THE EFFECT OF PAG STIMULATION EVOKED DORSAL ROOT REFLEXES IN

NEUROGENIC INFLAMMATION

by

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Presented to the Faculty of the Graduate School of

The University of Texas at Arlington in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE IN PSYCHOLOGY

THE UNIVERSITY OF TEXAS AT ARLINGTON

AUGUST 2009

ACKNOWLEDGEMENTS

Firstly, I'd like to thank my supervisor, Dr. Yuan B. Peng, not only for giving me the opportunity of working in his research group, but also for his continuous support, encouragement and guidance throughout this work and my life of graduate school. I'd also like to express my sincere appreciations to my thesis committee, Dr. Perry N Fuchs and Dr. Linda I Perrotti, for their time and valuable suggestions.

All the help and support I received from members (present and past) of my research group made my life easier, especially at the beginning of my work. Many thanks go to Chris Hagains, Jiwei He, Lara Kachlic, Magan Uhelski and Matthew Davis for being such good colleagues and friends. Also I need to thank the animal care facility for their assistance.

I was lucky to have the company of so many good friends, who were always there whenever I needed some help, during my time in Arlington. Those two years would definitely not have been the same without all those good friends.

I want to thank all faculty and staff of the Psychology Department for supporting me during last two years. I always appreciate the opportunity presented to me by the Graduate School and the University of Texas at Arlington in pursuing graduate studies at the UTA.

Finally, I would like to dedicate this work for my loving parents and my husband who always encouraged and supported me throughout my career and education.

July 10, 2009

ABSTRACT

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Neurogenic inflammation is defined as inflammation produced by the sensory nerves. It contributes to the cardinal signs of inflammation: rubor (redness), tumor (swelling), color (heat), and dolor (pain or itch). It suggested that neurogenic inflammation is due to dorsal root reflex (DRR) and axonal reflex, which are antidromic electrical impulses of primary afferents. However, the central regulatory mechanisms of neurogenic inflammation are still unclear. It is known that electrical stimulation of the periaqueductal gray (PAG) can induce release of GABA in the spinal cord. GABA can act on $GABA_A$ receptors on the central terminals of the primary afferents to generate DRRs. Therefore, it was *hypothesized* that cutaneous vasodilatation would increase from stimulation of the PAG and that the vascular response would be attenuated by applying bicuculline ($GABA_A$ receptor antagonist) to the L4-L6 dorsal root entry zones. Furthermore we predicted that the vascular response for PAG stimulation is due to activation of descending inhibition pathways rather than activation of autonomic nervous system. Seventeen adult Lewis rats were used: three animals to determine stimulation parameters; seven for the PAG stimulation group; and seven for the Bicuculline group. DRRs were recorded from L4 or L5 dorsal roots. Blood perfusion in both hind paws was measured by a Laser Doppler Imager. In the first 3 rats, fifteen images were taken as baseline measurements and continuous images

were taken for 30 minutes following stimulation of PAG (5V, 10V, 15V, and 20V; 1 Hz; 1.0 ms duration) for 20 sec. The PAG stimulation group followed the same methodology using the established optimum stimulation parameters, and the Bicuculline group differed from the PAG group by applying bicuculline to the dorsal roots entry zones before electrical stimulation. The mean arterial blood flow was calculated by selecting a region of interest (ROI). Two ROIs were outlined around each hind paw, and percent change scores were calculated to normalize mean responses. A 2 (group) x 2 (side) x 3 (stimulation) mixed ANOVA followed by Fisher's LSD was used for statistical analysis. The blood perfusion on both sides significantly increased following stimulation of PAG. Increased blood perfusion following PAG stimulation was attenuated by bicuculline. The results were consistent when compared between percentage values and mean blood perfusion values. One issue was a failure to observe an increase of DRRs following stimulation of PAG. There was no change in heart rate, suggesting no involvement of autonomic nervous system. The present study concluded that the DRR plays a significant role in causing cutaneous neurogenic inflammation, and descending inhibition can influence the development of neurogenic inflammation. Furthermore, these results demonstrate a role of the central nervous system in neurogenic inflammation.

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CHAPTER 1

INTRODUCTION

Inflammation is a complex biological response of the vascular immune systems to an injury or infection that occurs in the affected tissues. It is a type of protective response mechanism. Neurogenic inflammation is one type of the inflammatory process that involves the nervous system. Inflammatory agents stimulate the nervous system, which propagates electrical signals to higher centers related to nociception. Under certain circumstances, these electrical signals propagate antidromically through primary afferent fibers (PAFs) toward the peripheral terminals linking the immune system and neuroendocrine system for an integrative inflammatory response. Neurogenic inflammation is identified as a form of response contributing to pain and wound healing in injury if the system functions effectively. However, excess and inadequate responses lead to pathological conditions, such as migraine and diabetes. This inflammatory process includes vasoactive neuropeptides such as substance P (SP), calcitonin gene related peptide (CGRP), and neurokinins released by peripheral terminals of myelinated (Aδ) and unmyelinated (C fibers) PAFs. Clinically this inflammatory response can be observed as erythema, oedema, and pruritis in healthy skin and as pathological conditions, such as psoriasis and dermatitis (Myers, Campana, & Shubayew, 2006). Most physiological and clinical studies have been done to understand the peripheral and central regulatory mechanisms of neurogenic inflammation as evidence for efferent functions of sensory (afferent) nerves, but the supraspinal components of neurogenic inflammation are still not fully understood. The overall aim of this study is to explore the mechanisms of periaqueductal gray (PAG) activation in cutaneous neurogenic inflammation. It is known that electrical stimulation of the PAG can induce the release of GABA in the spinal cord (Lin, Peng, & Willis, 1994; Lin, Peng, & Willis, 1996c; Peng, Lin, & Willis, 1996a). GABA can then act on $GABA_A$ receptors located on the central terminals

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of the primary afferents, which may generate dorsal root reflexes (DRRs) (Eccles, Schmidt, & Willis, 1963; Peng, et al, 2001). Therefore, it was hypothesized that cutaneous neurogenic inflammation would be detected following stimulation of the PAG, and this inflammatory response could then be attenuated by blocking $GABA_A$ receptors (applying bicuculline). Furthermore, it was predicted that the vascular response to PAG stimulation would be due to activation of descending pain inhibition pathways but not the autonomic nervous system (stress analgesic mechanism).

1.1 Neurogenic inflammation and Antidromic vasodilatation

Cutaneous neurogenic inflammation is a skin response brought on by PAFs activated by tissue injury. Neurogenic inflammation has three major components (Lewis & Zotterman, 1927): flare (vasodilatation), wheal (plasma extravasation due to increase of permeability of blood vessels), and redness due to dilatation of small vessels. These three components are known as the triple response (Chahl, 1988). Neurogenic inflammation, however, is not limited to the triple response. Other signs of inflammation include pain and high temperature. The vascular response of neurogenic inflammation is mediated by vasoactive neuropeptides, such as SP and calcitonin gene-related peptide (CGRP). These neuropeptides are released by the peripheral terminals of PAFs, more specifically C-fibers and Aδ fibers. SP, neurokinin A, & neurokinin B (tachykinins) cause plasma extravasation due to an increase of space between the endothelial cells of venules, by contracting endothelial cells (Fuller, el al., 1987; Jacques, Couture, Drapeau, & Regoli, 1989). SP also causes vasodilatation (Carpenter & Lynn, 1981). CGRP acts as a vasodilator in neurogenic inflammation (Fuller et al., 1987). CGRP reduces SP degradation. Furthermore, SP and CGRP facilitate the release of histamine, prostaglandins and serotonin by degranulating mast cells (Barnes, 2008). These neuropeptides act as neurotransmitters, immune and inflammatory mediators and in turn modulate the inflammatory process.

Evidence for three neurological components of neurogenic inflammation have been shown in previous studies: DRR; axonal reflex; and the sympathetic nervous system. PAFs

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mainly convey the peripheral information to the spinal cord and stimulate ascending somatosensory pathways to higher centers to generate perception. These fibers also have the ability to convey action potentials antidromically to the periphery leading to the release of the vasoactive neuropeptides that cause neurogenic inflammation. This antidromic nerve conduction can either be axonal reflex and/or dorsal root reflex. Most studies have suggested that neurogenic inflammation is a result of axonal reflex and DRR. Bruce found evidence that suggests vasodilatation is due to collaterals of sensory neurons in the peripheral terminals; this was thought to be axonal reflexes (Bruce, 1913). It was theorized that the flare component of the triple response in cutaneous inflammation is mediated by neurotransmitters released through axon collaterals, i.e. axonal reflexes (Lewis et al., 1927). This suggests that when one branch from an axon of a sensory neuron is stimulated by an irritant or trauma, other branches terminate on blood vessels signals for producing neurogenic inflammation. The findings of previous experiments have suggested that DRR has significant effect on neurogenic inflammation in terms of blood perfusion. Further they could reduce blood perfusion with blocking these antidromic impulses by applying $GABA_A$ and NMDA receptor antagonists (Lin, Wu, & Willis, 1999). There is also evidence for enhancement of antidromic vasodilatation followed by electrical stimulation of Aδ and C-fibers (Lynn, 1988), and attenuation of antidromic vasodilatation followed by the elimination of C-fibers following treatment of capsaicin (Low & Westerman, 1989). Inflammation also activates the sympathetic nervous system. The sympathetic nervous system may causes vasoconstriction and can reduce the inflammatory signs(Lin et al., 1999). The symptoms of neurogenic inflammation are a balance among these three mechanisms.

1.2 Dorsal root reflexes

Nociceptive impulses generated in the periphery propagate through PAFs and enter the spinal cord. In the spinal cord they excite GABAergic interneurons leading to the release of GABA. GABA then stimulates $GABA_A$ receptors in the central terminals of PAFs and causes primary afferent depolarization (PAD) by opening chloride channels (figure A.1). Furthermore, it

was suggested that interneurons facilitate PAD by opening K^+ channels by increasing extracellular concentration of K^+ (Willis, 1999). This PAD can generate DRR, which propagates through myelinated and unmyelinated PAFs toward the periphery. It was first indentified by Gotch and Horsley in 1891 (Willis, 1999). PAD was measured by intracellular recording from PAFs and by extracellular field potentials (Eccles et al., 1963). The DRRs were recorded in Aδ fibers and C-fibers (Willis, 1999).

Increases of antidromic vasodilatation after dorsal root stimulation have also been observed (Hinsey & & Gasser, 1930). $GABA_A$ antagonists applied to the spinal dorsal horn have been found to reduce DRRs, which in turn reduced the amount of flare caused by capsaicin; this indicates that the spinal cord can modulate neurogenic inflammation through DRRs (Lin et al., 1999). Another neurotransmitter involved in DRR generation is serotonin. The $5\text{-}HT_3$ agonist ondansetron has been used to increase DRRs following its application to the spinal dorsal horn (Peng et al., 2001).

There is electrophysiological and chemical evidence for spinal cord regulation in inflammatory conditions. Increased responses of dorsal horn neuronal activity has been identified in an acute arthritis model (Schaible, Schmidt, & Willis, 1987). The increase of glutamate in dorsal horn neurons following arthritis indicated that the central nervous system participates in peripheral inflammatory conditions (Sluka & Westlund, 1992).

1.3 Gamma Amino Butyric Acid (GABA)

GABA is the major inhibitory neurotransmitter in the central nervous system. GABA is produced in the gray matter by converting glutamic acid to GABA and it is highly concentrated in the brain and spinal cord, especially in the vesicles in presynaptic terminals. There are two types of GABA receptors: $GABA_A$ (ionotropic) and $GABA_B$ (metabotropic). Both types of receptors are found extensively in the dorsal horn, especially in the superficial laminae, central terminals of PAFs and projection neurons. In the central nervous system, these receptors open chloride channels and hyperpolarize the neuron by facilitating Cl^- influx because the equilibrium potential for Cl^- is more negative than the resting membrane potential. However, primary

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afferent neurons have a high concentration of intracellular Cl^- due to Na^+-K^+-Cl^- co-transporter; therefore, the opening of Cl^- channels facilitates a Cl^- efflux from PAFs to depolarize the plasma membrane and increase excitability (Willis, 1999). $GABA_B$ receptors are linked with Ca^{2+} or K^+ ion channels. They act through a second messenger system (Cooper & Bloom, 2003; Curtis, et al, 1997). The $GABA_B$ receptors facilitate presynaptic inhibition in dorsal horn ganglionic cells by blocking voltage-gated Ca^{2+} channels (Curtis et al., 1997). Furthermore, they observed that $GABA_B$ receptors alone did not affect PAD (Curtis & Lacey, 1994). GABA facilitates postsynaptic inhibition by acting on $GABA_A$ receptors in projection neurons in the dorsal horn. It facilitates presynaptic inhibition by acting on $GABA_B$ receptors in PAFs.

Previous research suggests that GABA depolarizesand increases the membrane conductance of dorsal root ganglionic cells in Aδ and C-fibers (Curtis, Headley, & Lodge, 1984; Nishi, Minota, & Karczmar, 1974); depolarize and increase the excitability of PAFs in the dorsal root ganglion (Curtis et al., 1984); and reduce the dorsal root potentials and increase the dorsal root reflexes (Eccles et al., 1963). Application of GABA on dorsal horn of the spinal cord depolarizes all PAFs (A-delta, A-beta and C), providing evidence for site of drug action (Evans, 1985). There is electrophysiological evidence for enhancement of DRRs after topical application of 1mM of GABA on dorsal root entry zones and attenuation of DRRss after application of $GABA_A$ antagonists in the same manner (Peng et al., 2001). Several groups have found that the effect of $GABA_B$ receptor antagonists on DRRs is weak or absent (Willis, 1999). The preliminary studies demonstrated an increase of cutaneous blood perfusion following application of GABA to the entry zones of dorsal roots and a reduction of the vascular response to GABA after a dorsal root rhizotomy (Herath, et al, 2008).

1.4 5-HT$_3$ receptors

Besides GABA, some other neurotransmitters (glycine, excitatory amino acids, and serotonin) cause primary afferent depolarization. Serotonin is one of two major neurotransmitters involved in primary afferent depolarization (Peng, Kenshalo, & Gracely, 2003).

Immunocytochemical studies have revealed that serotoninergic neurons project from midbrain structures, especially from the PAG and nucleus raphe magnus (NRM), to the spinal cord (Cooper et al., 2003). Furthermore 5-HT$_3$ receptors are highly concentrated in the superficial dorsal horn, at all levels of spinal cord and on PAFs (Peng, Lin, & Willis, 1996c). Similar to GABA$_A$ receptors, 5-HT$_3$ receptors depolarize the central end of PAFs and cause presynaptic inhibition by opening Na$^+$ and K$^+$ channels (Derkach, 1989). There is electrophysiological evidence for an increase of antidromic nerve conduction in Aδ and C fibers following application of 5-HT$_3$ agonists and a decrease of antidromic nerve conduction with 5-HT$_3$ antagonists (Peng et al., 2001).

1.5 Periaqueductal gray area

The (PAG) is the area that surrounds the entire length of the cerebral aqueduct. It is involved in a wide variety of biological functions including fear and anxiety, defense behaviors, vocalization, respiration, and cardiovascular control. Furthermore, this area is widely known as a center for modulating nociceptive signals to the brain for decreasing pain. Many investigators have done deep brain stimulation studies of the PAG for for the treatment and understanding of chronic pain. Morphologically the PAG can be divided into four divisions: medial, dorsolateral, ventrolateral, and dorsal. The dorsolateral and ventrolateral areas are highly concentrated with neurons (Beitz, 1985). The PAG has afferent connections with the forebrain, diencephalon, and nociceptive neurons, which explains its functional characteristics. Retrograde axonal tracing has been used to trace prefrontal cortical projections to the PAG (An, et al, 1998). This method also demonstrated input from the medial and posterior prefrontal cortex, orbital areas, and dorsomedial areas. They also found that some subcortical areas, the central nucleus, the ventral part of the basal nucleus of amygdala have efferent connections to the PAG. The investigations for afferent connections of the PAG revealed that it has direct projections to basal forebrain areas, hypothalamus, nucleus cuneiformis, substantia nigra, ventral tegmental area, locus ceruleus, parabrachial nuclei, medullary reticular formation and NRM (Beitz, 1982). The PAG has two types of efferent projections, one ascending and the other descending in nature.

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There are three types of ascending projections: ascending ventral projections, dorsal periventricular projections, and ventral periventricular projections. The ascending ventral projections communicate with the ventral tegmental region, hypothalamus and amygdala. The dorsal periventricular projections connect with thalamic nuclei and ventral periventricular projections connect with the hypothalamus and preoptic area (Eberhart, Morrell, Krieger, & Pfaff, 1985). One of the important descending projections of the PAG goes to the NRM, which continues to descend to the dorsal horn of the spinal cord. This communication link plays an important role in descending pain inhibition. The PAG has descending projections to a variety of brain stem nuclei including the locus ceruleus, pontine nuclei and the parabrachial neuclei which contain noradrenergic neurons (Mantyh, 1983; Millan, 2002). The connection between the PAG and NRM contains serotonergic neurons (Millan, 2002). These afferent and efferent connections with limbic system, thalamus, NRM, RVM, locus ceruleus and dorsal horn neuron help to explain the pain regulating ability of the PAG. Furthermore, these connections suggest that the PAG acts as a communicator between the forebrain, brain stem and spinal cord.

There is plenty of electrophysiological and chemical evidence for pain modulation after stimulating various supraspinal structures, but there is little evidence for exact supraspinal sites for the origin of descending pain inhibition or facilitation pathways. Noradrenalin and serotonin are the most investigated neurotransmitters in the descending pain pathways; however, the PAG is one of the main supraspinal structures, which initiates descending inhibition. Electrical stimulation of the PAG causes release of GABA, serotonin, norepinephrine, glycine and opioids in the spinal cord. These neurotransmitters cause inhibition of dorsal horn neuron activity by activating $GABA_A$, $5HT_1$, $Alpha_2$, NMDA and opioid receptors (mu, delta) in dorsal horn respectively (Peng, Lin, & Willis, 1996a; Peng, Lin, & Willis, 1996c). It indicates that multiple neurotransmitters and multiple classes of receptors are involved in PAG induced descending pain control. The major descending analgesic mechanisms are serotonergic system, noradrenergic system and opiate system.

There is anatomical evidence for serotonin receptors distributed in the superficial laminae of the dorsal horn throughout spinal cord and on the central ends of PAFs (Basbaum & Fields, 1984). It was assumed activation of serotonergic pathway by electrically stimulating PAG and NRM stimulate GABAergic interneuron by activating $5HT_3$ receptors and inhibit projection neurons and PAF via $5HT_1$ receptors (Fields & Basbaum, 1978). The activation of GABAergic interneurons facilitates pain inhibition by releasing GABA onto projection neurons of the dorsal horn. PAG descending inhibition via serotonin neurons in the NRM has been confirmed by electrophysiological studies. Morphine induced and electrically induced PAG pain inhibition can be attenuated following application of $5HT_1$ and $5HT_3$ receptor antagonists using electrophysiological and behavioral studies. Furthermore, they observed increase of descending inhibition with $5HT_{1A}$ agonist (Millan, 2002). Serotonin may directly cause PAD by activating $5HT_3$ receptors and indirectly by activating GABAergic interneuron. Glycine has a synergistic effect with GABA for spinal antinociception. Also pain inhibition via PAG stimulation can be started with opiate neurons. They excite GABAergic inhibitory interneurons in the spinal cord and facilitate descending pain inhibition (Millan, 2002). $GABA_A$ antagonists and glycine antagonists attenuate PAG induced pain inhibition (Lin et al., 1994; Lin, Peng, & Willis, 1996b); however, the $GABA_B$ receptor does not appear to play a significant role in PAG descending inhibition. There is anatomical evidence for GABAergic efferent neurons from the NRM to the spinal cord (Reichling & Basbaum, 1990). GABA may also be released by bulbospinal neurons and GABAergic interneurons by stimulating $5HT_3$ receptors during descending pain inhibition.

There is little evidence linking supraspinal structures to the generation of DRRs. It has, however, been observed that DRRs increase following electrical stimulation of PAG (Peng et al., 2001). This appears to be because stimulation of the PAG leads to the release of serotonin in the spinal cord. Most neurotransmitters released in response to PAG stimulation induce descending pain inhibition indirectly by exciting GABAergic interneurons. The release of GABA during activation of PAG descending pain modulation induces dorsal root reflexes.

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1.6 PAG stimulation and cardiovascular effects

The PAG acts as a vasomotor center through its involvement in regulation of blood pressure, vascular tone, regional blood flow, and heart rate. The PAG has afferent and efferent connections with other major vasomotor areas, such as RVM, hypothalamus, NTS, and raphe nuclei. The evidence for cardiovascular changes following PAG stimulation suggest that the PAG contains neurons that excite both hypertension and hypotension responses. There is evidence for increased heart rate, vasodilatation in hind limbs and hyperpnoea following stimulation of the dorsal PAG (Lovick, 1985) and increased arterial blood pressure following stimulation of NRM. Furthermore, it was observed that stimulation of deeper areas in the PAG have less autonomic effect. It was suggested that increase of arterial blood pressure might be a defensive mechanism, due to activation of sympathetic nervous system via the dorsal PAG (Zhuo & Gebhart, 1997). Contrary to that, one clinical study observed the relationship of blood pressure changes with PAG stimulation. A linear relationship of reduction of arterial blood pressure with reduction of pain due to PAG stimulation was observed. It was speculated that these results may be caused by reduced arterial blood pressure following pain reduction, due to PAG stimulation and reduction of sympathetic drive. However, a relationship of reduction of pain due to PAG stimulation with peripheral blood flow could not be elicited (Green et al, 2006).

Neurophysiological and neuroanatomical studies suggest that there are functional differences between cell columns of the PAG. Stimulation of ventral columns facilitates hypotension responses with bradycardia, and stimulation of dorsal and lateral columns induce hypertension responses with tachycardia. The hypotension and bradycardia following stimulation of ventral PAG was explained by an increase of parasympathetic drive. Increase of peripheral blood flow due to DRRs may cause hypotension. Serotonin, inhibitory amino acids, and noradrenalin play a major role in cardiovascular modulation similar to pain modulation during PAG simulation (Rossi, Maione, & Berrino, 1994). The results of cardiovascular changes due to PAG stimulation has varied between studies. These studies though had some methodological variations: level of anesthesia, site of stimulation, etc.

Even though most studies concentrate on cardiovascular changes following PAG stimulation, the response of peripheral cutaneous blood perfusion following PAG stimulation has not been demonstrated. The theory of activation of a defense mechanism during PAG stimulation suggests that blood perfusion to the skin would be reduced, due to redirection of regional blood flow distribution. However, cutaneous blood perfusion should increase following PAG stimulation considering the dorsal root reflex concept. Therefore, the present study assumed that cutaneous neurogenic inflammation would be increased with PAG stimulation, due to an increase of DRRs. Furthermore, it was designed to demonstrate the role of GABA in PAG stimulation induced cutaneous neurogenic inflammation.

<u>1.7 Experimental hypothesis and objectives</u>

This investigation was conducted to better understand the link between the immune system and nervous system during inflammatory responses. The overall aim of this study is to determine the role of central regulatory mechanisms in cutaneous neurogenic inflammation. To examine the central regulatory mechanisms, we evoked DRRs by electrically stimulating the PAG. Cutaneous blood perfusion was measured as cutaneous neurogenic inflammation.

Specific aim 1*: Determine the contribution of PAG stimulation in neurogenic inflammation*

The first two experiments were run to test the hypothesis that cutaneous neurogenic inflammation would be increased with PAG stimulation. The first experiment was carried out to determine the optimum stimulating parameters for the second experiment. It was predicted that peripheral blood perfusion would increase with the increase of stimulating voltage.

Electrical stimulation of PAG activates descending inhibitory pain pathways through the NRM. Most neurotransmitters, including serotonin, noradrenalin, and glycine, stimulate GABAergic interneurons and facilitate the release of GABA. Then GABA acts on $GABA_A$ receptors on PAFs and induces DRRs. Serotonin acts on $5\text{-}HT_3$ receptors in PAFs and induces DRRs directly. DRR causes the release of vasoactive substances in the peripheral tissues and facilitates neurogenic inflammation.

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However, some studies suggest that PAG stimulation can increase arterial blood pressure due to sympathetic activation. Therefore, increase of arterial dilatation might be a balance between sympathetic activation and DRR.

Specific aim 2*: Determine the effect of spinal GABA on the response of neurogenic inflammation induced by PAG stimulation.*

It was also hypothesized that the blood perfusion increase from PAG stimulation would be attenuated by application of bicuculline to the spinal cord. This hypothesis was proposed based on the fact that the vascular response for stimulation of the PAG would be due to activation of descending inhibition pathways rather than activation the autonomic nervous system.

As explained above, most supraspinal descending mechanisms stimulate the release of GABA in the spinal cord. Therefore, bicuculline, a competitive $GABA_A$ receptor antagonist, was applied on the spinal cord to block $GABA_A$ receptors. It was expected that it would reduce PAD and inhibit PAG stimulated DRR. Therefore this experimental component had minimal effects from DRRs on cutaneous neurogenic inflammation. However, it was expected that the effect of sympathetic drive would remain unchanged.

CHAPTER 2

METHODS

All National Institutes of Health guidelines for the care and use of laboratory animals were followed in this study. All surgical procedures and treatments were conducted according to the guidelines of the Committee for Research and Ethical Issues for the study of pain (Zimmermann, 1983).

2.1 Subjects

Nineteen adult (10-12 month old) male, Lewis rats were used for this study. Rats weighed about 450 – 550g each. Lewis rats for this study were supplied from the animal laboratory in the psychology department of the University of Texas at Arlington. Prior to experimentation, these animals were properly monitored by visits from a veterinarian. They were on a 12 hour light: dark cycle, and had free access to food and water. The animal housing was maintained at 70°F and 60% humidity.

2.2 Materials

Vasodilatation was measured using Laser Doppler Imager (PeriScan PIM Π, Perimed AB, Sweden). It measures the arterial blood flow in the microvasculature of the dermis in Arbitrary Unit (AU). The same intensity of voltage (8.5 V) and lights were used throughout the experiment during Laser Doppler imaging for all animals. The mean arterial blood flow was calculated by selecting a region of interest, which included whole paw. The percent change was calculated using average mean values at baseline [(Blood perfusion at any time point – average blood perfusion of baseline images)/average blood perfusion of baseline images X 100)] to avoid individual differences.

2.3 Surgical procedures

Before surgical procedures, animals were anesthetized with (50 mg/kg) intraperitonial injections of pentobarbital. After experiment, animals were euthanized by pentobarbital overdose, which is approved by the American Veterinary Medical Association. The level of anesthesia was assessed by examining motor reflexes via the tail. Surgical sites were prepared by shaving using an electrical razor.

2.3.1 Laminectomy

The anesthetized animals were kept in prone position on a surgical table. The skin incision was made over spinous processes in the lumbosacral area. The ligaments and connective tissues between spinal process and transverse processes were removed. In order to expose the spinal cord and L4 – L6 dorsal roots spinous process, transverse processes and lamina were removed.

2.3.2 Tracheotomy and Jugular vein cannulation

The animal was turned into a supine position. The skin incision was made in the midline of the neck area. The trachea was exposed by splitting muscles around trachea. The tracheotomy tube (PE 50 tube) was inserted by making transverse incision over tracheal cartilage. Nylon threads were used to stabilize the tube in the trachea.

The jugular vein was exposed by separating surrounding connective tissues. The vein was cut at a $45°$ angle and a plastic anesthesia line (PE 10 tube) was inserted. The tube was stabilized using nylon thread and the location of tube was confirmed by withdrawing blood through tube. Pentobarbital mixed with normal saline (50 mg sodium Pentobarbital + 9 ml 0.9% normal saline) was administered through a jugular vein catheter continuously at a rate of 1.2 ml per hour in order to maintain continuous anesthesia throughout the experiment.

2.4 Data acquisition

A stereotaxic frame was used to hold the animal to prevent movements throughout the experiment. The spinal cord was exposed by removing the dura, and it was kept in a mineral oil pool throughout the recording of action potentials to avoid desiccation. All animals' end tidal

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CO$_2$ was maintained (controlled ventilation) around 40 mmHg, and body temperature was maintained at 37℃ to reduce individual variations. A feedback controlled electric heating blanket was used to maintain body temperature at 37℃. Heart rate was recorded throughout the experiment.

A small fiber from dorsal roots at either L4 or L5 was separated from distal end, and the proximal stump was wrapped around the recording electrode to record DRRs. Data acquisition was done using CED 1401Plus data acquisition system (Cambridge Electronic Design Ltd, UK) and SPIKE 2 computer software. Dorsal root reflexes were recorded as number of action potentials per second. In the analysis of electrophysiological data, the spikes were differentiated on the basis of their waveform. Heart rate was recorded as heart beat per second.

Vasodilatation was measured by a Laser Doppler Imager. The intensity was kept at 8.5 volts. Images of plantar surface for both hind paws were taken continuously. Each image took 117 sec to be collected.

2.4.1 Experiment 1

This experiment was conducted in three adult Lewis rats to determine the best stimulating parameters. After surgical preparation, animals were fixed into the stereotaxic frame and the dura was removed. The bipolar concentric electrode was placed in the PAG (7 mm posterior to bregma, 0.2 mm lateral to midline, and 4.5 – 5.0 mm deep from cerebral cortex) for electrical stimulation. Then a fiber from L4 or L5 was teased, and recording of DRRs was started. Fifteen images were taken as baseline measurements. The PAG was stimulated with 5V, 1 Hz, 10 ms duration for 20 sec. Images were collected for thirty minutes. Then the PAG was stimulated again in the same manner with increasing voltage at 10V, 15V and 20V. After data analysis, the optimal combinations of stimulus parameters were determined for Experiments 2 and 3.

2.4.2 Experiment 2

This experiment was designed to determine the effect of PAG stimulation evoked DRRs in cutaneous neurogenic inflammation. Eight Lewis rats were used in this study. The animals

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were prepared surgically the same as in Experiment 1. Stimulus intensity was set at 15V, 1Hz, with 10 ms duration for 20 seconds. Stimulus frequency, intensity and duration were determined by observing the response patterns of DRR recordings and vascular responses to PAG stimulation in the previous experiment. DRRs were recorded ipsilateral to PAG stimulation at L4 or L5 throughout experiment.

First, 15 baseline images were taken. The imaging was continued after PAG stimulation for one hour. Simultaneously, DRRs were recorded at the L4 or L5 level.

2.4.3 Experiment 3

This study was proposed to examine if application of bicuculline would attenuate the response of cutaneous neurogenic inflammation from PAG stimulation evoked DRRs. Eight Lewis rats were used for this group. The animals were prepared surgically the same as before. The rats in this group were paralyzed using pancuronium bromide before the data collection, and they were ventilated using an artificial respirator. DRRs were recorded the same as before. Fifteen images were taken as a baseline. Bicuculline (0.05mM, 100 microliter, Sigma-Aldrich) was applied to the dorsal root entry zone by using cotton swab. The concentration of bicuculline was decided by using previous studies (Peng et al., 2001). Ten minutes later, PAG stimulation (same parameters as above) was delivered. Images were collected continuously for one hour.

2.5 Histology

At the end of the experiment, rats were euthanized and their brains were extracted to be immersed in 10% formalin. The brains were frozen, cut into coronal sections, mounted on glass slides, and stained with thionin for preparation of histological examination. The stimulating electrode placements were verified by examining the slides under light microscopy and photographed with a digital camera (Figure A.2). Two rats were removed from the analysis (one rat from each group) because a proper electrode tract could not be verified in histological examination.

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2.6.1 Main analysis for hypothesis testing

The dependent variables in this study were percentage change of arterial dilatation, Heart rate and frequency of DRR, which are continuous variables. The mean arterial dilatations were calculated using select regions of interest which included whole paw. The percent change was calculated using average mean values of the baseline group. Frequency of DRRs was calculated as action potentials per second and heart rate was calculated as heart beat per second. Independent variables are Group (electrical stimulation only and bicuculline), Side (ipsilateral and contralateral) and Stimulation (baseline and electrical stimulation).

2.6.2 Analysis for multiple stimulating parameters: peripheral blood perfusion could be increased with stimulation of PAG due to generation of DRR and vascular response could be increase with increase of voltage due to increase of DRR.

Hypothesis 1: The electrical stimulation of the PAG would cause cutaneous neurogenic inflammation, due to generation of DRRs. Vasodilatation would increase with increased voltage and increased DRR output.

This is a 2 (side) X 5 (stimulation) mixed design. A mixed ANOVA was used to determine the trend of vascular responses to increases of voltage over four assessments. Post hoc Fisher LSD tests were used to examine the difference between groups at each level (STATISTICA, StatSoft, OK). The dependent variable in this study was percent change in arterial dilatation. Independent variables were Side (ipsilateral and contralateral) and Stimulation (depends on Voltage). The percentage values and mean blood perfusion values were analyzed separately.

DRRs were calculated as action potentials per second and used as a dependent variable. Repeated Measures ANOVA was performed to examine the effect of stimulation on DRR generation.

This group did not have recordings for heart rate.

2.6.3 Analysis for effect of PAG stimulation and bicuculline: The blood perfusion could be increased with PAG stimulation and that vascular response could be attenuated by bicuculline.

Hypothesis 2: Arterial dilatation would be increased with PAG stimulation. Furthermore, arterial dilatation would be attenuated by applying bicuculline to the spinal cord.

This was a 2 Side (contralateral and ipsilateral) X 2 Treatment Condition (baseline and stimulation) X 2 Group (Stimulation only and Bicuculline) mixed design. A mixed ANOVA was used to examine the effect of PAG stimulation on cutaneous neurogenic inflammation after pre-treatment with bicuculline.

Hypothesis 3: The vascular response following PAG stimulation occurred due to activation of descending inhibitory pathways rather than activation of autonomic nervous system.

It was assumed that the stimulation of PAG caused activation of descending inhibitory pathways and caused release of GABA. It was expected that the increase of GABA facilitated generation of DRRs. A Mixed ANOVA was used to determine the effect of both stimulation and bicuculline on the generation of DRRs.

Heart rate was analyzed with a Repeated Measures ANOVA after averaging heart rate for every five data points. The effect of PAG stimulation and bicuculline on the autonomic nervous system was examined.

CHAPTER 3

RESULTS

Data screening for this study was performed as grouped data (stimulation only group, bicuculline group, and baseline and stimulation group) because a mixed ANOVA was planned for data analysis. Prior to analysis, the data were examined for accuracy of data entry, missing values, and assumptions for ANOVA.

3.1 The electrical stimulation of PAG could cause neurogenic inflammation and inflammatory signs could be increase with increase of stimulating Voltage

The first study was conducted to determine the optimum electrical stimulating parameters for the second experiment. The first part of the hypothesis predicted that bilateral arterial dilatation would be increased with stimulation of the PAG, due to generation of DRRs. Furthermore, it was predicted that the blood perfusion effect would increase with voltage increases, due to increases of generation of DRRs. Mixed ANOVA was performed to determine the effect of Electrical Stimulation, Treatment Side, Voltage, and their interactions for blood perfusion. The Fisher LSD test was conducted to compare individual differences.

There was a significant main effect of Electrical Stimulation on mean blood perfusion, $F_{(71, 284)} = 14.47$, $p < 0.001$. Neither Treatment Side, $F_{(1, 4)} = 0.31$, $p > 0.05$ nor Treatment Side X Electrical Stimulation, $F_{(71, 284)} = 0.29$, $p > 0.05$ were significant (Figure A.3). The blood perfusion was significantly affected by increase of voltage. The analysis of percentage change values revealed that there was a significant effect of Stimulation $F_{(71, 284)} = 3.07$, $p < 0.001$. However there were no significant effect of Side $F_{(1, 4)} = 14.47$, $p > 0.05$ nor Side X Stimulation $F_{(71, 284)} = 0.41$, $p > 0.05$, (Figure A.4).

The posthoc analysis revealed that 10V, 15V, and 20V segments of Voltage had significantly higher blood perfusion compared to the Baseline segment ($p < 0.05$) and the 5V

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segment ($p < 0.05$). Also the 20 V and 15V segments were significantly higher than the 10 V segment ($p < 0.05$). The mean blood perfusion values for 20V had significantly higher blood perfusion than 15 V ($p < 0.05$). The results were consistent for mean blood perfusion values and percentage values except that there was no significant difference between the 15V and 20V segments ($p > 0.05$, NS) in percentage change values.

3.2 The PAG stimulation did not significantly increase generation of DRR.

DRRs in the first experiment were analyzed using Repeated Measures ANOVA and revealed that there was no significant effect of Electrical Stimulation on generation of DRRs, F (73, 365) = 0.96 ($p > 0.05$) (Figure A.5 & A. 6).

3.3 The PAG stimulation increased cutaneous blood perfusion, which were attenuated by spinal bicuculline application

The second experiment was conducted to determine the effects of PAG stimulation on peripheral blood perfusion. Based on the above experiment, PAG stimulation parameters were adjusted to 15V, 1Hz, 10ms duration for 20sec. In the third experiment, it was assumed that the vascular response due to PAG stimulation could be attenuated by application of bicuculline. The analysis was a 2 (Group) X 2 (Side) X 2 (Stimulation) design and mixed ANOVA with Fisher LSD posthoc test. There was a significant main effect of Stimulation on mean blood perfusion, F (44, 308) = 5.01, $p < 0.001$ and a significant interaction between Stimulation and Group, F (44, 308) = 2.49, $p < 0.001$. However there were no significant differences of mean blood perfusion between Sides, F (1, 7) = 0.54, NS; or Group F (1, 7) = 2.36, $p > 0.05$. Furthermore there were no significant interactions between Group and Side F (1, 7) = 0.75, $p > 0.05$; or Stimulation, Group and Side, F (44, 308) = 1.05, $p > 0.05$.

The posthoc analysis revealed that the significant increase of blood perfusion following stimulation of PAG occurred twenty minutes after stimulation, compared to baseline ($p < 0.05$) (Figure A.7). There were no significant difference of baseline blood perfusion between the stimulation only group and bicuculline group ($p > 0.05$). The stimulation only group had significantly higher blood perfusion forty minutes after stimulation compared to the bicuculline

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group ($p < 0.05$). According to statistical analysis, stimulation did not cause any significant increase of blood perfusion compared to its baseline after application of bicuculline ($p > 0.05$) (Figure A.8).

The results for percentage change blood perfusion values confirmed that there was significant effect of Stimulation on percentage change blood perfusion, $F (44, 396) = 1.94$, $p < 0.01$; and there was no significant effect for Side, $F (1, 9) = 2.10$, $p > 0.05$. Furthermore, the effect of Bicuculline was not statistically significant, $F (1, 9) = 1.12$, $p > 0.05$. There were no significant interactions for Stimulation, Group and Side, $F (44, 396) = 0.64$, NS; or Stimulation and Group, $F (44, 396) = 0.90$, $p > 0.05$. However the percentage change blood perfusion had a significant interaction between Side and Stimulation, $F (44, 396) = 1.44$, $p < 0.05$.

The individual group comparisons revealed that there were no significant differences between baseline groups of both sides ($p > 0.05$) and both groups ($p > 0.05$). Similar to mean blood perfusion values, the blood perfusion was significantly increased twenty minutes after stimulation of the PAG in the ipsilateral side ($p < 0.05$) and forty minutes after stimulation in contralateral side ($p < 0.05$) compared to their baselines (Figure A.9). In agreement with the raw data, the normalized values indicated that there was no significant difference of blood perfusion after PAG stimulation in contralateral side compared to its respective baseline in the bicuculline group ($p > 0.05$, NS). However the percentage change blood perfusion was significantly increased in the last four images of the ipsilateral side of the bicuculline group compared to its baseline ($p < 0.05$) (Figure A.10).

3.4 The vascular response for stimulation of PAG was due to activation of descending inhibition pathways rather than activation of autonomic nervous system

A mixed ANOVA for DRRs revealed no significant effects for PAG Stimulation $F (29, 580) = 1.16$, NS, (Figure A.11 & A. 12); or Bicuculline $F (1, 20) = 0.16$, NS, (Figure A.13 & A.14), on generation of DRRs. Furthermore, there was no significant interaction between Stimulation and Group $F (29, 580) = 1.48$, $p > 0.05$.

The heart rate recordings were analyzed using a Repeated Measures ANOVA to determine the effect of PAG stimulation on the autonomic nervous system. There was no significant impact of PAG stimulation on heart rate for Stimulation in stimulation only group, $F_{(9, 18)} = 2.38$, $p > 0.05$ (Figure A.15). However there was a significant effect of PAG stimulation for Bicuculline group, $F_{(11, 44)} = 3.31$, $p < 0.05$ (Figure A.16 & A.17). Further analysis revealed that the heart rate was significantly reduced after PAG simulation compared to baseline in the bicuculline group ($p < 0.05$); however there was no significant effect of bicuculline on heart rate ($p > 0.05$, NS).

CHAPTER 4

DISCUSSION

The present study was designed to understand the supraspinal regulatory mechanisms for neurogenic inflammation. In this study, neurogenic inflammation was measured as a peripheral blood perfusion increase. There was evidence for the release of GABA and serotonin at the spinal cord level following activation of descending pain inhibition. The electrophysiological studies suggested that GABA and serotonin could act on $GABA_A$ receptors and $5HT_3$ receptors in PAFs and facilitate depolarization (Willis, 1999). The increase of DRRs and cutaneous neurogenic inflammation following application of GABA on to the entry zones of dorsal roots were observed in preliminary studies (Herath, et al, 2008). Therefore we hypothesized that the activation of descending inhibitory pain pathways by stimulation PAG would facilitate cutaneous neurogenic inflammation through DRRs. Furthermore, we assumed that the vascular response for PAG stimulation could be attenuated by blocking DRRs using bicuculline.

4.1 The electrical stimulation of periaquiductal grey area facilitate cutaneous neurogenic inflammation

The second experiment showed that there was a significant increase of peripheral blood perfusion following electrical stimulation of the PAG. According to the results, the vascular response was bilateral. Furthermore, the results of the third part of the study suggested that the vascular response of PAG stimulation could be attenuated by applying bicuculline.

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<u>4.2 The vascular response of PAG stimulation is due to activation of descending inhibition</u>
<u>pathways rather than activation of autonomic nervous system</u>

The electrophysiological recording for DRR did not support the hypothesis of increased DRRs following PAG stimulation. We observed the expected pattern of response in two cells out of eight in the first experiment, two cells out of thirteen in second experiment, and three cells out of ten in the third experiment (Figure A.5, A.11, and A.13). However the reduction of the vascular response following PAG stimulation in the bicuculline group strongly supports an involvement of descending inhibition pathways in cutaneous neurogenic inflammation.

The heart rate recording in the second group showed that there was no changes of heart rate following PAG stimulation. The heart rate of the bicuculline group showed a significant decrease of heart rate after PAG stimulation. However, three rats out of five were responsible for this pattern of response (Figure A.17).

The PAG is one of most investigated brain areas for pain regulation. Previous studies stated that the PAG descending inhibition pathway operates mainly through the use of opiate, serotonergic and GABAergic mechanisms (Millan, 2002; Peng et al., 1996a; Peng et al., 1996c). The investigation of neural mechanisms of the generation of DRR following PAG stimulation revealed that electrical stimulation of the PAG facilitates the release of GABA and serotonin; it also facilitates primary afferent depolarization through $GABA_A$ and $5HT_3$ receptors (Peng et al., 2003). However, there was no increase of DRR following electrical stimulation of the PAG in this study. The lack of response may be due to inactivation of the fibers with time, or inaccurate selection of fibers might be an alternative explanation for this absent effect. Since mechanical teasing of a strand from a dorsal root can elicit strong electrical activity, it was designed to have only one strand to be teased in one animal to minimize the damage. Therefore, it is possible that the fibers in that strand may not have been responsive to PAG stimulation. The finding that no DRRs were elicited by PAG stimulation in this study does not rule out the possibility of other remaining intact dorsal root fibers that may have conducted antidromic propagation to the

periphery. In fact, a bilateral attenuation of the increase of PAG stimulation blood perfusion following bicuculline supports this assumption.

GABA is the major inhibitory neurotransmitter in central nervous system. However it has an excitatory effect within the PAFs. GABA and serotonin have been highly investigated in the context of DRR. There is evidence for generation of DRR following application of GABA and serotonin (Peng et al., 2001) and a reduction of DRRs following application of a $GABA_A$ antagonist (bicuculline) or $5HT_3$ antagonist (ondansetron) (Curtis et al., 1994; Eccles et al., 1963; Peng et al., 2001; Wall, 1994). The link between neurogenic inflammation and DRR has also been repeatedly observed (Rees, et al, 1996; Sluka et al., 1992). In agreement with the above arguments, the results of the present study stated that there was an increase of signs of cutaneous neurogenic inflammation following activation of the descending inhibition system. The attenuation of the vascular response following application of bicuculline further supported the proposed neural mechanism. The non-significant vascular response for the bicuculline group following PAG stimulation suggests a role for 5-HT_3 receptors in generation of DRR, considering the slight increase of blood perfusion that was left intact.

The cardiovascular response and pain response following stimulation of PAG was studied in several groups; however the pattern of cardiovascular response for PAG stimulation was not consistent within and between experimental designs. An increase of heart rate and blood pressure was observed following stimulation of the dorsal and dorsolateral PAG (Lovick, 1985). Some studies observed a decrease of heart rate and blood pressure following stimulation of the PAG (Green et al., 2006). They used stress analgesic mechanisms to explain the down regulation of the cardiovascular response and flight or fight response for an increase of the vascular response. However, there was no evidence for cutaneous blood perfusion following PAG stimulation. According to the above theories, skin blood perfusion has to be reduced following activation of a defense mechanism. We do not have strong evidence to support an activation of the autonomic nervous system following stimulation of PAG in this

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study; but stimulation of the PAG did not significantly increase the heart rate as suggested, nor did it trigger a strong autonomic response in this study. On the contrary, only three rats showed a significant reduction of heart rate following stimulation of the PAG.

Only the bicuculline group was paralyzed and ventilated due to unexpected tonic movements following application of bicuculline. The experimental design in the stimulation only group was not treated with saline equivalent to bicuculline. The present study consists of those limitations. It has been observed that bicuculline and pancuronium increase heart rate and cardiac output (Melnikov, et al., 2009; Hong & Henry, 4009). In the present study, a decreased heart rate in the bicuculline group was observed.

The understanding of central nervous system pathways, receptors and mechanisms for peripheral inflammation has implications in constructing new therapeutic targets for inflammatory diseases, such as psoriasis as well as chronic pain conditions like migraine and peripheral vascular disease.

In conclusion, the results of the present study showed that DRR has significant role in generation of cutaneous neurogenic inflammation, and descending inhibition pathways can regulate signs of neurogenic inflammation. Furthermore, this study supports a role of the central nervous system for influencing neurogenic inflammation.

APPENDIX A

FIGURES
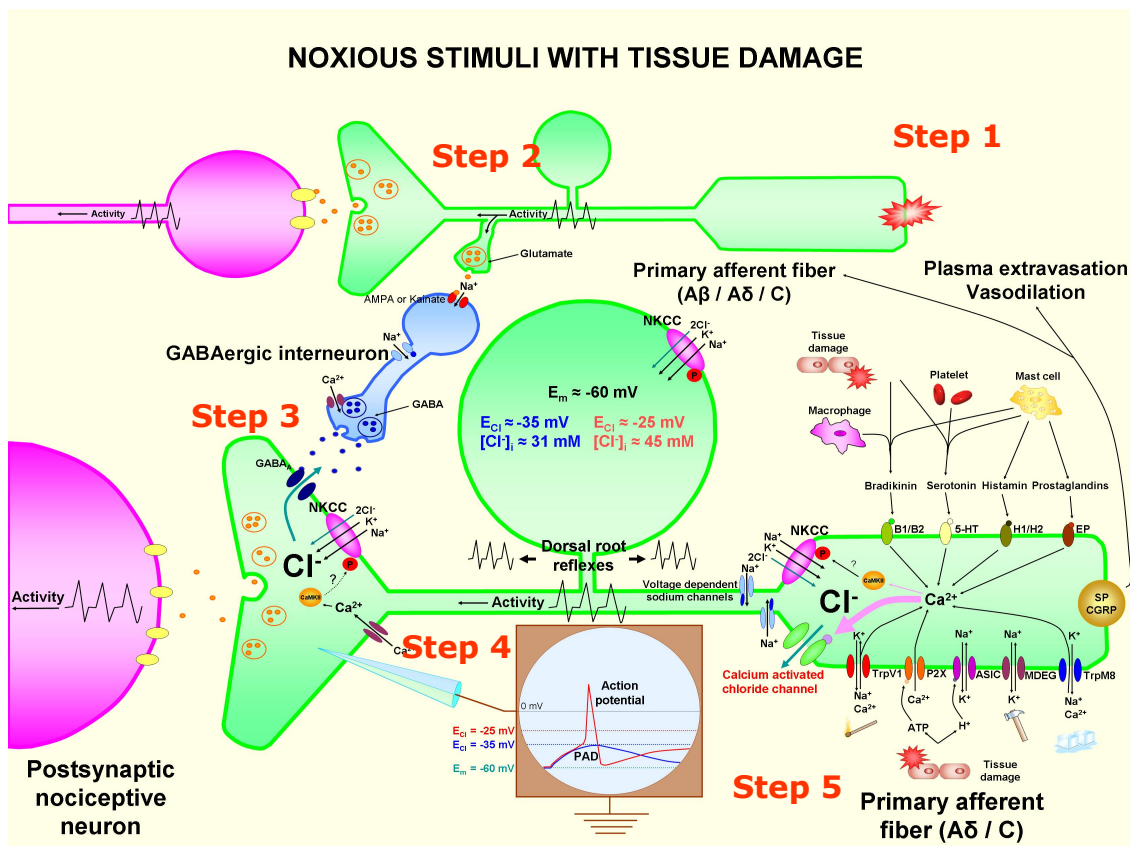
Figure A.1. The generation of dorsal root reflexes

This diagram explains changes in molecular level (Granados et al, 2004).

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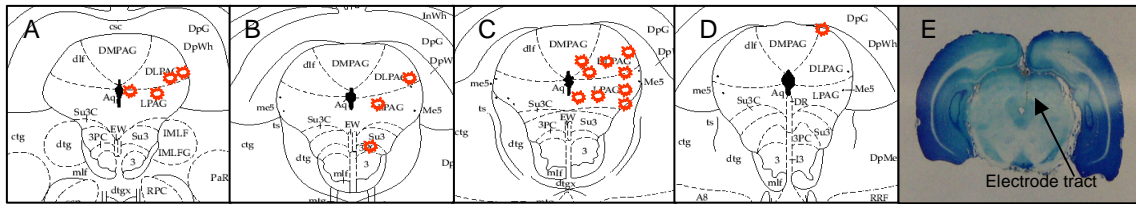
Figure A. 2. Histology

Image A, B, C, and D are coronal section at 6.3mm, 6.7mm, 6.8mm, and 7.0 mm anterior to the bregma respectively (Paxinos and Watson, 1998). The electrode tips were marked in the diagram. Image E represent histology slide.
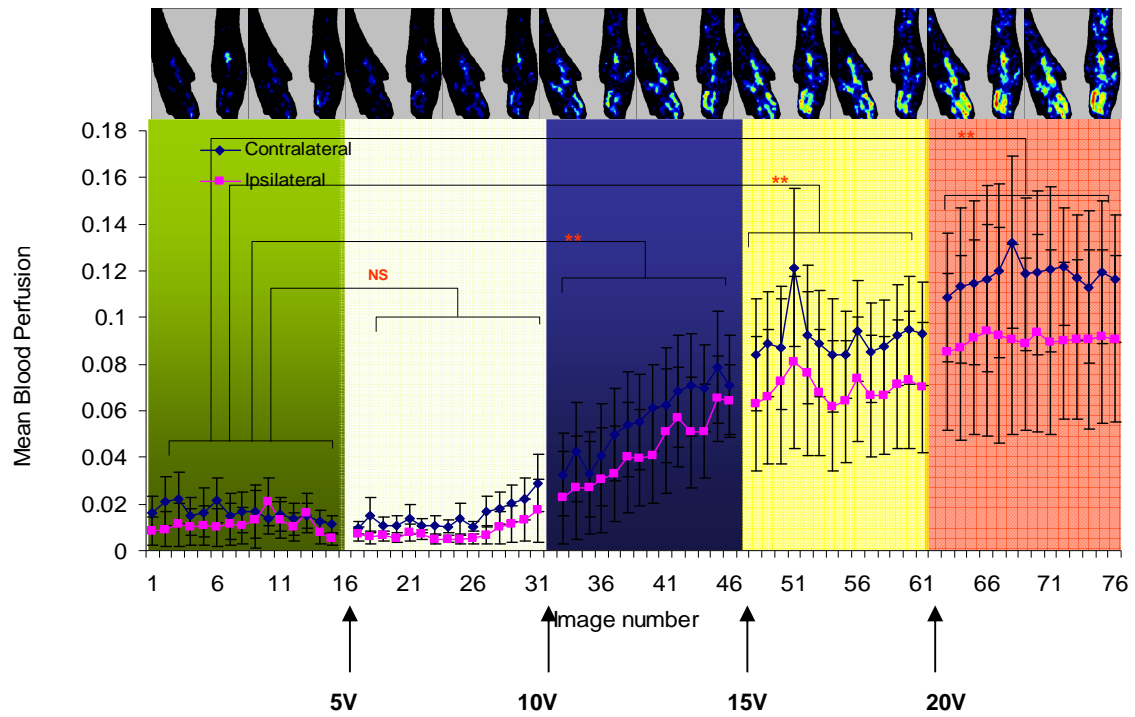
Figure A. 3. The Mean Blood Perfusion changes for multiple stimulating parameters (n=3).

The upper panel shows the Laser Doppler images from one rat. The pattern of vascular response for multiple stimulation parameters was demonstrated. (Note: **p<0.01 , NS -not significant)
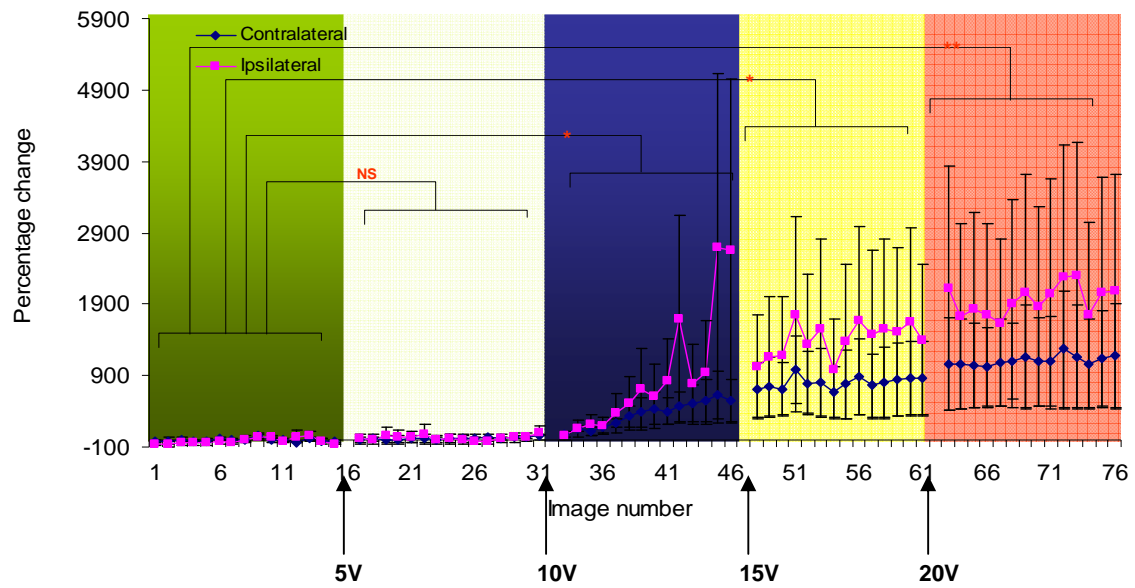
Figure A. 4. The percentage change blood perfusion values for multiple stimulating parameters

The percentage change blood perfusion in both hind paws for four different stimulating parameters were demonstrated (n=3). The results of individual group comparison were summarized. (Note: **$p<0.01$, * $p<0.05$, NS -not significant)
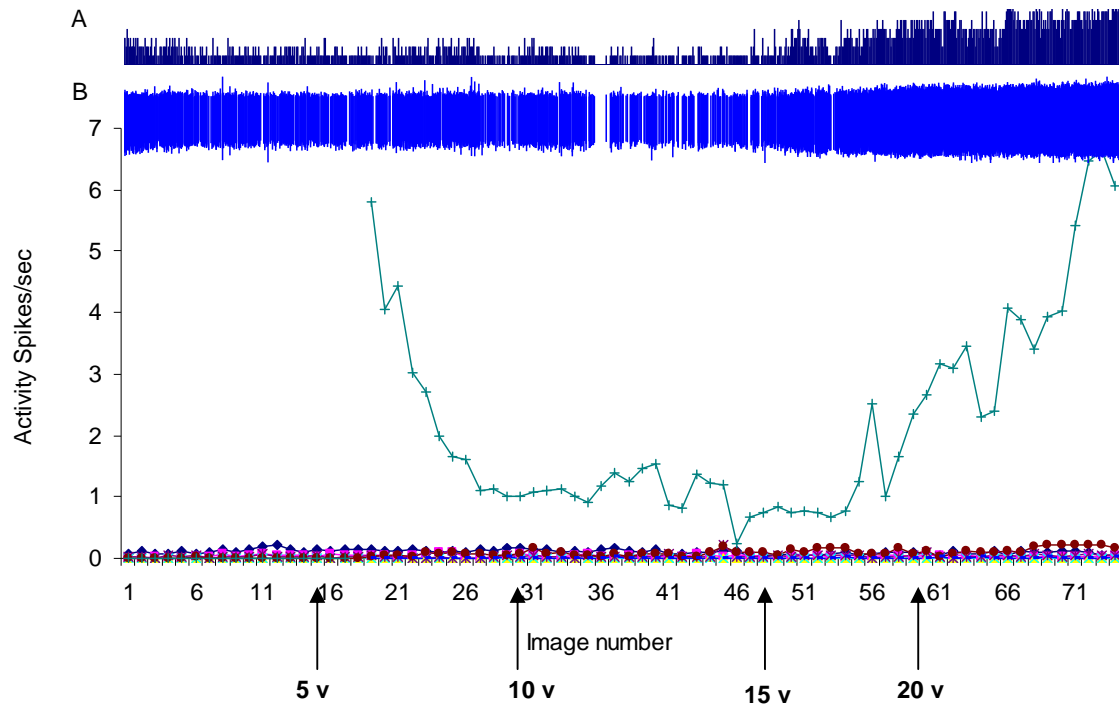
Figure A. 5. The DRR response of group of fibers for PAG stimulation

A. The upper panel shows rate of action potentials (action potentials per second) in one cell. The each vertical line in lower panel represents a dorsal root reflex. B. Each line represents response of DRR in one cell. Two out of eight cells showed increase of DRR following stimulation.
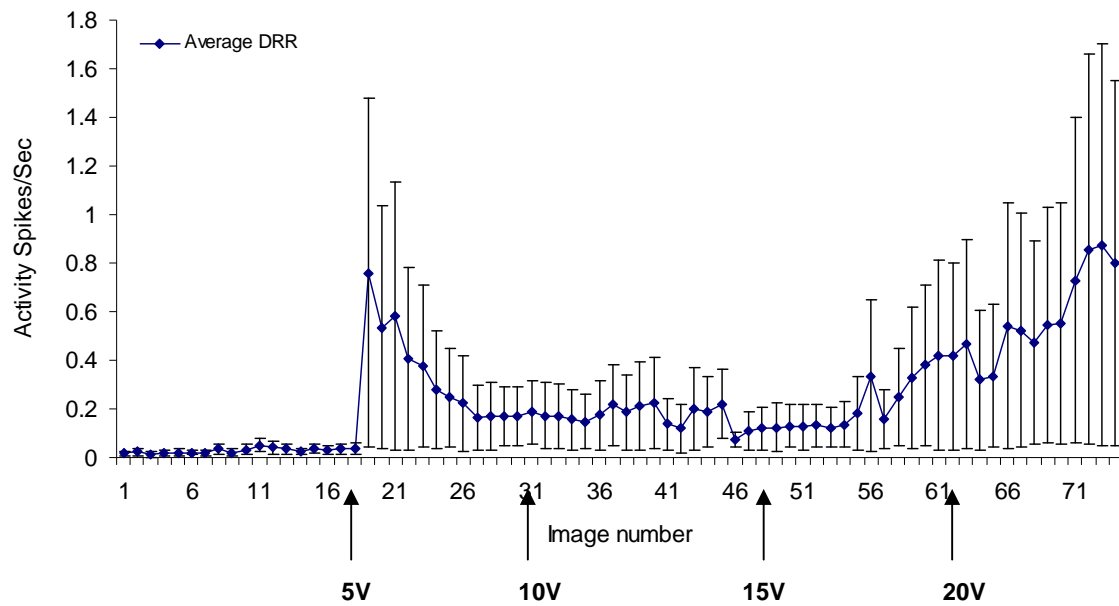
Figure A. 6. Summary for DRR recordings

The DRR response for four different stimulating parameters was collected. The average values of DRR were presented in this figure.
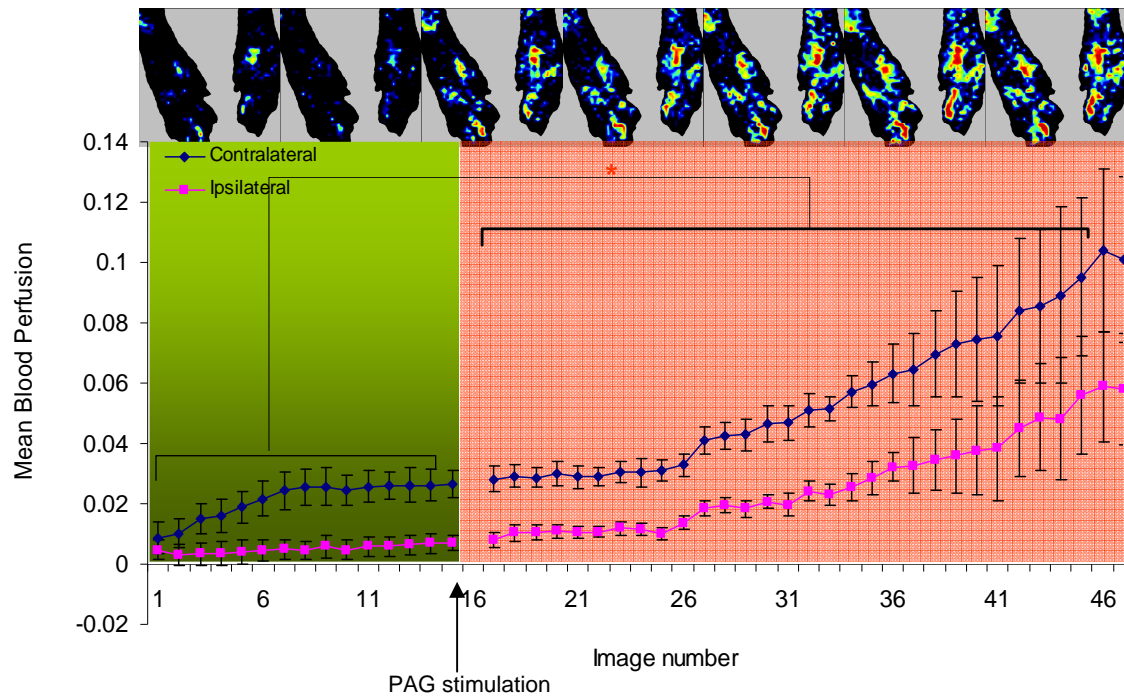
Figure A. 7. The Mean Blood Perfusion values for stimulation only group

The vascular response for PAG stimulation was demonstrated (n=7). The upper panel consists of Laser Doppler Images from one rat. The grey color area represents baseline blood perfusion and orange color area represents blood perfusion following PAG stimulation. (Note: * p<0.05)
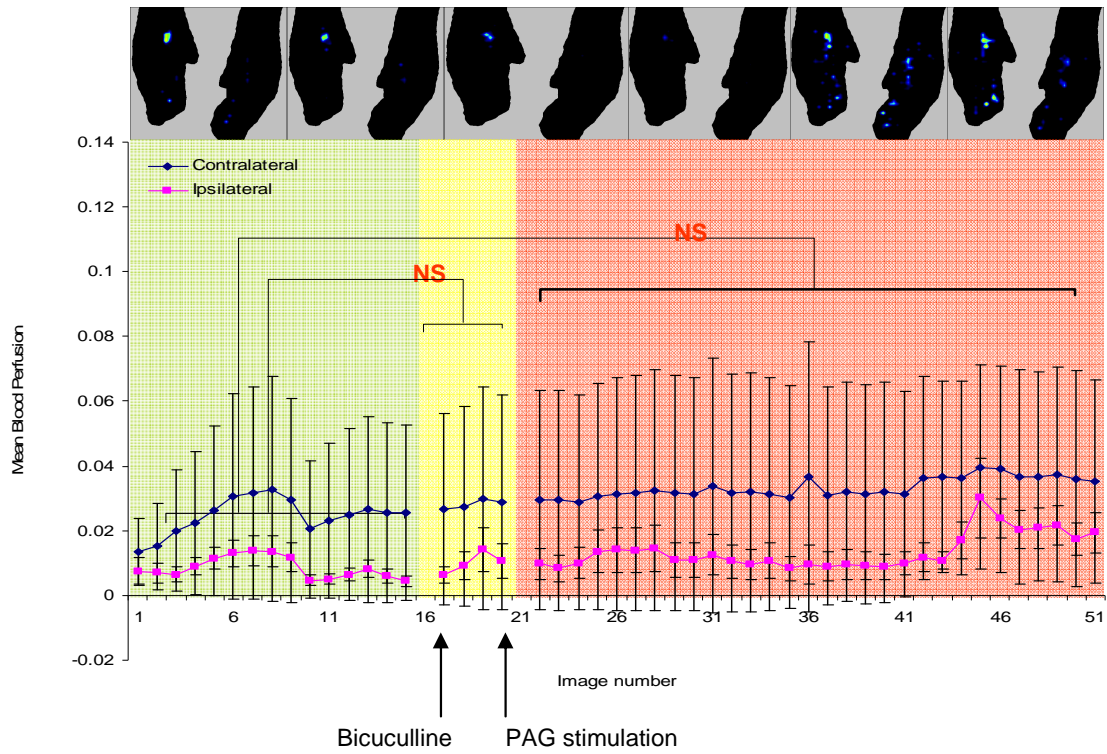
Figure A. 8. The Mean Blood Perfusion in Bicuculline group

The vascular response for PAG stimulation after application of bicuculline was graphically presented (n=7). The above panel is an example for Laser Doppler Image recordings in one rat. (Note NS – Not significant)
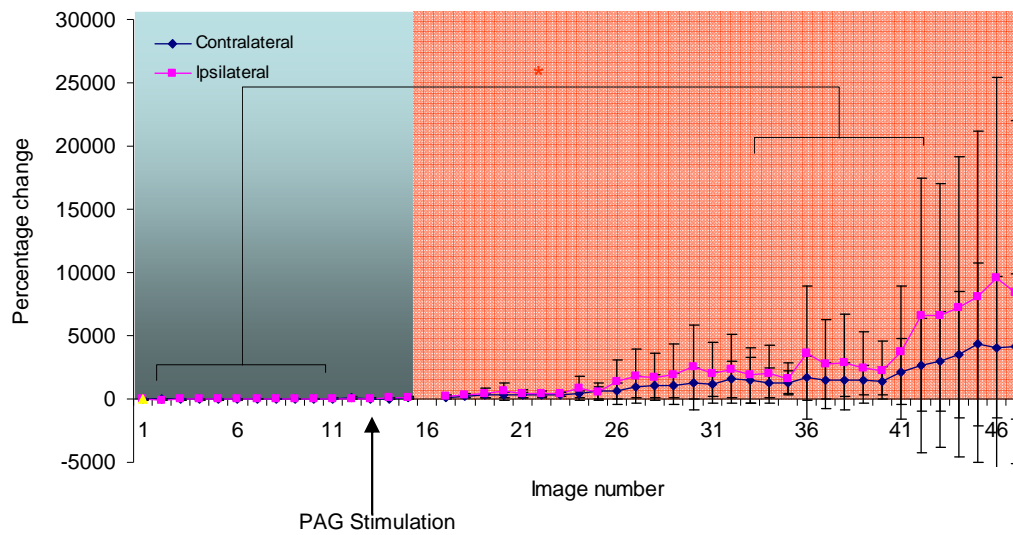
Figure A. 9. The percentage change blood perfusion for PAG stimulation

The normalized vales for peripheral blood perfusion for PAG stimulation were demonstrated (n=7). (Note: * p<0.05)
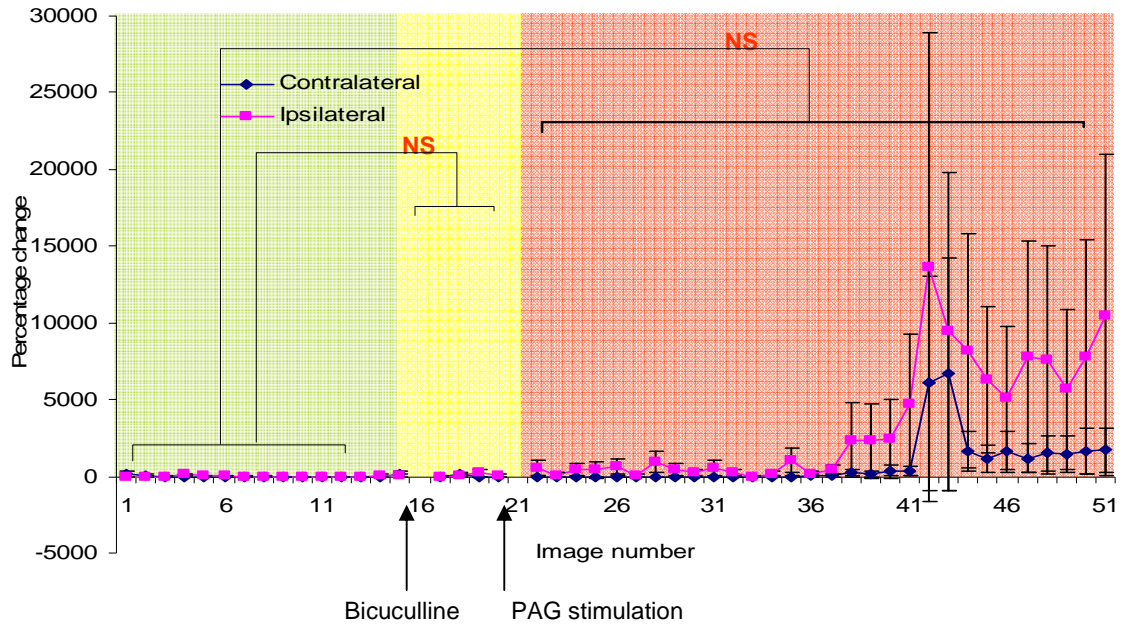
Figure A. 10. The percentage change blood perfusion in Bicuculline group

This graph shows that normalized values of blood perfusion following application of bicuculline and PAG stimulation (n=7). (Note: NS- not significant)
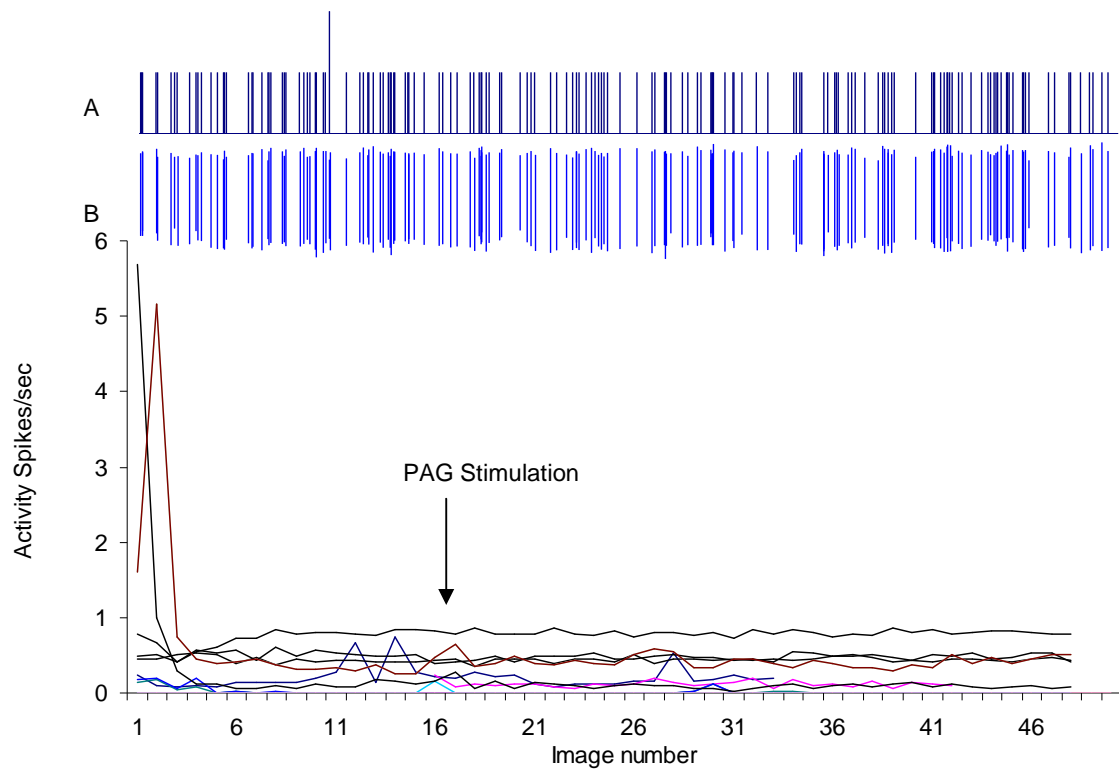
Figure A. 11. The DRR response of group of fibers for PAG stimulation

A. The upper panel represents rate of DRR (spikes/sec) and each line in lower panel represents DRR. B. Each line indicates individual cell response of DRR for PAG stimulation. Two out of thirteen cells were showed expected increase of DRR following stimulation.
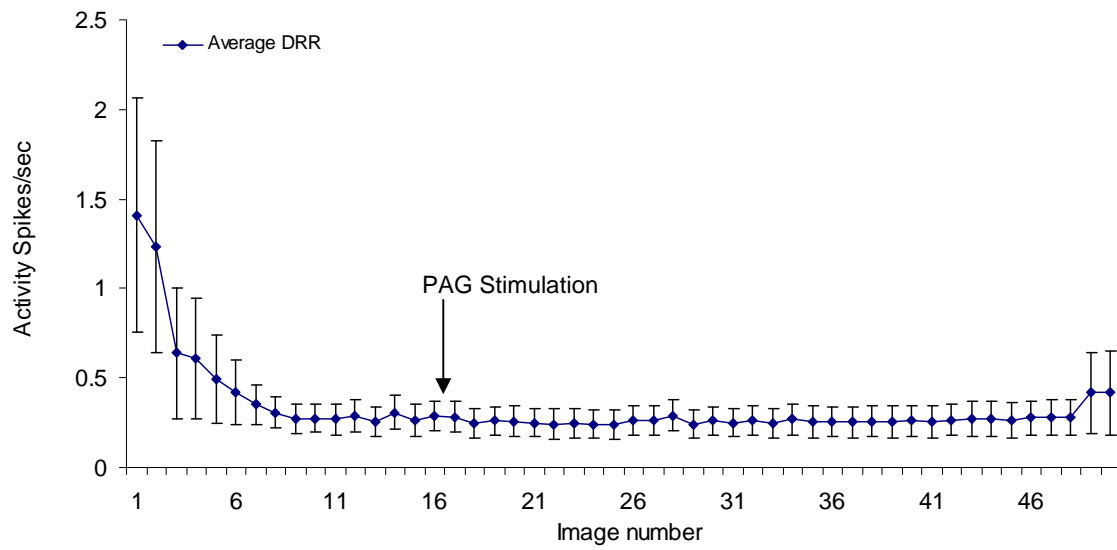
Figure A. 12. The mean values of DRR for PAG stimulation

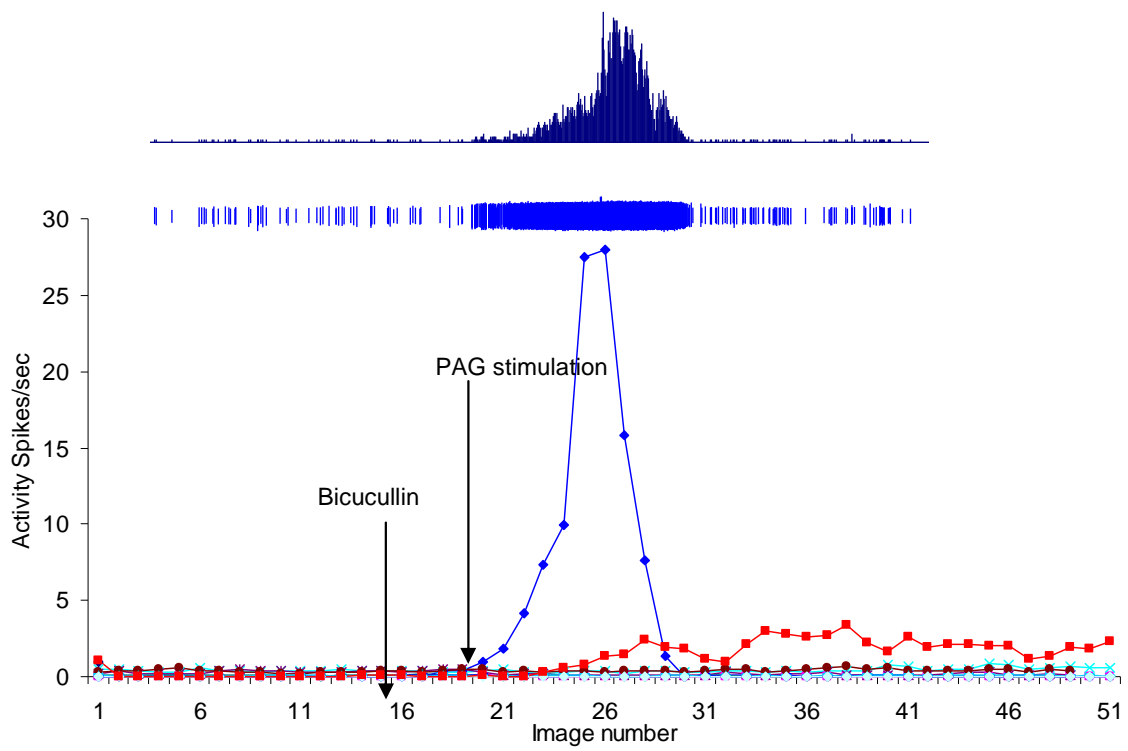The summary of DRR recordings for PAG stimulation was presented (n=13).

Figure A. 13. The group of fibers with DRR in bicuculline group

A. The rate of DRR was presented in upper panel. Each vertical line indicates DRR in lower panel. B. Each line represents DRR response for individual cell. Three out of ten cells showed increase of activity after PAG stimulation. (n=10)
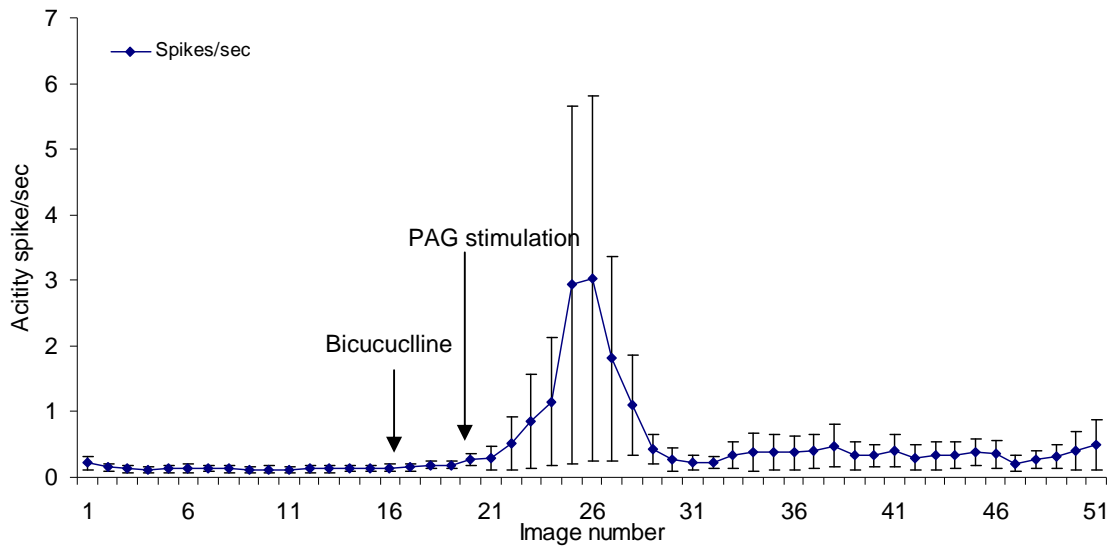
Figure A. 14. The mean DRR values for bicuculline group

The average DRR response values for ten cells were presented. The one cell out of ten cells responsible for the following pattern of DRR response
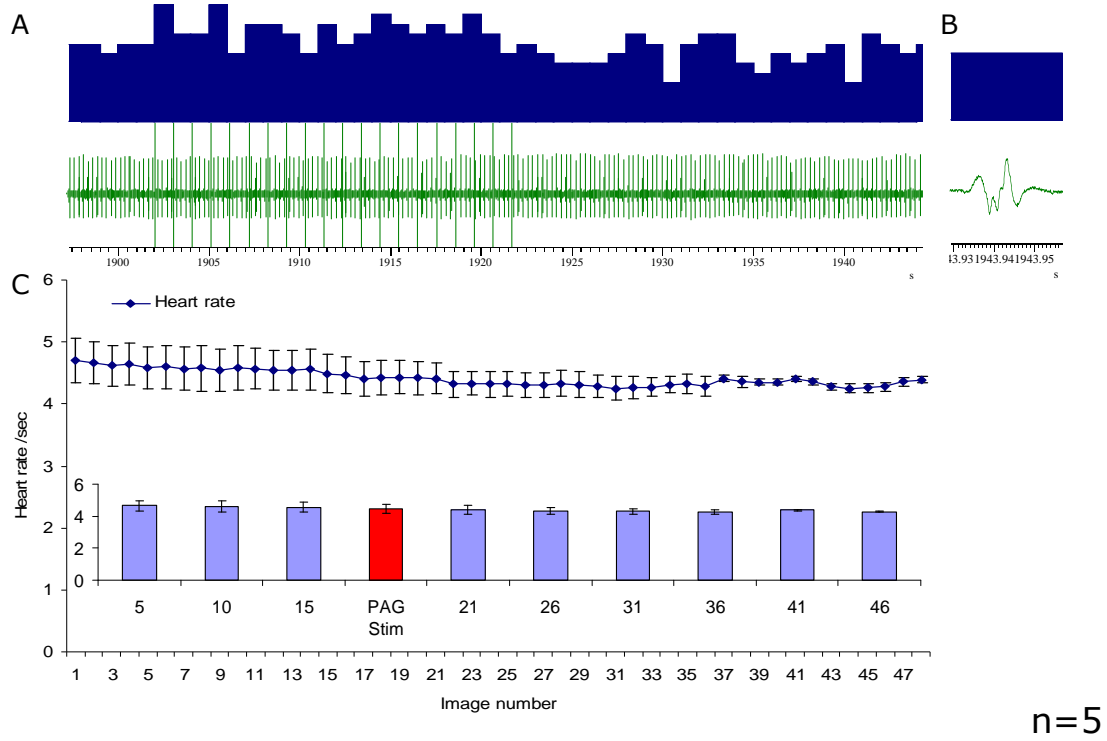
Figure A. 15. Heart rate recordings in stimulation only group

The diagram A is an example for heart rate recording. The recording of individual heart rate was presented in figure B. The graph C indicates average heart rate response for PAG stimulation.
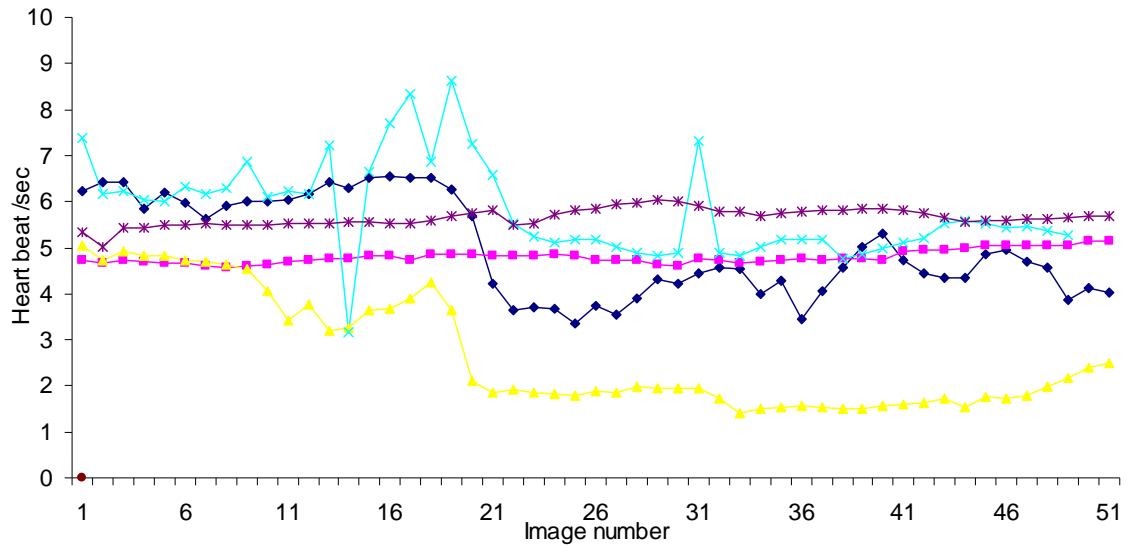
Figure A. 16. The heart rate recordings of individual rats in bicuculline group

The panel A is an example for heart rate recording. Each line in figure B represents heart rate response for individual rat in bicuculline group.
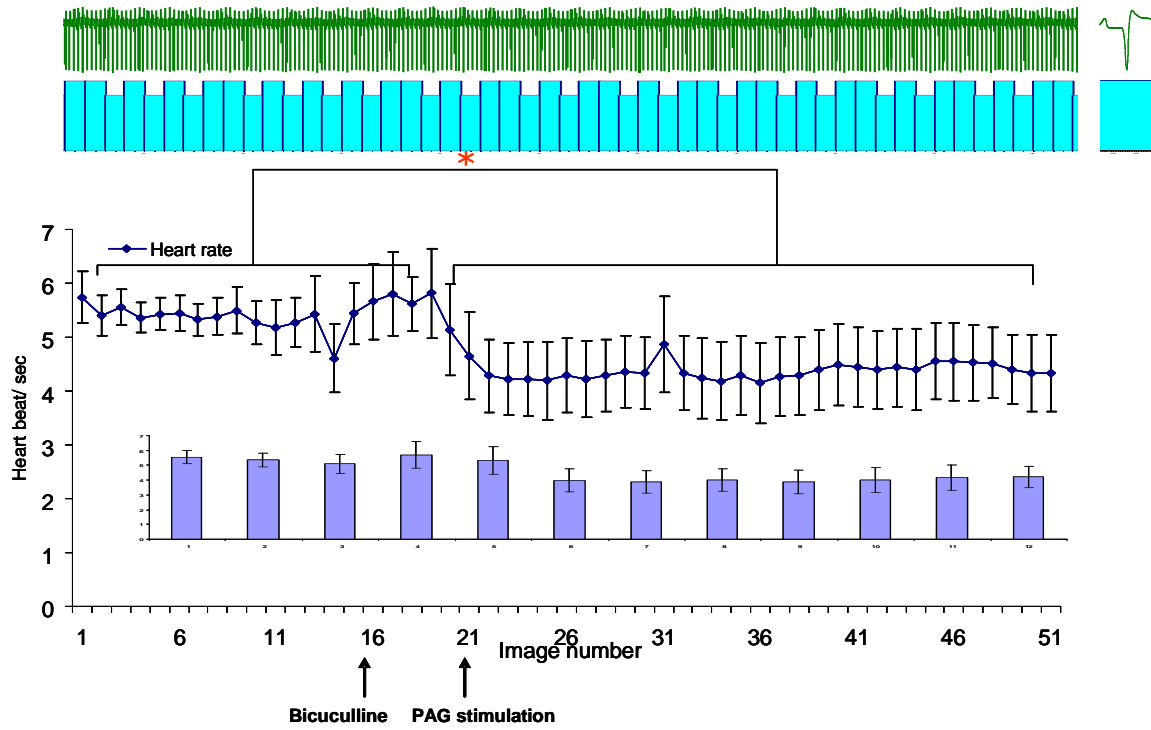
Figure A. 17. The summary for heart rate response in bicuculline group

The three out of five rats were made significant difference in heart rate.

REFERENCES

Akil, H., Mayer. D. J., & Liebeskind. J. C. (1976). Antagonism of stimulation-produced analgesia by naloxone, a narcotic antagonist. <u>Science</u>. 191(4230), 961-962.

An, X., Bandler, R., Ongur, D., & Price, J. L. (1998). Prefrontal cortical projections to longitudinal columns in the midbrain periaqueductal gray in macaque monkeys. <u>J.Comp Neurol</u>., 401, 455-479.

Melnikov, A. L., Malakhov, K. Y., Helgesen, K. G., & Lathrop. D. A. (2009). Cardiac effects of non-depolarizing neuromuscular blocking agents pancuronium, vecuronium in isolated rat atria. <u>General </u>33, 313-317.

Barnes, P., Brown, M. J., Dollery C. T., Fuller R. W., Heavery D. J., & Ind P. W (2008). Histamine is released from skin by substance P but does not act as the final vasodilator in the axon reflex. <u>British Jounal of pharmacology. </u>. 88, 741-745.

Basbaum, A. I., & Fields, H. L. (1984). Endogenous pain control systems: brainstem spinal pathways and endorphin circuitry. <u>Annu.Rev.Neurosci</u>., 7, 309-338.

Beitz, A. J. (1982). The organization of afferent projections to the midbrain periaqueductal gray of the rat. <u>Neuroscience, </u>7, 133-159.

Beitz, A. J. (1985). The midbrain periaqueductal gray in the rat. I. Nuclear volume, cell number, density, orientation, and regional subdivisions. <u>J.Comp Neurol., </u>237, 445-459.

Bruce, A. N. (1913). Vasodilator- Axonal reflex. <u>Journal of Experimental Physiology. </u>6, 339-354.

44

Carpenter, S. E., & Lynn, B. (1981). Vascular and sensory responses of human skin to mild injury after topical treatment with capsaicin. Br.J.Pharmacol., 73, 755-758.

Chahl, L. A. (1988). Antidromic vasodilatation and neurogenic inflammation. Pharmacol.Ther., 37, 275-300.

Cooper, J. R., Bloom, F. E., &. Roth. R. H. (2003). The Biochemical basis of Neuropharmacology. (4th ed) Oxford University Press.

Curtis, D. R., Gynther, B. D., Lacey, G., & Beattie, D. T. (1997). Baclofen: reduction of presynaptic calcium influx in the cat spinal cord in vivo. Exp.Brain Res., 113, 520-533.

Curtis, D. R., Headley, P. M., & Lodge, D. (1984). Depolarization of feline primary afferent fibres by acidic amino acids. J.Physiol, 351, 461-472.

Curtis, D. R. & Lacey, G. (1994). GABA-B receptor-mediated spinal inhibition. Neuroreport, 5, 540-542.

Derkach, V., Surprenant. A., & North, R. A. (1989). 5-HT3 receptors are membrane ion channels. Nature. 339, 706-709.

Eberhart, J. A., Morrell, J. I., Krieger, M. S., & Pfaff, D. W. (1985). An autoradiographic study of projections ascending from the midbrain central gray, and from the region lateral to it, in the rat. J.Comp Neurol., 241, 285-310.

Eccles, J. C., Schmidt, R., & Willis, W. D. (1963). Pharmacological studies on presynaptic inhibition. J.Physiol, 168, 500-530.

Evans, R. H. (1985). Kainate sensitivity of C-fibres in rat dorsal roots. Journal of Physiol (Lond). 342-358.

Fields, H. L. & Basbaum, A. I. (1978). Brainstem control of spinal pain-transmission neurons. Annu.Rev.Physiol, 40, 217-248.

Fuller, R. W., Conradson, T. B., Dixon, C. M., Crossman, D. C., & Barnes, P. J. (1987). Sensory neuropeptide effects in human skin. Br.J.Pharmacol., 92, 781-788.

Granadose-Soto, V., Arguelles, C. F., & Avarez-Leefmans, F. J. (2004) Peripheral and central antinorciceptive action of Na+ K+ Cl- cotransporter blockers on formalin induced norciception in rats, (poster) Society for Neuroscience.

Green, A. L., Wang, S., Owen, S. L., Xie, K., Bittar, R. G., & Stein, J. F. (2006). Stimulating the human midbrain to reveal the link between pain and blood pressure. Pain, 124, 349-359.

Herath, P. M., Hagains, C. E., Kachlic L. A., He, J. W., Liu, H., & Peng, Y. B.(2008) Increased blood perfusion after GABA evoked dorsal root reflex, (abstract) Society for Neuroscience.

Hinsey, J. C., & & Gasser, H. S. (1930). The component of the dorsal root mediating vasodilation and the Sherrington contracture. American Journal of Physiology . 92, 679-689.

Hong, Y., & Henry, J. L., (1990). Spinal mediation of the increases in arterial pressure and heart rate in response to intrathecal administration of bicuculline. Brain Res. 513(1), 86-93.

Jacques, L., Couture, R., Drapeau, G., & Regoli, D. (1989). Capillary permeability induced by intravenous neurokinins. Receptor characterization and mechanism of action. Naunyn Schmiedebergs Arch.Pharmacol., 340, 170-179.

Lewis, T., & Zotterman, Y. (1927). Vascular reactions of the skin to injury: Part VIII. The resistance of the human skin to constant currents, in relation to injury and vascular response. J.Physiol, 62, 280-288.

Lin, Q., Peng, Y., & Willis, W. D. (1994). Glycine and GABAA antagonists reduce the inhibition of primate spinothalamic tract neurons produced by stimulation in periaqueductal gray. Brain Res., 654, 286-302.

Lin, Q., Peng, Y. B., & Willis, W. D. (1996a). Antinociception and inhibition from the periaqueductal gray are mediated in part by spinal 5-hydroxytryptamine(1A) receptors. J.Pharmacol.Exp.Ther., 276, 958-967.

Lin, Q., Peng, Y. B., & Willis, W. D. (1996b). Inhibition of primate spinothalamic tract neurons by spinal glycine and GABA is reduced during central sensitization. J.Neurophysiol., 76, 1005-1014.

Lin, Q., Peng, Y. B., & Willis, W. D. (1996c). Role of GABA receptor subtypes in inhibition of primate spinothalamic tract neurons: difference between spinal and periaqueductal gray inhibition. J.Neurophysiol., 75, 109-123.

Lin, Q., Wu, J., & Willis, W. D. (1999). Dorsal root reflexes and cutaneous neurogenic inflammation after intradermal injection of capsaicin in rats. J.Neurophysiol., 82, 2602-2611.

Lovick, T. A. (1985). Ventrolateral medullary lesions block the antinociceptive and cardiovascular responses elicited by stimulating the dorsal periaqueductal grey matter in rats. Pain, 21, 241-252.

Low, A., & Westerman, R. A. (1989). Neurogenic vasodilation in the rat hairy skin measured using a laser Doppler flow meter. Life Sci., 45, 49-57.

47

Lynn, B. (1988). Neurogenic Inflammation. Skin Pharmacology. 1, 217-224.

Mantyh, P. W. (1983). Connections of midbrain periaqueductal gray in the monkey. II. Descending efferent projections. J.Neurophysiol., 49, 582-594.

Millan, M. J. (2002). Descending control of pain. Prog.Neurobiol., 66, 355-474.

Myers, R. R., Campana, W. M., & Shubayew V.I. (2006). The role of neuroinflammation in neuropathic pain: mechanisms and therapeutic targets. Drug Discov Today. 11, 8-20.

Nishi, S., Minota, S., & Karczmar, A. G. (1974). Primary afferent neurons: the ionic mechanism of GABA-mediated depolarization. Neuropharmacology, 13, 215-219.

Peng, Y. B., Kenshalo, D. R., & Gracely, R. H. (2003). Periaqueductal gray-evoked dorsal root reflex is frequency dependent. Brain Res., 976, 217-226.

Peng, Y. B., Lin, Q., & Willis, W. D. (1996a). Effects of GABA and glycine receptor antagonists on the activity and PAG-induced inhibition of rat dorsal horn neurons. Brain Res., 736, 189-201.

Peng, Y. B., Lin, Q., & Willis, W. D. (1996b). Involvement of alpha-2 adrenoceptors in the periaqueductal gray-induced inhibition of dorsal horn cell activity in rats. J.Pharmacol.Exp.Ther., 278, 125-135.

Peng, Y. B., Lin, Q., & Willis, W. D. (1996c). The role of 5-HT3 receptors in periaqueductal gray-induced inhibition of nociceptive dorsal horn neurons in rats. J.Pharmacol.Exp.Ther., 276, 116-124.

Peng, Y. B., Wu, J., Willis, W. D., & Kenshalo, D. R. (2001). GABA(A) and 5-HT(3) receptors are involved in dorsal root reflexes: possible role in periaqueductal gray descending inhibition. J.Neurophysiol., 86, 49-58.

Rees, H., Sluka, K. A., Lu, Y., Westlund, K. N., & Willis, W. D. (1996). Dorsal root reflexes in articular afferents occur bilaterally in a chronic model of arthritis in rats. J.Neurophysiol., 76, 4190-4193.

Reichling, D. B. & Basbaum, A. I. (1990). Contribution of brainstem GABAergic circuitry to descending antinociceptive controls: II. Electron microscopic immunocytochemical evidence of GABAergic control over the projection from the periaqueductal gray to the nucleus raphe magnus in the rat. J.Comp Neurol., 302, 378-393.

Rossi, F., Maione, S., & Berrino, L. (1994). Periaqueductal gray area and cardiovascular function. Pharmacol.Res., 29, 27-36.

Schaible, H. G., Schmidt, R. F., & Willis, W. D. (1987). Enhancement of the responses of ascending tract cells in the cat spinal cord by acute inflammation of the knee joint. Exp.Brain Res., 66, 489-499.

Sluka, K. A., & Westlund, K. N. (1992). An experimental arthritis in rats: dorsal horn aspartate and glutamate increases. Neurosci.Lett., 145, 141-144.

Wall, P. D. (1994). Control of impulse conduction in long range branches of afferents by increases and decreases of primary afferent depolarization in the rat. European Journal of Neuroscience, 6, 1136-1142.

Willis, W. D., Jr. (1999). Dorsal root potentials and dorsal root reflexes: a double-edged sword. Exp.Brain Res., 124, 395-421.

Yang, S. W., Zhang, C., Zhang, Z. H., Qiao, J. T., & Dafny, N. (1996). Sequential mediation of norepinephrine-and dopamine-induced antinociception at the spinal level: involvement of different local neuroactive substances. Brain Res.Bull., 41, 105-109.

Zhuo, M., & Gebhart, G. F. (1997). Biphasic modulation of spinal nociceptive transmission from the medullary raphe nuclei in the rat. J.Neurophysiol., 78, 746-758.

Zimmermann, M. (1983). Ethical guidelines for investigations of experimental pain in conscious animals. Pain, 16, 109-110.

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