INTRODUCTION

INTRODUCTION TO PAIN

Pain being the subjective sensation with no objective measures is hard to define and remains one of the most common reasons for seeking the medical treatment. As per the International Association for the Study of Pain, pain is defined as an "unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (International Association for the Study of Pain: www.iasp-pain.org). This definition emphasizes that pain is a complex experience that includes multiple dimensions.

It is well known that pain is the body's normal response to injury and normal defense mechanism. It serves an evolutionary role, warns an individual of potential injury or illness and thus prevents the worsening of an existing pathology. Without pain sensation, chances of getting subjected to repeated injury increases which may lead to poor healing or no healing at all (Hainline, 2005). Pain is mainly divided into two main categories: acute and chronic pain.

Acute pain or nociceptive pain resulting mainly from activation of nociceptors in peripheral tissues is a normal sensation triggered in the nervous system to alert about the possible injury and the need to be taken care of (Acute Pain Management Guideline, 1992). Pain arising from various sources like cutaneous (skin), deep somatic (muscle, bone), or visceral structures (organs within chest and abdomen) could be acute in nature (Acute Pain Management Guideline, 1992). There are specific anatomical and physiological changes that occurs immediately following tissue damage. Normally, healing occurs but in the instance wherein these changes persist long after injury, transition of acute pain into chronic pain takes place (Kyranou and Puntillo, 2012).

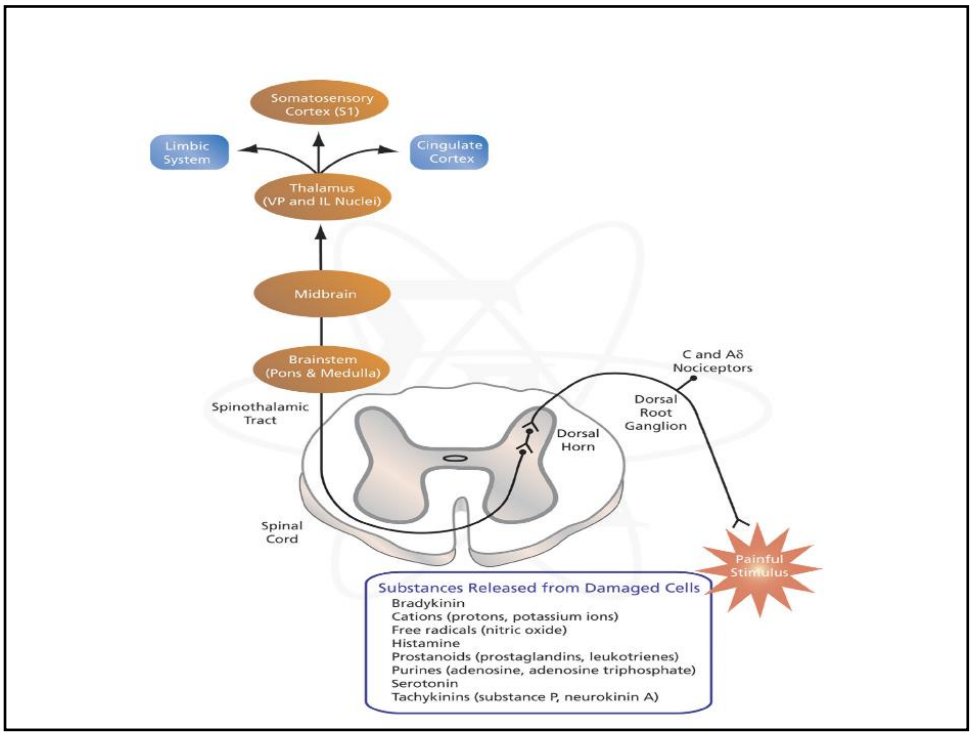
Chronic pain persists and pain signal keep firing in the nervous system for weeks, months, even years. Chronic pain differs from acute pain with respect to its underlying mechanisms. Chronic pain may not have identifiable ongoing injury or inflammation (Wang and Wang, 2003).

Classification of Pain

From an experimental perspective, pain can be further classified into three types - each mediated by different mechanisms: nociceptive pain, inflammatory pain and neuropathic pain (Henry, 2008)

Nociceptive pain: It mainly results from activation of nociceptors in peripheral tissues through intense thermal, mechanical or chemical stimuli. These nociceptors are the primary afferent terminals of nerves that generate impulses to the spinal cord through dorsal root ganglion (DRG). The part of the signal makes synaptic contact with secondary neurons from brainstem to increase the conscious alertness to the pain signal and the other part of signal which goes to thalamus increases our perception of the pain signal. Pain signal in thalamus further travels to somatosensory cortex to help the organism localize the source of pain and part of the signal crosses over to hypothalamus and limbic system as well (Figure. 1) to trigger the behavioural and emotional response to pain stimulus (Voscopoulos, 2010).

Figure 1: Main pathway for sensory information from the periphery to the cortex and limbic system.



This figure has been adopted from: http://www.cgl.ucsf.edu/Outreach/bmi219/slides/images/ascending_pain_pathway.Par.0001.File.png

Nociceptors are categorized by their receptive modality and by their response to that stimulus (Gold and Gebhart, 2010). Classically there are two types of nociceptors - Aδ fibres and C fibres. Aδ fibres are medium diameter myelinated afferents that mediate fast pain. C-fibres are small diameter unmyelinated fibres and conduct slow pain. They are present in skin, muscles, tendons, joints and viscera with varying degrees of density and serve to detect cutaneous, somatic and visceral pain (Gold and Gebhart, 2010). Nociceptors are excited only when stimulus intensities reach the noxious range, indicating the presence of biophysical and molecular properties which enable them to selectively detect and respond to potentially injurious stimuli (Basbaum *et al.*, 2009).

Inflammatory Pain: It mainly arises from tissue injury (e.g. inflammed joints) and subsequent inflammatory process i.e. increased excitability of peripheral nociceptive sensory fibres produced by the action of inflammatory processes (Linley *et al.*, 2010). It is adaptive in nature i.e., it triggers physiologic responses that promote healing. But under certain pathological circumstances, sensory nervous system may go overboard in being protective by exhibiting increased response to non-painful stimuli and causes pain that is not beneficial. It now detects non-painful stimuli as highly painful (allodynia) or responds in an exaggerated manner to painful stimuli (hyperalgesia) (Linley *et al.*, 2010). Plasticity in nociceptors and central pain pathways could be responsible for this abnormal development. Inflammatory pain typically improves as a function of "healing" and resolution of the inflammation (Xu and Yaksh, 2011).

Since it is believed that inflammatory mediators play the inducing role in inflammatory pain, in routine practice, non-steroidal anti-inflammatory drugs (NSAIDs) and opiates are normally found to be efficacious in inflammatory pain (Xu and Yaksh, 2011). It is widely known that prostaglandins (PG) play a key role in the generation of the inflammatory response and PG is synthesized by the sequential action of enzymes and the last in this series is cyclooxygenase (COX). NSAIDs are found to be more effective in inflammatory pain as they directly inhibit PG production by intervening arachidonic acid pathway through COX (Ricciotti and FitzGeralt, 2011). However, there are reports citing deleterious side effects due to chronic use of NSAIDs which poses major challenge for the control of inflammatory pain (Wallace, 2008).

Neuropathic Pain: It mainly arises from injury or irritation to the nerve. Any damage to neurons in the peripheral nervous system (PNS) and central nervous system (CNS) involves

sensitization of these systems resulting in neuropathic pain (Xu and Yaksh, 2011). Peripheral sensitization results in an increased stimulation of peripheral nociceptors that amplifies pain signals to the central nervous system. Central sensitization results in hyperstimulation of neurons originating in the dorsal horn of the spinal cord, increasing pain signals to the brain and thereby increasing pain sensation. This damage could be due to numerous causes - traumatic injury or surgical intervention, infection (herpes zoster), or disease (cancer, diabetic neuropathy, multiple sclerosis, etc.). It is chronic debilitating condition characterized by allodynia, hyperalgesia and spontaneous pain (Vranken, 2012).

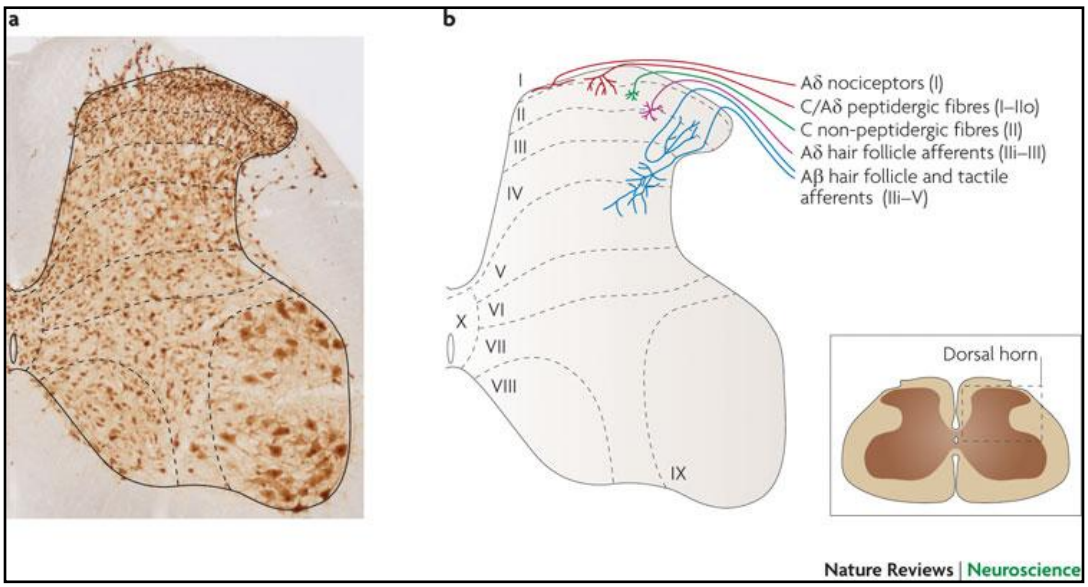
Unlike, inflammatory pain, neuropathic pain does not show reversal in spite of resolution of injury associated with the sensation (Kehlet *et al.*, 2006). Hence, the treatment options available today for the management of neuropathic pain are as diverse as its etiologies. Therapeutic strategies have been described as being based on "trial and error" (Hansson *et al.*, 2001). Current treatments used for chronic neuropathic pain hints general insensitivity to NSAIDs and relative resistance to opioids, but higher doses of opioids are found to be effective albeit with severe side effects (Kalso *et al.*, 2004). Approved treatment for neuropathic pain includes ion-channel blocking drugs (e.g. anticonvulsants gabapentin and pregabalin), antiarrhythmic drugs (mexiletine), tricyclic antidepressants (TCAs) (e.g. amitriptyline), serotonin-norepinephrine reuptake inhibitors (SNRIs) (e.g., duloxetine). These therapeutic approaches are found to be either poorly efficacious or accompanied with side-effects (Dray, 2008). These observations emphasize on requirement for novel drugs which are effective in relieving the neuropathic pain and at the same time do not elicit severe side effects upon chronic administration.

Mechanism of Neuropathic Pain:

Most of the information we have today about the pathophysiology of neuropathic pain, has been generated from various animal models. They indicate towards the peripheral and central pathophysiological processes taking place following nerve injury which could be responsible for generation of neuropathic pain (Millan, 1999). Peripheral sensitizations works on the peripheral nociceptors and central sensitization takes place along the central path leading from dorsal horn to brain. Along with sensitization process, abnormal interactions between the sympathetic and sensory pathways also play a crucial role in generation of neuropathic pain (Millan, 1999).

Pain Processing: Primary afferent neurons (Aβ-, Aδ- and C-sensory neurons) are responsible for transmitting sensory information from periphery to CNS (dorsal horn of the spinal cord) via dorsal root ganglion (DRG) and are also responsible for transducing the external stimuli (mechanical, chemical and electrical information) into electrical activity (Gold and Gebhart, 2010). Under physiological circumstances, C (dull pain-slow pain) and Aδ fibres (sharp pain-fast pain) transmit nociceptive information from PNS to CNS and Aβ-fibres are capable of responding only to non-noxious mechanical stimuli (Vranken, 2012). These sensory neurons may interact with secondary neurons in the gray matter of the dorsal horn of spinal cord. Gray matter of spinal cord horn is partitioned into 10 laminae (I-X). Aδ- fibers project to lamina I (marginal layer) and C-fibers project o lamina II (substantia gelatinosa). Aβ-fibers project in laminae III and V (nucleus propius) (Figure 2.). Along with transmission, second order neurons modulate pain signal in the dorsal horn into excitatory and inhibitory types (Willis and Westlund, 1997 and Biella *et al.*, 1997). Once the pain signal reaches dorsal horn, it travels to thalamus through ascending pathways and interacts with limbic circuits on its way to cortex (Price, 2002).

Figure 2: Rexed's laminae of the spinal cord



This diagram indicates the main laminae for the termination of fibers. The central terminals of primary afferents (Aδ, C and Aβ) occupy highly ordered spatial locations (I-V) in the dorsal horn. This figure has been adopted from: http://www.nature.com/nrn/journal/ v11/n12/ images/ nrn2947-f1.jpg

Ascending projection neurons extend, contralaterally to supraspinal targets including caudal ventrolateral medulla, the nucleus of the solitary tract, the lateral parabrachial area, the periaqueductal gray matter and the thalamus (Vranken, 2012). Functionally, important

ascending pathways are tractus spinothalamicus lateralis which takes care of sensory discriminative dimensions of pain and spino-parabrachio-amygdaloid pathway and spino-parabrachio-hypothalamic pathway which takes care of emotional-cognitive dimensions of pain (Vranken, 2012).

Descending pathways originate from the cortex and/or midbrain and provide modulatory feedback signals at the level of the spinal cord and regulate the nociceptive experience (White *et al.*, 2007; Vranken, 2012). There is a delicate balance of excitation and inhibition signal which is important for representation of pain stimulus appropriately all along the pain processing pathway - right from the primary afferent nociceptors, to the dorsal horn of the spinal cord, to the supraspinal processing sites and descending pathways. Any miscommunication at any of the above junctures may lead to transition of acute pain into chronic pain (Todd, 2010; Dubin and Patapoutian, 2010).

Peripheral Sensitization: Sensitization of primary afferent neurons occurring due to tissue injury or inflammation or direct activation, triggers a myriad of processes. It results in the release of chemicals and inflammatory mediators, such as bradykinin, prostaglandins, 5-hydroxytryptophane (5-HT), nitric oxide (NO), acetylcholine, adenosine-5'-triphosphate (ATP) and protons from neurons, blood vessels and immune cells collectively termed as 'inflammatory soup' (McMahon *et al.*, 2006; Basbaum *et al.*, 2009). These mediators in turn act on primary afferent neurons causing pain or sensitization directly or indirectly by interactions with nociceptors often via ion channels (Na^+, TRPV1receptors, ATP-gated ion channels or purinergic receptors) or by receptor-mediated second messenger action (e.g., bradykinin and NGF) or through inflammatory cells via release of cytokines (IL-1β, TNF-α etc) through different mechanisms (Woolf and Salter, 2000). It includes triggering of prostaglandin E_2 (PGE_2) release from inflammatory cells, inducing nerve growth factor (NGF) and up-regulating of substance P (SP) levels in neurons (Dayer *et al.*, 1986; Hart *et al.*, 1991; Safieh-Garabedian *et al.*, 1995; Mizumura, 1997; Ritner *et al.*, 2009). NGF produces profound hypersensitivity to heat and mechanical stimuli through Phospholipase C (PLC), mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) signaling pathways (Chuang *et al.*, 2001). Primary afferent neurons express one or more cell surface receptors capable of recognizing and responding to each of these pro-inflammatory agents. Sensory neurons (Aδ- and C-fibers) which usually responds to only noxious stimuli under normal physiology, gets sensitized and becomes abnormally sensitive under pathological condition (tissue injury, inflammation or direct activation). This could result in

interaction of C-fibres and Aβ-fibers giving rise to mechanical allodynia (Attal and Bouhassira, 1999; Oaklander, 2008). Following nerve damage, Aβ-fibers undergoes phenotypic switch and express Substance P and Calcitonin-gene related peptide (CGRP), normally expressed by C- and Aδ-fibers. This results in abnormal pro-nociceptive actions following innocuous mechanical stimulation and generates the state of central hyperexcitability by release of SP, CGRP and brain-derived neurotrophic factor (BDNF) and thus creates a neurogenic inflammation (Bolay and Moskowitz, 2002; Schaible *et al.*, 2005).

In brief, peripheral mechanisms display plasticity and undergo major changes in processing post inflammation and nerve damage. Silent nociceptors become active, chemicals within inflammatory soup act directly or indirectly on nociceptors via ion channels and receptor mediated second messenger systems. Up and down regulation and reorganization of many pain processing factors (ion channels) in damaged and undamaged nerve fibers contributes to a state of hyperexcitability, low threshold to external stimuli and increased spontaneous firing (Woolf, 1991; Ma and Pei, 2007).

Central Sensitization: Central sensitization is a process through which a state of hyperexcitability is established in the CNS, leading to enhanced processing of pain messages and persists for weeks at different stages of acute and chronic inflammation (Woolf, 1983; Mense, 1993). It represents a dramatic functional shift in the way organisms perceive somatosensory inputs. Increased responses to noxious stimulation, lowering of threshold of nociceptive specific spinal cord neurons, increased responses to stimuli applied to non-inflamed tissue surrounding the inflamed site, expansion of the receptive field are some of the typical change observed in individual neurons subjected to external stimuli (Schaible *et al.*, 1987). The major mechanisms involved in central sensitization are alteration in glutamatergic neurotransmission/N-methyl-D-Aspartate (NMDA) receptor-mediated hypersensitivity, loss of tonic inhibitory controls (disinhibition) and glial-neuronal interactions (Basbaum *et al.*, 2009).

Glutamate/NMDA receptor-mediated sensitization: Activation of N-methyl-D-Aspartate receptor (NMDAR) is an essential step in initiating and maintaining central sensitization. Under normal conditions, the NMDAR channel is blocked in a voltage-dependent manner by a magnesium (Mg^{2+}) ion sitting in the receptor pore. Sustained release of glutamate, neuropeptides like SP, CGRP by nociceptors leads to removal of Mg^{2+}, resulting in entry of Ca^{2+} into the neuron, activating intracellular pathway and thus contributing the maintenance

of central sensitization (Mayer *et al.*, 1984; Woolf and Thompson, 1991; Ma and Woolf, 1995). In addition to primary hyperalgesia, central sensitization contributes to secondary hyperalgesia, wherein inputs from Aβ afferents, normally responding to light touch now gets engaged in pain transmission circuits, resulting in profound mechanical allodynia (Campbell *et al.*, 1988).

Loss of GABAergic and glycinergic controls - Disinhibition: The inhibitory neurotransmitters GABA and glycine act on ionotropic, chloride permeable $GABA_A$ or glycine receptors or metabotropic (G-protein-coupled) $GABA_B$ receptors. Under normal conditions, the intracellular concentration of chloride ions in neurons is kept low so that the opening of $GABA_A$ or glycine activated chloride channels causes the entry of negatively charged chloride ions into neurons thereby hyperpolarizing them (Fein, 2012). In the gate theory of Pain, it's postulated that functional loss of GABAergic, glycinergic inhibitory interneurons (disinhibition) located in the dorsal horn would result in increased pain (Melzack and Wall, 1965). Shift in the transmembrane chloride ion gradient causing normally inhibitory anionic synaptic currents to an excitatory gradient could lead to disinhibition (Coull *et al.*, 2003). Disinhibition enhances spinal cord output in response to painful and non-painful stimuli, contributing to mechanical allodynia (Torsney and McDermort, 2006; Keller *et al.*, 2007). Going with this theory, Sivilotti and Woolf (1994) and Malan *et al.* (2002) performed experiments in rodents by spinal administration of GABA (Bicuculline) or glycine (Strychnine) receptor antagonists, which produced behavioral hypersensitivity similar to that observed in post peripheral injury inflicted animals. Modulation of glycinergic signaling involving spinal cord action of prostaglandins could also result in disinhibition (Harvey *et al.*, 2004).

Glial-neuronal interaction: After peripheral nerve injury, CNS glial cells (microglia, oligodendrocytes and astrocytes) in the dorsal horn gets activated and release pro-inflammatory mediators that modulate pain processing by affecting either pre-synaptic release of neurotransmitters and/or post-synaptic excitability (Vallejo *et al.*, 2010). In addition to these, neurotrophins (responsible for inducing mechanical allodynia) and glially derived neurotrophic factors (BDNF) are also released. These mediators influence the spinal processing and thus contribute in the generation of neuropathic pain by increasing excitatory and reducing inhibitory transmission and enhancing descending facilitation in the dorsal horn (Vanegas and Schaible, 2001; Marchand *et al.*, 2005; Coull *et al.*, 2005). Activation of microglia leads to initiation of enhanced cytokine expression (TNF-α, IL-β and IL-6)

responsible for inflammatory responses in the CNS (Vallejo *et al.*, 2010) and accelerates the release of nociceptive neurotransmitters thereby increasing the excitability of nociceptive second-order neurons creating changes in the spinal cord (Vranken, 2012).

Role of Glial/Non-Neuronal Cells in Neuropathic Pain: As mentioned earlier glia cells also known as non-neuronal cells are of three types and it make up approximately 70% of total cell population in the CNS. Glia cells including astrocytes and microglia play crucial role in initiation and maintenance of neuropathic pain and inflammatory pain. They have close interactions with neurons and thus modulate pain transmission particularly in pathological conditions (Inoue and Tsuda, 2009; McMahon and Malcangio, 2009; Milligan and Watkins, 2009). Oligodendrocytes facilitate fast nerve conduction by synthesizing myelin sheaths for multiple neurons (Peters, 1964).

Astrocytes play a major role in neuronal activity by making connections with neuronal synapses (Bushong *et al.*, 2002), vasculature (Abott *et al.*, 2006) and also other astrocytes (Blomstrand *et al.*, 1999) with their long processes extending in all directions. They clear neurotransmitters (Glutamate and GABA) from synaptic cleft and have the ability to release proinflammatory cytokines (IL-1β, IL-18, TNF-α), excitatory aminoacids and growth factors (Araque *et al.*, 1999). Various studies have shown up-regulation astrocytes specific marker - Glial Fibriliary Acidic Protein (GFAP) indicating activation of astrocytes under pathological conditions (Garrison *et al.*, 1991; Garrison *et al.*, 1994; Hutchinson *et al.*, 2007).

Historically microglial cells were thought to support neurons structurally and maintain homeostasis in CNS. Gradually, CNS glial cells were acknowledged for their various roles important for survival of host; such as providing immune surveillance, clearing debris, regulating ionic and chemical composition of the extracellular space, but studies conducted in early 1990s brought glia in limelight with respect to their role in varying pain states by using the astrocyte-specific marker GFAP (Garrison *et al.*, 1991; Garrison *et al.*, 1994; Hutchinson *et al.*, 2007). Since then, number of studies have been carried out to pinpoint the glial activation using preclinical animal models of chronic pain (Colburn and DeLeo,1999; Sweitzer *et al.*, 1999; Raghvendra *et al.*, 2004) using various markers such as ionized calcium binding adapter molecule 1 (Iba-1) (Banati, 2002; Ji and Suter, 2007; Guasti *et al.*, 2009), major histocompatibility complex II (MHC II) and integrin alpha (ITGAM, also known as cluster of differentiation molecule 11b or CD11b) (Hayes *et al.*, 1987; Eriksson *et al.*, 1993). There are also studies where usage of selective glial inhibitors (Minocycline, Fluorocitrate)

showed reduction in hyperalgesic or allodynic behavior thus showing importance of glia in pain transmission (Fonnum *et al.*, 1997; Raghvendra *et al.*, 2003; Ledeboer *et al.*, 2005).

Once activated, microglia undergoes functional and morphological changes, proliferation and migration to the site of injury (Tsuda *et al.*, 2008). Under pathological conditions, microglia cells get changed into activated state from resting stage. Microglia in activated state has bigger soma with lesser numbers of shorter processes (Figure 3). Their density increases in the damaged area (Frommann, 1878; Kettenmann *et al.*, 2011). Functionally, they start producing and releasing various neuroexcitatory substances such as reactive oxygen species(ROS), nitric oxide (NO), prostaglandins, excitatory amino acids, growth factors and pro-inflammatory cytokines (TNF-α,IL-6, IL-1) (Watkins and Maier, 2000; Watkins *et al.* 2001a; Watkins and Maier 2003a) hence, they play a crucial role in generation of central sensitization. Chemokines such as fractalkine, interferon-inducible protein of 10kDa (IP-10) and monocyte chemoattractant protein-1 (MCP-1) play major role in activating the neuron-glia signalling pathway which results in release of proinflammatory cytokines and NO (Milligan *et al.*, 2008; Gao and Ji, 2010). Up-regulation of Toll like receptors (TLR) 1 , 2 and 4 in CNS was observed in case of nerve injury with subsequent increase in production of pro-inflammatory cytokines (TNF-α and IL-1) and chemokines (MCP-1) (Tanga *et al.*, 2005; Owens *et al.*, 2005) indicating its link with microglia activation (Figure 4).

Figure 3: Morphology of microglia post nerve injury: Transition from the resting state to the activated state in the pathological condition

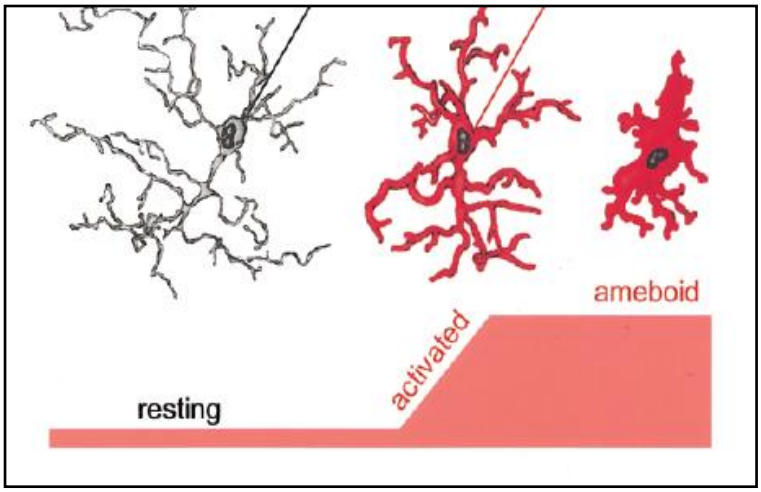


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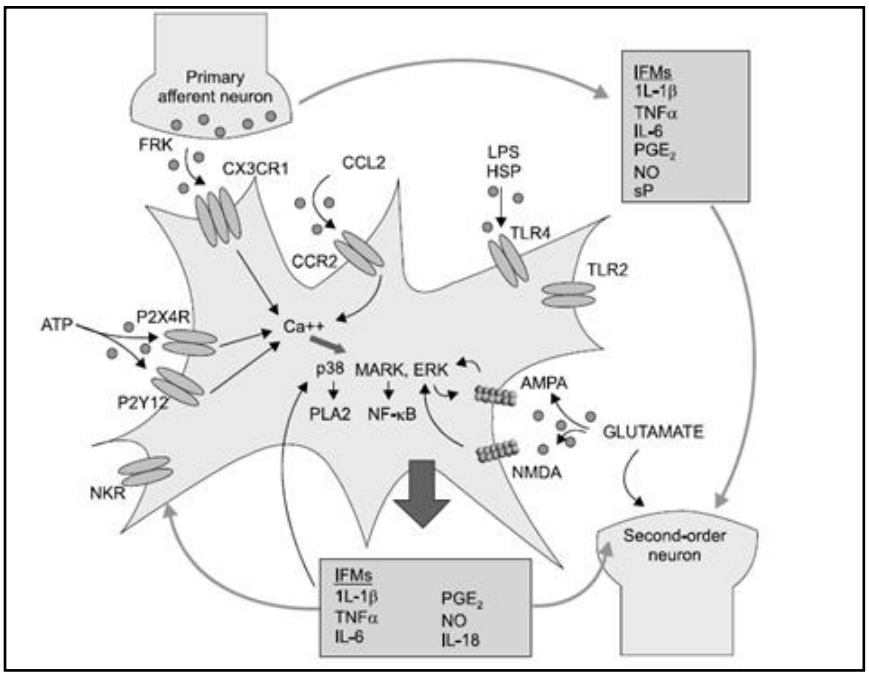
Figure 4: Response of activated microglia cells in neuropathic pain



Upregulation of multiple receptors such as chemokine receptors CX3CR1, P2X/Y, CCR2 and spinal microglia resulting due to nerve injury leads to release of fractalkine and neurotransmitters from the primary afferent neuron which in turn activates microglia. Activation of microglia causes the increase in intracellular calcium and initiates p38 MAPK/ERK pathway. Activated microglia release several proinflammatory cytokines, chemokines and other agents who will act as a mediator for modulating neural transmission by affecting presynaptic release of neurotransmitters and/or postsynaptic excitability (Jo *et al.*, 2009). This image has been adopted from – DOI:10.3344/kjp.2009.22.1.1

Role of Cytokines in Neuropathic pain: Cytokines are pivotal mediators in the multistep response taking place in the body to counteract xenobiotic insults. Both microglia and astrocytes release a variety of pro-inflammatory cytokines (IL-1, IL-6, TNF-α), which play a crucial role in mediating or maintaining hyperalgesia and allodynia (DeLeo *et al.*, 1996; DeLeo and Yezierski, 2001; Milligan *et al.*, 2001; Sweitzer *et al.*, 2001b; Watkins *et al.*, 2001a, b; Milligan *et al.*, 2003). They increase the sensitivity of damaged neurons to nociceptive and non-nociceptive stimuli (Sommer and Schafers, 2004) and are thought to contribute to pain state by direct action on primary afferent neurons and by indirect actions via activation of signaling pathways in immune cells (Thacker *et al.*, 2007). There are several studies carried out using immunosuppressive (methotrexate) and immunomodulatory (propentofylline) drugs with an aim of suppressing neuropathic pain via suppression of glial-dervied pro-inflammatory cytokines in spinal cord. In spite of being successful in reducing enhanced pain response, they are not considered as viable option for chronic administration in human being due to the negative impact on the peripheral immune system (Sweitzer *et al.*, 2001c; Hashizume *et al.*, 2000).

Il-1β, one of the many pluripotent proinflammatory cytokines, is produced and secreted by immune cells (macrophages, monocytes and microglia) under pathological conditions (Giri *et al.*, 1985; Ferrari *et al.*, 1997; Mackenzie *et al.*, 2001; Clark *et al.*, 2006). Upregulation of IL-1 β mRNA has been observed in animal models of chronic constriction injury (CCI) (Okamoto *et al.*, 2001; Kleinschnitz *et al.*, 2004) and in injured sciatic nerve after experimental transaction (Shamash *et al.*, 2002). Moreover, increase in spinal protein levels of IL-1 β were reported after peripheral neuropathy and inflammation (Sweitzer *et al.*, 1999). Although the mechanism of action of IL-1β in the peripheral neuropathy is still unclear, electrophysiologic evidence suggests that 1L-1 β may directly excite nociceptive fibres (Fukuoka *et al.*, 1994; Reeve *et al.*, 2000), increase their response to heat stimuli (Obreja *et al.*, 2002) and modulate sensory neuron transmission via increased release of the nociceptive neuropeptide SP (Malcangio *et al.*, 1996; Inoue *et al.*, 1999) and CGRP (Fukuoka *et al.*, 1994; Hou *et al.*, 2003). There are studies demonstrating reduced pain-associated behavior in neuropathic mice after CCI by neutralizing antibodies to IL-1 Type 1 receptor (Sommer *et al.*, 1999; Schafers *et al.*, 2001).

Another prominent proinflammatory mediator is TNF-α which is known to initiate a cascade of cytokines and growth factors activation. Increased TNF-α mRNA (Wagner and Myers, 1996) and protein expression (Sommer and Schafers, 1998; George *et al.*, 1999) observed in

injury induced animal models of neuropathic pain points towards it correlation with the development of allodynia/hyperalgesia. Its influence on altered pain processing seems to be mediated directly and/or indirectly by the phosphorylation of extracellular regulated kinase (Takahashi *et al.*, 2006) and p38 MAPK (Schafers *et al.*, 2003) which may in turn mediate mechanical allodynia via a modulation of tetrodotoxin-resistant Na^+ channels (Jin and Gerean, 2006). Studies indicate, impairment of TNF- α signalling reduces hyperalgesia/ allodynia after spinal nerve ligation (SNL) (Schafers *et al.*, 2003), CCI (Lindenlaub *et al.*, 2000; Sommer *et al.*, 2001a, b) and partial transaction of the sciatic nerve (Sommer *et al.*, 2001a).

Moreover, IL-6 has also shown to be involved in pathophysiology of neuropathic pain post CCI (Cui *et al.*, 2000; Murphy *et al.*, 1999) and partial nerve ligation (PNL) (Cui *et al.*, 2000). Increased IL-6 mRNA levels were reported in DRG after CCI. Attenuation of thermal hyperalgesia and mechanical allodynia has been observed in IL-6 knockout mice post CCI (Murphy *et al.*, 1999). Ramer *et al.* (1998) opined that IL-6 induced adrenergic sprouting, due to its direct excitatory effect on nociceptive neurons, could be one of the contributing factors for the generation of neuropathic pain.

It is now well understood that anit-inflammatory cytokines (IL-4, IL-10, IL-13 and TGFβ) provide negative feedback to pro-inflammatory production and release. IL-10 is known to selectively suppress the production and release of proinflammatory cytokines (IL-1, IL-6 and TNF-α) (Moore *et al.*, 1995; Wieseler-Frank *et al.*, 2005), up-regulates the production of other anti-inflammatory substances (Il-4, IL-13), and down-regulates the expression of proinflammatory cytokine receptors (Moore *et al.*, 2001). Based on this fact, there are several studies carried out using IL-10 as the target for treating neuropathic pain. Gene therapy to induce spinal IL-10 production has been found to be effective in preventing or reversing enhanced pain in preclinical animal models of neuropathic pain (Moore *et al.*, 1995; Watkins *et al.*, 2003b; Milligan *et al.*, 2004; Milligan *et al.*, 2005).

Animal Models of Pain

Due to high prevalence of pain worldwide and its impact on socio-economic life, discovery and development of novel analgesics is a subject of extensive research in both academia and industry. Rodent behavioral pain models are used at all stages of this work, right from identification of novel targets and mechanisms to estimation of efficacy and therapeutic window (Berge, 2011). Experimentally animal models for pain can be divided into three

types: animal models for acute nociceptive pain, inflammatory pain and neuropathic pain

Animal models for acute nociceptive pain: These animal models are useful in assessing and predicting the analgesic properties of novel compounds in naïve rats. It includes acute tests such as hot-plate, tail-flick, paw-pressure tests and require a high intensity stimulus (such as thermal, mechanical or chemical) to a convenient body part (e.g., hind paw, tail or abdomen) and is not tested in pre-injured animals. The response measured is immediate or within few seconds (easy to score as a response to stimulus), it uses the Aδ- and C-fiber input and is also known to activate the spinal dorsal horn bearing nociceptive specific cells (Eaton, 2003). Animal models for acute nociception are of two types: Somatic nociception and visceral nociception. Models for somatic nociception includes i) tail flick model where the thermal stimulation is given on the tail till the tail flicks and the time taken for the tail flick is measured (Bass and Vander Brook, 1952) ii) paw pressure model where the mechanical pressure is exerted on the paw until the threshold for paw withdrawal is reached (Green and Young, 1951). Models for visceral nociception includes abdominal constriction assay often known as writhing test where the animal is exposed to chemical stimuli (acetic acid or phenoquinone, i.p.) and abdominal constrictions are recorded (accompanied by slight twisting of trunk and followed by bilateral extension of the hind limbs) post stimuli from 5-20 minutes (Collier *et al.*, 1968).

For our research purpose, we studied abdominal constriction assay using acetic acid as the chemical stimuli and is described in chapter 1 in detail.

Models of inflammatory pain: These tests use an irritant, foreign chemical agent as the nociceptive stimulus. Unlike, acute nociceptive pain models, they do not measure the threshold response only but they also quantitatively measure the resulting behavior after the stimulus (Eaton, 2003). Examples of inflammatory pain models include intraplantar injections of either carrageenan (Tonussi and Ferreira, 1992) or complete Freund's adjuvant (CFA) or formalin (Dubuisson and Dennis, 1977), rheumatoid arthritis (either induced by collagen or adjuvant), osteoarthritis (induced by monosodium iodoacetate -MIA). Inflammatory pain models are carried out to understand chronic pain mechanisms at molecular, cellular, electrophysiological and anatomical levels. It helps us to understand pathophysiological mechanisms underlying pain and allow preclinical evaluation of potential analgesics and safety and efficacy of various NSAIDS (Fiorucci *et al.*, 2001) including the

COX-1 and COX-2 inhibitors commonly used for inflammatory pain (Giulianao and Warner, 2002).

To study the anti-inflammatory properties of the compound of our interest, we studied formalin test, carrageenan induced paw edema test and osteoarthritis model induced by MIA and detailed description of these models is provided in chapter 2.

Models for neuropathic pain: Neuropathic pain is the most difficult type of pain to treat. Clear understanding of what leads to such persistent pain and testing of pharmacological agents in such conditions can help us to develop better treatment of neuropathic pain. In this scenario, animal models can provide useful and essential system. Numerous animal models have been developed to simulate specific human painful conditions, mostly by producing disease or traumatic injuries that is followed by pain behavior.

Surgical animal models of neuropathic pain: Several animal models have been developed using surgical intervention to induce peripheral nerve injury. Wall *et al.* (1979) developed the first model where sciatic nerve was subjected to complete transaction at mid thigh level resulting into autotomy. Ethics issue related to this model led to development of now widely used chronic constriction injury model by Bennett and Xie (1988). This method employs four loosely tied catgut ligatures around the sciatic nerve proximal to the sciatic trifurcation, leading to intraneural edema, focal ischemia and an axonal degeneration resulting in chemical and heat evoked hyperalgesia accompanied with cold and mechanical allodynia lasting till two months (Bennett and Xie, 1988; Attal *et al.*, 1990). Seltzer *et al.* (1990) developed the partial sciatic nerve ligation (PSL) model by tightly ligating 1/3 to 1/2 of the sciatic nerve with a single ligature resulting in to mechanical allodynia, heat-evoked hyperalgesia and spontaneous pain lasting up to seven months. Kim and Chung (1992) developed spinal nerve ligation (SNL) model which employs tight ligation of L5 and L6 spinal nerve close to their respective ganglia resulting into mechanical and heat evoked hyperalgesia lasting till 4 months (Choi *et al.*, 1994). Recently developed model includes transaction of three branches of sciatic nerve (tibial, sural and common peroneal) (Lee *et al.*, 2000a) where transaction of tibial and sural nerves resulted in the mechanical allodynia, chemical hyperreactivity and spontaneous pain. Behavioral read outs observed in these models are found to be similar to the sensation felt by neuropathic pain patients (Bennett and Xie, 1988; De Vry *et al.*, 2004). These models are also found to be responding well to drugs used in clinics (Attal *et al.*, 1991; Hunter *et al.*, 1997; De Vry *et al.*, 2004).

Disease induced neuropathic pain models: There are various types of disease which have neuropathic pain sequelae and this condition has compelled to develop animal models with those specific disease conditions. Examples are diabetes, shingles, and cancer. Diabetes mellitus is the leading cause of neuropathy (Dyck *et al.*, 1993; Simmons and Feldman, 2002). Chronic pain associated with shingles is mimicked in model of post-herpetic neuralgia by employing the administration of varicella-zoster virus (VZV), the virus responsible for the pathologic condition with persistent allodynia and hyperalgesia (Sadzot-Delvaux *et al.*, 1990; Fleetwood-Walker *et al.*, 1999). The most commonly used model to study diabetes induced neuropathic pain is streptozotocin-induced diabetic neuropathy model, where single intraperitonal injection of streptozotocin kills insulin secreting islet cells causing hyperglycemic condition and inducing long lasting thermal and mechanical hyperalgesia and cold and thermal allodynia (Forman *et al.*, 1986; Wuarin-Bierman *et al.*, 1987; Courteix *et al.*, 1993; Courteix *et al.*, 1994). Several studies have been performed to model cancer pain. They are chemotherapy-induced peripheral neuropathy models (inducing agents such as Vincristine (Aley *et al.*, 1996), Taxol (Cavaletti *et al.*, 1995), Cisplatin (Authier *et al.*, 2000)), cancer invasion pain model (Shimoyama *et al.*, 2002) and bone cancer pain models (Schwei *et al.*, 1999; Wacnik *et al.*, 2001; Walker *et al.*, 2002).

CCI and diabetes induced peripheral neuropathic pain are the models selected for our research purpose and chapter 3 carry the detailed description of these models.

Behavioral Readouts: Unlike, human beings, animals cannot speak out or describe the pain sensation being felt. Hence, there needs to be a way to assess the pain behavior observed in animals and this has to be through their response to painful or non painful stimuli. Commonly used assessment parameters in neuropathic pain studies includes heat hyperalgesia where paw withdrawal latencies to a radiant heat stimulus are measured, cold allodynia where response (paw withdrawal, licking, flinch, stumping) to cutaneous administration of acetone is recorded and mechanical hyperalgesia or allodynia where paw withdrawal threshold to mechanical stimuli or increasing pressure is measured. We studied thermal hyperalgesia, mechanical allodynia and cold allodynia for our experiments and are described in more details in relevant chapters.

Prevalence of Pain

Globally, it has been estimated that 1 in 5 adults suffer from pain and that another 1 in 10 adults are diagnosed with chronic pain each year (International Association for the Study of

Pain: www.iasp-pain.org). The significant prevalence of pain worldwide has great impact on socio-economic life. Pain is a costly health issue in terms of lost productivity and income. Reports quote that pain alone costs US ~$ 100 billion annually in terms of lost productivity and income due to absenteeism (American Pain Foundation; McCarberg and Billington, 2006). According to internet based survey performed by Johannes *et al* (2010), chronic pain is a considerable burden in US. Accordingly, discovery and development of novel analgesics is subject to extensive research in both academia and industry.

The need of the hour-Drugs with better efficacy and lesser side effects

Chronic pain and especially neuropathic pain has a profound effect upon individual's economies and society in general but still remains under-diagnosed and under-treated. The treatment options available for the management of neuropathic pain are as diverse as its etiologies (Dworkin, 2002). Although number of drugs from various class like anticonvulsants (gabapentin, pregabalin), TCAs (amitriptyline), SNRIs (duloxetine) are prescribed either alone or in combination but they are either poorly efficacious or accompanied with severe side effects (Dray, 2008). Also there are reports hinting towards neuropathic pain patients showing general insensitivity towards NSAIDs and relative resistance to opioids. Although higher doses of opioids are found to be efficacious, they have their own side effects which are very severe in nature (Kalso *et al.*, 2004). These observations emphasize on requirement for novel drugs which are effective in relieving the neuropathic pain and at the same time do not elicit severe side effects upon chronic administration.

In the era of new therapeutic agents, plants still remain to be the major possible source of future drugs and chemicals. They continue to be the source of lead structures for synthetic modifications and optimization of bioactivity. Due to limited efficacy and severe side effects associated with currently available agents, medicinal products derived from plants, marine organisms etc are preferred and are becoming part of integrative health care systems in industrialized nations (Qadrie *et al.*, 2009). A dramatic increase is seen in the number of patients opting for complementary and alternative medicine and consuming extracts from biological compounds from folklore medicine (Smith and Mills, 2001). Along with better pain relieving property than that of currently available options such as NSAIDs, analgesics, opiates etc, herbal medicinal products are believed to have lesser side effects. Even when exact mechanism of action of herbal medicinal product remains elusive, it is for sure that

most of them might exhibit their efficacy/potency through several pathways which include inhibition of cyclooxygenase (COX) and /or lipoxygenase (LOX), inhibition of cytokine release, inhibition of elastase or hyaluronidase, and may also induce antioxidative activity as opined by Cameron *et al.* (2009a, b).

Biological Immune Response Modulator (BIRM)

The botanical tested for its pain relieving property, the Biological Immune Response Modulator, abridged 'BIRM', was a kind gift by Dr. Edwin Cevallos Arellano of BIRM Inc. (Quito, Ecuador). BIRM is an aqueous extract of dried roots of a plant *Solanum dulcamara* grown in Ecuador and is marketed as a greenish brown suspension with a mild bittersweet smell. *S. dulcamara* also known as bittersweet nightshade is native to Africa, Europe and Asia, spreading throughout the world. The inactive ingredients in BIRM comprise 16% solid particles of root fibres and the remainder, a lipid-free liquid. BIRM is prepared by aqueous extraction of dried roots followed by oxidation/reduction of the extract. During this process, the amount of roots and the timing of redox reaction are carefully controlled so as to minimize batch-to-batch variation.

Unlike other drugs, BIRM does not act over the immune system, but might achieve its efficacy by modulating or balancing the immune system. This allows usage of BIRM not only in the disease with diminished immune system but in autoimmune diseases too where the immune system is overloaded and needs to be stabilized.

Earlier studies using BIRM have shown cytotoxic and anti-metastatic properties in prostate cancer model (Dandekar *et al.*, 2003) and the mother tincture of *S. dulcamara* inhibits the production of PGE_2 by downregulating the inducible isotype of cyclooxygenase enzyme – COX-2 (Jaggi *et al.*, 2004). The formulation is also being dispensed as a natural remedy for a variety of maladies including HIV-1 infection and also against cancer (Cevallos, 1994). However, very little systematic information is currently available on the therapeutic potential of BIRM. Baring the two isolated attempts mentioned at the beginning of this paragraph, no studies have been undertaken to investigate the structure-function correlation of ingredients in BIRM.

BIRM as a Potent Analgesic Agent: The Rationale for selection

Owing to the apparent lack of effective pain relieving agents coupled with severe side effects reported among the therapeutic options available currently, people look upon for alternate treatment for relief from chronic and debilitating pain.

In search of a product having broad range of efficacy in ameliorating pain (inflammatory or otherwise) with minimal side-effects, we came across various reports depicting 'magical properties' of a plant extract marketed by BIRM Inc. (Quito, Ecuador) under the trade name 'Biological Immune Response Modulator' (BIRM). Although, this plant (*S. dulcamara*) is widely distributed globally with presence in Africa, Asia and Europe, it is mainly found in Amazon region of South America. The natives of Ecuador found it to be efficacious in mitigating various diseases including cancer and AIDS, the claim in the recent past has been well supported by the published scientific data (Dandekar *et al*., 2003). In addition, there was also a report from Jaggi and coworkers (2004) indicating the inhibitory action of mother tincture from *Solanum dulcamara* - the source of BIRM, on PGE_2 by downregulating the cyclooxygenase activity in *in vitro* conditions. Furthermore, as the name suggests, BIRM is an immune system modulator and believed to maintain the optimal immune response under various pathological conditions.

The polyunsaturated fatty acid derived eicosanoid - the PGE_2 is well accepted as a mediator in the neuro-inflammation process and also, at least in part, in the genesis of neuropathic pain, the later however, might also involve mediation by spinal microglia as well as immune system in its maintenance. The BIRM with its reported inhibitory effect of eicosanoid biosynthesis and immunomodulatory potential should be able to attenuate the acute as well as chronic inflammatory and neuropathic pain better than the currently available analgesic compounds and that too with minimum or no adverse effect. The above notion prompted us to carry out a systematic study wherein the efficacy of BIRM in pain management was evaluated using animal models widely accepted by regulatory authorities for nociceptive pain, inflammatory pain and neuropathic pain.