggest that contralateral and ipsilateral orofacial hyperalgesia are mediated by NK1-R activation in the RVM.

Figure 4.2: Inhibition of NK1-R in the RVM attenuates inflammatory bilateral hyperalgesia. Experiment sequence (**A**) shows CFA (0.05 ml; 1:1 oil/saline) injection into the masseter muscle 24 hours prior to RP67580 (**B, C**) and L-733,060 (**D, E**) microinjection into the RVM (0.5 ml). +: lowest dose vs. saline. #: middle dose vs. saline. *: highest dose vs. saline. +,**#,*:** p<0.05**;** ++,##,**: p<0.01; +++,###,***: p<0.001 (ANOVA with repeated measures and Student Neuman-Keuls post-hoc test).

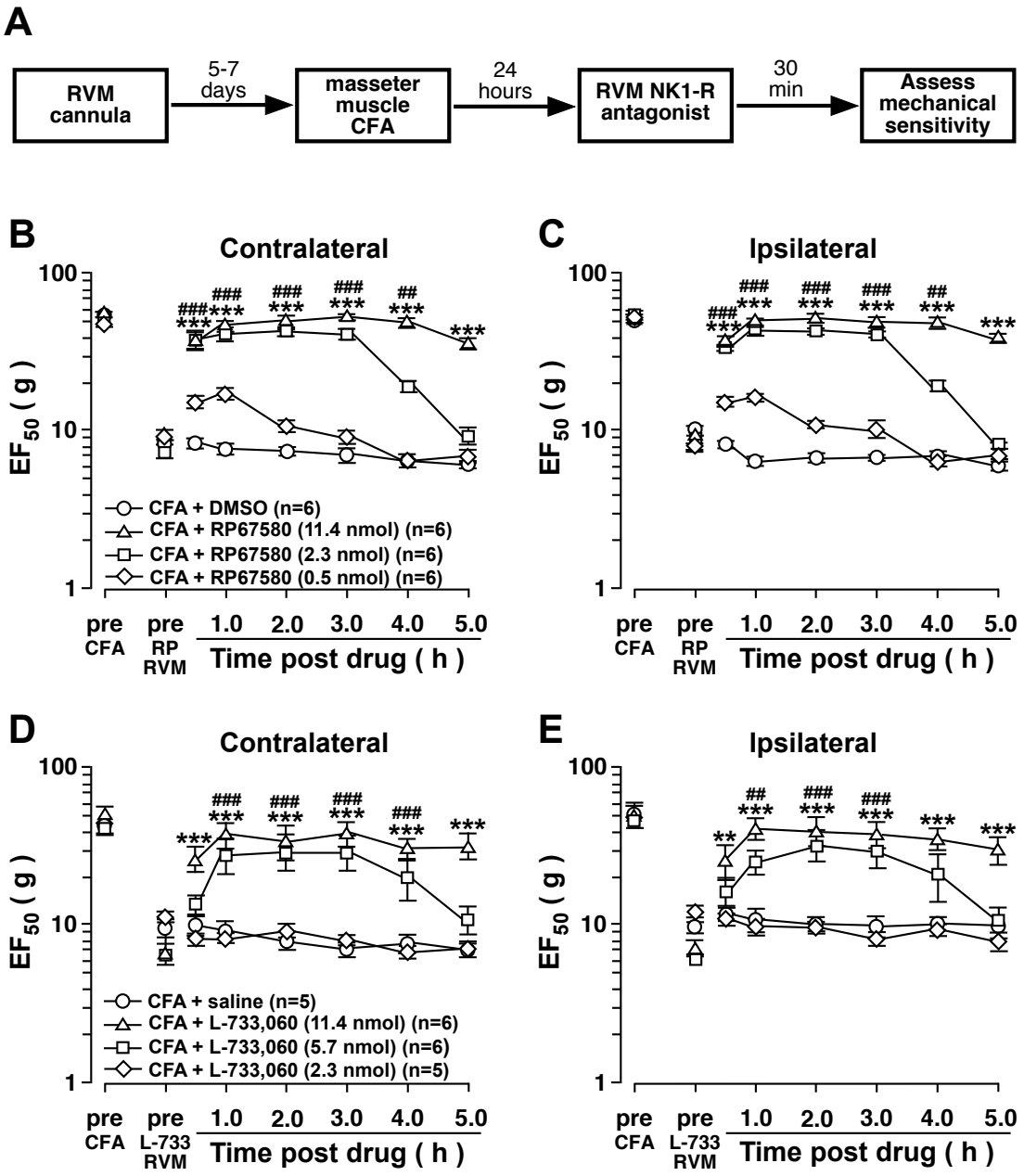
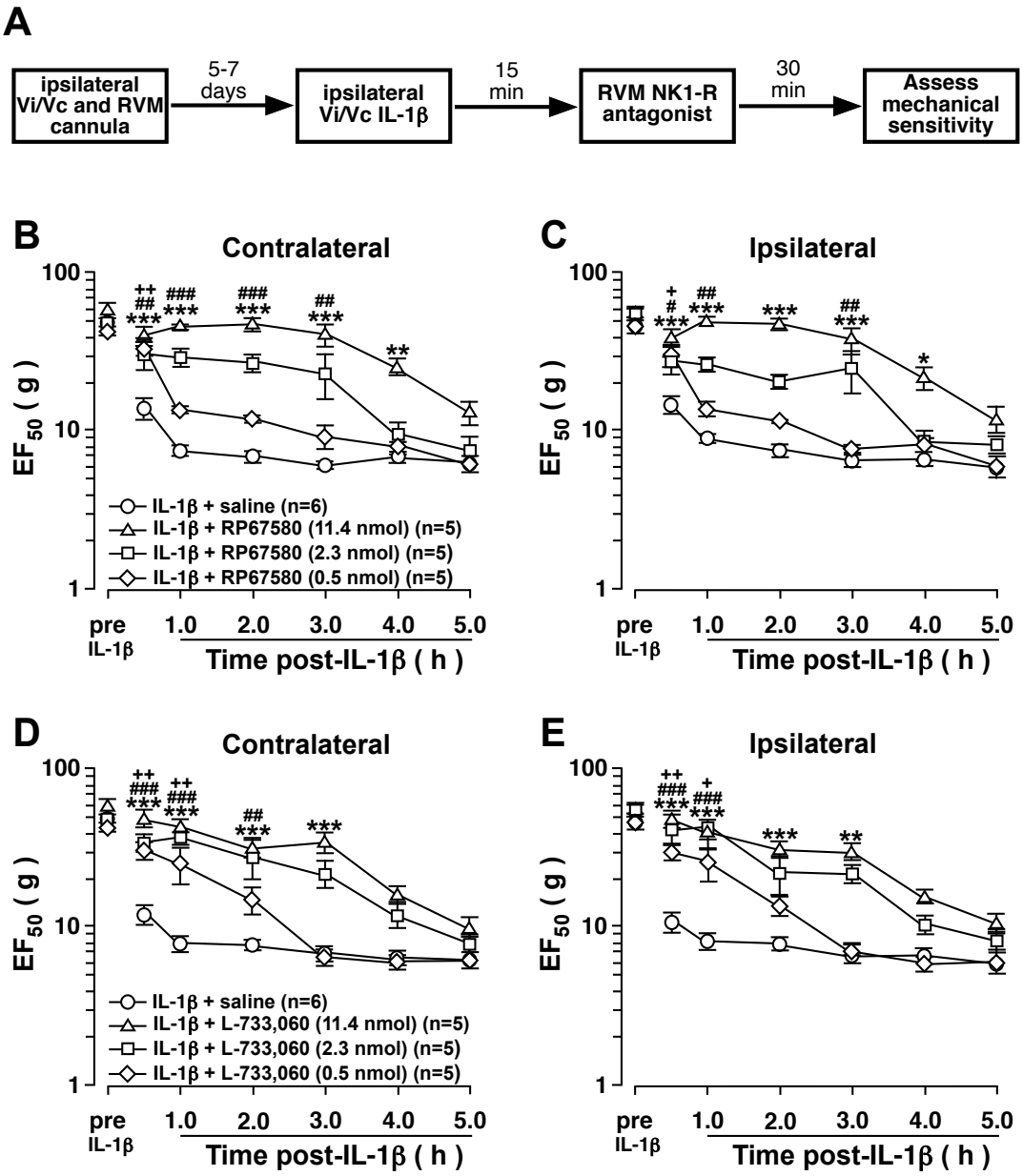


Figure 4.3: Inhibition of NK1-R in the RVM attenuates IL-1β-induced bilateral hyperalgesia. Experiment sequence (**A**) shows IL-1β (160 fmol) microinjection into the Vi/Vc 15 minutes before RP67580 (**B, C**) and L-733,060 (**D, E**) microinjection into the RVM (0.5 ml). +: lowest dose vs. saline. #: middle dose vs. saline. *: highest dose vs. saline. +,**#,*:** p<0.05**;** ++,##,**: p<0.01; +++,###,***: p<0.001 (ANOVA with repeated measures and Student Neuman-Keuls post-hoc test).



4.4 Discussion

The RVM is a critical site in descending modulation of pain (Mayer et al. 1971; Fields et al. 1991). Much research has focused on the descending inhibitory affects of the opioid system within the RVM (Fang et al. 1989; Boyer et al. 1998). Our lab has shown that upregulation and phosphorylation of AMPA receptors in the RVM can lead to descending inhibition of inflammatory pain (Guan et al. 2003; Guan et al. 2004). However, there has been mounting evidence of RVM-mediated descending facilitation (Wiertelak et al. 1997; Urban et al. 1999; Ren and Dubner 2002; Porreca et al. 2002; Heinricher et al. 2009; LaGraize et al. 2010).

We have shown that excitotoxic lesions of RVM neurons before intra-Vi/Vc IL-1β microinjection and intra-masseter CFA injection prevented the development of contralateral hyperalgesia (Sugiyo et al. 2005; Shimizu et al. 2009a). Glia activation in the RVM participates in descending facilitatory contribution to inflammatory and neuropathic hyperalgesia (Roberts et al. 2009; Wei et al. 2008). Research has shown that neuropathic pain may be maintained by cholecystokinin release in the RVM (Kovelowski et al. 2000). Research has also shown that neuropathic pain may induce RVM neuronal cell death, which could result in a net increase in descending facilitation (Leong et al. 2011). Brain -derived neurotrophic factor (BDNF) activation of tyrosine kinase receptor–B in the RVM is involved in the development of persistent pain (Guo et al. 2006).

Substance P has been shown to be involved in RVM descending facilitation of pain (Pacharinsak et al. 2008; Brink et al. 2012). NK1-R activation in the RVM enhances excitatory glutamatergic inputs to RVM neurons in rats with persistent pain (Zhang et al.

2009). NK1-R antagonism in the RVM attenuated CFA-induced thermal hyperalgesia in the hind paw (Hamity et al. 2010). Previous studies have shown that NK1-R expression is increased in the RVM following hind paw inflammation and direct injection of substance P into the RVM can induce bilateral thermal hyperalgesia in the hind paw (LaGraize et al. 2010). It was also shown that substance P-induced hyperalgesia can be mediated by 5-HT, NMDA, and $GABA_A$ receptors at the spinal level (LaGraize et al. 2010). We have now shown that NK1-R inhibition in the RVM attenuated inflammatory orofacial hyperalgesia bilaterally. These results suggest that NK1-R activation in the RVM contributes to descending facilitation that mediates inflammatory orofacial hyperalgesia to the ipsilateral and contralateral side.

It is surprising, however, that intra-Vi/Vc IL-1β-induced ipsilateral orofacial hyperalgesia was not attenuated but rather slightly enhanced after excitotoxic lesions of RVM, which suggests that IL-1β-induced ipsilateral hyperexcitability was not dependent on a descending facilitatory drive. This result differs from the results obtained from NK1-R inhibition in the RVM after IL-1β-induced ipsilateral hyperalgesia. This result is also different from the model of masseter muscle inflammation, in which RVM lesions abolished ipsilateral hyperalgesia at 1–3 d after inflammation (Sugiyo et al. 2005) and NK1-R inhibition in the RVM attenuated ipsilateral hyperalgesia up to 5 hours after injection. The lack of an effect of RVM lesions on IL-1β-induced ipsilateral hyperalgesia may be explained by a removal of a descending net inhibitory effect on the ipsilateral side after IL-1β injection. It has been shown that there are dynamic changes in descending pain modulation after inflammation of the rat hind paw, which involves an ipsilateral enhanced inhibition (Ren and Dubner 1996; Terayama et al. 2000). These

results also suggest differential descending control of primary and secondary hyperalgesia. While there is a dynamic balance between descending inhibition and facilitation on primary hyperalgesia, the descending modulation of secondary hyperalgesia may be predominantly facilitatory (Ren and Dubner 2002; Vanegas et al. 2004).

Chapter 5: Descending 5-HT activation of the contralateral Vi/Vc in contralateral hyperalgesia

5.1 Introduction

It has been established that descending neurons of the RVM are the major part of the descending serotonin (5-HT) pathway in the spinal cord (Millan 2002). Literature has shown that 5-HT is involved in the descending modulation of pain in the spinal cord (Sommer 2006; Bardin 2011). A conditional knockout of serotonin-containing neurons in mice resulted in an attenuation of opioid-mediated analgesia (Zhao et al. 2007). Selective lesions to serotonergic neurons in the RVM caused enhanced inflammatory pain (Wei et al. 1999).

Recent evidence suggests that the descending serotonin system also participates in descending facilitation of pain. Ablation of 5-HT-containing neurons that project to the spinal cord inhibits nocifensive behavior in rats after noxious stimulation to the hind paw (Svensson et al. 2006; Rahman et al. 2006). We have also shown that the serotonin system is involved in descending facilitation of pain in the spinal cord after tissue and nerve injury. Through the depletion of 5-HT in the RVM by RNA interference (RNAi), we showed that the 5-HT system is involved in the facilitation of spinal hyperalgesia (Wei et al. 2010).

However, these opposing effects may be mediated by the expression of the large family of 5-HT receptor subtypes (Lopez-Garcia 2006). Descending 5-HT activation of 5-HT1A receptors in the spinal cord contributes to anti-nociception (Buritova et al. 2005;

Viisanen and Pertovaara 2010). Activation of 5-HT7 receptors in the spinal cord is involved in the antinociceptive effects of systemic morphine (Dogrul and Seyrek 2006). Spinal 5-HT2A and 5-HT2B receptor activation may contribute to neuropathic pain (Aira et al. 2010). Similarly, 5-HT2A and 5-HT2C receptor activation in the spinal cord has been shown to increase hyperalgesia induced by peripheral inflammation (Kjorsvik et al. 2001).

Spinal 5-HT3 receptor activation has been shown to be pro-nociceptive (Ali et al. 1996; Zeitz et al. 2002). Inhibition of 5-HT3 receptors in the spinal cord reduced the evoked responses of dorsal horn neurons to mechanical and thermal stimuli (Ali et al. 1996; Suzuki et al. 2002). Activation of 5-HT3 receptors in the spinal cord contributes to the development of hyperalgesia after spinal cord injury (Oatway et al. 2004; Chen et al. 2009). Intra-RVM CCK induced hyperalgesia was prevented by inhibition of spinal 5-HT3 receptors (Dogrul et al. 2009).

These data suggest that descending 5-HT from the RVM is facilitatory upon activation of spinal 5-HT3 receptors. We hypothesize that descending 5-HT activation of 5-HT3 receptors in the contralateral Vi/Vc leads to the development of contralateral orofacial hyperalgesia.

5.2 Specific Methods

5.2.1 shRNA

The shRNA plasmids containing the 5-HT synthesizing enzyme Trytophan

Hydroxylase – 2 (Tph-2) or a scrambled sequence control was administered into the RVM with a 1μL Hamilton syringe (0.5 μg/0.5 μl) over 5 minutes. Fifteen minutes after the injection, the syringe was slowly removed and a pair of Teflon-coated silver positive and negative electrodes spaced 3 mm apart were placed in a rostrocaudal direction at the injection site for electroporation (7 square-wave pulses; 50 ms, 40 V, 1 Hz). The rats were allowed to heal for 3 days before unilateral masseter injection of CFA. Behavioral tests were performed 24 hours post-CFA.

5.2.2 Western Blot

Naïve and treated rats were anesthetized with 50mg/kg pentobarbital sodium (i.p) and decapitated. The RVM tissue was removed as previously described (Guo et al. 2007). The RVM tissue was homogenized and centrifuged at 20,200 x g for 10 min at 4°C, and the supernatant was removed. The protein concentration was determined. Each sample contained proteins from one animal. The proteins (50 mg) were separated on a 7.5% SDS-PAGE gel and blotted to a nitrocellulose membrane (GE Healthcare Biosciences, Pittsburgh, PA). The blot was incubated with rabbit anti-Tph-2 antibody overnight at 4°C. The membrane was washed with TBS and incubated for 1 h with anti-goat IgG horseradish peroxidase (HRP) (1:3000; Santa Cruz Biotechnology, Santa Cruz, CA) in 5% milk/TBS. The immunoreactivity was detected using enhanced chemiluminescence (ECL) (GE Healthcare, Pittsburgh, PA). The loading and blotting of equal amount of proteins were verified by reprobing the membrane with anti-β-actin antiserum (Sigma, St. Louis, MO). The ECL-exposed films were digitized, and densitometric quantification of immunoreactive bands was performed using U- SCAN-IT gel (version 4.3, Silk

Scientific). Photoshop software was utilized to construct the figure from the raw western blot data.

5.2.3 Immunohistochemistry

Rats were anesthetized at various time points after gene transfer with 50mg/kg of pentobarbital sodium (i.p) and perfused transcardially with 0.9% saline solution followed by 4% paraformaldehyde in 0.1M phosphate buffer solution. The brainstem and cervical spinal cord (C1-C2) was removed and immersed in the same fixative overnight at 4°C and transferred to 30% sucrose (w/v) in 0.1 M phosphate buffer solution for cryoprotection. After several days, 30 mm thick coronal sections of the tissue sample were sectioned with a cryostat at -20°C. Free floating tissue sections of the Vi/Vc were incubated with rabbit anti-5-HT antibody (1:4000, Immunostar, Hudson,WI) or rabbit anti-c-fos antibody overnight at room temperature. After serial washes, the sections staining for 5-HT were incubated with Cy2-conjugated goat anti-rabbit IgG (1:500, Jackson ImmunoResearch, West Grove, PA). The sections staining for Fos protein were incubated with Nickel DAB. Control staining procedure was performed by omission of the primary antibody. Photoshop software was utilized to construct the figure from the original microscope photos.

5.2.4 5-HT3 receptor antagonist

Cannulae were implanted into the ipsilateral and contralateral Vi/Vc. CFA (0.05ml; 1:1 oil/saline) was injected into the ipsilateral masseter muscle 5-7days after cannula implantation. 24 hours after CFA injection, 5-HT3 receptor antagonist Y-25130

was injected into the Vi/Vc. Mechanical sensitivity was tested 0.5, 1, 2, 3, 4, and 5 hours after L-733,060 and RP67580 microinjection. Baseline mechanical sensitivity was tested before CFA injection and before L-733,060 and RP67580 microinjection.

5.3 Results

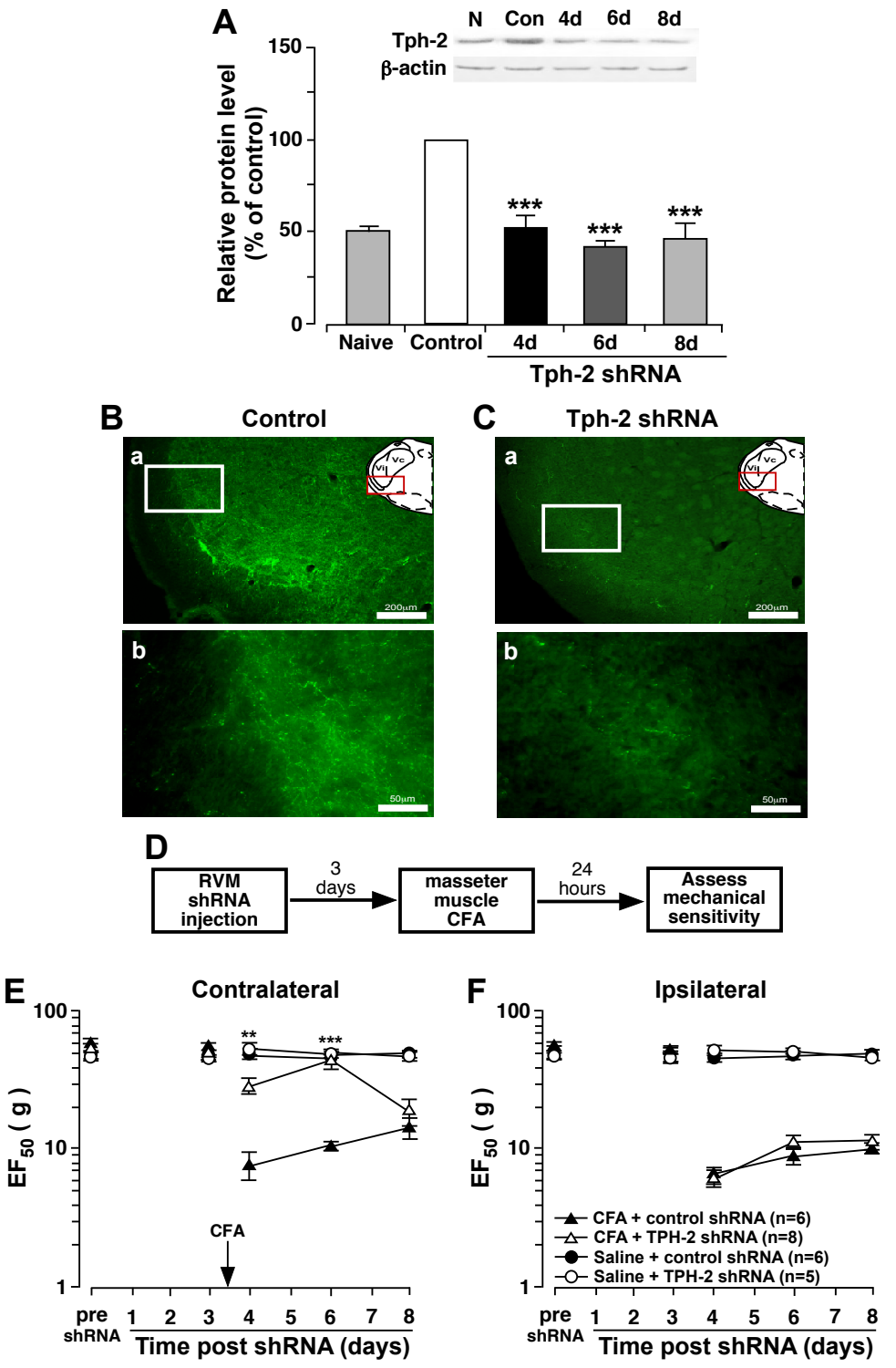
5.3.1 5-HT depletion in the RVM inhibits the development of CFA-induced contralateral orofacial hyperalgesia

The results suggest that NK1-R activation of RVM neurons may lead to descending facilitation of pain on the contralateral side and ipsilateral side. We previously showed that depletion of 5-HT in the RVM neurons inhibits injury-induced hyperalgesia (Wei et al. 2010) and an intrathecal 5-HT3 receptor antagonist blocked intra-RVM substance P-induced hyperalgesia (LaGraize et al. 2010). Thus, we tested the hypothesis that CFA-induced masseter inflammation could ultimately result in 5-HT-dependent descending facilitation to the contralateral Vi/Vc.

To evaluate the role of 5-HT in the development of contralateral orofacial hyperalgesia, down regulation of Tph-2, a rate limiting enzyme for 5-HT synthesis in RVM neurons was achieved by RNA interference. Since the RVM is a midline structure that projects bilaterally, RNA interference should result in bilateral 5-HT depletion at the level of the Vi/Vc. In CFA-induced hyperalgesic rats, western blot showed that Tph-2 expression in the RVM was significantly down regulated at 4-8 days after Tph-2 shRNA administration when compared to control ($n=4$; $p<0.001$) (Figure 5.1A). Immunostaining

at 4 days after shRNA administration shows a reduction of 5-HT immunoreactivity in the ventral Vi/Vc axons of CFA-induced hyperalgesic rats when compared to control shRNA (Figure 5.1B, C). 5-HT depletion inhibits the development of CFA-induced contralateral orofacial hyperalgesia 4-6 days after CFA injection (Figure 5.1E). However, 5-HT depletion did not inhibit development of ipsilateral hyperalgesia after CFA masseter muscle injection (Figure 5.1F). These data suggest that 5-HT-dependent descending facilitation of pain is necessary for the development of contralateral hyperalgesia but not the ipsilateral hyperalgesia.

Figure 5.1: 5-HT depletion in the RVM prevents the development of contralateral hyperalgesia. RNAi of Tph-2 in the RVM prevents the development of CFA-induced contralateral hyperalgesia. **A.** Western blot analysis of Tph-2 in the RVM. CFA (0.05 ml; 1:1 oil/saline) injected unilaterally into the masseter muscle 3 days after shRNA administration. Tph-2 protein expression was measured in rats at 4 day (n=4), 6 days (n=4) and 8 days (n=4) after shRNA was administered. Tph-2 protein expression was measured in rats given control shRNA (n=4) 4 days after the shRNA was administered. **B,C.** Immunohistochemical fluorescent staining of 5-HT in the Vi/Vc of shRNA treated rats (n=3) 24 hours after CFA administration shows decreased 5-HT expression in the Vi/Vc following Tph-2 depletion in rats as compared to control shRNA (n=3). CFA was administered 3 days after shRNA administration. (Ba: control shRNA,10x; Bb: control shRNA,40x. Ca: Tph-2 shRNA,10x; Cb: Tph-2 shRNA,40x). **D.** Timeline of behavioral testing. **E,F.** Mechanical hyperalgesia was tested in 5-HT depleted rats with CFA-induced hyperalgesia. Control or Tph-2 shRNA was administered and CFA was injected 3 days after. Mechanical hyperalgesia was inhibited in 5-HT depleted rats as compared to control on the contralateral side (**E**), but not the ipsilateral side (**F**). *: CFA + Tph-2 shRNA vs. CFA + control shRNA, $p<0.05$; **: $p<0.01$; ***: $p<0.001$ (ANOVA with repeated measures and Student Neuman-Keuls post-hoc test).


A
N
Con
4d
6d
8d
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β-actin
Relative protein level (% of control)
150
100
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8d
Tph-2 shRNA
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C
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RVM shRNA injection
3 days
masseter muscle CFA
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F
Ipsilateral
100
10
1
EF50 (g)
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CFA
pre shRNA
1 2 3 4 5 6 7 8
Time post shRNA (days)
CFA + control shRNA (n=6)
CFA + TPH-2 shRNA (n=8)
Saline + control shRNA (n=6)
Saline + TPH-2 shRNA (n=5)

5.3.2 Vi/Vc 5-HT3 receptor activation is necessary for CFA-induced contralateral orofacial hyperalgesia

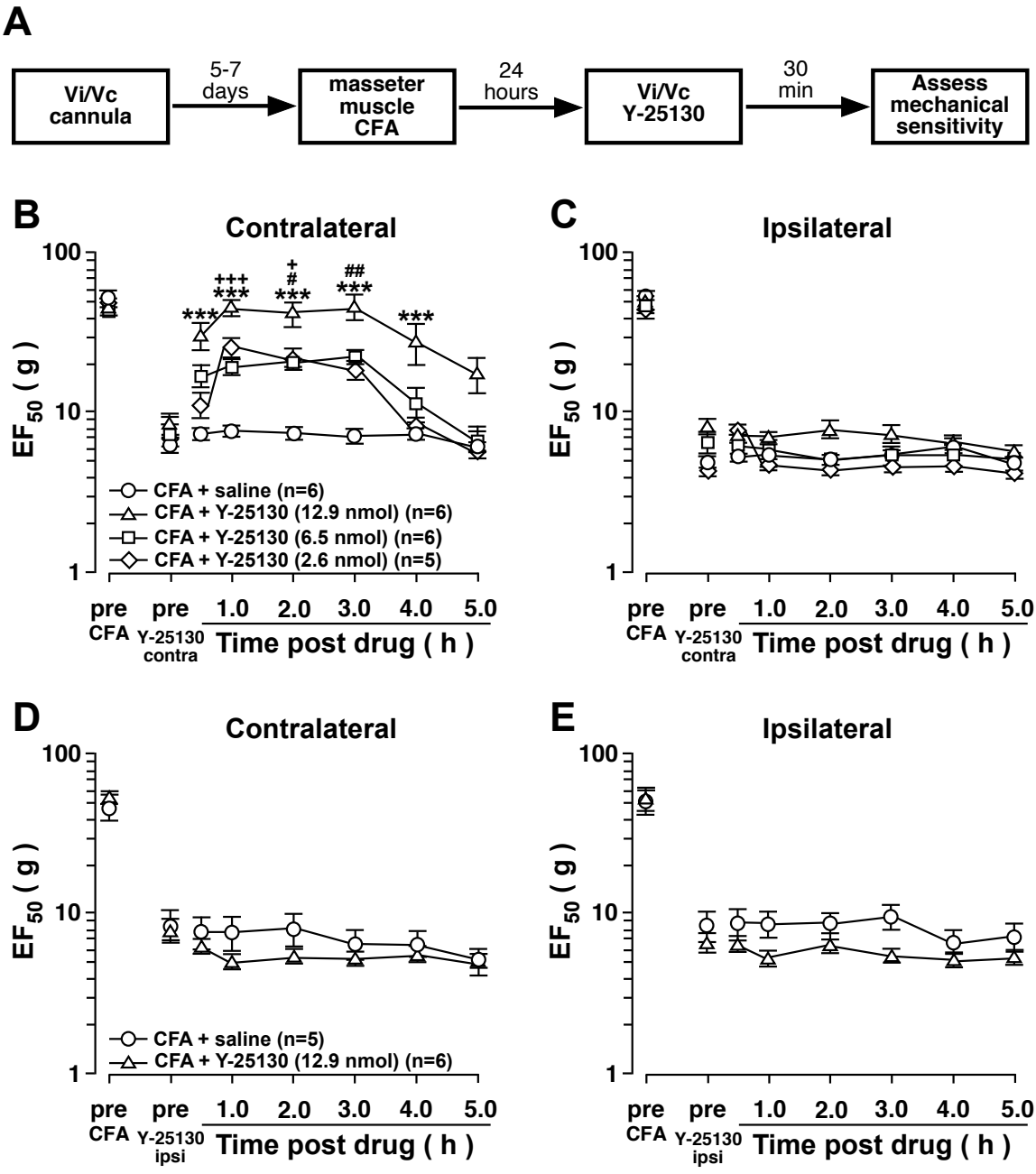
Previous studies have shown that 5-HT3 receptor expression is upregulated in the spinal cord and trigeminal nuclei in animal models of persistent pain (Rahman et al. 2006; Doucet et al. 2007). In similar models of persistent pain, hyperalgesia is attenuated following 5-HT3 receptor inhibition (Oatway et al. 2004; Rahman et al. 2006; Zeitz et al. 2002).

To test whether 5-HT3 receptors are involved in contralateral orofacial hyperalgesia, a 5-HT3 receptor antagonist, Y-25130 was microinjected into the contralateral Vi/Vc following CFA masseter muscle injection. Contralateral orofacial hyperalgesia was dose-dependently attenuated following Y-25130 (n=6) (Figure 5.2A). In contrast, Y-25130 microinjected into the contralateral Vi/Vc did not attenuate the ipsilateral hyperalgesia (Figure 5.2B). These results suggest that 5-HT3 receptor activation in the contralateral Vi/Vc facilitates the CFA-induced contralateral hyperalgesia.

To test whether the 5-HT3 receptor was involved in ipsilateral orofacial hyperalgesia, Y-25130 was microinjected into the ipsilateral Vi/Vc following CFA masseter muscle injection. Microinjection of Y-25130 into the ipsilateral Vi/Vc did not attenuate either ipsilateral or contralateral hyperalgesia (Figure 5.2C, D). These results provide evidence that coincides with the results seen in Figure 5.1 that showed 5-HT depletion did not affect the development of ipsilateral hyperalgesia and that ipsilateral hyperalgesia is predominantly driven via the ipsilateral Vc. These results suggest that descending 5-HT activation of 5-HT3 receptors in the ipsilateral Vi/Vc is not the

predominant mechanism involved in the development of ipsilateral orofacial hyperalgesia.

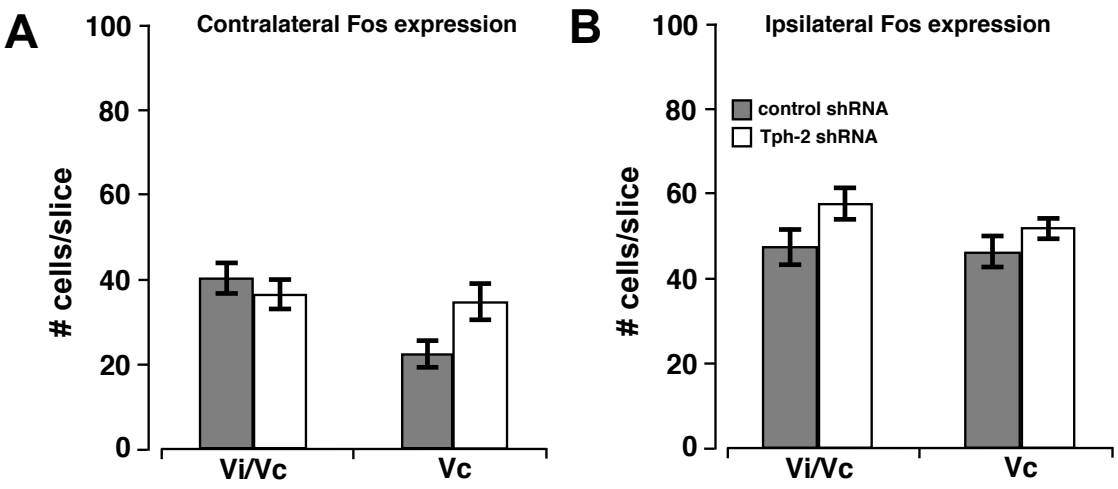
Figure 5.2: Vi/Vc 5-HT3 receptor inhibition attenuates contralateral hyperalgesia. **A.** Experiment sequence shows Vi/Vc cannulation 5-7 days before CFA masseter muscle injection. After 24 hours, the 5-HT3 receptor antagonist was injected into the Vi/Vc. Inhibition of the 5-HT3 receptor in the contralateral Vi/Vc attenuates CFA-induced contralateral hyperalgesia. CFA (0.05 ml; 1:1 oil/saline) was injected into the masseter muscle and 24 hours later, Y-25130 was microinjected (0.5 ml) into the contralateral Vi/Vc (**B, C**). Y-25130 microinjection into the contralateral Vi/Vc attenuated contralateral hyperalgesia (**B**) but not ipsilateral hyperalgesia (**C**). Y-25130 microinjection into the ipsilateral Vi/Vc (**D, E**) did not attenuate ipsilateral or contralateral hyperalgesia. +: lowest dose vs. saline. #: middle dose vs. saline. *: highest dose vs. saline. +,**#,*:** $p<0.05$**; ++**,##,**: $p<0.01$; +++,###,***: $p<0.001$

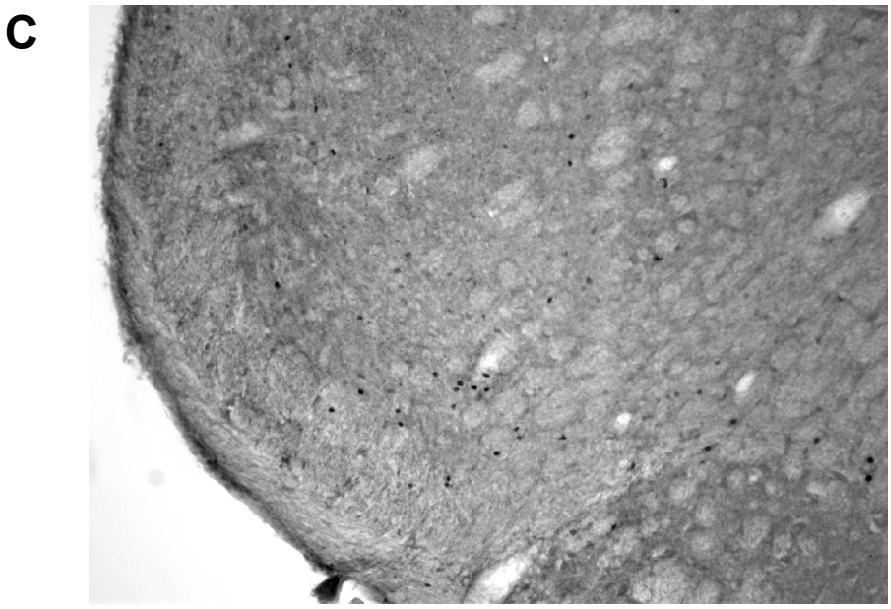

A
Vi/Vc cannula
5-7 days
masseter muscle CFA
24 hours
Vi/Vc Y-25130
30 min
Assess mechanical sensitivity
B
Contralateral
C
Ipsilateral
D
Contralateral
E
Ipsilateral
EF50 (g)
100
10
1
CFA + saline (n=6)
CFA + Y-25130 (12.9 nmol) (n=6)
CFA + Y-25130 (6.5 nmol) (n=6)
CFA + Y-25130 (2.6 nmol) (n=5)
CFA + saline (n=5)
CFA + Y-25130 (12.9 nmol) (n=6)
pre CFA
pre Y-25130 contra
pre Y-25130 ipsi
1.0
2.0
3.0
4.0
5.0
Time post drug (h)

5.3.3 Fos protein expression is not significantly different between the administration of control and Tph-2 shRNA in CFA-induced hyperalgesic rats

Previous research showed that CFA-induced masseter inflammation also results in bilateral neuronal activation in the Vi/Vc while ipsilateral neuronal activation is seen in the Vc (Imbe et al. 1999). We hypothesized that the activation of contralateral Vi/Vc neurons was to due descending facilitation from the RVM. Following the behavioral data (Figure 5.1) which shows a significant attenuation in contralateral hyperalgesia after 5-HT depletion we tested for Fos protein expression in neurons of the Vi/Vc and Vc to see if neuronal activation corroborates the behavior. We found that Fos protein expression in the Vi/Vc and the Vc showed no difference in Fos protein expression bilaterally (Figure 5.3A, B).

Figure 5.3: 5-HT depletion does not significantly reduce Fos protein expression in the contralateral ventral Vi/Vc. A. Immunohistochemical analysis of contralateral Fos protein expression in CFA-induced hyperalgesic rats after control and Tph-2 shRNA administration. **B.** Immunohistochemical analysis of ipsilateral Fos protein expression in CFA-induced hyperalgesic rats after control and Tph-2 shRNA administration. **C.** 10x magnified photograph of Fos protein staining in a CFA-induced hyperalgesic rat after control shRNA.





5.4 Discussion

In the spinal cord, administration of a 5-HT3 receptor antagonist inhibits evoked responses of dorsal horn neurons after noxious stimulation (Rahman et al. 2006) and intra-RVM cholecystokinin-induced mechanical and thermal hyperalgesia (Dogrul et al. 2009). Similarly, intrathecal administration of a 5-HT3 receptor antagonist attenuates formalin-induced hind paw flinching and spinal ERK activation (Svensson et al. 2006). We have previously shown that after the depletion of endogenous 5-HT while maintaining the viability and function of serotonergic neurons, descending facilitation is dependent on the activation of serotonergic neurons in the RVM (Wei et al. 2010). The present study shows that descending serotonergic facilitation is necessary for the development and maintenance of contralateral orofacial hyperalgesia after CFA-induced masseter inflammation. Furthermore, activation of contralateral Vi/Vc 5-HT3 receptors is necessary for the induction of CFA-induced contralateral orofacial hyperalgesia.

An interesting observation from this study is that shRNA treatment of Tph-2 in the RVM, which down regulates 5-HT bilaterally, did not affect ipsilateral hyperalgesia. One would expect that hyperalgesia would be attenuated bilaterally. Similarly, 5-HT3 receptor activation only appears to be necessary in the contralateral Vi/Vc for the development of contralateral hyperalgesia and does not affect the development of ipsilateral hyperalgesia. One possible explanation is that ipsilateral hyperalgesia is dominated by hyperexcitability and central sensitization in the Vc driven by peripheral input which masks the loss of descending facilitation seen through the 5-HT depletion and 5-HT3 receptor inhibition.

A recent study has shown that descending 5-HT3 activation in the Vc is involved in secondary hyperalgesia on the ipsilateral side in neuropathic pain conditions (Okubo et al. 2013). The results from Okubo et al. are different from my results in that they show an ipsilateral involvement with the descending serotonergic system as well as a Vc involvement in the mechanism of secondary hyperalgesia. However, 5-HT3 receptor activation in the ipsilateral STN is involved in the mechanism of secondary hyperalgesia similar to the contralateral Vi/Vc 5-HT3 receptor activation I have shown. Furthermore, Okubo et al. also show that the primary hyperalgesia is predominantly dependent on peripheral input. The Vc lesion experiment (Figure 3.4) provides evidence that the primary afferents of the masseter muscle are necessary for the development of the primary hyperalgesia seen on the ipsilateral side.

An alternative explanation is that the absence of attenuation of ipsilateral hyperalgesia may be related to the finding of varying degrees of descending inhibitory and facilitatory input into the contralateral and ipsilateral medullary/spinal dorsal horn. It has been shown that descending pain modulation is in dynamic balance between inhibitory and facilitatory signals (Vanegas and Schaible 2004). After hind paw inflammation, there is a time-dependent enhancement of descending inhibition on the ipsilateral side (Ren and Dubner 1996; Terayama et al. 2000). Descending 5-HT projections from the RVM also produce dual inhibitory and facilitatory effects due to activation of different 5-HT receptor subtypes (Bardin 2011). The net effect of descending modulation may be facilitatory on the contralateral side and inhibitory on the ipsilateral side in this masseter inflammation model. Thus, depletion of descending 5-HT on the ipsilateral side would reduce predominant inhibitory signals and the hyperalgesia

would be sustained.

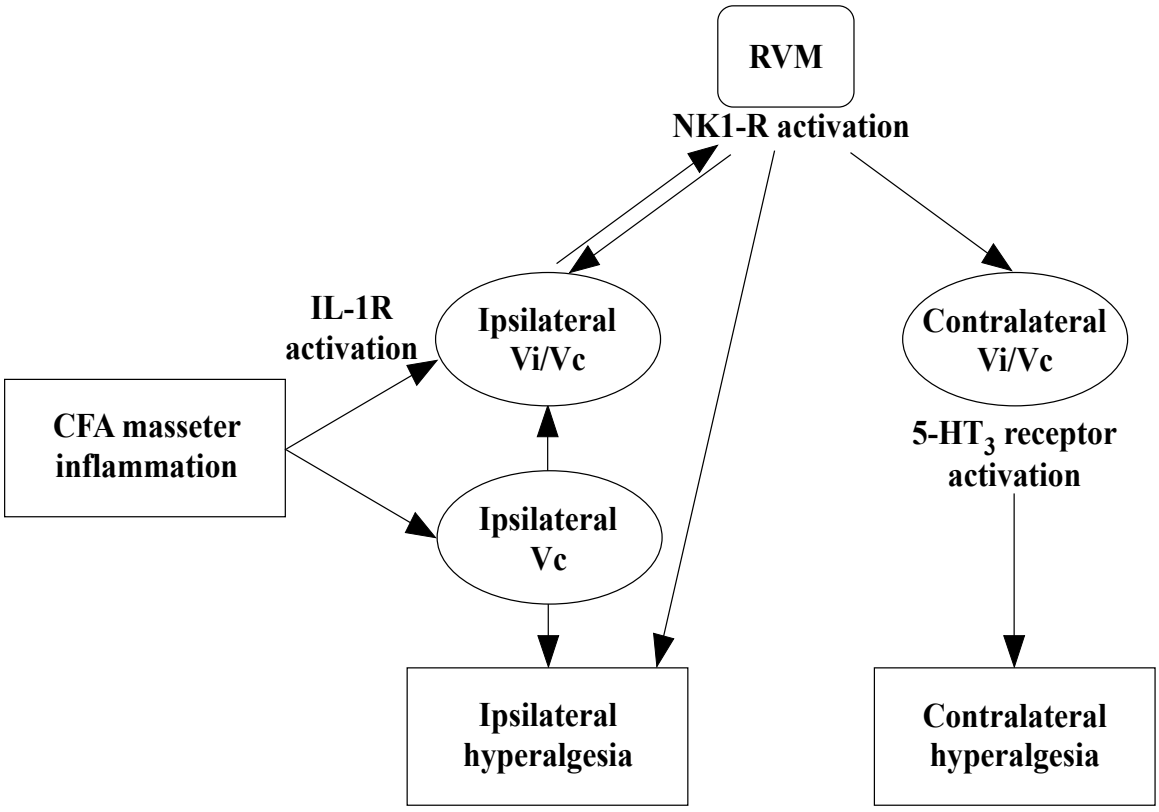
The Fos protein expression experiment did not generate further support for the behavioral data. However, the results should be explained with caution. Measuring Fos protein expression as a means of nociceptive activation comes with its own pitfalls. Fos protein expression is the expression of immediate early gene *c-fos* and is thus thought to suggest neuronal activation. It is known that not all activated neurons express Fos proteins such is seen in the dorsal root ganglion. Thus, this negative result may not be indicative of a lack of activation. Nevertheless, the behavioral data still provides evidence that suggests that 5-HT activation of 5-HT3 receptors in the contralateral Vi/Vc is involved in the development of contralateral hyperalgesia.

Chapter 6: General Discussion

6.1 Conclusion

Overall, our results support the hypothesis that the Vi/Vc–RVM circuitry mediates the development of hyperalgesia on the contralateral side after ipsilateral deep tissue injury. CFA-induced inflammatory orofacial hyperalgesia produces an increase in IL-1β activation of IL-1R in the ipsilateral Vi/Vc. IL-1R activation in the Vi/Vc ultimately leads to NK1-R activation in the RVM. This leads to activation of descending serotonin-containing RVM neurons that project to the Vi/Vc bilaterally. Activation of 5-HT3 receptors in the contralateral Vi/Vc results in deep tissue orofacial pain in the contralateral masseter muscle (Figure 6.1).

Figure 6.1: Vi/Vc-RVM circuitry is necessary for the development of contralateral hyperalgesia. The proposed pathway involved in the development of contralateral orofacial hyperalgesia following unilateral deep tissue inflammatory hyperalgesia. Unilateral CFA masseter inflammation leads to increased IL-1β activation of IL-1R in the ipsilateral Vi/Vc. Activation of the RVM through NK1-R activation leads to descending serotonergic facilitation of pain to the contralateral Vi/Vc. Activation of 5-HT3 receptors in the contralateral Vi/Vc leads to contralateral orofacial hyperalgesia. The arrows are not indicative of direct synaptic connections.



6.2 Ipsilateral STN

Our earlier research has shown that masseter muscle injection of CFA produces an inflammatory response and upregulation of IL-1β expression in astrocytes of the Vi/Vc transition zone (Guo et al. 2007). Glia research has shown that increased IL-1β expression and release due to noxious stimulation after injury can occur from microglia and astrocytes of the spinal trigeminal nucleus (STN) (Ren and Torres 2009). IL-1β activation of IL-1R results in PKC activation and phosphorylation of the NMDA receptor subunit GluNR1. Phosphorylation of the GluNR1 subunit increases NMDA

receptor activity leading to increased excitatory responses (Guo et al. 2007). This mechanism of IL-1R activation and NMDAR modulation in the ipsilateral STN is thought to participate in the development of ipsilateral orofacial hyperalgesia. However, our results suggest that Vi/Vc activation is not a predominant contributor. Lesion of the Vc suggests that Vc activation from masseter afferent neurons is the main mechanism involved in ipsilateral hyperalgesia.

The ascending pathway involved in the development of contralateral orofacial hyperalgesia remains unclear. Ascending Vi/Vc transition zone neurons have been shown to project to the submedius nucleus of the thalamus (Sm), the VPM of the thalamus, and the RVM (Ikeda et al. 2003; Sugiyo et al. 2005). It is possible that Vi/Vc activation leads to activation of RVM neurons, which then activates the descending serotonergic facilitation to the Vi/Vc of the contralateral side. It is also possible that ipsilateral Vi/Vc activation leads to activation of the sensory cortex via the thalamus, which then activates the descending modulatory PAG-RVM circuit. Activation of the RVM could then result in descending serotonergic activation of the contralateral Vi/Vc.

However, regardless of the ascending system involved, our research suggests that the primary mechanism involved in the development of ipsilateral and contralateral orofacial hyperalgesia are different. We have shown that ipsilateral hyperalgesia is unaffected when descending serotonergic facilitation is inhibited. On the other hand, contralateral hyperalgesia is unaffected with the lesion to the ipsilateral Vc. Similarly, ipsilateral hyperalgesia persisted after RVM lesion. This same lesion did produce an attenuation to the contralateral hyperalgesia. Thus it appears that the Vi/Vc-RVM circuitry is the primary pathway involved in contralateral orofacial hyperalgesia while the

masseter afferent – Vc activation is the primary pathway involved in ipsilateral orofacial hyperalgesia.

6.3 RVM

Activation of the descending modulatory circuit involves the activation of NK1-R containing neurons in the RVM. We showed that NK1-R activation in the RVM attenuates bilateral orofacial hyperalgesia. This data suggests that NK1-R activation plays a facilitatory role. Supporting this hypothesis, substance P can be released from neurons in the PAG (Nakaya et al. 1994).

We further showed that serotonin released from the RVM is involved in the facilitation of hyperalgesia on the contralateral side. Interestingly, research suggests that RVM neurons containing serotonin do not express NK1-R (Zhang and Hammond 2009). It is possible that substance P activation of NK1-R occurs on RVM interneurons which then activates serotonin release. This possibility would suggest that the downstream action of NK1-R-containing neurons is within the RVM before lateralization of the descending projections, which may result in a bilateral effect. Another possibility would be that the descending serotonin system modulates the glutamatergic synapse of the NK1-R-containing RVM neurons in the STN. This possibility would suggest that the downstream action of NK1-R-containing neurons is within the STN, which may result in a unilateral effect. These possibilities are purely speculations and would require further research to determine the cellular mechanisms involved.

6.4 Contralateral STN

Pain referred to the contralateral side is a phenomena that appears exclusive to the trigeminal system (Ambalavanar et al. 2006; Shimizu et al. 2009a). CFA injection in to the orofacial region resulted in hyperalgesia in the contralateral face as well as the contralateral hind paw. While hind paw inflammation induced by large amounts of intramuscular CFA can lead to referred pain, the pain remains localized to the ipsilateral side (Urban et al. 1999). It is possible that this is due to a difference in descending mechanisms. We have suggested that the RVM-Vi/Vc transition zone circuitry may be the difference between the trigeminal and spinal system.

Our previous research showed that CFA-induced masseter inflammation also results in bilateral neuronal activation in the Vi/Vc while ipsilateral neuronal activation is seen in the Vc (Imbe et al. 1999). We hypothesized that the activation of contralateral Vi/Vc neurons was to due descending facilitation from the RVM. Our results provide behavioral evidence that mirrors the Fos protein data previously seen in CFA-induced hyperalgesic rats.

We showed that descending serotonin activation of 5-HT3 receptors in the contralateral Vi/Vc transition zone was involved in the development of contralateral hyperalgesia. Our results also suggest that removal of descending serotonergic facilitation is not enough to decrease the neuronal activation in the ipsilateral Vi/Vc. Depending upon the site of modulation, 5-HT3 receptor activation can have varying effects. Similar to other neuromodulators, presynaptic modulation can lead to increased release of various neurotransmitters (Funahashi et al. 2004). Postsynaptic modulation can lead to

depolarization (Ronde and Nichols 1998). It is possible that the descending serotonin activation of 5-HT3 receptors in the contralateral Vi/Vc leads to modulation of the neurotransmitters released from primary afferent fibers of the masseter muscle. It is also possible that activation of 5-HT3 receptors modulate the excitability of the second order neurons projecting to the supraspinal structures of the brain. Lastly, it is possible that the activation of 5-HT3 receptors in the Vi/Vc can modulate the descending signals of the NK-1 containing neurons.

Although we have shown that depletion of 5-HT from the RVM resulted in an attenuation of the contralateral hyperalgesia, the mechanism remains unknown. Further experimentation would be needed to establish whether masseter muscle inflammation results in increased synaptic release of 5-HT and/or changes in 5-HT receptor expression.

6.5 Research implications

Conditions of chronic pain have been shown to persist in patients for a lifetime despite the removal or resolution of the injury (van Wilgen and Keizer 2012). Many instances of TMJ pain have also persisted through the life of a patient despite the therapies given (Sidebottom et al. 2011). The hyperalgesia seen in chronic pain patients is believed to be a product of neuronal plasticity and central sensitization (Ren and Dubner 1999; Woolf and Salter 2000). Animal models of chronic pain research have always attempted to mimic the symptoms of hyperalgesia and the central sensitization seen in these chronic pain patients. Intramuscular CFA injection has been shown to induce profound inflammatory hyperalgesia that lasts up to 3 weeks (Ren 1999; Ambalavanar et

al. 2005; Shimizu et al. 2009a). Nerve injury models can produce hyperalgesia that persists up to 2 months after injury (Bennett and Xie 1988). The current animal models of persistent pain have shown neuronal and glial changes in the central nervous system following injury (Sugiyo et al. 2005; Guo et al. 2007; Shimizu et al. 2009a; Shimizu et al. 2009b; Wei et al. 2010).

However, differentiation between primary and secondary hyperalgesia is not very precise in current animal models of pain. When testing for secondary hyperalgesia one must test sites at varying distances from the site of injury. Therefore it is unknown whether the site tested is in fact referred or within the receptive field of neurons directly activated by the injury. Our research provides a novel model of testing descending modulation in pain without confounding factors such as the field of innervation and possible crossover innervation that is seen with unilateral pain. This model allows clear differentiation between primary and secondary hyperalgesia.

Current models also fail to differentiate the mechanism being tested of the secondary hyperalgesia seen. It is unknown whether the secondary hyperalgesia seen is a result of segmental activation due to direct spread to local neurons not receiving input from the injured zone or due to the involvement of descending mechanisms. Our research shows that the secondary hyperalgesia is seen at the contralateral site due to descending mechanisms and not to cross innervation of primary afferent neurons or brainstem neurons. This allows one to develop experiments involving the descending modulatory mechanism in secondary hyperalgesia.

6.6 Clinical implications

Our research focuses on the involvement of descending modulation of secondary hyperalgesia in the orofacial region. Clinical symptoms of secondary hyperalgesia can be seen amongst chronic pain disorders (Goldberg 1997; Schultz and Melzack 1999; Pourrier et al. 2010). It is possible that pain outside the zone of injury seen in human chronic pain conditions may involve descending mechanisms as well. This would suggest that primary and secondary hyperalgesia in humans may result from different pathways and mechanisms suggesting that different treatment modalities might be necessary to alleviate all the pain.

Our research also suggests that the ascending nociceptive system from primary afferents to the Vc is the dominant mechanism involved in primary hyperalgesia (pain at the site of injury). Although it is possible that descending facilitation is involved in primary hyperalgesia, the role of the Vi/Vc appears minimal. Our findings suggest that the influence of the descending modulation evoked by the activation of Vc may differ from that evoked by activation of the Vi/Vc transition zone. The former may be related to sensory discriminative features and the latter to more reflexive behaviors.

While our model focuses on the secondary hyperalgesia seen on the contralateral side, usually TMJD is seen unilaterally in human presentations. It is our speculation that the contralateral hyperalgesia seen in rats is mainly due to the robust inflammatory response that is seen after CFA administration. The severity of inflammation seen in rats after CFA appears to be greater than the inflammatory response seen in the human condition. It is possible that the development of contralateral pain in humans may be

dependent on the severity of the injury in the TMJ. Upon further speculation, the uniqueness of the TMJ and the mandatory bilateral function of the TMJ provide an anatomical possibility for why contralateral hyperalgesia may be necessary for protection from further injury to the joint. If the joint is severely injured unilaterally, bilateral symptoms of pain would prevent all attempts at movement.

6.7 Future Directions

This research provides evidence that descending modulatory mechanisms in pain are involved in the development of secondary hyperalgesia in the trigeminal system. More specifically, this research shows that the Vi/Vc-RVM circuitry is involved in orofacial pain evoked from outside the zone of injury. However, many questions still remain.

Much is unknown regarding the neuronal mechanisms involved in the supraspinal structures involved with this model of secondary hyperalgesia. It is still unknown what the ascending circuitry is that leads to the activation of the RVM. We are unsure of supraspinal structures and chemical mediators involved after ipsilateral Vi/Vc activation. It is also unknown how the substance P/NK1-R system of the RVM modulates the descending serotonin system.

It is also unknown whether the Vi/Vc-RVM circuitry is involved in other models of secondary hyperalgesia. Is descending facilitation from the RVM involved in secondary hyperalgesia on the ipsilateral side? Is descending facilitation from the RVM

involved in secondary hyperalgesia models when the injury is not located in the orofacial region? To answer these questions, behavioral experiments involving manipulation of the descending system would need to be tested in other persistent pain models involving secondary hyperalgesia.

However despite the many questions still left unanswered, we have provided a novel model for testing the role of descending modulation in pain outside the zone of injury. While it has been hypothesized that descending modulatory signals differ between the primary site of injury and sites of secondary pain, it has been difficult to test. This model of pain provides a clear behavioral testing site for secondary hyperalgesia that is clearly distinct in innervation from the site of injury.

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