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DOES PERIPHERAL KNEE JOINT INFLAMMATION INCREASE NEUROGENESIS IN THE SPINAL CORD AND HIPPOCAMPUS?

by

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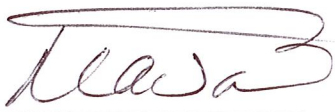

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Finally, This thesis is only a beginning of my journey.

AN ABSTRACT OF THE THESIS OF

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Title: Does Peripheral Knee Joint Inflammation Increase Neurogenesis in the Hippocampus and Spinal Cord?

Background: Osteoarthritis is a degenerative joint disease that involves swelling and damage of the affected joints causing pain, and hypersensitivity to mechanical and thermal stimulation. Peripheral and central neuronal sensitization, including the up-regulation of many excitatory postsynaptic receptors, ion channels and astrocytic proliferation, have provided a mechanistic explanation for the heat hyperalgesia and mechanical allodynia that develop post inflammation. Along with nociceptive behaviors associated with peripheral inflammation, the body also learns to adjust certain behaviors in response to altered environmental demands, such as guarding the inflamed site and changing gait patterns. Learning to adjust requires the hippocampus, and leads to neuroplasticity and neurogenesis in this area. Though it is well established that neurogenesis occurs in the hippocampus, new studies have recently shown that under normal conditions, adult spinal cord neurons can also undergo neurogenesis at a constant rate, and the newly born cells migrate to the superficial dorsal horn. However, whether this rate changes under pathological conditions is still not yet confirmed.

Hypothesis and Aim: In light of these findings, we hypothesize that the observed central neuronal sensitization associated with peripheral inflammation, might be attributed to increased cellular proliferation that lead to reorganization of nociceptive neuronal circuits in the dorsal horn of the spinal cord as well as neurons in higher brain areas, particularly those involved in memory and learning. The aim of the present study is to examine whether knee joint inflammation, using an animal model of osteoarthritis, promotes cellular proliferation in the spinal cord as well as the dentate gyrus (DG) of the hippocampal region.

Methods: Adult Male Sprague-Dawley rats were used in this study. To induce knee joint inflammation, a mixture of 3% kaolin and carrageenan (K/C; 0.1ml) was injected in the synovial cavity of the left knee joint. A group of rats receiving intra-articular injection of saline served as control. Changes in sensory and motor behaviors were assessed in all rats prior to injection and at 4, 8, 24 and 48 hours post induction of inflammation. Nociceptive behavioral tests such as mechanical allodynia and heat hyperalgesia were performed. In addition, motor coordination in rats was tested using the rotarod test.

To assess the severity of the inflammation, knee joint circumference of each rat was measured prior to and at 4, 8, 24 and 48 hrs post injection. All rats received an intraperitoneal injections of 5'-Bromo-2'- deoxyuridine (BrdU) (200 mg/kg; ip) before induction of inflammation. Rats were then sacrificed at 8, 24, and 48 hrs after the injection and perfused transcardially with 4% paraformaldehyde. Brain and spinal cord tissues were removed, post fixed and subsequently processed for immunofluorescence staining. Newly born neuronal cells immuno-positive for

BrdU and neuronal nuclear antigen (NeuN), a mature neuronal marker, were identified using laser scanning confocal microscopy. The effects of peripheral inflammation on neurogenesis in the spinal cord and the dentate gyrus (DG) of the hippocampus were compared between groups and analysed.

Results and Conclusions: Injection of K/C into the knee joint of adult rats produced swelling of the knee and nociceptive behaviors that peaked at 8hrs. Immunofluorescent staining revealed an increase in BrDU positive neurons both in the dorsal horn of the spinal cord and the dentate gyrus of the hippocampus. The increase in cellular proliferation was shown to correlate with the severity of inflammation. The results of the present study provide new insights into the role of cellular proliferation in the development of peripheral inflammatory pain.

CONTENTS

ILLUSTRATIONS

ABBREVIATIONS

CNS	Central Nervous System
PNS	Peripheral Nervous System
DRG	Dorsal Root Ganglion
SP	Substance P
CGRP	Calcitonin Gene Related Peptide
NKA	Neurokinin A
ET-3	Endothelin 3
NMDA	N-Methyl-D-Aspartate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
DG	Dentate Gyrus
GC	Granule Cells
BrdU	Bromodeoxyuridine/5-bromo-2’deoxyuridine
NSCs	Neural Stem Cells
PAN	Posterior Articular Nerve
MAN	Medial Articular Nerve

c-AMP	Cyclic adenosine monophosphate
LTP	Long term potentiation
IL-1B	Interleukin 1 beta
CA1	Cornu Ammonis 1
ALDH2	Aldehyde dehydrogenase 2
PBS	Phosphate Buffered Saline
L3-L4	Lumbar 3-Lumbar 4
HCL	Hydrochloric acid
K/C	Kaolin and Carrageenan
AIA	Antigen Induced Arthritis
DCX	Doublecortin expressing cells
GABA	Gamma Aminobutyric Acid
NKCC1	Sodium-Potassium-Chloride Cotransporter 1
KCL	Potassium Chloride
SCI	Spinal Cord Injury
NeuN	Nuclear Neuronal Antigen

CHAPTER I

INTRODUCTION

A.NEUROGENIC INFLAMMATION

Neurogenic inflammation is a pathophysiological process that involves a complex biological response of the immune, vascular and neural systems (Geppetti, et al., 2008). It is initiated through the release of peptides and inflammatory mediators from the peripheral nervous system (PNS), particularly from small-diameter C afferent fibers. Although many studies highlighted the contribution of primary afferent C fibers, more evidence support the role of sympathetic postganglionic terminals in the development and maintenance of neurogenic inflammation (Levine et al., 1985). Substance P (SP) and Calcitonin gene related peptide (CGRP) are two of several released peptides that act on postcapillary venules, and cause plasma extravasation, vasodilatation and hypersensitivity of sensory neurons (Schaible et al., 2011). The release of peptides can also cause antidromic activation of C fibers which results in more neuropeptide release at peripheral terminals that further activate immune cells, thereby inducing a positive feedback loop that drives and facilitates inflammation. The CNS integrates the information received from the periphery and subsequently, modulates the tissue response to inflammatory mediators. Neurogenic inflammation has been implicated in several diseases including asthmas, rhinitis, migraine headache, psoriasis, fibromyalgia, and arthritis (Kodji et al., 2018; Littlejohn, et al., 2018; Tomljenovic et al., 2018). While it occurs in many tissues, it has been chiefly studied in the skin (Viana, 2018; Hagains, et al., 2010). Over the past two decades, accumulating research findings have supported the hypothesis that neurogenic inflammation is a major contributor to the development and maintenance of arthritis (Krustev et al., 2015; Walsh et al., 2015). Joints are greatly innervated by neuropeptides-expressing primary afferent and autonomic fibers (Davenport, et al., 2016;

Franco et al., 2015). When peptides are released into the synovial cavity of knee joints, they exert proinflammatory actions on articular tissues (Spilberg, 1975; Hernanz et al., 1993). Moreover, blocking the activity of the articular nerve has been shown to reduce the joint susceptibility to arthritis (Cambridge et al., 1992; Niissalo et al., 2002). Taken together, these lines of evidence highlight the pathophysiological role of neurogenic inflammation as one of the underlying mechanisms of arthritis.

B.NEUROGENIC INFLAMMATION IN OSTEOARTHRITIS

Osteoarthritis is a common degenerative joint disease that affects more than 30 million middle age to elderly people across different regions of the world (Kwoh, 2012; Pereira et al., 2015). It is characterized by a progressive loss of the articular cartilage, subchondral bone thickening, synovial inflammation and in severe cases, deterioration of the entire joint (Sophia et al., 2009; Loeser et al., 2012; Funck and Cohen, 2015)

The pathological changes existing in OA are the result of the action of biomechanical forces associated with a number of extracellular and intracellular events that lead to a breakdown in intra-articular tissues (Pereira et al., 2015; Thomas et al., 2017).

Typically, these events are accompanied by joint pain and inflammation, tenderness, occasional effusion, rigidity, crepitus, and limitation of movement (Scanzello, 2017). At the outset of the disease, the pain emerges as a result of prolonged activity; however, as the condition progresses, pain becomes chronic and worsens in severity over time (Hunter et al., 2008). Stiffness is usually very common in the morning, and last not more than thirty minutes after beginning daily activities, but may often return after periods of inactivity. Osteoarthritic patients also suffer from joint bolting and trembling as well as a cracking noise of the shoulder and knee joint during movement (Song et al., 2018). All the aforementioned symptoms have been shown to affect their daily activities due to pain and stiffness (Peat et al., 2001). Some

people report increased pain associated with cold temperature, high humidity, or a drop in barometric pressure (McAlindon et al., 2007). Overall, the symptoms will eventually lead to loss of joint function and diminish the patient's quality of life. Interestingly, the prevalence of osteoarthritis and the severity of symptoms are higher in women than in men (Sokka et al., 2009). By some estimates, nearly 10 percent of men and about 18 percent of women over the age of sixty are affected by the condition. Research suggests that estrogen has an effect on the cushioning cartilage that is situated between the bones of the joints to allow smooth joint movement (Richette et al., 2003). Although estrogen protects the joint cartilage from inflammation, women lose that protection after menopause when the levels of estrogen drop. Treatment with estrogen improves both synovitis and joint destruction in ovariectomized mice via activation of estrogen receptors α (ERα) (Engdahl et al, 2014).

Despite extensive pharmacological and physiological studies seeking therapeutic interventions for this disease, unfortunately, there are still no effective treatments for osteoarthritis.

C.NEUROGENIC INFLUENCES IN OSTEOARTHRITIS

- ***Joint Innervation***

The anatomy of the rat knee joint is as intricate as that of the human knee. Two sets of primary articular nerves were identified in relation to the knee joint: a posterior (PAN) and a medial (MAN) articular nerve (Hildebrand et al., 1991; Dold et al., 2017). The PAN is larger and emerges as a single trunk from the tibial nerve in the popliteal fossa, at or slightly distal to the level of the branches to the gastrocnemius muscle. The PAN then courses through the popliteal fat towards the joint capsule, at right angles to the popliteal vessels (Heppelmann, 1997).

Afferent fibers within articular nerves fall within three distinct groups: heavily myelinated Aβ (group II) fibers, thinly myelinated Aδ (group III) fibers and unmyelinated C

(group IV) fibers. Synovial joints are innervated with the three groups, in addition to unmyelinated sympathetic postganglionic fibers (Longo et al., 2013). The vast majority of articular fibers are unmyelinated, comprising sensory C fibers and sympathetic efferent fibers in nearly equal numbers. Only 20% of these fibers in a typical articular nerve are myelinated, mainly involving Aδ fibers with relatively few of the larger diameter Aβ fibers (Samuel, 1949).

The articular capsule of the joint receives an extensive network of nerve fibers with free complex or encapsulated nerve endings. A similar innervation has been found in tendons, ligaments, deep fascia and periosteum (Fox et al., 2009).

Articular afferent fibers are grouped on the basis of their response to mechanical stimuli. The first group includes low threshold afferents activated by innocuous stimuli, such as movement within the normal range, which presumably have a role in proprioception; the majority of rapidly conducting Aβ fibers falls into this category (Wojtys, 1990). The second group includes afferents activated mainly or purely by noxious stimuli such as movement exceeding the normal range; these are Aδ and C fibers and, in so far as they respond mainly to potentially damaging events within the joints, may be classed as nociceptors. Finally, some fibers do not react to any mechanical stimulus applied to the normal joint whatsoever; these have been termed mechano-insensitive afferents or silent nociceptors.

Recent immunohistochemical studies have shown that the knee synovial tissue is also richly supplied with sensory nerve fibers. The exact modality of nerve distribution in the synovium has been examined using the modified gold chloride method that has a high specificity for neural elements. This type of staining revealed an extensive neural network that comprises free nerve endings and several mechanoreceptors. These receptors are activated by relatively narrow ranges of stimulus intensity. The sensitivity of these receptors is governed by a critical interaction with the local microenvironment as well as by factors released from the immune and nervous systems.

In osteoarthritic synovium, SP immunoreactive nerve fibers were widely distributed. Substance P was found in free nerve endings and nerve fiber bundles with or without accompanying blood vessels, and in the perivascular neural network. The incidence of SP-positive free nerve fibers or endings was predominantly found on the medial side. Substance P-positive free nerve endings in the medial synovium showed distinct axonal branching, while some containing the same peptide were encircled by several monocytes forming a cluster (Wojtys, 1990).

The distribution of the nerves containing CGRP showed almost the same pattern as those containing SP. The main location of CGRP was in large or medium nerve fiber bundles. The incidence of CGRP-immunoreactive free nerve fibers was most prevalent in synovium from the medial compartment, followed by the lateral compartment, and the suprapatellar pouch (Hanesch et al., 1997).

Given the intricate innervation of the knee joint, there has been a growing recognition that neurogenic mechanisms play an important role in the development and maintenance of osteoarthritis. It appears that the inflammatory responses present in the joint encompass a series of vascular and non-vascular inflammatory reactions, triggered by the activation of primary sensory neurons and the subsequent release of neuropeptides, such as SP, CGRP, Neurokinin A (NKA), and endothelin-3 (ET-3) from sensory nerve terminals into the joint (Bileviciutea et al., 1997).

These neuropeptides trigger the release of histamine from adjacent mast cells, which in turn stimulates more peptide release suggesting a bidirectional link between the immune and the nervous system (Bileviciutea et al., 1997). The presence of SP and CGRP in the joint results in vasodilatation, plasma extravasation and hypersensitivity of articular sensory receptors (Lam and Ferrell, 1991).

- ***Joint Pain***

Joint pain is the main symptom in osteoarthritis, and is associated with poor functional outcomes when compared with other chronic conditions (McDougall, 2006). Twenty five percent of elderly people have a persistent episode of knee pain that requires frequent treatment with steroidal and non-steroidal anti-inflammatory drugs. Pain associated with osteoarthritis may present with different clinical features and can be broadly categorized into three stages: 1) Onset pain triggered by an activity. 2) Mid OA characterized by constant and intermittent pain, with or without a neuropathic component and 3) advanced OA characterized by a dull aching pain, interrupted by episodes of unpredictable throbbing and intense pain. The pain is typically made worse by prolonged activity and relieved by rest (McDougall, 2006; Philpott et al., 2017).

A number of studies have shown that OA pain originates in the free axonal endings innervating the synovium, periosteum bone, and tendons, but not the cartilage and that it involves both peripheral and central neurophysiological mechanisms (Blom et al., 2004; Sun et al., 2017). The nociceptive events involve not only peripheral neurotransmitters, mediators and modulating factors, but also central regulators of pain pathways. It is an intricate state of affairs where local and extraneous factors originating both within and external to the individual are at play. Pain perception arises in response to a cascade of underlying neurophysiologic phenomena involving transduction of stimuli, transmission of encoded information, and subsequent modulation of this activity at both peripheral and more central levels. For that reason, the intensity of arthritic pain is correlated poorly with joint degradation because pain is multidimensional and governed by numerous environmental, and psychological factors (Buckwalter et al., 1998).

Several lines of evidence highlight the importance of neural mediators released from the synovium as being critical to symptoms development in OA (Kidd, 2012). The

manifestation of knee pain has been shown to result from activation of specific peripheral receptors present at the terminal ends of articular nerves. The sensitivity of these receptors is governed by a critical interaction between peripherally released neurotransmitters and inflammatory mediators in response to joint injury (Pinho-Ribeiro, et al., 2017). Several cellular mechanisms by which these changes occur entail early post-translational changes, activation of receptor ion channels and subsequently longer lasting transcription-dependent mechanisms. Along with peripheral changes, exaggerated responses to normal stimuli together with expansion of receptive field size producing tenderness and referred pain in areas away from the site of injury also occur at the spinal level (Hylden et al., 1989). Substantial number of neurotransmitters and neuromodulators has been proposed to influence the nociceptive system.

D.K/C MODEL OF OSTEOARTHRITIS

Despite extensive clinical management of osteoarthritic patients, the cause and pathogenesis of OA are still not fully understood (Chen et al., 2017). Many scientists attempted to model this joint disease in experimental animals to gain profound insight into the underlying cellular and molecular mechanisms leading to its development (Ren and Dubner, 1999; Teeple et al., 2013). Several animal models of OA were developed in small and large animals; however, rodents were the most widely used to model this type of inflammation since they have a complex articular innervation similar to that found in humans (Vandamme, 2014; McCoy, 2015).

Over 20 different OA animal models have been developed using different induction methods; nevertheless, kaolin and carrageenan-induced arthritis is a well established and most used model of acute monoarthritis that partly mimics the initial inflammatory stages of OA

(Neugebauer, 2013; Kuniyu et al., 2016). It is mainly characterized by degeneration of hyaline articular cartilage and subsequent inflammation and pain.

Taking into consideration the progressive degenerative nature of inflammatory disorders, the majority of studies using this model use a constricted time frame. The K/C arthritis model has been used in several animals, including cats (Schaible et al., 1987) monkeys (Dougherty et al., 1992; Vierboom, et al., 2005), rats (Neugebauer, 2013; Radhakrishnan et al., 2003), mice (Little and Zaki, 2012), and guinea pigs (Meacock, 1990) to study inflammatory pain mechanisms in the peripheral and central nervous systems. Using this model, the animal exhibits nociceptive behaviors, inflammatory changes in the synovium, and neuroplasticity with a well-defined time-course of acute onset followed by a plateau phase that persists for at least 1 week (Covacu and Brundin, 2017).

kaolin, is a type of clay used to mechanically destroy the intra-joint structures which can not be achieved in inflammatory models using carrageenan alone. However, when kaolin and carrageenan mixture is injected into the synovial cavity of the knee joint, it results in damage to the cartilage, and subsequent inflammation of the synovia.

E.MODULATION OF NEUROGENIC INFLAMMATION AT THE PERIPHERAL LEVEL

It is now evident that afferent fibers far from being stagnant as first assumed, are changing structures able to vary their responses to a wide range of stimuli including noxious stimulation. There appears to be a dynamic interaction between sensory fibers and the molecules present in the surrounding environment that can decrease the excitation threshold and cause sensitization by increasing transduction that is secondary to repeated stimulation. Peripheral sensitization is well illustrated in the joint, where inflammatory mediators elicit

profound changes in the response properties of afferents to movement and other chemical mediators (Levine et al., 1985).

Several hours after induction of experimental arthritis with a mixture of kaolin and carrageenan, the response pattern of articular sensory fibers changes dramatically. The majority becomes sensitized and shows an increased responsiveness to stimuli applied to the joint. First, the high threshold primary afferent fibers become sensitized to a normal range in movements, and have resting activity even when mechanical stimulation is absent. Secondly, a proportion of the initially mechano-insensitive sensory fibers develop responsiveness to mechanical stimuli. It is relevant that the time-course of these changes in articular fibers mirrors the development of pain behavior in awake animals (Schaible et al., 2002).

Injection of carrageenan/kaolin mixture into the knee joint causes an immediate increase in glutamate and nitric oxide metabolites in the knee joint, which persists for hours (Lawand et al., 2000). The increase is prevented by intraarticular administration of lidocaine suggesting that the glutamate is released from neuronal endings in the joint (Lawand et al., 2000). This release is accompanied by synthesis and release of inflammatory mediators and neuropeptides such as SP and CGRP, which cause edema and rapid infiltration of neutrophils within the first few hours (Schaible, 2012). These local changes in the joint are likely responsible for the sensitization of peripheral nociceptors and sensory afferents, which contribute to the development of primary and secondary hyperalgesia, and sensitization of spinal dorsal horn neurons (Schaible et al., 2002).

Many, if not all, of the inflammatory mediators released into the affected joint fluid exhibit strong and complex effects on primary afferent fibers, either by directly activating receptors, or by sensitizing others to mechanical, thermal and other stimuli. In either case, the resultant effect of these mediators depends ultimately on the responses of membrane ion channels, which may be directly linked to membrane receptors (receptor-gated ion channels)

or controlled indirectly through activation of intracellular second messengers (Miller et al., 2015).

F.MODULATION OF NEUROGENIC INFLAMMATION AT THE SPINAL CORD LEVEL

Many studies have reported that repeated and sustained sensory input from small C and Aδ primary afferent fibers originating in the knee joint triggers a sustained but reversible enhancement in the excitability and synaptic efficacy of central nociceptive neural circuitries (Latremoliere and Woolf, 2009). Increased barrage from primary afferents neurons release glutamate and neuropeptides centrally leading to interruption of descending inhibition. Increased glutamate release depolarizes N-methyl-D-aspartate (NMDA) receptors in dorsal horn neurons and reduces their excitation threshold leading to augmentation of receptor fields, and initiation of sustained ongoing discharges (Latremoliere and Woolf, 2009; Woolf and Salter, 2000). In this specific type of spinal central sensitization, increased release of glutamate ends in increased calcium levels and kinase stimulation, which in turn modulates descending and facilitating pathways of the CNS and leads to the development of hyperalgesia and Allodynia (Woolf and Salter, 2000; Schaible, 2007; Yam et al., 2018).

In models of inflammatory pain, increased afferent input, induced by peripheral inflammatory mediators, triggers large increases in neurotransmitter content in the dorsal horn of the spinal cord. Released glutamate, SP and CGRP have been measured over time in the dorsal horn (Sluka et al., 1992; Sluka et al., 1994a, b; Duggan, 1995). After induction of knee joint inflammation, glutamate begins to increase within 15 min, ramps upward for 4 h, and remains elevated for over 8 h. This increase is highly correlated with the time course of hyperalgesic behaviors observed in a kaolin/carrageenan model of inflammation (Sluka et al., 1994a). Additionally, Substance P and CGRP are also greatly released from the central

terminals of nociceptors and were shown to contribute to the development of hyperalgesia associated with inflammation. Activation of CGRP receptors on central terminals of primary afferent neurons further facilitates transmitter release and increases glutamate activation of AMPA receptors (Zhang, 1993; Duggan, 1995). The effects of these receptors are mediated by cAMP-dependent mechanisms. Substance P activates the three subtypes of neurokinin receptors coupled to phospholipase C and triggers the generation of intracellular messengers resulting in depolarization of neuronal membrane and enhancing the function of AMPA and NMDA receptors. Moreover, it was shown that activation of neurokinin-1 receptors by SP release in the dorsal horn triggers the synthesis of prostaglandins whereas neurokinin-3 receptor activation leads to nitric oxide synthesis (Zhang et al., 2007). Both substances act as retrograde messengers across excitatory synapses and enhance nociceptive processing in the spinal cord (Wu et al., 2001). These central changes have been shown to profoundly modulate the neurogenic inflammation.

G.MODULATION OF NEUROGENIC INFLAMMATION AT THE CORTICAL LEVEL

Neurogenic inflammation is processed at different levels of the central nervous system (McColl et al., 2014). Widely distributed and interconnected neural networks in the spinal cord and brainstem modulate the peripheral inflammatory response and its behavioral correlates. Nuclei in the brainstem exert their effect on nociceptive neurons in the spinal cord through activation of descending inhibitory and excitatory pathways (Mello and Dickenson, 2008). On the other hand, cortical neuronal circuitries in different areas in the brain have been proven to process, integrate and modulate inflammatory pain. The involvement of the anterior cingulate cortex, insular cortex, somatosensory cortex and motor cortex in processing peripheral inflammation have been strongly demonstrated. Similar to the brainstem, the cortex exerts its

modulatory effect through direct or indirect descending projections to dorsal horn neurons of the spinal cord and to the trigeminal nucleus. Indirect projections from the cortex involve many neuronal circuitries in the amygdala, hypothalamus, periaqueductal gray, and medulla. While these structures are shown to be significantly involved in regulating the pain and inflammatory response, greater emphasis has now been placed upon the role of the hippocampus, an integral component of the limbic system, in pain processing and its interaction with the immune system. Anatomical studies have demonstrated that several projections from the hippocampus to a number of limbic and cortical areas are involved in the processing of the motivational-affective component of pain (Hofbauer et al., 2001).

In early studies by Lico et al., (1974), the involvement of the hippocampus in modulating the perception of pain was examined. They found that stimulation of dorsal hippocampus could exert an analgesic and algesic effects. Furthermore, Prado and Roberts (1985) reported that electrical stimulation of the dorsal and medial hippocampus caused strong antinociception while stimulation of the lateral and ventral hippocampus elicited none. Evidence for the role of the hippocampus in processing inflammatory pain comes from studies by Abbott and his colleagues in which they reported that electrical stimulation of the dorsal hippocampus resulted in a prolonged analgesia following formalin injection in the skin. In addition, functional, anatomical, and biochemical changes have been reported in the hippocampus of chronic pain patients and animal pain models making it a key structure in the limbic system neural circuitry that modulate the affective component of pain (Covacu and Brundin, 2017).

H.EFFECT OF PERIPHERAL INFLAMMATION ON HIPPOCAMPAL NEUROGENESIS

Neurogenesis is the plastic capacity of the brain to constantly generate new neurons from neural stem cells (NSCs) and integrate them into its own pre-existing circuitry (Baptista, et al., 2018; Toda and Gage, 2017). For long, it has been thought that neurogenesis occurs only during brain development (Sorrells et al., 2018). After several decades of debate (Eckenhoff and Rakic 1988; Rakic 1985) and evidence validating the existence of dividing cells in the adult brain (Ming and Song, 2011), it is only recently that an agreement among scientists has been reached that new neurons are added to the adult mammalian brain. The development of synthetic thymidine analogue 5-bromo-2′- deoxyuridine (BrDU) and other markers with the aid of confocal microscopy has helped to determine the existence of adult neurogenesis. Adult neurogenesis was eventually confirmed in the dentate gyrus (DG) of the hippocampus of rodents (Seki and Arai 1999) and in the DG of the human hippocampus (Knoth et al. 2010; Spalding et al. 2013). In the mammalian brain, new neurons are continually born throughout adulthood in two conventional niches: The subgranular zone (SGZ) of the dentate gyrus of the hippocampus and the subventricular zone (SVZ) of the striatum. A number of studies have shown that newly born neurons in the SVZ of rodents are interneurons that migrate to the olfactory bulb to contribute to the processing of olfaction. In contrast, very few olfactory bulb neurons are generated after birth in humans. Recently, much attention has been given to the study of neurogenesis in the dentate gyrus (Lieberwirth et al., 2016). Although newly born cells in this area die shortly after birth, many of them become functionally integrated into pre-existing circuitries. The function of newly integrated neurons in adult brain function remains to be determined. Mounting evidence indicates that adult neurogenesis plays a crucial role in learning and memory by contributing newly born granules cells (GC) to the dentate gyrus (Kropff et al., 2018). Numerous studies have suggested multiple mechanisms for the

relationship between enhanced neurogenesis and ameliorated cognition. New neurons in the hippocampus have been shown to increase memory capacity, or add information about time to memories (Harrison et al., 2014). While some experiments directed at blocking neurogenesis have not yielded conclusive results, a slew of studies have demonstrated neurogenic-dependence in some types of learning. The act of learning has been demonstrated to be associated with increased neuronal survival although the overall findings that adult neurogenesis is important for any kind of learning remains to be investigated.

Many intrinsic and extrinsic factors affect neurogenesis and cellular proliferation in the hippocampus. While central inflammation is one important factor that was shown to be detrimental for hippocampal neurogenesis, the influence of peripheral inflammation on cellular proliferation or neurogenesis has not received much attention (Whitney et al., 2009).

Despite the long-standing belief that the brain is an immune-privileged organ, it is now clear that the immune system plays a vital role in many central neuronal processes involved in learning and memory, including central sensitization, synaptic plasticity, long-term potentiation (LTP) and neurogenesis. It appears that the immune system is capable, through the release of pro-inflammatory molecules, to alter synaptic functions and neural plasticity within the CNS (Di Filippo et al., 2009). Recently, it has been demonstrated that peripheral inflammation causes the synthesis of pro-inflammatory cytokines, which through different routes, can communicate with neurons in the CNS to modulate the plastic changes of neuronal circuits in the hippocampus and to induce a range of behavioral/cognitive changes (Bablioni et al., 2010, Dantzer et al., 2008). There are numerous mechanisms through which peripheral inflammation can provoke changes in cytokine levels within sensitive brain regions. Circulating cytokines may be actively transported across the blood-brain barrier or via the circumventricular organs and vascular endothelium. Research studies have shown that the hippocampus, one of the central vagus nerve targets, show enhanced activity within 2–3 hours

of peripheral inflammatory response in both rodents and humans; whereas electrical stimulation of vagal afferents, results in a rapid increase in IL-1β expression within the hippocampus (Schneider et al., 1998; Hosoi, et al., 2000).

Activation of the immune system in the periphery has also been shown to trigger the release of high levels of pro-inflammatory mediators such as cytokines and prostaglandins from glial and immune cells, not only peripherally but also in the brain, and exerts negative effects on memory, neural plasticity and neurogenesis by activating microglia in the CA1 hippocampal region. Recently, accumulating evidence revealed that activated microglia reduces neurogenesis and cellular proliferation in the dentate gyrus of the hippocampus by inducing apoptosis of newly born neurons and preventing their migration and integration into existing neuronal circuits. Collectively, these data provide strong evidence to support the impact of peripheral inflammation on hippocampal function.

I.EFFECT OF PERIPHERAL INFLAMMATION ON SPINAL CORD NEUROGENESIS

Mounting evidence has indicated the presence of neurogenic potential in the spinal cord, from which multipotent precursors can be differentiated. Some of these precursor cells have stem cell markers, and exhibit different proliferation rates. In the spinal cord, cellular proliferation was found to occur at low levels; however, many external factors such as the addition of growth factors, hormones or other signaling molecules increase the proliferation and differentiation of these precursor cells (Rusanescu, 2016). Unlike the hippocampus, precursor cells transplanted into the spinal cord of adult rats remained mostly undifferentiated, with some exhibiting glial markers suggesting the presence of inhibitory factors in the spinal cord that suppress neuronal differentiation.

In spinal cord injury, proliferation of cells was observed in the adult spinal cord, specifically in the white matter. However, these cells were mostly glial progenitors that give rise to either astrocytes or oligodendrocytes (Sweitzer et al., 1999; Horner and Palmer, 2003). Recently, research studies have demonstrated the presence of adult neural stem cells at the lining of the central canal and their ability to proliferate in vitro (Martens et al., 2002). It was shown that infusion of growth factors into the fourth ventricle can increase the number of BrdU-expressing cells in the central canal, most of which are nestin-positive; nevertheless, no mature neurons were detected. Following spinal cord injury, the number of proliferating ependymal cells located outside the ependymal layer has been found to increase and migrate towards the site of injury, to contribute to the glial scar formation along with reactive astrocytes (Meletis et al., 2008). Subsequently, some of the proliferating cells differentiate into oligodendrocytes to aid in the re-myelination process of damaged axons.

Recent immunohistochemical studies (Gould, 2007) using EdU, a thymidine analogue, and specific neuronal markers demonstrated that adult-generated spinal cord neural stem cells could differentiate into mature neurons. Newly born cells in the ependymal layer of the spinal cord migrate along lamina IV toward the superficial layers of the dorsal horn and become mature neurons. In sciatic nerve injury, increased migration of neural progenitor cells from the ependymal layer and conversion into functional neurons has been demonstrated. More importantly, these newly differentiated cells were shown, three months after injury, to be concentrated in lamina I/II of the dorsal horn on the side ipsilateral to the injury, indicating their integration into pre-existing circuitry and their involvement in the modulation of pain perception. Moreover, Rusanescu and Mao (2015) observed that these cells exhibited enhanced excitability and increased expression of immature neuronal markers, at a time that coincide with the manifestation of increased nociceptive sensitivity following injury. Interestingly, many genes such as aldehyde dehydrogenase 2 (ALDH2), Notch3 and c-kit, that

stimulate neuronal differentiation are also involved in the regulation of nociceptive sensitivity. Together, these observations may implicate the spinal cord newly proliferating cells in the development of central sensitization, a mechanism underlying increased nociceptive sensitivity associated with neural injury and peripheral inflammation.

HYPOTHESIS AND AIM OF THE STUDY:

Neurogenic inflammation associated with OA results in a broad range of sensory (hyperalgesia and allodynia) and motor (limping) symptoms, in addition to plastic changes in the CNS. These changes include neuronal hyperexcitability, increased synaptic efficacy and enhanced nociceptive sensitivity. Whether cellular proliferation is linked to these plastic changes remains to be determined. In the present study, we hypothesize that peripheral neurogenic inflammation triggers cellular proliferation in the spinal cord and the hippocampus, two areas involved in processing inflammatory pain. To test this hypothesis, we aim to examine the effect of neurogenic inflammation, using an animal model of osteoarthritis, on cellular proliferation in the dorsal horn of the spinal cord and the dentate gyrus (DG) of the hippocampus using behavioral and immunohistochemical studies

CHAPTER II

MATERIALS AND METHODS

Adult Male Sprague-Dawley rats (n=24) were used in this study. All experimental procedures were approved by the institutional animal care and use committee at the American university of Beirut and followed the ethical guidelines for experimental pain on conscious animals.

A.Experimental Protocol

Rats were divided into three experimental groups according to time of sacrifice following induction of inflammation. The groups were sacrificed at 8 hrs (n= 4), 24hrs (n=4) and 48 hrs (n=4) post inflammation. Saline-injected and naive rats (were perfused at the same time frame with a total of n=4 in each group.

B.Induction of Inflammation

Rats were briefly anesthetized using Isoflurane. A 1 ml Syringe was used to inject a 0.1ml of a mixture of 3% kaolin and carrageenan solution into the synovial cavity of the left knee. Control rats received the equivalent volume of saline in the left knee as well.

C.Brdu Administration

Bromodeoxyuridine (5-bromo-2'-deoxyuridine,broxuridine), a thymidine analogue, is a synthetic nucleoside that is commonly used in the detection of proliferating cells in living tissues. 5-Bromodeoxycytidine is deaminated to form BrdU. BrdU, when injected intraperitoneally, will get incorporated into the newly synthesized DNA of replicating cells (during the S phase of the cell cycle during which DNA is replicated), substituting for thymidine during DNA replication. Antibodies specific for BrdU can then be used to detect the

incorporated chemical (see immunohistochemistry), thus labeling cells that were actively replicating their DNA. BrdU powder (Sigma-Aldrich B5002-IG) was weighed and dissolved in 0.9% warm saline. The solution was properly dissolved and filtered using a 0.2mm filter unit. All rats received three BrdU injections at two-hour interval (66mg/Kg/300ml/injection, ip), 12 hrs prior to the administration of the inflammatory agent to ensure maximal availability.

D.NOCICEPTIVE BEHAVIORAL TESTING

All rats were tested for nociceptive behaviors prior to and at 4, 8, 24 and 48 hrs post induction of inflammation. Tests were conducted during the light phase of the cycle. All animals were put in the experimental room for at least an hour for accommodation.

- ***Mechanical Allodynia Test***

Paw withdrawal frequency to a non-noxious stimulus was determined by applying a von Frey filament with a bending force of 2g to the plantar surface of the hindpaw. The tip of the filament was applied perpendicularly to the medial plantar surface from below the mesh grid until a withdrawal was observed. An increased response indicated the development of mechanical allodynia. Prior to testing, all rats were placed in a transparent chamber on a metal wire mesh floor and left for 30-min acclimatization period. Testing started by poking the paw 5 successive times with the filament. Five trials were used separated by 5 min time interval. Baseline values were obtained prior to injection of kaolin and carrageenan into the knee joint. After induction of inflammation, testing was repeated at 4, 8, 24 and 48hrs. Measurements were averaged for each animal and the responses of both the left and the right paws of all rat groups were recorded.

- ***Mechanical Hyperalgesia Test***

Paw withdrawal frequency to application of von Frey filament with a bending force of 15g to the plantar surface of the hindpaw was recorded. The 0.15N force applied by the filament has been shown to activate mechanoreceptors and nociceptors. Testing started by poking the plantar surface 5 successive times with the 15g filament until the animal elicits a behavioral response. Five trials were conducted separated by 5 min time interval. Baseline values were obtained and then testing was repeated at 4, 8, 24 and 48hrs post induction of inflammation. Measurements were averaged for each animal and the responses of both the left and the right paws of all rat groups were recorded.

- ***Heat Hyperalgesia Test***

Heat hyperalgesia test was done by measuring the foot withdrawal latency to radiant heat applied to the plantar surface of the hind paw. Approximately 15 min prior to testing, each rat was placed individually in a clear plastic cage placed atop an elevated 3-mm thick glass plate to accommodate. The height of the glass plate was adjusted so that heat applied to the plantar surface of the normal foot evoked a withdrawal response after approximately 20 s. To avoid conditioning limb withdrawal, heat stimuli were applied 3 times with a 5-min resting period between trials.

The heat stimulus consisted of a focused beam of light produced by a 50 W halogen light bulb encased in a vented container. The withdrawal latency was defined as the elapsed time, in seconds, from stimulus onset to paw withdrawal; a cut off of 20 s was imposed to avoid tissue damage.

E.Motor Behavioral Test

The rotarod test is one of the oldest used in assessing the effects of a drug on animal behavior. It provides a rapid and simple first estimation of whether the inflammatory agent has any effect on neuromuscular coordination. The rotarod consists of a horizontal rod turning at a constant or increasing speed. Animals placed on the rotating rod try to balance and remain on it to avoid falling off onto a platform 30 cm below. Vertical barriers were used to separate the animals from one another. Each rat was tested three times, with each trial lasting for 300 seconds and separated by at least a 10 min inter-trial period. Rats were placed on the rod and the apparatus was turned on to a fixed speed of 5 rpm. The latency to fall off the rotating rod was recorded.

F.Joint Circumference Measurement

In order to evaluate the severity of the joint inflammation, joint circumferences of all rats were assessed before and at 4, 8, 24, and 48 hrs post-inflammation. The circumference of the ipsilateral and contralateral knee joints was recorded using a flexible tape measure wrapped mid-patella around the center of the knee joint.

G.Animal Perfusion

Perfusion procedures were conducted at different time points according to the experimental protocol. All animals were deeply anesthetized with ketamine (ketalar:50mg/Kg) and Xyla (Xylazine;12mg/Kg) and then transcardially perfused with 0.9% saline solution followed by 4% paraformaldehyde (PFA) dissolved in 0.1 M phosphate buffer (PB, pH 7.4). After the brains and spinal cords were removed, tissues were post-fixed overnight in PFA and then transferred to 30% sucrose in PB solution for 48 h at 4 °C or until processing time. To visualize the distribution of proliferating cells in the entire hippocampus, we cut the brains

transversely in 40 µm sections using a freezing microtome in order to be stained for BrdU. The lumbar segment of the spinal cord for each rat was cut off and sectioned at 40-µm thickness.

H.Tissue processing

Perfused brains were sectioned coronally using a freezing microtome. The ventral aspect of the left side of the brain was marked by a small puncture. Brain sections were sliced serially from the rostral to the caudal extent of the hippocampus following the rostro- caudal coordinates of -2.12 to -6.6 mm with reference to bregma. The hippocampus was topographically divided into 3 sub-regions: rostral, intermediate and caudal according to the following coordinates: -2.12 to -3.7 rostral; -3.7 to -4.9 intermediate; and -4.9 to -6.3 caudal. Systematic sampling of brain sections was achieved following the fractionator method for unbiased stereology (Schmitz et al., 2014). Following this method, sections were collected in six wells containing 15mM sodium azide dissolved in PBS. Each well contained a specific number of slices designated to a specific region (rostral, intermediate, caudal). The 1^{st} section was placed in the first well, the 2^{nd} section in the second well and the 6^{th} in the sixth well and this was repeated so that the 7^{th} section gets placed in the first well and the difference between the 1^{st} and 7^{th} section would be 300mm. Consequently, each well represents a random depiction of each topographic area of the hippocampus (Chamaa et al., 2016). A random well was then picked from each region for immunofluorescent staining.

Spinal cord sections from L3-L4 segments were also cut at 40 µm using a freezing microtome. A total number of 180 slices were taken sequentially and placed in wells containing Sodium Azide (0.38g/100ml PBS) as described above. Sections were then processed for BrdU immunohistochemistry.

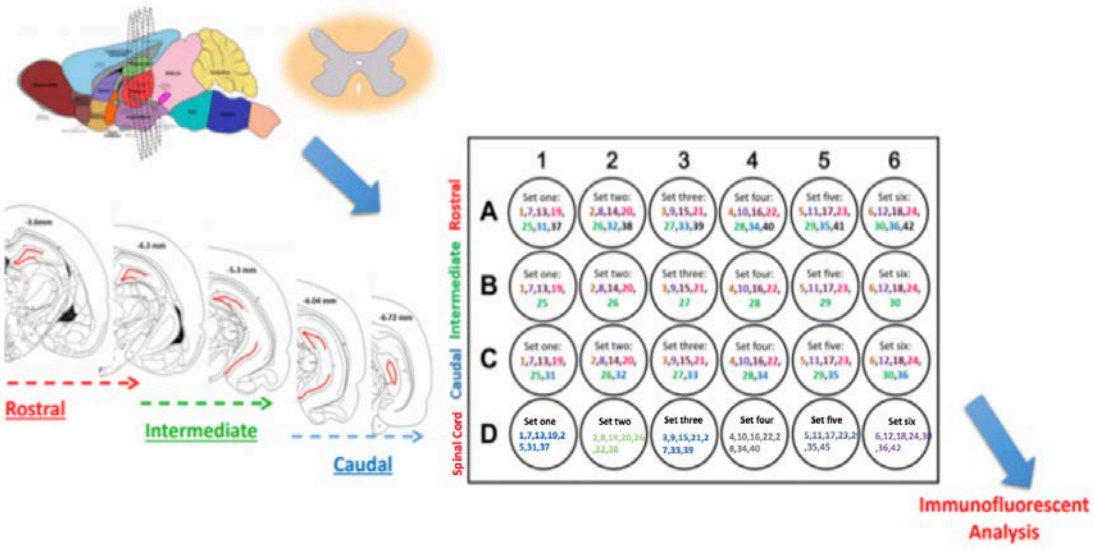


Figure 1: The fractionator Method: free floating sections of brain and spinal cord tissues (40µm) were distributed in a 24-well plate based on the topographical region (rostral, intermediate, caudal). The numbers in the wells designate the number of the section that is put in the well while cutting a brain.

I. Immunohistochemical Studies

One well is selected randomly for each rat for Immunofluorescence (Rostral/caudal/intermediate). Tissues were washed 3 times with 1x PBS for 5 min each, and treated with 2N HCL for 30 min at 37°C to allow DNA strands to open since thymidine analog is integrated in the DNA. Tissues were washed 1 time with 1x PBS for 5 min and then neutralized with borate buffer for 10 min at room temperature. Next, tissues were washed 3 times with 1x PBS for 5 min each, and blocked for 1 hour at 4°C with blocking solution, after which they were incubated overnight with rat-anti BrdU (1:100) diluted with blocking solution. The tissues were then washed 3 times with 1x PBS for 5 min each and incubated with secondary antibody (goat-anti rat Alexa 568, 1:200) for 2 hours at room temperature and placed on a shaker. Again, tissues were washed 3 times with 1x PBS for 5 min each and incubated with an antibody to the neuronal marker overnight (NeuN, 1:500). Tissues were then washed 3 times with PBS, 5 min each and incubated with secondary antibody (goat-anti mouse Alexa 488, 1:250) for 2 hours and placed on a shaker following by 3 times washing for 5 min each with

PBS. Lastly, tissues were mounted on slides and covered after the application of an anti-fading mounting medium.

J.Quantification of BrdU expressing cells

Using a laser scanning confocal microscope (Zeiss LSM 710), BrdU positive cells were visualized using the 40X-oil objective and counted to quantify the number of stem/progenitor cells. Sections were taken from a representative whole well. The final number of BrdU positive cells was multiplied by 6 to estimate the full count in each region of the hippocampus. These numbers were then added to calculate the total number of positive cells in the whole hippocampus. Furthermore, due to the laterality significance, the right versus left BrdU counts were summed and compared to each other. The total number of BrDU positive cells in all sections taken from the control group was compared to those taken under inflammatory conditions at different time points.

K.Statistical Analyses

Statistical analyses were performed using Sigma Plot software (Version 14, USA). All data are presented as means ± standard errors of the means (S.E.M). In normally distributed populations, significant changes in latency and withdrawal frequencies within groups over time were determined with one-way repeated measures analysis of variance (ANOVA) of the raw data followed by Bonferroni t-test for post-hoc analysis. In non-normally distributed populations, significant changes were calculated using Friedman's analysis of variance on ranks. Comparison of paw withdrawal latency to heat and mechanical stimulation before and after induction of inflammation at all testing times was done using paired *t*-tests. The immunohistochemical data were analyzed using one-way repeated measures ANOVA followed by Newman-Keuls post hoc test. Differences are considered significant at $p < 0.05$.

CHAPTER III

RESULTS

A.INDUCTION OF INFLAMMATION

A solution of 3% kaolin and carrageenan injected into the synovial cavity of the knee joint of rats produced acute inflammatory response characterized by swelling, plasma extravasation, and nociceptive behaviors (Ferrell and Russell, 1986). Edema formation was evident in the inflamed joint, as the joint circumference showed a significant increase compared to baseline values and to the unaffected knee. As shown in figure 1, baseline values were similar in all three groups. The joint circumference of the naïve rats remained almost constant at all time points. Similar observations were noticed for the saline-injected rats, despite the presence of insignificant variations at certain time points. In the inflamed group, the joint circumference of the inflamed rats at baseline was approximately 5.2 cm, comparable to the other two groups. However, it increased significantly at 4 hrs post injection (5.8cm), and continued to swell reaching a peak of 6.6cm at 8hrs. Thereafter, the joint circumference started to decrease while remaining significantly bigger than other groups at 24 hrs post injection, before returning to its original value at 48 hrs.

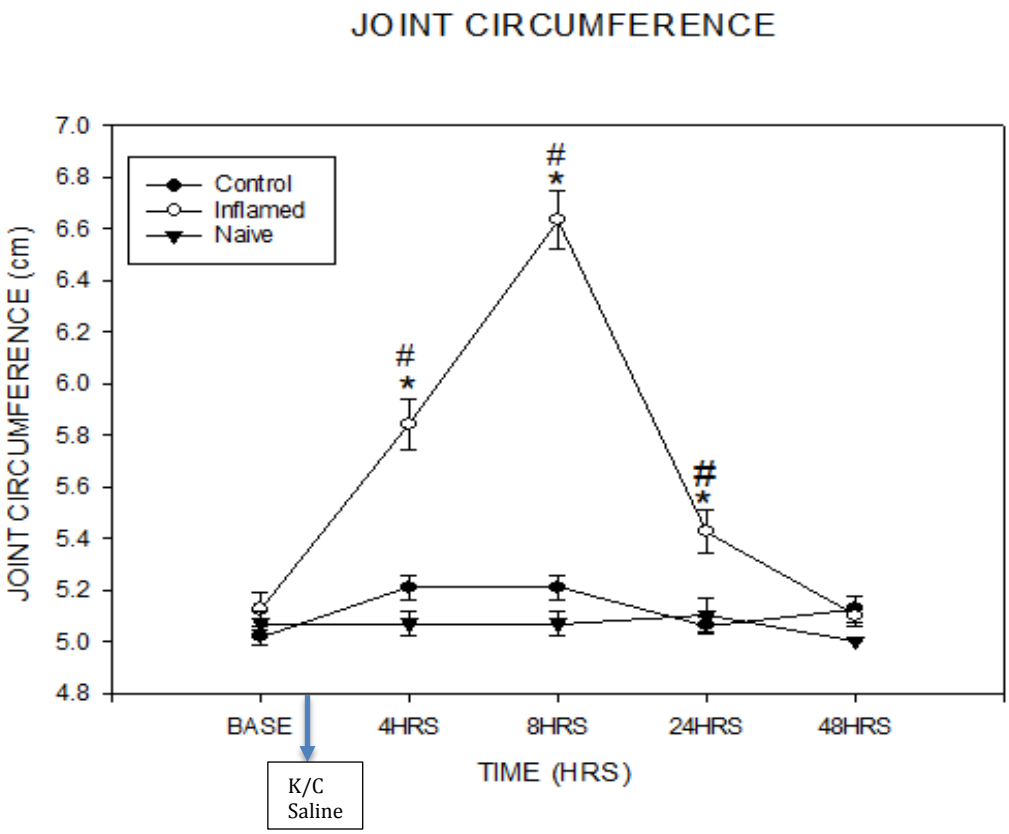


Figure 2: A line graph depicting the severity and progression of inflammation in rats over time. The joint circumferences are represented as the average circumference of the knee joint measured before and at 4 (n=24), 8 (n=24), 24 (n=16) and 48 hours (n=8) post injection of either saline or K/C. The joint circumferences of arthritic rats were compared to those of saline-injected and naïve rats. Data are presented as Mean ± SEM and were analyzed by one-way ANOVA with post hoc Bonferroni tests comparing joint circumferences at corresponding time points. A significant increase occurred for the arthritic rats compared with baseline values, * $p < 0.05$ and with other groups at corresponding time points, $^{\#}$ $p < 0.05$.

B.MOTOR BEHAVIORAL TESTING

The effect of acute peripheral inflammation on motor ability was assessed using a rotarod machine. The duration taken for the rats to fall off the wheels of the rotarod machine was recorded. This test was performed for the two rat groups (control and inflamed) at different time points (baseline, 4,8, 24 and 48 hrs). As shown in figure 3, the control rats maintained their motor abilities through the different time points, with time taken to lose their balance remaining almost unchanged at 170 sec. The inflamed rats had a significant reduction of their

motor balance, with a continuous decrease of their delay to fall from 180 sec at baseline, to 90 sec at 4 hrs then to 50 sec at 8 hrs post-injection. Subsequently, the inflamed rats regained their motor coordination after 24 hrs.

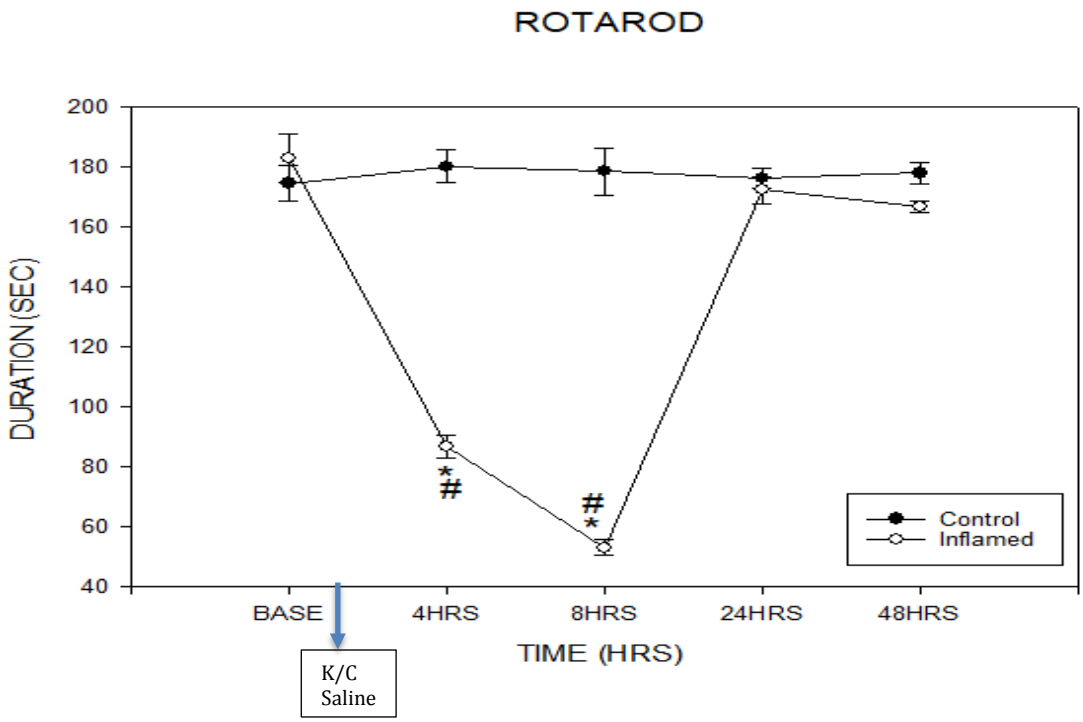


Figure 3: Time course of the effect of acute peripheral inflammation on motor coordination over time. A group of eight-week old Rats (130 g) was injected with the inflammatory agents Kaolin and carrageenan in the synovial cavity, while another was injected with saline and served as control. The duration taken for the rats to fall off the wheels of the rotarod machine was recorded. Data are expressed as mean ± SEM.
The # sign indicates statistically significant difference between inflamed and control groups at 4 and 8 hrs (# $P<0.05$). The * asterisk indicates statistically significant difference within group at each time point versus baseline values * $P< 0.05$.

C.NOCICEPTIVE BEHAVIORAL TESTING

- ***HEAT HYPERALGESIA***

To test the development of nociceptive behaviors following inflammation, the hind paws of rats were exposed to a painful stimulus (light beam produced by a 50 W halogen light bulb). Injection of kaolin and carrageenan into the knee joint significantly decreased the latencies at

4 and 8hrs when compared to baseline values (Figure 4). This decrease recovered gradually but remained significant at 48 hours. However, no change in the latency was observed in the intact contralateral paw when compared to its own baseline. In the control group, the saline-injected knee showed no significant difference from the contralateral paw.

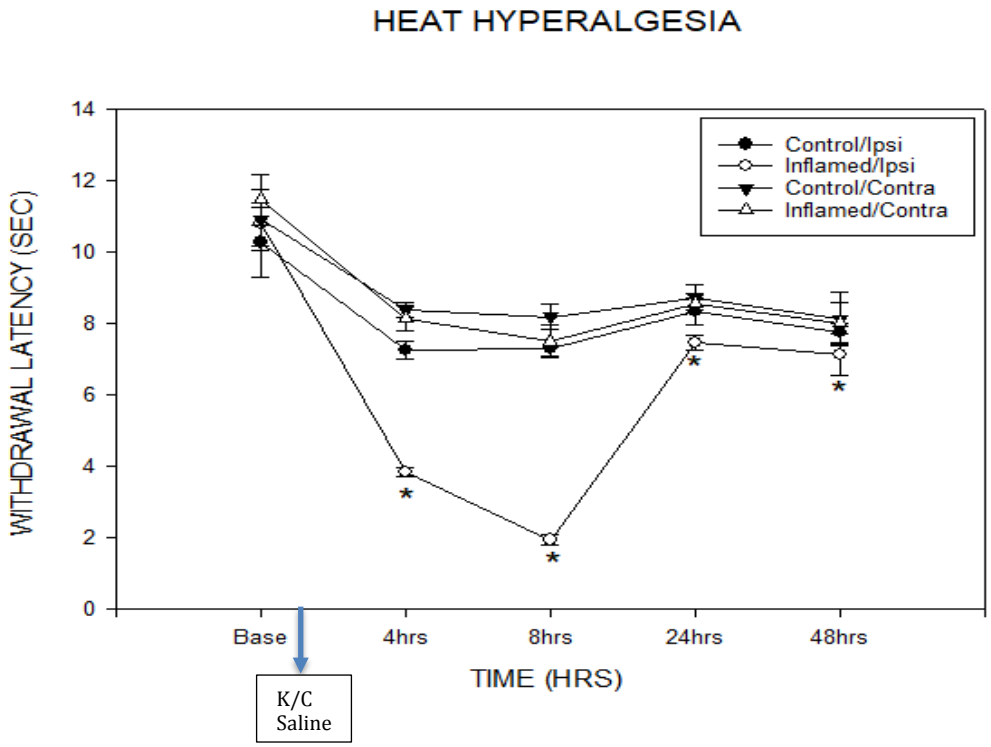


Figure 4: Time course of heat hyperalgesia. Rats injected with Kaolin and carrageenan in the synovial cavity developed heat hyperalgesia over 48 hrs. Data are expressed as mean ± SEM. The * asterisk indicates statistically significant difference within group at each time point versus baseline values * P< 0.05.

- ***MECHANICAL ALLODYNIA***

The development of mechanical allodynia was assessed in rats injected with K/C or Saline. A Von Fry filament with a bending force of 2g was used as an innocuous stimulus to activate mechanoreceptors. At 4 and 8hrs post induction of inflammation, rats injected with K/C showed an significant increase in paw withdrawal frequency in the ipsilateral side when compared to baseline or to the contralateral paw (Figure 5). In contrast, the saline-injected group exhibited no change in the frequency of paw withdrawal from baseline.

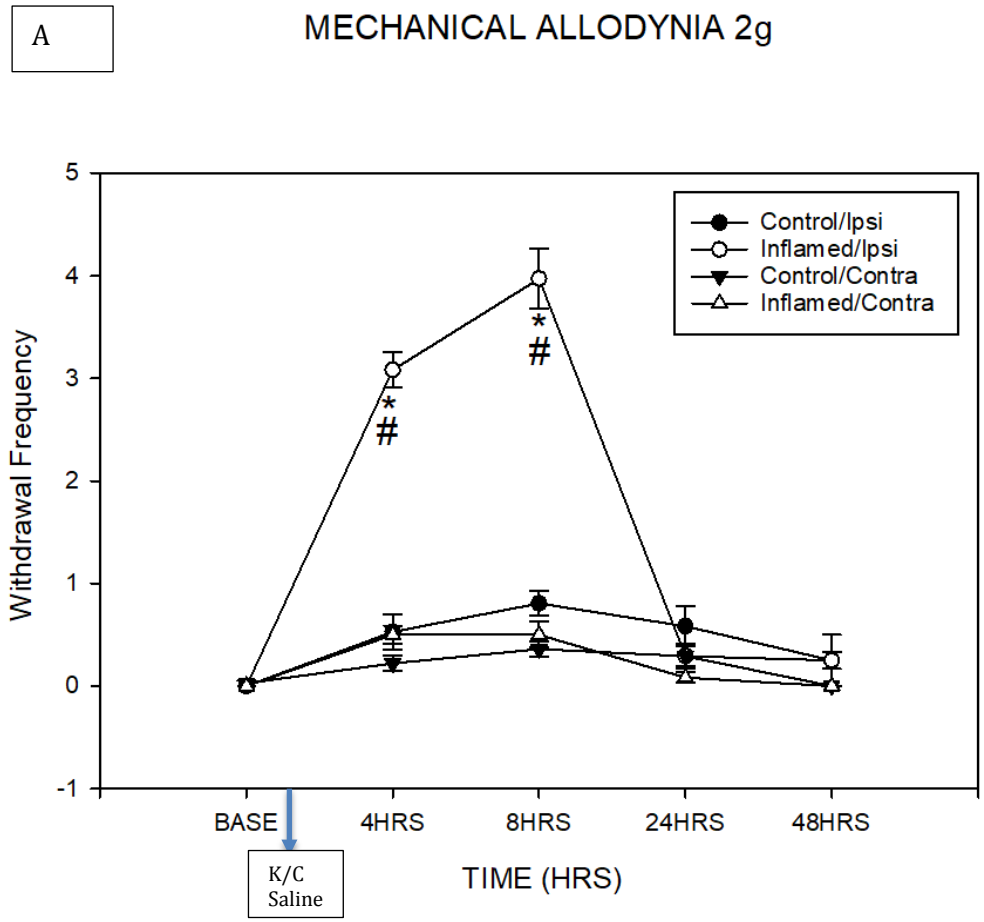


Figure 5: Time course of mechanical allodynia in arthritic (n=12) vs. control (n=12) rats. Eight week-old Rats (130 g) were injected with the inflammatory agents Kaolin and carrageenan in the synovial cavity. The paw withdrawal frequency to the innocuous stimulus was recorded before and after induction of inflammation. Data were expressed as mean ± SEM. The # sign indicates statistically significant difference between inflamed and control groups at 4 and 8 hrs (# $P<0.05$). The * asterisk indicates statistically significant difference within group at each time point versus baseline values * $P< 0.05$.

- ***MECHANICAL HYPERALGESIA***

The development of mechanical hyperalgesia was also assessed in rats injected with K/C or Saline. A Von Fry filament with a bending force of 15g was used as a stimulus that activates both mechanoreceptors and nociceptors. At 4 and 8hrs post induction of inflammation, rats injected with K/C showed a significant increase in the paw withdrawal frequency in the ipsilateral side when compared to baseline or to the contralateral paw (Figure 6). In contrast, the saline-injected group exhibited no change in the frequency of paw withdrawal from baseline.

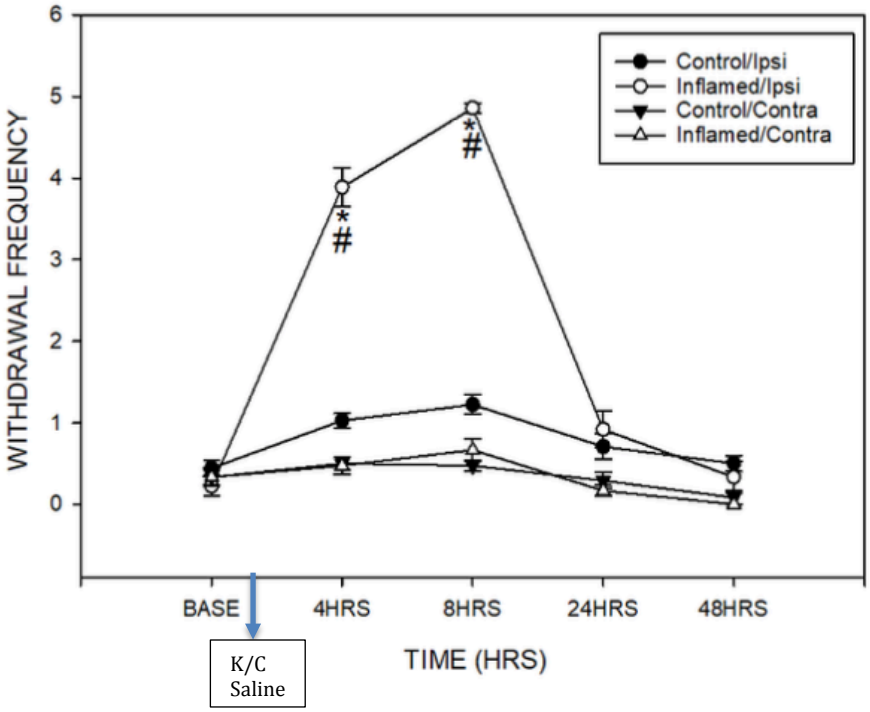


Figure 6: Time course of mechanical hyperalgesia in arthritic (n=12) vs. control (n=12) rats. Eight week-old Rats (130 g) were injected with the inflammatory agents Kaolin and carrageenan in the synovial cavity. The paw withdrawal frequency to a noxious stimulus was recorded before and after induction of inflammation. Data were expressed as mean $\pm$ SEM. The # sign indicates statistically significant difference between inflamed and control groups at 4 and 8 hrs (# $P<0.05$). The * asterisk indicates statistically significant difference within group at each time point versus baseline values * $P< 0.05$.

D.PLASMA EXTRAVASATION

Plasma extravasation, one of the distinguishing symptoms of neurogenic inflammation, is a phenomenon that leads to significant alteration in the control of the cutaneous circulation. Evans Blue, an azo dye, binds with high affinity to serum albumin to make a complex molecule that we often use to quantitatively assess the extent of extravasation associated with neurogenic inflammation. Under physiologic conditions the vascular endothelium is impermeable to albumin, so Evans blue-albumin complex remains within blood vessels. Under inflammatory conditions, vascular endothelial cells become permeable to albumin and allow for extravasation of Evans Blue in tissues. The high intensity blue color in tissues indicates significant plasma extravasation

As illustrated in figure 7, the concentration of the blue color reflected the intensity of the inflammation in the knee injected with kaolin and carrageenan and confirmed the manifestation of plasma extravasation. It is important to note that the inflammation was confined to the injected knee as no blue color was detected on the contralateral knee joint side.

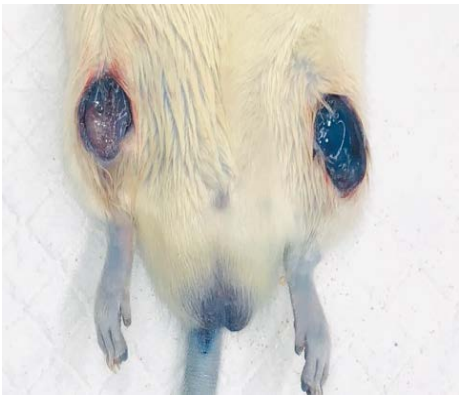


Figure 7: Plasma extravasation in the inflamed knee as detected by the Evans Blue dye. The inflammation was confined to the knee injected with K/C.

E.PROLIFERATING CELLS IN THE HIPPOCAMPUS

The effect of peripheral inflammation on proliferating cells in the dentate gyrus of the hippocampus was evaluated using immunohistochemical studies. The number of BrdU positive cells in control and inflamed rats was quantified at different time points. As seen in figures 8, the dentate gyrus contralateral to the injection site of both the control and inflamed group contained comparable numbers of BrdU positive cells at all time points (8, 24 and 48 hours). However, the number of BrdU positive cells contained in the ipsilateral hippocampus was significantly higher in the inflamed rats as compared to the control, at both 8 and 48 hours. A similar upsurge was noted at 24hrs for the ipsilateral hippocampus; nonetheless, it wasn't statistically significant.

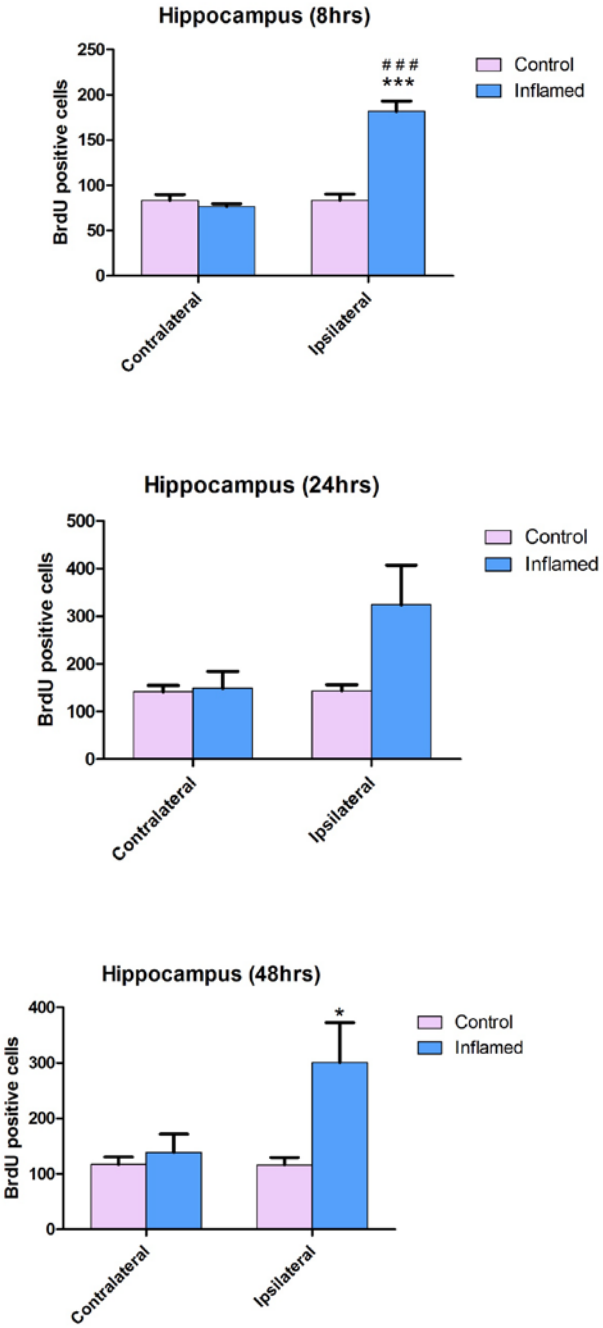


Figure 8: Total number of proliferating BrdU positive cells in the hippocampi of control and inflamed rats at 8, 24 and 48hrs post induction of inflammation. Eight week-old Rats were injected with a mixture of Kaolin and carrageenan in the synovial cavity of the knee joint, and the number of BrdU positive cells was counted. Data are expressed as means $\pm$ SEM. *Asterisks were used to indicate significant differences between inflamed and control groups; while $^{\#}$ pounds were used to show significant differences between the ipsilateral and contralateral side of the hippocampus. * indicates $p< 0.05$; ** indicates $p< 0.01$; *** indicates $p<0.001$; $^{\#\#\#}$ $p<0.001$

F.SPATIAL DISTRIBUTION OF BRDU CELLS IN THE HIPPOCAMPUS

In order to determine the spatial distribution of proliferating cells in the hippocampus, sections from the rostral, intermediate and caudal parts of the hippocampus were stained individually for both control and inflamed rats. For the Rostral region, the number of BrdU positive cells labeled in the ipsilateral side of inflamed rats was significantly higher, than that of the inflamed contralateral and control ipsilateral side, at the three different time points. Similar trends were observed for the intermediate and caudal hippocampal regions, with the absence of statistical significance at 24 hour for the former and 48 hours for the latter. Furthermore, the total number of BrdU positive cells was noticeably the highest in the caudal hippocampal regions at all time points.

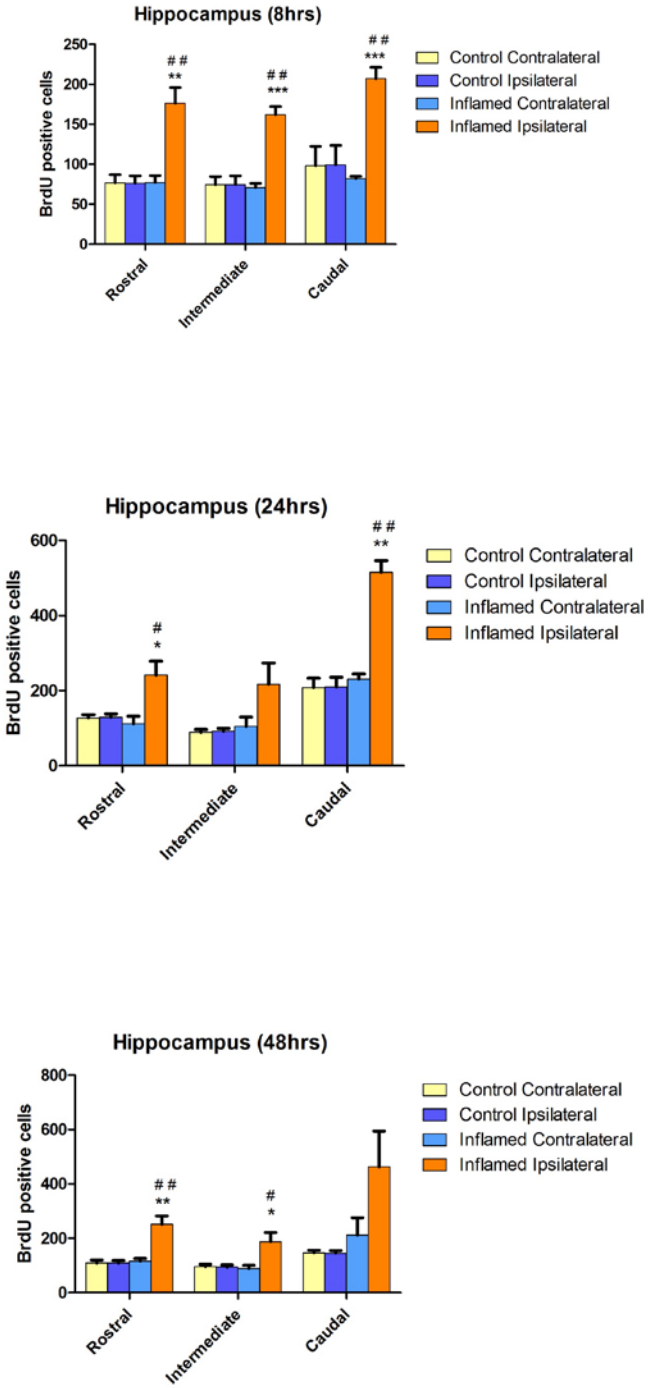
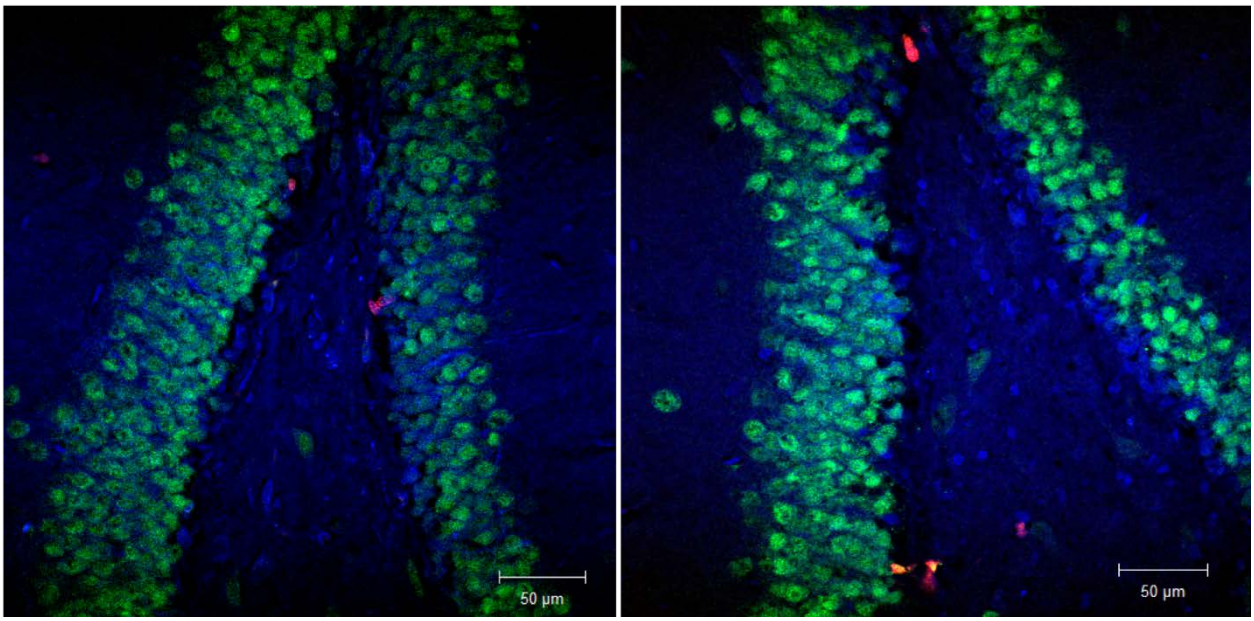


Figure 9: Quantitative comparisons of BrdU positive cells in the rostral, intermediate and caudal regions of the hippocampi of Control and inflamed rats at 8hrs (A), 24 hrs (B) and 48hrs (C) post induction of inflammation. BrdU-positive cells were more numerous in K/C -treated animals than in control animals at the three tested time points, specifically on the side ipsilateral to the injection site. Data are expressed as means ± SEM. *Asterisks were used to indicate significant differences between inflamed and control groups, while # pounds were used to indicate significant differences between both sides of the hippocampus contralateral and ipsilateral to the injection site.
* $p<0.05$; ** $p< 0.01$; *** $p< 0.001$; # $p <0.05$; ## $p<0.01$.

Representative images of the caudal region of the hippocampal dentate gyrus were shown in figure 10. Cells were stained with antibodies against NeuN, a marker for mature neurons, BrdU for proliferating cells and Hoechst for nuclei. The number of BrdU positive cells almost doubles in the inflamed caudal hippocampus (right column) compared to the control caudal hippocampus (left column). This amplification in the proliferation is seen at 8hrs (Fig. 10A), 24 hrs (Fig.10B) or 48 hrs (Fig. 10C), with the latter having the most significant increase. Cells co-labeled with BrdU and DAPI, indicate that BrdU was expressed in nuclei of cells. No mature neurons were seen in any of the sections due to the short waiting period between BrdU injection and perfusion. A minimum of twenty-eight days is necessary to allow maturation of proliferating cells into neurons.



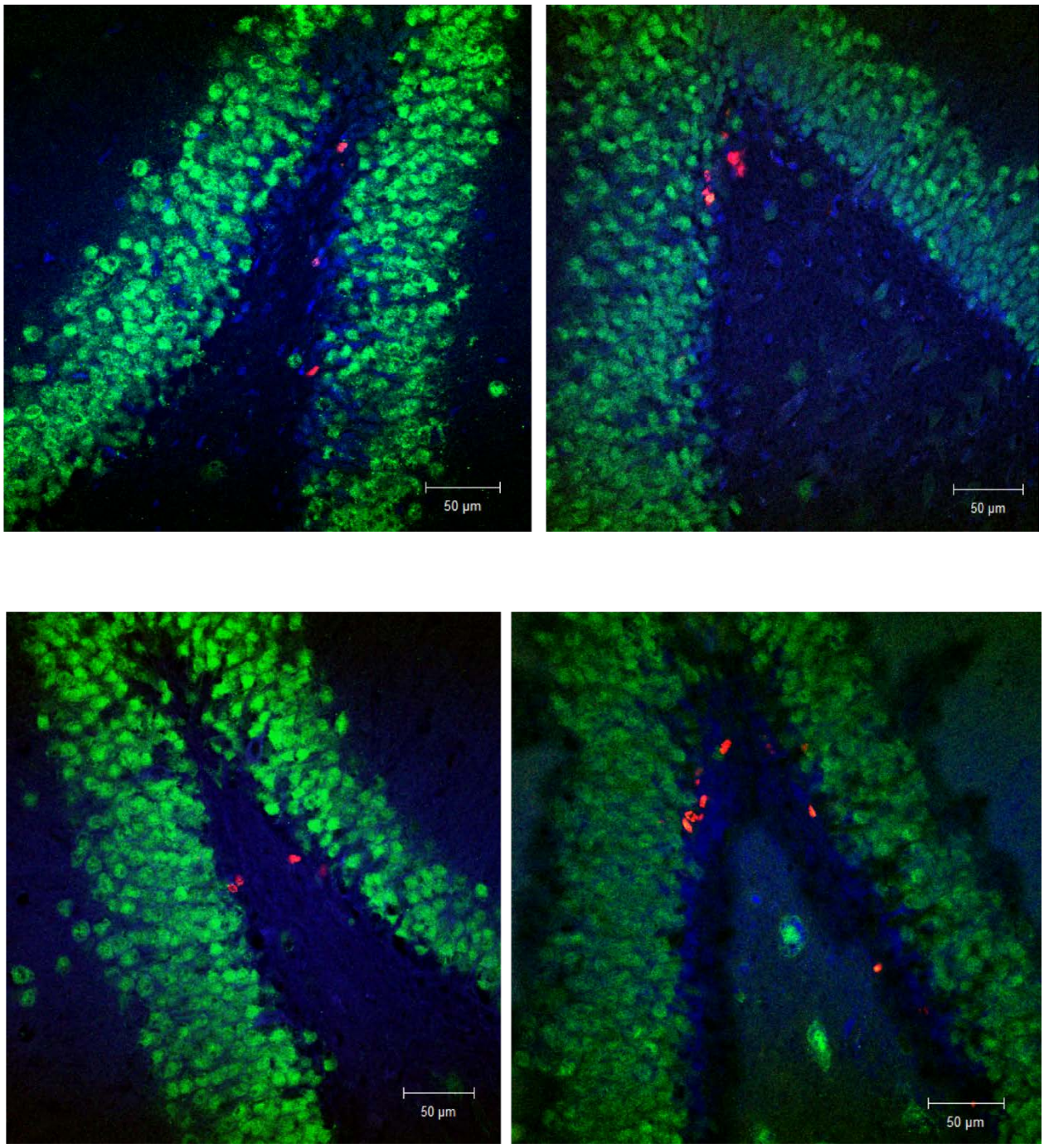


Figure 10: Representative images showing BrdU-positive nuclei (red), NeuN-positive (green) and Hoechst (blue) cells in the dentate gyrus in control and K/C-treated animals at 8 (A), 24 (B) and 48 (C) hrs post injection. At 8hrs, very few BrdU-positive nuclei can be seen dispersed throughout the dentate gyrus in the control group. After K/C administration, the number of BrdU-positive cells was visibly increased throughout the section. Most of the BrdU-labeled cells were located in the subgranular zone (SGZ) of the dentate gyrus. The number of BrdU-positive cells appears to be increased at 24 and 48hrs in inflamed animals compared with control animals. Scale bar, 50µm.

G.PROLIFERATING CELLS IN THE SPINAL CORD

The number of proliferating cells in the L3-L4 segment of the spinal cord was quantified. As seen in figure 11, BrdU positive cells were significantly higher in number after 8 hours of Kaolin and carrageenan injection as compared to the saline injected control. An upsurge of proliferation was also seen at 24 and 48hrs post-induction of inflammation, in all three studied areas of the spinal cord: Dorsal Column (Fig. 12A), Ventral Horn and Anterior Funiculus (Fig. 12C), but most notably in the Dorsal Horn (Fig. 12B).

In the dorsal column, BrdU positive nuclei were more numerous in inflamed rats as compared to the control group when seen at 8hrs following injection of kaolin and carrageenan in the knee joint. Interestingly, BrdU-expressing nuclei were mostly found in the dorsal horn of the spinal cord. Dense clusters of BrdU labeling were noted particularly in the superficial dorsal horn, namely the substantia gelatinosa. Additionally, BrdU labeled cells were occasionally noted in the ependymal layer of the central canal.

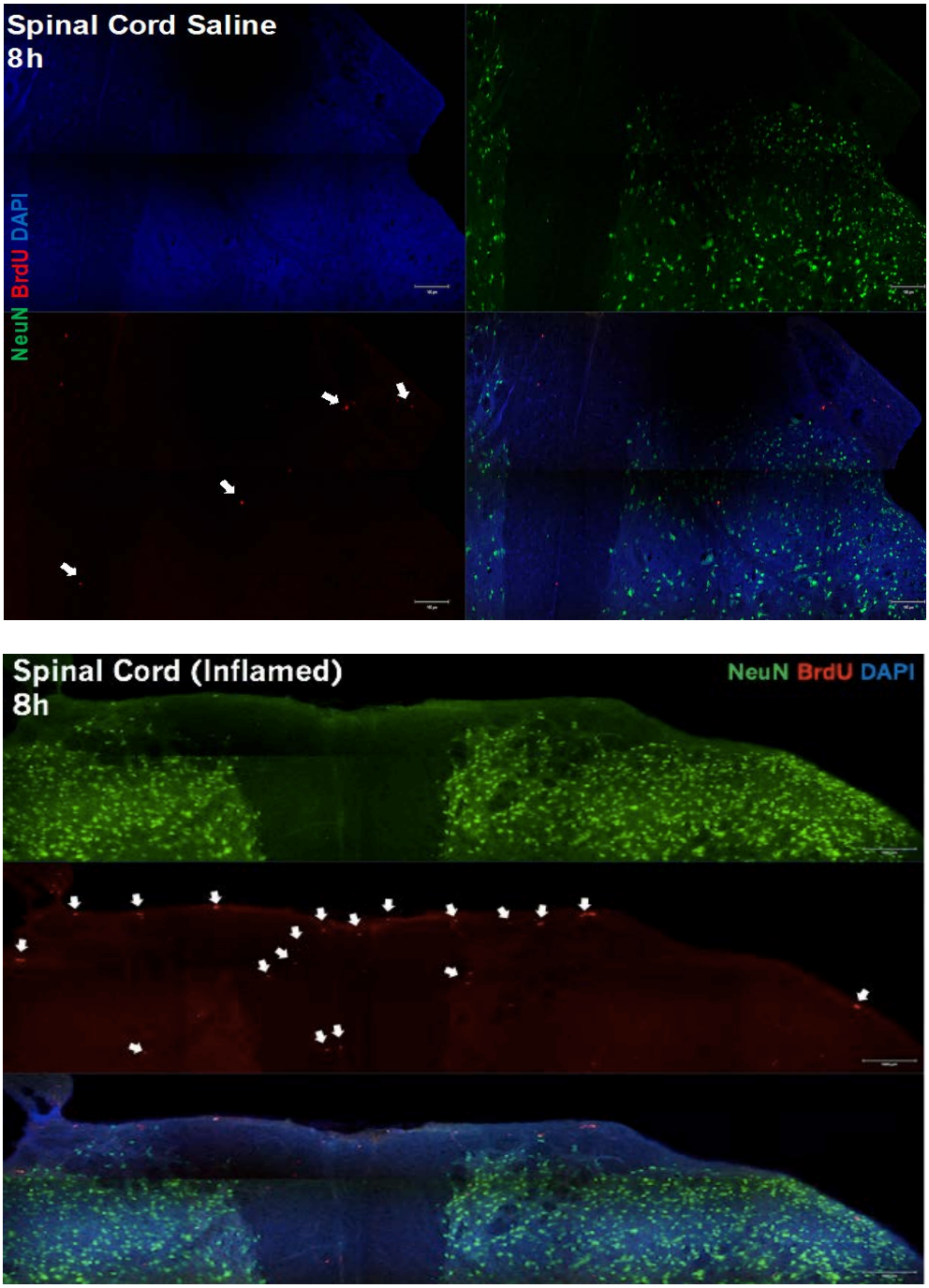
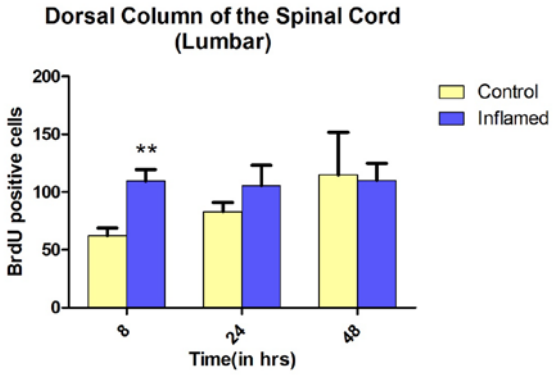
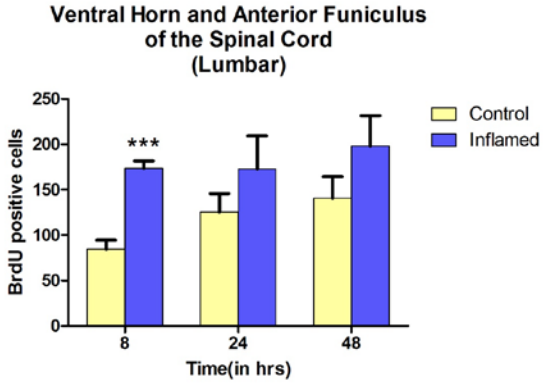


Figure 11: Representative images showing BrdU-positive nuclei (red), NeuN-positive (green) and Hoechst (blue) cells in the spinal cord in control and K/C-treated animals at 8 hrs post injection. Very few BrdU-positive nuclei can be seen dispersed throughout the spinal cord in the control group. After K/C administration, the number of BrdU-positive cells was visibly increased throughout the superficial layers of the dorsal horn. Scale bar, 50μm.

A.



B.



C.

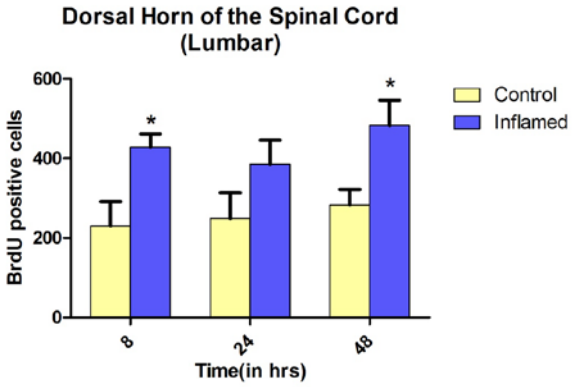


Figure 12: Total number of BrdU positive cells in different parts of the spinal cords at 8, 24, and 48-hrs post-induction of inflammation. K/C-treated rats showed a significant increase in the number of BrdU labeled cells in the dorsal column (A), dorsal horn (B) and ventral horn and anterior funiculus (C) of the spinal cord when compared to saline-treated rats at each time point. Data were expressed as means $\pm$ SEM. Asterisks were used to indicate statistical significant differences between control and inflamed groups. * $p<0.05$; ** $p<0.01$; *** $p<0.001$

CHAPTER IV

DISCUSSION

In the present study a rat model of osteoarthritis was used to investigate the effects of peripheral neurogenic inflammation on the proliferation of newly generated cells in specific regions of the central nervous system, namely the dentate gyrus of the hippocampus and the dorsal horn of the spinal cord. The results show that injection of kaolin and carrageenan into the synovial cavity of the knee joint induces nociceptive behaviors and impaired motor coordination accompanied by damage to the cartilage, synovial inflammation, knee swelling and plasma extravasation. The acute nociceptive behaviors and inflammatory responses peaked at 8hrs and lasted up to 24hrs. The development of hyperalgesia and allodynia post induction of inflammation confirms previous findings (Sluka et al., 1994a) and is indicative of neuroplastic changes at the peripheral and central levels. More importantly, our immunohistochemical results revealed a significant increase in the number of proliferating cells in the hippocampus and the spinal cord of rats at 8, 24 and 48 hrs post induction of knee joint inflammation. This increase was found in rats that exhibited significant nociceptive sensitivity at the inflamed knee joint and pronounced alteration in locomotor behavior. Moreover, as assessed by immunohistochemistry and confocal microscopy, proliferation of progenitor cells was observed in caudal, intermediate and rostral regions of the hippocampus, ipsilateral to the side of inflammation. While in the spinal cord, the enhanced cellular proliferation was chiefly noted in the dorsal horn of the spinal cord post inflammation. Based on our experimental data, it is not clear whether the increase in cellular proliferation can be attributed to inflammation or to nociceptive behaviors and altered locomotion since no selective intervention was attempted in this study. Relatively few in vivo studies of chronic peripheral inflammation have been done with regard to neurogenesis and almost all have

examined neurogenesis in the SGZ of the dentate gyrus of the hippocampus (Chesnakova et al., 2016). Our results are consistent with findings by wolf et al. (2009) showing that peripheral immune response increases proliferation of neural precursor cells in the adult hippocampus. By using an antigen-induced arthritis (AIA) as a model of immunity that lacks central inflammatory phenotype, as evident by the lack of microglial proliferative activity in all experimental animals, they found significantly enhanced numbers of proliferating DCX-positive precursor cells in the hippocampus of AIA animals compared with controls. In line with our findings, they demonstrated a bilateral increase in the number of proliferating cells in the hippocampus that was correlated with the progress and severity of the disease as measured by knee joint swelling. However, When we injected a mixture of K/C into the synovial cavity of the knee joint, proliferative activity in the SGZ was strongly increased in the ipsilateral hippocampus, while in the contralateral side, only a few proliferating cells were counted and their number was comparable with the control group. Given that no interventions were attempted in this study, it is undeniably difficult to determine the mechanisms underlying the unilaterality of proliferation. When articular nociceptive afferents project into the ipsilateral dorsal horn of the spinal cord, they transmit nociceptive information to cells that project contralaterally to the thalamus and higher brain areas; therefore, we expected either a bilateral increase in the number of BrDU positive cells or an increase on the side of the hippocampus contralateral to the inflammation. During inflammation, there are many factors at play that influence areas in the brain in order to alert it to the inflammation in the periphery. It has been shown that locally produced pro-inflammatory cytokines activate primary afferent pathways, and vagal nerve afferents that show sensitivity to peripheral cytokines since they are major constituents of an inflammatory reflex mechanism that controls inflammatory responses during pathogen invasion and tissue injury. Interestingly, Hosoi et al., (2000) showed that electrical stimulation of the vagus nerve afferents results in a rapid increase in IL-1β expression within

the hippocampus (Schneider et al., 1998). It is likely that humoral and neurally mediated routes may communicate peripheral inflammatory responses centrally supporting memory processes. It is now recognized that circulating pro-inflammatory cytokines, released by macrophages and primary afferent fibers, access the brain via the circumventricular organs and active transport systems (Dantzer et al., 2008). When the blood-brain barrier is disrupted by pro-inflammatory cytokines, it allows the circulation of activated lymphocytes and cytokines between the peripheral immune system and the brain (Sommer and Kress, 2004; Morris et al., 2015). Engagement of this peripheral immune system-to-brain communication eventually leads to activation of several neuronal circuitries in the (CNS), alteration of neurogenesis and modulation of the local immune response.

Over the last two decades, research has primarily focused on studying neurogenesis in the hippocampus, disregarding unconventional niches (Snyder and Cameron, 2012). There is indeed a paucity of studies examining the link between neurogenic inflammation and neurogenesis in the spinal cord. It has been generally thought that adult neural stem cells are concentrated in two conventional regions of the brain, the subgranular zone (SGZ) of the dentate gyrus, in the hippocampus, and the subventricular zone (SVZ) of the lateral ventricles. The existence of adult NSCs, which generate new neurons and restore damaged neural circuits, was indubitably a breakthrough that provided new prospects for the development of neural regenerative strategies. However, it was soon recognized that this newly discovered neurogenic potential was present in defined brain regions with restricted neuronal migration. Therefore, the search for new neurogenic niches was needed. So far, there is an increasing amount of evidence demonstrating the presence of other niches with lower rates of proliferation and differentiation in the CNS, including the spinal cord; however, there has been limited research on the role of these newly found niches in different neuropathological diseases.

Horner et al. (2000) have demonstrated the presence of neuron progenitor cells in the adult rodent spinal cord; however the differentiation of these progenitors into functionally integrated neurons was not confirmed, due to a lack of adequate imaging methods. Recently, there is growing recognition that the presence of immature neurons in the spinal cord plays a pivotal role in spinal cord physiology. In contrast to mature neurons, cortical immature neurons were shown to depolarize in response to GABA, due to a high intracellular chloride concentration. Similar observation was made in an animal model of peripheral nerve injury, in which a similar increase in chloride concentration, resulted in increased excitability of spinal cord neurons. This was shown to be due to an increased expression of the sodium–potassium–chloride cotransporter NKCC1 and a decrease in K-Cl co-transporter KCC2 expression, both of which regulate chloride homeostasis. Taken together, these two separate observations suggest that the neurons with increased excitability observed in the spinal cord dorsal horn after nerve injury might be in fact immature neurons. This was corroborated experimentally, by showing the enhanced expression of immature neuronal markers in the ipsilateral dorsal horn after peripheral nerve injury. Moreover, they showed that the time period of increased expression of these immature neuron markers correlates with the period of increased nociceptive sensitivity to mechanical and thermal stimulation following injury. A similar observation was made in our study, which showed an increased cellular proliferation in the dorsal horn of the spinal cord post induction of inflammation. The increase was also correlated with the severity of the inflammation. The number of BrDU expressing cells was higher in the dorsal horn than those expressed in the dorsal column or ventral horn of the spinal cord, suggesting a dynamic change in the organization of dorsal horn matrix as a result of inflammation. Most of the proliferating cells were found at the medial edge of the dorsal horn, or toward the superficial dorsal horn layers. Several mechanisms can account for the enhanced proliferation; It is important to consider the altered physiology of the dorsal horn following

neurogenic inflammation. A body of data has shown an increased release of glutamate, substance P and CGRP in the dorsal horn of the spinal cord in several animal models of inflammation (Bardoni et al., 2018). In 2007, Park et al. demonstrated that SP has the ability to stimulate neural stem cells proliferation in the normal brain, while it has also triggered ischemia-induced neurogenesis in the adult brain. More recently, new studies by Kim et al (2015) have highlighted the role of substance P in triggering cellular proliferation in the spinal cord and promoting neural regeneration after spinal cord injury. Their in vitro studies showed that a low dose of SP could continuously increase the proliferation of cultured spinal cord neural stem cells, from 6 hours to 5 days. In vivo, they found that intrathecal infusion of SP for 7 days could significantly increase the number of proliferating cells positive for both BrdU and Nestin in the spinal cord in adult rats, while infusion of an NK1-receptor antagonist significantly decreases the post-SCI induction of proliferation in the spinal cord. Notably, our previous studies using the kaolin and carrageenan model have also shown an increased release of SP in the dorsal horn of the spinal cord. Taken together, we can speculate that neurogenic inflammation induced by intra-articular injection of kaolin and carrageenan triggers a barrage of afferent inputs into the dorsal horn of the spinal cord inducing cellular proliferation through substance P release. Our findings suggest that the increased proliferation under inflammatory conditions could contribute to the reorganization of dorsal horn cells, causing plastic changes that enhance nociceptive sensitivity.

In summary, our results indicate that peripheral neurogenic knee joint inflammation induced by intra-articular injection of kaolin and carrageenan triggers the generation of new cells in the dentate gyrus of the hippocampus and the dorsal horn of the spinal cord suggesting a pivotal role for these newly born cells in the development of nociceptive and adaptive behaviors associated with inflammation.

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